

Mechanisms of Over-Active Endothelium-Derived Contracting Factor Signaling
Causing Common Carotid Artery Endothelial Vasomotor Dysfunction in
Hypertension and Aging

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Background and Purpose: The endothelium is a single-cell layer positioned at the blood-vascular wall interface, where in response to blood-borne signals and hemodynamic forces, endothelial cells act as central regulators of vascular homeostatic processes including vascular tone, growth and remodeling, inflammation and adhesion, and blood fluidity and coagulation. Agonist- or flow-stimulated endothelium-dependent vasorelaxation becomes impaired in states of cardiovascular disease (CVD) risk and has been identified as a possible biomarker of overall endothelial dysfunction leading to vascular dysregulation and disease pathogenesis. Accordingly, it is important to elucidate the mechanisms accounting for this endothelial vasomotor dysfunction. Upon stimulation, endothelial cells can synthesize and release a variety of endothelium-derived relaxing factors (EDRFs), the most prominent of which is nitric oxide (NO) derived from NO synthase (NOS). In addition, under certain CVD risk conditions including hypertension and aging, stimulated endothelial cells can become a prominent source of endothelium-derived contracting factors (EDCFs) produced in a cyclooxygenase (COX)-dependent manner. Consequently, endothelial dysfunction may be caused by under-active EDRF signaling and/or competitive over-active EDCF signaling. Much attention has been given to elucidating the mechanisms of under-active EDRF signaling and its role in causing endothelial dysfunction, wherein excess reactive oxygen species (ROS) accumulation and oxidative stress under CVD risk conditions have been recognized as major factors in reducing NO bioavailability thus causing under-active EDRF signaling and endothelial dysfunction. Less attention however, has been given to elucidating the mechanisms of over-active COX-mediated EDCF signaling and its role in causing endothelial dysfunction. Moreover, while COX-mediated EDCF signaling activity has been investigated in some segments of the vasculature, most notably the aorta, it has not been well-investigated in the common carotid artery (CCA), a highly accessible cerebral blood flow conduit particularly advantageous in exploring the roles of the endothelium in vascular pathogenesis. It was the global purpose of this thesis to gain a better understanding of the cellular-molecular mechanisms accounting for endothelial dysfunction in the CCA of animal models known to exhibit COX-mediated EDCF signaling activity, in particular essential (spontaneous) hypertension and aging.

Experimental Objective and Approach: This thesis comprises three studies. Study I and Study II investigated the CCA of young-adult (16-24wk old) normotensive Wistar Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats. Study III investigated the CCA of Adult (25-36wks old) and Aging (60-75wks old) Sprague Dawley (SD) rats treated in vivo (or not; CON) with L-

buthionine sulfoximine (BSO) to chronically deplete the cellular anti-oxidant glutathione (GSH) and increase ROS accumulation and oxidative stress. The global objective and approach across these studies was to systematically examine the relative contributions of NOS and COX signaling pathways in mediating the acetylcholine (ACh)-stimulated endothelium-dependent relaxation (EDRF) and contractile (EDCF) activities of isometrically-mounted CCA in tissue baths in vitro, with a particular focus on elucidating the mechanisms of COX-mediated EDCF signaling activity. An added objective was to examine the in vivo hemodynamic characteristics of the CCA in each animal model investigated, serving both to identify the pressure-flow environment that the CCA is exposed to in vivo and to provide assessment of potential hypertension, aging, and oxidative stress effects on large artery hemodynamics.

Key Findings: Study I hemodynamic analysis confirmed a hypertensive state in young adult SHR while also exposing a reduction in mean CCA blood flow in SHR compared to WKY accompanied by a multi-faceted pressure-flow interaction across the cardiac cycle relating to flow and pressure augmentation. Study III hemodynamic analysis found that neither aging nor chronic BSO-induced GSH depletion affected CCA blood pressure or blood flow parameters in SD rats.

Study I and II demonstrated that a COX-mediated EDCF response impaired ACh-stimulated endothelium-dependent vasorelaxation in pre-contracted CCA from young adult SHR, while EDRF signaling activity, predominantly mediated by NO, remained well-preserved compared to WKY. Examining ACh-stimulated contractile function specifically from a quiescent (non pre-contracted) state revealed that EDCF activity did exist in WKY CCA but could be completely suppressed by NO-mediated EDRF signaling activity, whereas the similarly robust NO-mediated EDRF signaling activity in SHR CCA could not fully suppress its >2-fold augmented EDCF activity vs. WKY CCA. Further pharmaco-dissection of ACh-stimulated contractile function in the SHR-WKY CCA model revealed that the EDCF signaling activity was completely dependent on the COX-1 (but not COX-2) isoform of COX and was almost exclusively mediated by the thromboxane-prostanoid (TP) sub-type of the prostaglandin (PG) G-protein coupled receptor family and by Rho-associated kinase (ROCK), a down-stream effector of the molecular switch RhoA. Furthermore, it was found that while exogenous ROS-stimulated CCA contractile function was similarly >2-fold augmented in SHR vs. WKY and dependent on COX-1 and TP receptor and ROCK effectors, ACh-stimulated CCA EDCF signaling activity was only minimally affected by in-bath ROS manipulating compounds. Additional biochemical and molecular analysis revealed that ACh stimulation was associated with PG over-production from an over-expressed COX-1 in SHR CCA, and with CCA plasma membrane localization and activation of RhoA.

Study III demonstrated that a COX-mediated EDCF response impaired ACh-stimulated endothelium-dependent vasorelaxation in pre-contracted CCA from Aging SD rats, while EDRF signaling activity, predominantly mediated by NO, remained well-preserved compared to Adult SD rats. Specific examination of ACh-stimulated contractile function revealed that EDCF activity did exist in Adult CCA but could be completely suppressed by NO-mediated EDRF signaling activity, whereas the similarly robust NO-mediated EDRF signaling activity in Aging CCA could not fully suppress its >3-fold augmented EDCF activity vs. Adult CCA. Further pharmacodissection of ACh-stimulated contractile function in the Adult-Aging SD rat CCA model revealed that EDCF signaling activity was completely dependent on COX-1, but while exogenous ROS was able to elicit a COX-dependent CCA contractile response, in-bath ROS manipulating compounds were found to be without effect on ACh-stimulated CCA EDCF signaling activity. Furthermore, biochemical analysis revealed that aging was not associated with a change in tissue (liver and vascular) GSH content or ROS accumulation. Chronic in vivo BSO treatment was effective in depleting tissue GSH content and increasing ROS accumulation, to a similar extent, in both Adult and Aging SD rats. However, regardless of age, neither ACh-stimulated NO-mediated EDRF signaling activity nor COX-mediated EDCF signaling activity were affected by these BSO-induced perturbations.

Conclusions and Perspective: In the CCA of animals at the early pathological stages of either essential hypertension (young adult SHR) or normotensive aging (Aging SD rats), endothelial vasomotor dysfunction can be caused solely by over-active EDCF signaling, apparently disconnected from changes in NO bioavailability or oxidative stress. While NO and ROS may act, respectively, as negative and positive modulators of the established COX-PG-TP receptor-RhoA-ROCK cell-signaling axis mediating endothelium-dependent contractile activity, these factors do not appear to be essential to the mechanism(s) underlying the development of over-active EDCF signaling. Further elucidation of the cellular-molecular causes of over-active EDCF signaling, and its patho-biological consequences, in the SHR-WKY and Adult-Aging SD rat CCA models of EDCF activity established and hemodynamically characterized in this thesis, may help to identify new or more effective targets to be used in prevention or treatment strategies to combat the pathogenesis of CVD.

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List of Abbreviations

A23187	Calcymycin (calcium ionophore)
AII	Angiotensin II (AT ₁ receptor agonist)
AA	Arachidonic Acid
AC	Adenylate cyclase
ACh	Acetylcholine (muscurinic receptor agonist)
AI	Augmentation index
Amplex Red	10-acetyl-3,7-dihydroxyphenoxazine (fluorescent probe used in conjunction with horseradish peroxidase (HRP) to detect hydrogen peroxide (H ₂ O ₂))
Apocynin	NADPH oxidase inhibitor
AUC	Area under the curve
BCA	Bicinchonic acid
BH ₄	Tetrahydrobiopterin
BSA	Bovine serum albumin
[Ca ²⁺] _i	Intra-cellular Ca ²⁺ concentration
CaM	Calmodulin
CAT	Catalase
CCA	Common carotid artery
Celecoxib	Selective cyclooxygenase (COX)-2 inhibitor (orally active; indicated for the clinical treatment of osteoarthritis, rheumatoid arthritis, and acute pain)
CVD	Cardiovascular disease
CYP450	Cytochrome P450 monooxygenase
DAG	Diacylglycerol
DBP	Diastolic blood pressure
DCFH-DA	2'-7'-dichlorodihydrofluorescein-diacetate (fluorescent probe; considered a general reactive oxygen species (ROS) detector)
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EC ₅₀ or EC ₅₀	Concentration of drug effective in eliciting 50% of the maximum amplitude (Max Amp) of the drug-stimulated response
ECM	Extra-cellular matrix
EDCF	Endothelium-derived contracting factor
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factor

EETs	Epoxyeicosatrienoic acids
-Endo	Endothelin-denuded
eNOS	Endothelial nitric oxide synthase (NOS)
ET-1	Endothelin-1 (ET _A receptor agonist)
Fasudil	Selective Rho kinase (ROCK) inhibitor (orally active; indicated for the clinical treatment of cerebral vasospasm following stroke; in pre-clinical and clinical development for treatment of various conditions including pulmonary hypertension and angina)
FMD	Flow-mediated dilation
GF109203X	Broad-spectrum protein kinase C (PKC) inhibitor
GPCR	G-protein coupled receptor
GPx	Glutathione peroxidase
GSH	Glutathione
H1152	Selective Rho Kinase (ROCK) inhibitor
H ₂ O ₂	Hydrogen peroxide
HETEs	Hydroxyeicosatetraenoic acids
HR	Heart rate
HRP	Horseradish peroxidase
IMT	Intima-media thickness
Indo or INDO	Indomethacin (non-selective cyclooxygenase (COX) inhibitor)
iNOS	Inducible nitric oxide synthase (NOS)
IP ₃	Inositol triphosphate
K ⁺ channels	Big-, intermediate-, and small-conductance Ca ²⁺ -activated channels, ATP-sensitive channels, and inwardly-rectifying channels
L-NAME or LN	Non-selective nitric oxide synthase (NOS) inhibitor
L-NMMA	Non-selective nitric oxide synthase (NOS) inhibitor
LOX	Lipoxygenase
LV	Left ventricle
MAPK	Mitogen activated protein kinase
Max Amp	Maximum amplitude
MEGJ	Myo-endothelial cell-cell gap junction
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MnTMPyP	Manganese porphyrin reactive oxygen species (ROS) scavenger (considered a superoxide dismutase (SOD) + catalase (CAT) mimetic)

MYPT1	Myosin targeting subunit of myosin light chain phosphatase (MLCP)
NADPH	Nicotinamide adenine dinucleotide phosphate
ND	No drug (control)
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS	Neuronal nitric oxide synthase (NOS)
NO	Nitric oxide
NS398	Preferential cyclooxygenase (COX)-2 inhibitor
O ₂ ^{•-}	Superoxide anion
OH [•]	Hydroxyl radical
ONOO ⁻	Peroxynitrite
PDBu	Phorbol-12,13-dibutyrate (protein kinase (PK)C activator)
PE	Phenylephrine (α_1 -specific receptor agonist)
PEG	Polyethylene glycol
PG	Prostaglandin
PGS	Prostaglandin (PG) synthase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLA	Phospholipase A
PLC	Phospholipase C
PP	Pulse pressure
PVDF	Polyvinylidene fluoride
PWV	Pulse wave velocity
RLC ₂₀	Regulator light chain 20 (kDa)
ROCK	Rho kinase
ROS	Reactive oxygen species
S18886	Terutroban (selective thromboxane-prostanoid (TP) receptor antagonist; orally active; in Phase III/IV clinical development for secondary stroke prevention related to atherosclerosis)
SBP	Systolic blood pressure
SC560	Selective cyclooxygenase (COX)-1 inhibitor
SD	Sprague Dawley rat
SR	Sarcoplasmic reticulum
sGC	Soluble guanylate cyclase
SC560	Selective cyclooxygenase (COX)-1 inhibitor

SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SHR	Spontaneously hypertensive rat
SP-SHR	Stroke Prone Spontaneously hypertensive rat (SHR)
SNP	Sodium nitroprusside (nitric oxide (NO) donor)
SOD	Superoxide dismutase
STZ	Streptozotocin
SQ29548	Selective Thromboxane-Prostanoid (TP) receptor antagonist
TBS-T	Tris-buffered saline with Tween-20
Tempol	Nitroxide reactive oxygen species (ROS) scavenger (considered a superoxide dismutase (SOD) mimetic)
Tiron	Chemical scavenger of superoxide anion ($O_2^{\cdot-}$)
TP	Thromboxane-prostanoid
TX	Thromboxane
U46619	Selective thromboxane-prostanoid (TP) receptor agonist
VAS	Valeryl salicylate (preferential cyclooxygenase (COX)-1 inhibitor)
VSM	Vascular smooth muscle
WKY	Wistar Kyoto rat
X:XO	Xanthine : Xanthine oxidase (exogenous reactive oxygen species (ROS) generating system)
Y27632	Selective Rho kinase (ROCK) inhibitor

Chapter 1

General Background and Introduction

1.1 Thesis Briefing

1.1.1. Global Rationale

Endothelial cells line the lumen of all blood vessels where they play a pivotal role in regulating local vascular homeostasis, from vascular tone and stiffness, to growth and remodeling, to oxidative stress and inflammation, to blood fluidity and coagulation. Endothelial dysfunction, characterized by impaired endothelium-dependent vascular smooth muscle (VSM) relaxation, is now known to be a hallmark of cardiovascular disease (CVD), and an independent predictor of future cardiovascular events. In response to a variety of chemical or hemodynamic stimuli, the endothelium can produce both endothelium-derived relaxing (EDRF) and contracting (EDCF) factors. While much attention has been given to elucidating the cell-signaling mechanisms of EDRF activity and its role in endothelial dysfunction, less attention has been given to EDCF signaling. However, in both animal models and humans, EDCF signaling activity is gaining recognition as an important contributor to endothelial dysfunction under various conditions of CVD and CVD risk.

1.1.2. Global Purpose

The global purpose of this thesis is to gain a better understanding of the cellular-molecular mechanisms accounting for endothelial dysfunction in animal models known to be characteristic of EDCF signaling activity, in particular essential (spontaneous) hypertension and aging, both of which impart substantial CVD risk. The focus of all experiments in this thesis was on the common carotid artery (CCA), a blood flow conduit to the cerebral circulation that is of importance both physiologically and clinically, and highly accessible and advantageous to investigate experimentally, but under-studied in regard to EDCF activity and vascular (dys)function. In addition to characterizing its vasomotor properties, a further aim of this thesis was to characterize the hemodynamic properties of this CCA model in the different animals studied.

1.1.3. Description of Experimental Studies

There are three experimental chapters in this thesis (Chapters 2-4), each comprising a separate Study. The first two studies (Thesis Study I and II) investigated the CCA of young adult normotensive Wistar Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats, a model of human essential hypertension. The third study (Thesis Study III) investigated the CCA of adult

and aging Sprague Dawley (SD) rats, an animal model commonly used to study candidate aspects of vascular pathogenesis via experimental manipulation.

Thesis Study I is described in Chapter 2, presented with permission as the published article *Impaired Hemodynamics and Endothelial Vasomotor Function via Endoperoxide-Mediated Vasoconstriction in the Carotid Artery of Spontaneously Hypertensive Rats*, found in the April 2009 issue of *American Journal of Physiology – Heart and Circulatory Physiology*. This study explored the in vivo pressure-flow environment of the CCA in WKY and SHR and the endothelium-dependent relaxation (EDRF) and contractile (EDCF) responses of WKY and SHR CCA in vitro, the latter of which established the SHR CCA as an appropriate experimental model to explore the mechanisms of EDCF signaling activity in endothelial dysfunction.

Thesis Study II is described in Chapter 3, presented with permission as the published article *RhoA-Rho Kinase Signaling Mediates Endothelium- and Endoperoxide-Dependent Contractile Activities Characteristic of Hypertensive Vascular Dysfunction*, found in the May 2010 issue of *American Journal Physiology – Heart and Circulatory Physiology*. This study expands upon the vasomotor findings of Thesis Study I by further exploring the cell-signaling mechanisms of EDCF activity in the CCA of SHR vs. WKY rats, with a particular focus on VSM receptor signaling effectors.

Thesis Study III is described in Chapter 4, presented as an unpublished manuscript *Effect of Glutathione Depletion and Aging on Endothelium-Derived Relaxing and Contracting Factor Activity in the Common Carotid Artery of Sprague Dawley Rats*. This study describes the in vivo pressure-flow environment and in vitro endothelium-dependent relaxation (EDRF) and contractile (EDCF) responses in the CCA of Adult and Aging SD rats, and explores how chronic experimental manipulation of anti-oxidant status and oxidative stress may affect age-associated CCA EDCF signaling activity.

1.1.4. Preface to General Background and Introduction, and General Summary and Perspectives

The remainder of **Chapter 1 – General Background and Introduction** is organized as follows. **Section 1.2 – Endothelial Function: Paradigm of Its Importance**, **Section 1.3 – Endothelium-Dependent Vasorelaxation: A Display of Endothelial (Dys)function**, and **Section 1.4 – Methods Used to Evaluate Endothelial Vasomotor (Dys)function** outline the overall importance of the endothelium and how its functions can be measured. **Section 1.5 – Endothelial Function and Dysfunction: Balancing EDRF and EDCF Signaling Activities** provides two background reviews of the current literature: one regarding Under-Active EDRF Signaling Mechanisms (1.5.1 and 1.5.2) and the other regarding Over-Active EDCF Signaling Mechanisms (1.5.3) of Endothelial

(Dys)function Under CVD Risk Conditions, each review finishing with a summary. **Section 1.6 – CCA: An Advantageous Model to Study Vascular (Dys)function and the Role of Endothelial Cells** describes the CCA circulation and its (patho)physiological importance, and highlights advantages of the CCA as an experimental model. Then, **Section 1.7 – Specific Rationale, Objectives, and Hypotheses of Thesis Studies** provides a specific Introduction to the underlying rationale for each thesis study and their particular experimental objectives and hypotheses.

Following the experimental chapters (2-4), **Chapter 5 – General Summary and Perspectives** summarizes the key findings, conclusions, and novel contributions of each thesis study (**Section 5.1**), which is followed by a brief perspective on the general conclusions of the thesis studies and future directions of the research area (**Section 5.2 and 5.3**), and then closing remarks (**Section 5.4**).

1.2 Endothelial Function: Paradigm of Its Importance

Endothelial cells have an important function in maintaining vascular homeostasis (Figure 1.1). Because of its location at the blood-wall interface, the endothelium is able to directly sense and respond to vascular perturbations in homeostasis caused by blood-borne signals and hemodynamic forces. Through the synthesis and release of a wide variety of autocrine/paracrine factors, endothelial cells can influence a multitude of cellular and non-cellular constituents of the vascular wall and blood, including VSM, elastin and collagen matrix proteins, platelets and clotting factors, and inflammatory/immune cells. Under physiological conditions, the balanced production of endothelial-derived factors acts to maintain a 'healthy' degree of vascular tone, inflammation, growth and remodeling, and blood and vascular fluidity. However, under pathological conditions, characterized by exposure to CVD risk factor(s), the endothelium can adopt a pro-constrictory, pro-inflammatory, pro-oxidant, proliferative/fibrotic, and pro-thrombotic phenotype. This CVD phenotype thus predisposes the vasculature to multiple cellular-molecular dysfunctions that contribute significantly to overt disease manifestations, including: *atherosclerosis* – i.e. plaque formation, progression, and rupture ((125, 218, 391, 410); reflecting a pro-inflammatory/pro-oxidant/pro-thrombotic phenotype); *arteriosclerosis* – i.e. increased arterial stiffness ((10, 145, 412); reflecting a proliferative/fibrotic/pro-constrictory phenotype); and downstream *organ/tissue hypo-perfusion* ((393, 395); also reflecting a pro-constrictory/proliferative/fibrotic phenotype). In turn, these states of vascular consequence may lead to a number of adverse structural-functional complications (e.g. myocardial infarction, stroke, left ventricular (LV) overload, and end-organ micro-vascular damage) and conditions (e.g. ischemic heart disease, cardiomyopathy, heart failure, renal insufficiency, and cerebral deterioration) associated with profound morbidity and mortality.

1.3 Endothelium-Dependent Vasorelaxation: A Display of Endothelial (Dys)function

Endothelial (dys)function can be characterized by the ability of the stimulated endothelium to elicit relaxation of the underlying VSM, termed endothelium-dependent vasorelaxation. This was recognized shortly after the discovery that endothelial cells could actually produce vasoactive factors, before which time the endothelium was considered as an inert diffusional barrier.

In 1980, Furchgott and Zawadzki published their seminal work demonstrating that upon stimulation with the muscarinic receptor agonist acetylcholine (ACh), an isolated donor strip of rabbit aorta with endothelium could release a diffusible substance they termed 'endothelium-

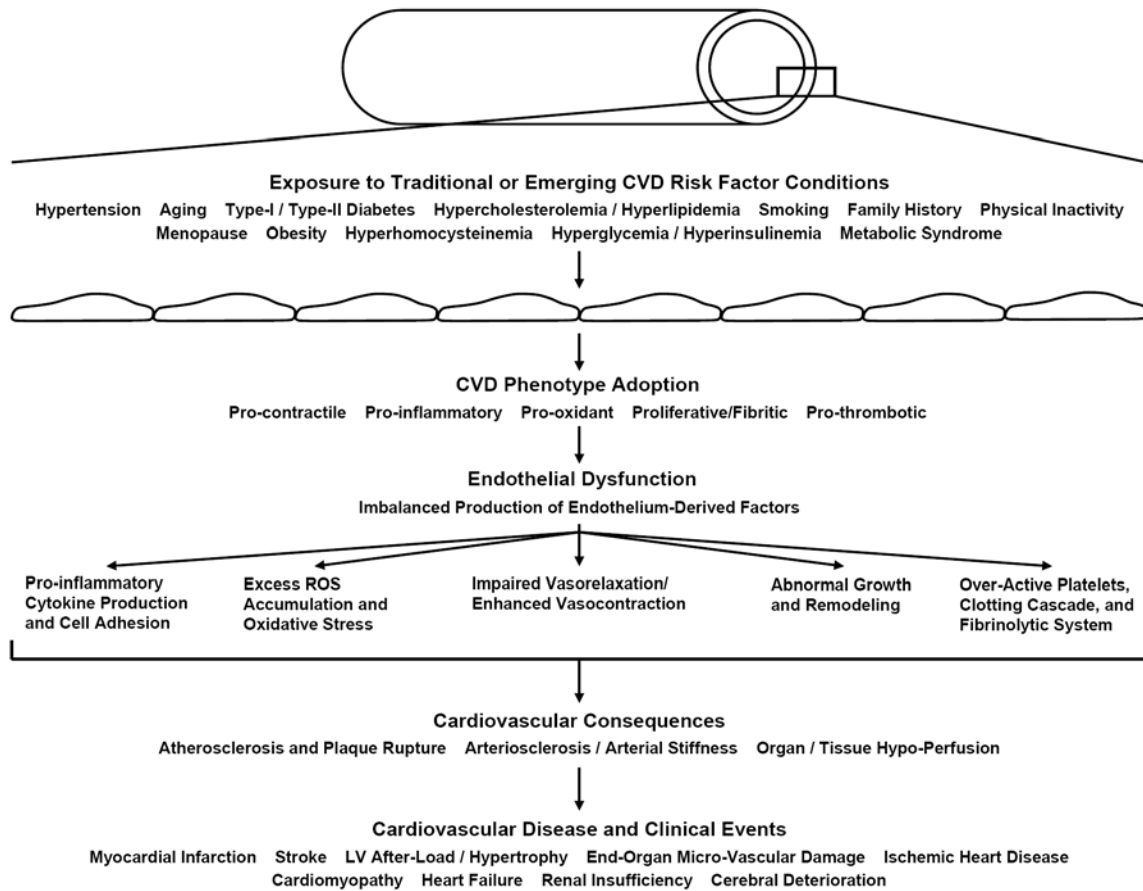


Figure 1.1. Paradigm of the importance of endothelial function. Refer to Section 1.2 and Section 1.3 for details.

-derived relaxing factor', or EDRF, which could elicit vasorelaxation in an isometrically-mounted adjacent strip denuded of endothelium (120). It was further discovered that this endothelium-dependent vasorelaxation response could be elicited not only by ACh but also by a wide range of more physiological stimuli, including shear stress (caused by the flow of viscous blood across the endothelium), circulating hormones (e.g. catecholamines, vasopressin), products of platelet activation (e.g. serotonin, adenosine diphosphate (ADP)), autocooids (e.g. histamine, bradykinin, prostaglandins (PGs)), and thrombin (384). Of pathophysiological interest were the subsequent observations that endothelium-dependent vasorelaxation could be blunted by states of known CVD risk. By 1988, it had already been shown that impaired endothelium-dependent vasorelaxation existed in the isolated conduit aortic or iliac vasculature of various rodent models of hypertension (199, 221, 229, 232, 415) and in hypercholesterolemic rabbits (167, 175, 390) and diet-induced atherosclerotic monkeys (116), as well as in vivo in the conduit coronary vasculature of atherosclerotic patients (225). Moreover, in the aorta of SHR, endothelium-

dependent contractile activity was found (230, 231). These early reports prompted the suggestion that endothelial dysfunction could be an early marker of CVD (174).

Since then, a plethora of experimental and clinical research has confirmed that endothelial dysfunction can be an early, sensitive biomarker of CVD and discriminating target for its risk factors ((125, 391, 405, 410); PubMed citations in response to the search term ‘endothelial dysfunction’ from 1988 to 2010 exceed 30000, excluding reviews; www.pubmed.gov). In both humans and animal models studied in vivo and in vitro, impaired endothelium-dependent vasorelaxation has been found to be a hallmark of overt CVD states/conditions and associated with each of its *major (traditional) risk factors*, including: advanced age, family history, hypertension, hypercholesterolemia/hyperlipidemia, type-I/type-II diabetes, smoking, physical inactivity, and menopause; as well as its many *emerging risk factors*, including: obesity, hyperhomocysteinemia, hyperinsulinemia, hyperglycemia, systemic inflammation, and the metabolic syndrome. The presence of multiple CVD risk factors is related to a progressive worsening of endothelium-dependent vasorelaxation (32, 406), while many lifestyle (i.e. dietary factors and physical activity) and pharmacological (i.e. drug) interventions that reduce CVD risk factors also improve endothelium-dependent vasorelaxation (308, 410).

The presence of clinical and sub-clinical *indices of atherosclerosis*, including coronary plaque, CCA intima-media thickness (IMT), and circulating inflammatory biomarkers such as C-reactive protein (125, 134, 392, 410), are independently associated with the extent of impaired endothelium-dependent vasorelaxation, as are *indices of arterial stiffness*, including CCA-femoral (aortic) pulse-wave velocity, CCA pressure wave reflection and augmentation index (AI), and CCA or brachial distensibility (99, 249, 412), as well as *indices of major target organ damage*, including LV hypertrophy, reduced renal function, and cerebral decline or white matter hyperintensities (69, 190, 393). Furthermore, clinical trials have firmly established that impaired endothelium-dependent vasorelaxation, assessed via agonist- or flow-stimulation in either the conduit macro-vasculature or resistance micro-vasculature of the coronary or more accessible arm/forearm circulation, is of *prognostic significance*, independently predicting future CVD-related clinical events in various populations (125, 308, 391, 394).

Thus, it has become apparent that endothelium-dependent vasorelaxation provides an important window to display both the (patho)biological and (patho)mechanical activities of endothelium-derived factors in the vasculature, which besides acting acutely to affect arterial or arteriolar tone, may also signal to either prevent or promote a sustained CVD phenotype and function (Figure 1.1). Accordingly, it is an ongoing priority in vascular research to gain a better understanding of the endothelial-VSM cell-signaling mechanisms controlling vasomotor activity

and exactly how these mechanisms may become dysfunctional in each state of CVD risk. This work has been successful in, and continues to hold the promise of, identifying new or more effective targets or indices to be used in prevention and treatment strategies aimed at combating specific CVD pathologies and their consequences (93, 103, 106, 302, 361, 393).

In this thesis, the endothelium-derived factors and cellular-molecular mechanisms accounting for endothelial-VSM cell-signaling and vasomotor (dys)function were investigated in states of hypertension and aging, both of which are known to impart substantial CVD risk.

1.4 Methods Used to Evaluate Endothelial Vasomotor (Dys)function

1.4.1 Human In Vivo Approaches

In humans, macro-vascular endothelial vasomotor (dys)function is evaluated clinically in the coronary circulation using *quantitative angiography* to measure diameter changes of the conduit epicardial artery in response to endothelial agonist(s) infused through an intra-coronary arterial catheter, or in response to increased blood flow induced by agonist-mediated dilation of the downstream resistance vasculature. In addition, coronary micro-vascular endothelial (dys)function can be assessed by using *intra-coronary arterial Doppler ultrasound* to measure the hyperemic response to vasoactive agonist infusion. Because the coronary circulation is the most prominent site of atherosclerosis and CVD-related clinical events, these approaches have been considered as ‘gold standard’ methods in evaluating human endothelial dysfunction. However, their utility is greatly limited by accessibility and risk (308, 394). Consequently, assessment of the more accessible arm vasculature has been used extensively to study endothelium-dependent vasomotor (dys)function in humans.

Micro-vascular endothelial (dys)function can be assessed using *strain-gauge venous occlusion plethysmography* or *Doppler ultrasound* to measure vasodilation and subsequent hyperemia of the forearm in response to the brachial artery infusion of agonist(s). Although still moderately invasive and risky, this approach is particularly valuable experimentally because of the ability to reliably generate dose-response relationships to agonists that may be mechanistically dissected using co-infused pharmacological inhibitors and/or antagonists. Accordingly, most studies investigating the cell-signaling mechanisms accounting for endothelium-dependent vasomotor activity in humans have been performed using this forearm blood flow technique (394).

Because of its relative accessibility and non-invasiveness, echo-tracking ultrasound imaging of *flow-mediated dilation (FMD)* in the macro-vascular brachial artery, stimulated by forearm occlusion and subsequent reactive hyperemia, is also widely used to assess human

endothelial (dys)function. Although this approach is considerably limited by technical expertise, high day-to-day variability, and sometimes dubious stimulus-response parameters and cell-signaling mechanisms (308, 394), it is still very useful because it can readily identify populations with impaired endothelial function, thus bolstering its use both experimentally and clinically.

Indeed, a significant correlation, albeit modest, has been found between impaired endothelium-dependent vasodilation measured as brachial artery FMD, agonist-stimulated forearm hyperemia, and/or agonist-stimulated coronary artery dilation within the same patient/participant (11, 12), thus supporting the concept that endothelial dysfunction is systemic in nature and as such may be evaluated indiscriminately. However, it has become apparent from experimental investigations in both humans and animal models of disease that heterogeneity does exist throughout the vasculature regarding the extent to which endothelium-dependent vasodilation/relaxation is affected by particular CVD risk factors, as well as the exact cell-signaling mechanisms accounting for endothelial function and dysfunction. Accordingly, these heterogeneities must be appreciated when evaluating endothelial function, since different tests could provide disparate clinical information about the health or pathology of the vasculature (394).

1.4.2 Animal In Vitro Approaches

In animals, the function and cell-signaling activities of a particular segment within the macro-vasculature or micro-vasculature can be investigated in great detail. The most widespread means of evaluating endothelial vasomotor (dys)function remains to be the *classic vascular myography approaches* pioneered by Furchgott and contemporaries (120, 281), where a segment of the arterial macro-vasculature (e.g. aorta, coronary epicardial, CCA, brachial, renal, or femoral artery) or arteriolar micro-vasculature from a certain organ or tissue (e.g. the coronary, cerebral, renal, mesenteric, or limb skeletal muscle circulatory bed) is excised from an animal (or a human, if possible; e.g. see Refs. (418, 419)) and evaluated in vitro (see Figure 1.2). In a temperature-controlled organ bath filled with aerated physiological buffer, the dose-dependent relaxation and/or contractile responses to vasoactive agonist(s) can be measured as the tension lost (i.e. relaxation) or gain (i.e. (pre-/re-)contraction) by an isometrically-mounted segment (ring) of isolated vasculature (308). Alternatively, *FMD* can be measured in an isolated vascular segment mounted on perfusion pipettes and situated in an organ bath on a microscope stage, thus allowing for specific manipulations of pressure and flow while continuously measuring arterial diameter via video microscopy (308). While these approaches do have the inherent limitation of being

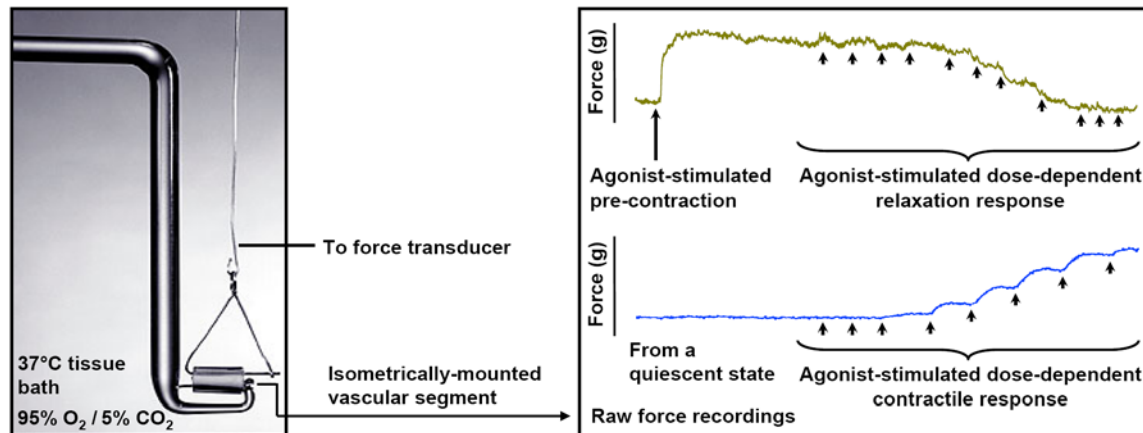


Figure 1.2. Isometrically-mounted vascular myography approach to evaluating endothelial vasomotor (dys)function. Refer to Section 1.4 (and the experimental chapters) for details. Image of vascular segment supports from http://www.radnoti.com/products/tissue_bath_accessories#s1908 (accessed on 09/12/2010).

isolated from specific in vivo (patho)physiological conditions that may significantly affect vascular function (e.g. sympathetic activation, the precise biochemical milieu of circulating and local factors, and, in the former more so than in the latter, the prevailing stresses of pressure and flow), they for this same reason have the distinct advantage of providing a precisely controlled and easily manipulatable environment in which to clearly identify and thoroughly examine vascular cell-signaling mechanisms (e.g. via pharmacological or genetic manipulations and/or environmental clamping) and to specifically evaluate the intrinsic vascular effects of (patho)physiological conditions (e.g. CVD risk factors or overt CVD conditions) and/or potential therapeutic interventions (e.g. drugs or lifestyle factors).

In each of the three studies of this thesis, an isometrically-mounted vascular myography approach was used as the primary means by which to investigate the endothelial-VSM cell-signaling mechanisms controlling vasomotor function and dysfunction in the CCA of rats. Details of how this methodological approach was employed are described in the Materials and Methods section of each experimental chapter.

1.5 Endothelial Function and Dysfunction: Balancing EDRF and EDCF Cell-Signaling Activities

Following the work of Furchgott and Zawadski (120), it was discovered that the stimulated endothelium can elicit vasorelaxation via the synthesis and release of various EDRFs, including nitric oxide synthase (NOS)-derived nitric oxide (NO), cyclooxygenase (COX)-derived prostaglandin (PG)_{I₂}, as well as non-NOS/non-COX-derived substances classified as

endothelium-derived hyperpolarizing factors (EDHFs) (101, 384). Also important was the discovery by Vanhoutte and colleagues that the stimulated endothelium can under certain circumstances elicit concomitant vasoconstrictive activity via the synthesis and release of contracting factors, termed EDCFs, which is abrogated by the inhibition of COX (385).

Therefore, impaired agonist-/flow-stimulated endothelium-dependent vasorelaxation/dilation can be caused by *under-active EDRF signaling*, *over-active EDCF signaling*, or both (Figure 1.3).

In relation, much more is known regarding the cell-signaling mechanisms of EDRF activity (Figure 1.4) and its role in the pathogenesis of endothelial dysfunction and CVD. A current review of this knowledge is provided in Sections 1.5.1 and 1.5.2 below (Review #1). Following that, Section 1.5.3 provides a review of the current knowledge regarding the cell-signaling mechanisms of EDCF activity (Figure 1.5) and its role in the pathogenesis of endothelial dysfunction and CVD (Review #2).

For abbreviated overviews of this knowledge relating to EDRF and EDCF signaling activities, refer to the summaries located at the end of Section 1.5.2 and Section 1.5.3, respectively.

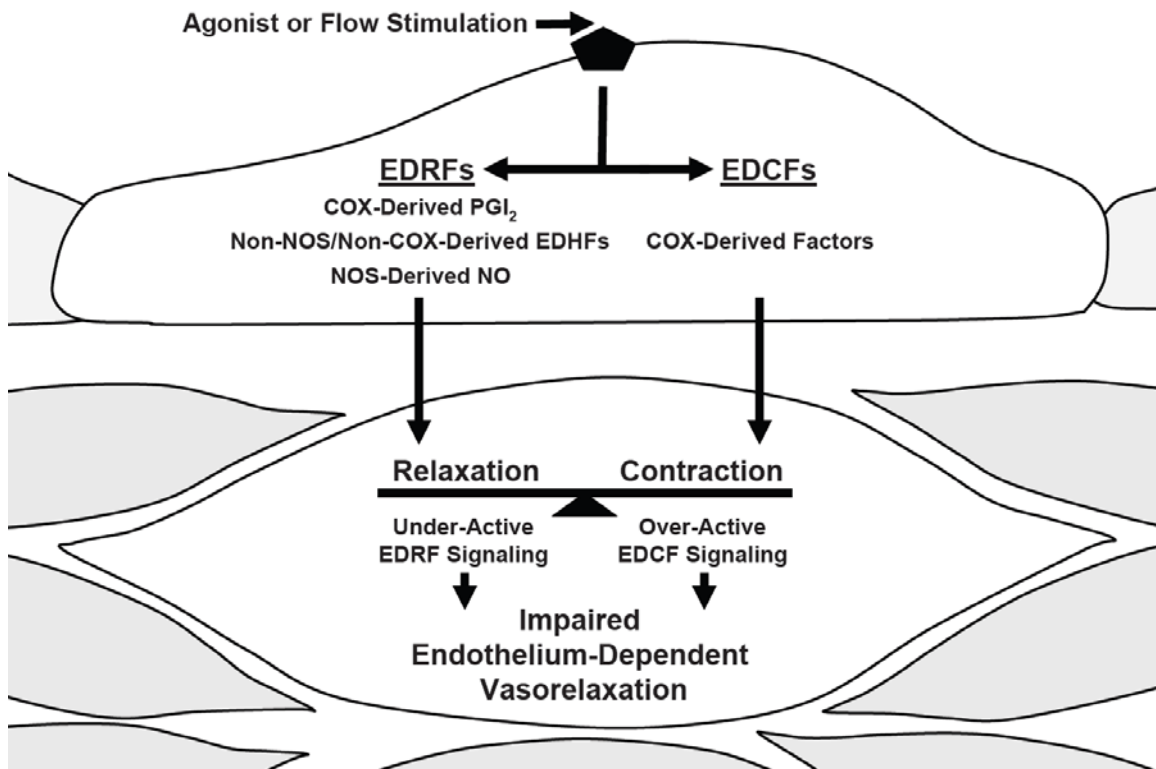


Figure 1.3. Imbalance between EDRFs and EDCFs causing impaired agonist- or flow-stimulated endothelium-dependent vasorelaxation.

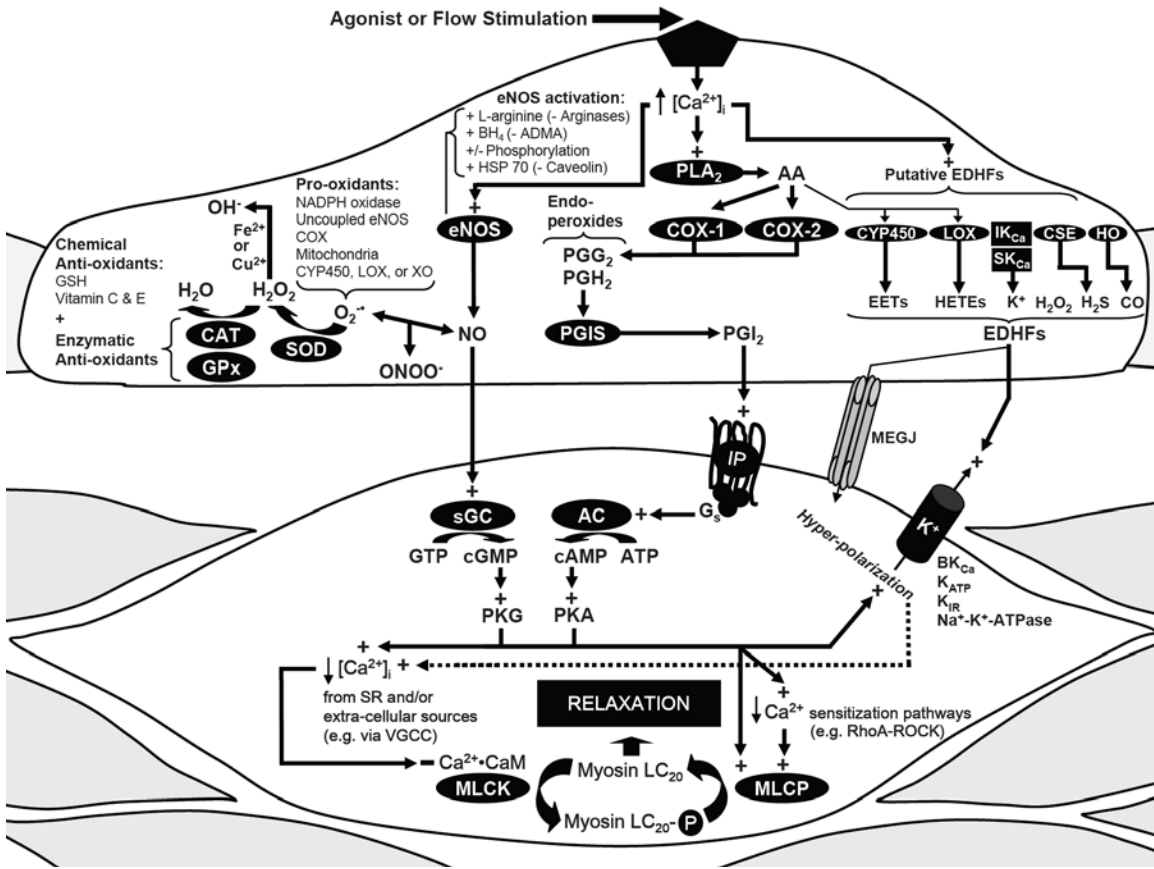


Figure 1.4. Outline of EDRF cell-signaling activity. Refer to the Section 1.5.1-1.5.2 Review for details.

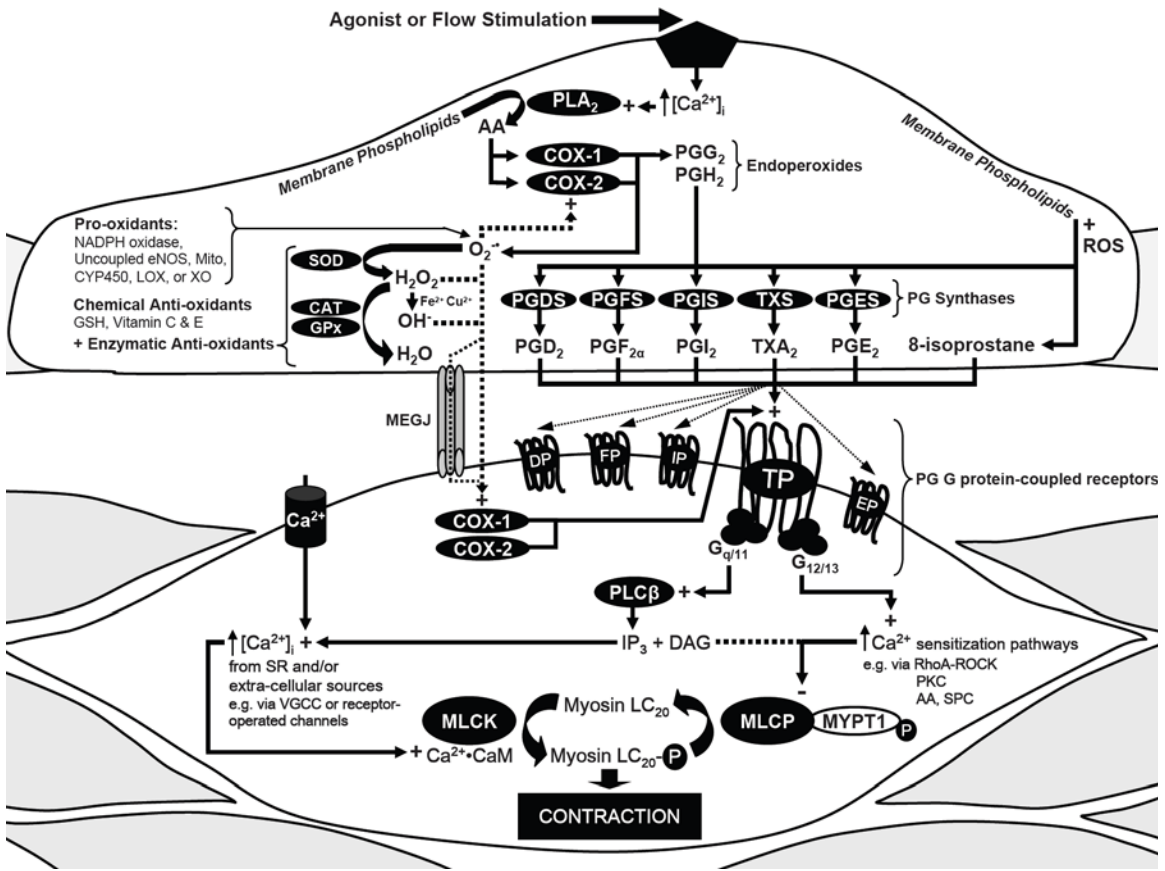


Figure 1.5. Outline of EDCF cell-signaling activity. Refer to the Section 1.5.3 Review for details.

Background Review #1

Under-Active EDRF Signaling Mechanisms of Endothelial (Dys)function

Under CVD Risk Conditions

1.5.1 EDRF Cell-Signaling Pathways

Overview

Endothelium-dependent vasomotor activity is initiated by a rise in endothelial intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to agonist stimulation of endothelial cell-surface receptors or shear stress disruption of the endothelial cell-surface matrix. In turn, this may elicit the activation of Ca^{2+} -dependent enzymes or ion channels within the endothelium, leading to the production/activation of multiple potential EDRFs that either diffuse, or travel through cell-to-cell (i.e. *myo-endothelial gap junctions (MEGJ)*, to the underlying VSM and elicit relaxation via multiple potential mechanisms (Figure 1.4).

In VSM, contractile tone is dictated by the phosphorylation state of the *20-kD regulatory light chain subunits of myosin (RLC₂₀)*, which in turn is controlled by the opposing activities of *myosin light chain kinase (MLCK)* and *myosin light chain phosphatase (MLCP)* (341, 342, 348). An increase in $[Ca^{2+}]_i$ leads to the formation of Ca^{2+} -*calmodulin (Ca²⁺-CaM)* complexes that bind to and stimulate MLCK, which phosphorylates RLC₂₀, thus increasing acto-myosin ATPase activity resulting in cross-bridge cycling and contraction. In addition to this classic Ca^{2+} -dependent mechanism, contractile tone can be activated and sustained through a Ca^{2+} -*independent mechanism*, typically involving stimulation of protein kinase signaling pathways, which act to phosphorylate and inhibit MLCP, and/or phosphorylate and activate MLCK, thus increasing the Ca^{2+} -CaM-MLCK:MLCP activity ratio, resulting in Ca^{2+} *sensitization* – i.e. an increase in contraction without an concomitant increase in $[Ca^{2+}]_i$; (348). Thus, EDRFs can elicit vasorelaxation via decreasing $[Ca^{2+}]_i$ and/or de-activating Ca^{2+} sensitization. As with contractile stimulation, these relaxation effects may occur via *pharmaco-mechanical* (i.e. membrane potential-independent, receptor-mediated) *coupling* and/or *electro-mechanical* (i.e. membrane polarization-mediated) *coupling* mechanisms (341).

NOS-Derived NO

Throughout the vasculature, NO has been found to act as both a potent EDRF and a pivotal mediator in the protection against a CVD phenotype and function (see *Under-Active EDRF Signaling and Endothelial Dysfunction*, below, for details). NO is a free radical synthesized from the amino acid *L-arginine* by the reductase and oxygenase activities of the NOS enzyme, which requires the presence of molecular oxygen and the co-factors nicotinamide adenine dinucleotide

phosphate (NADPH), flavin adenine dinucleotide, flavin mononucleotide, heme, and tetrahydrobiopterin (BH₄). NOS has three isoforms: *neuronal NOS (nNOS, or NOS1)*, *inducible NOS (iNOS, or NOS2)*, and *endothelial NOS (eNOS, or NOS3)*. While both eNOS and nNOS are constitutively expressed, iNOS expression is typically very low or seemingly absent unless induced by cellular perturbations such as pro-inflammatory stimuli (e.g. cytokines). In endothelial cells, NO production is largely mediated by the abundantly expressed eNOS isoform, which is activated by stimulus-induced Ca²⁺-CaM binding as well as protein phosphorylation (20).

The main physiological target of NO is *soluble* (i.e. cytosolic) *guanylate cyclase (sGC)* that converts GTP to *cGMP*, thus activating *cGMP-dependent protein kinase I (cGKI, also called protein kinase G, PKG-I)*. NO has been found to elicit VSM relaxation by decreasing [Ca²⁺]_i, and thus MLCK activity (68, 70). This may occur through pharmaco-mechanical coupling mediated by sGC-cGMP-PKG-dependent, or sometimes -independent (i.e. direct) mechanisms, involving both the stimulation of Ca²⁺ efflux/uptake, via increasing plasma membrane Ca²⁺-ATPase and Na⁺-Ca²⁺ exchanger activity and sarcoplasmic reticulum (SR) Ca²⁺-ATPase activity, and the inhibition of Ca²⁺ release from the SR, via decreasing IP₃-receptor activity. NO may also decrease [Ca²⁺]_i and tone through electro-mechanical coupling mechanisms involving the stimulation of various *plasma membrane K⁺ channels* – most notably large-conductance Ca²⁺-activated channels (BK_{Ca}) and ATP-sensitive channels (K_{ATP}), either directly (via thiol group modification – i.e. nitrothiosylation) or by PKG-dependent phosphorylation, thus resulting in VSM hyperpolarization, which acts to inhibit plasma membrane (or SR) *voltage-gated Ca²⁺ channel* activity and Ca²⁺ influx (39). Besides affecting these ion regulatory targets, there is evidence that the targeting of Ca²⁺ sensitization pathways is an important means by which NO elicits VSM relaxation (65, 212, 277). This may occur through pharmaco-mechanical coupling mediated by a sGC-cGMP-PKG-dependent mechanism involving the de-activation of protein kinase signaling pathways acting to inhibit MLCP activity, most notably the *RhoA-Rho-associated kinase (ROCK)* signaling pathway (342). The effect of EDRF signaling on RhoA-ROCK and MLCP activity is further introduced below when describing the rationale for Thesis Study II (see *Rationale, Objectives, and Hypotheses of Thesis Studies*, and Chapter 3). Altogether, these multiple and redundant actions decreasing both [Ca²⁺]_i and Ca²⁺ sensitization explain the potent vasorelaxation properties of NO.

COX-Derived PGI₂

Even before the work of Furchgott and Zawadzki, Moncada demonstrated that PGI₂ was produced by the vascular wall where it could act as both a vasorelaxant and a potent endogenous inhibitor of platelet aggregation and thrombosis (263, 264). PG production in mammalian cells occurs via

three sequential biosynthetic steps ((380); see both Figure 1.4 and 1.5). Stimulus-induced activation of phospholipases, in particular cytosolic Ca^{2+} -dependent *phospholipase A₂* (*PLA₂*), leads to the breakdown of cell-membrane phospholipids and the intracellular liberation of arachidonic acid (AA), which can be metabolized by several enzyme systems, including cytochrome P450 monooxygenases (CYP450s), lipoxygenases (LOXs), and COXs, each known to have tissue- and cell-specific distributions. Liberated AA is metabolized to the *endoperoxide* PGG₂ and then PGH₂ by the cyclooxygenation and peroxidation activities of COX. This enzyme has two isoforms: *COX-1*, which in most tissues is constitutively expressed, and *COX-2*, which relative to COX-1 often has a low level of expression unless, like iNOS, it is induced by pro-inflammatory cellular perturbations. The endoperoxide PGH₂, itself bio-active, is a short-lived intermediary, next metabolized to one of various bio-active *PGs*, namely PGI₂, PGE₂, PGD₂, PGF_{2 α} , or thromboxane (TX)A₂, by their respective terminal *PG synthases* (PGSSs; e.g. PGI synthase for PGI₂, TX synthase for TXA₂). In healthy endothelial cells, PGI₂ is the major COX-derived metabolite of AA (105, 111).

Like all PGs, PGI₂ exerts its vasoactive effects via activation of *seven trans-membrane G-protein-coupled receptor(s)* (GPCR). *PG receptors* are classified into the sub-types IP, EP, DP, FP, and TP, based on their sensitivity for PGI₂, PGE₂, PGD₂, PGF_{2 α} , and TXA₂, respectively. Hence, PGI₂ is the preferential activator of IP receptors, while TXA₂ is the preferential activator of TP receptors. The target-cell/tissue effect of any GPCR agonist depends on the type of G-protein(s) and downstream signal transduction effectors coupled to the receptor it activates (341). PGI₂-activated IP receptors are typically coupled to the *G_S* class of the heterotrimeric G-protein family, which stimulates *adenylate cyclase* (*AC*) that converts ATP to *cAMP*, thus activating *cAMP-dependent protein kinase* (also called *protein kinase A*; *PKA*). Acting through this *G_S-AC-cAMP-PKA* signaling cascade, PGI₂ has been found to elicit VSM relaxation by both decreasing $[\text{Ca}^{2+}]_i$ and de-activating Ca^{2+} sensitization (and thus decreasing MLCK activity and increasing MLCP activity) through a number of the same pharmaco-mechanical or electro-mechanical coupling mechanisms as NO (i.e. the targeting of VSM Ca^{2+} and/or K^+ regulatory proteins, and protein kinase signaling pathways, as described above).

Non-NOS/Non-COX-Derived EDHFs

Traditionally, endothelium-dependent vasorelaxation responses that were found not to be mediated by NOS-derived NO or COX-derived PGI₂ were supposed to be mediated by a then unknown factor termed EDHF, since VSM hyper-polarization was a characteristic feature of these non-NOS/non-COX-mediated relaxation responses (108, 110). It is now known that the stimulated endothelium may cause VSM hyper-polarization, subsequent inhibition of voltage-

gated Ca^{2+} channels, and relaxation, through diverse electro-mechanical coupling mechanisms that indeed may or may not involve the release of ‘factors’ per se (101). Briefly, this latter mechanism has been shown to involve the stimulation of endothelial Ca^{2+} -activated K^+ channels (intermediate-conductance (I) K_{Ca} and small-conductance (S) K_{Ca}) leading to *endothelial hyperpolarization*, which can be transferred to the VSM via MEGJ, thus resulting in VSM hyperpolarization directly. Furthermore, endothelial hyperpolarization may cause accumulation of K^+ ions in the endothelial-VSM space, which can elicit VSM hyperpolarization by stimulating VSM plasma membrane inward-rectifying K^+ channels and/or Na^+/K^+ -ATPase. In regard to a true ‘factor’-mediated EDHF response, the EDRFs NO and PGI_2 , as described above, may elicit vasorelaxation in part through VSM hyperpolarization. Additionally, numerous non-NOS/non-COX-derived EDRFs have been discovered that elicit vasorelaxation predominantly through VSM hyperpolarization, and are thus considered EDHFs. These include certain species of *epoxyeicosatrienoic acids (EETs)* and *hydroxyeicosatetraenoic acids (HETEs)* derived from the metabolism of AA by specific isoforms of *CYP450s* and *LOXs*, respectively. Other putative EDHFs may include *hemeoxygenase (HO)-derived carbon monoxide (CO)*, *cystathionine β -synthase/cystathionine γ -lyase (CSE)-derived hydrogen sulfide (H_2S)*, as well as vasoactive peptides such as *C-type natriuretic peptide*. In addition, *hydrogen peroxide (H_2O_2)*, a *reactive oxygen species (ROS)* formed by the spontaneous or enzymatic reduction of superoxide anion (O_2^-) generated as a by-product of many endothelial enzymes (see *Pivotal Role of NO Bioavailability and Oxidative Stress in the Pathogenesis of Endothelial Dysfunction*, below, for details), may also act as an EDHF. While the exact electro-mechanical coupling mechanisms remain to be elucidated, these EDHF factors all share the ability to effectively stimulate VSM plasma membrane K^+ channels, in particular BK_{Ca} and/or K_{ATP} (101, 108, 110)

In the studies of this thesis, the relative contributions of NOS, COX, and non-NOS/non-COX signaling pathways in mediating agonist-stimulated, endothelium-dependent vasorelaxation responses were systematically investigated using pharmacological inhibitors of NOS and COX, alone or in combination.

1.5.2 Under-Active EDRF Signaling and Endothelial Dysfunction

EDRF Signaling Activity Under Physiological Conditions

The relative contribution of NO, PGI_2 , and different EDHFs to EDRF signaling activity can vary considerably depending on the vascular bed/territory (organ/tissue) or segment (macro-vascular vs. micro-vascular) of the particular animal or human model investigated, and sometimes the particular receptor agonist or physical stimuli employed (109), likely reflecting exposure (and

resulting adaptation) to differing local neuro-humoral, metabolic, and hemodynamic environments throughout the vasculature in combination with differing genetic backgrounds. The *L-arginine analogs* N^{ω} -nitro-L-arginine methyl ester (L-NAME) and N^{ω} -monoethyl-L-arginine (L-NMMA) are competitive inhibitors of NOS routinely used in vitro and in vivo, respectively, to assess the NO component of endothelial cell-signaling in vascular function and dysfunction. By and large, in smaller peripheral arteries and arterioles of the coronary, cerebral, renal, mesenteric, and limb skeletal muscle vascular territories, etc., EDRF activity is found to be mediated substantially in part by NO signaling, as evidenced by a ~40-60% reduction in endothelial agonist-/flow-stimulated vasorelaxation/dilation following acute NOS inhibition, while COX-mediated and in particular non-NOS/non-COX-mediated EDHF signaling also contribute substantially (101). In larger more central arteries, including the aorta, epicardial, CCA, brachial, renal, and femoral arterial conduits, the contribution of NO signaling to EDRF activity is generally found to be even more substantial, as evidenced by a ~75-95% reduction in endothelial agonist-/flow-stimulated vasorelaxation/dilation following acute NOS inhibition. Hence, both in the resistance micro-vasculature – where moment-to-moment changes in blood flow and pressure are primarily regulated, and even more so in the conduit macro-vasculature – the focal site of pulsatile hemodynamic buffering, arterial stiffness, and atherosclerotic plaque development, NO is recognized as the most prominent EDRF produced by the endothelium. As such, the role of NO signaling activity in regulating vascular homeostasis, and thus protecting against a CVD phenotype and function, has been studied extensively, including its contribution to under-active EDRF signaling in states of overt CVD and CVD risk.

NO, Vascular (Dys)regulation, and Under-Active EDRF Signaling Under Pathological Conditions

Pathophysiological and Pathobiological Impact of NO Signaling Activity

An acute and/or chronic loss of NO production in vivo, provoked by in vivo pharmacological inhibition (via L-NAME or L-NMMA infusion) or genetic deletion (via gene knockout) of eNOS, can significantly decrease organ (216) and limb blood flow (394, 395), elevate blood pressure (298), increase large artery stiffness (412), and accelerate atherogenesis and plaque rupture (30, 72), thus clearly demonstrating the (patho)physiological impact of NO signaling activity. Using a multitude of in vitro, ex vivo, and in vivo approaches, it has been firmly established that the bio-activity of NO possesses far-reaching abilities to prevent the development of pro-constrictory, pro-inflammatory, pro-oxidant, proliferative/fibrotic, and pro-thrombotic phenotypes within the constituents of the vascular wall and blood that lead to dysregulation and pathobiological events (30, 391, 410). Beyond acting as a potent EDRF, NO can inhibit the synthesis or action of

vasocontractile factors; the release of pro-inflammatory cytokines/chemokines; the expression of adhesion molecules; the recruitment and activation of macrophages and T lymphocytes; the oxidation of LDL; VSM de-differentiation, proliferation, and migration; endothelial and VSM apoptosis; extracellular matrix degradation; vascular wall collagen deposition; as well as the activation of platelets and the fibrinolytic system. As a result, maintaining a 'healthy' level of NO signaling activity appears to be of governing importance in protecting against the pathogenesis of a CVD phenotype and function. As described next, this is strongly supported by the fact that endothelial vasomotor dysfunction found under conditions of CVD risk, as well as overt CVD, is often caused by reduced EDRF activity accounted for by alterations in NO signaling.

Cell-Signaling Pathways Responsible for Reduced EDRF Activity Under Pathological Conditions

Indeed, acute NOS inhibition via L-NAME incubation or L-NMMA infusion can often essentially eliminate or significantly diminish the impaired agonist/flow-stimulated vasorelaxation/dilation responses that inevitably develop in both the conduit artery and resistance artery/arteriolar vasculature of animals and humans exposed to each traditional and emerging CVD risk factor – including advanced age, hypertension, hypercholesterolemia and atherosclerosis, type-I/type-II diabetes and obesity, and hyperhomocysteinemia (64, 93, 109, 308, 395), signifying that reduced NO-mediated signaling activity is largely responsible for the endothelial dysfunction found under pathological conditions. In contrast, while attenuation of EDHF-mediated signaling responses have undoubtedly been found to independently contribute to impaired endothelium-dependent vasorelaxation/dilation under certain pathological conditions – including states of hypertension, diabetes/obesity, and advanced age (93, 103, 226), the EDHF component of EDRF signaling activity is instead often found to be augmented, at least during early exposure to pathological conditions, in recompense for a loss of NO-mediated signaling. This is consistent with findings in eNOS knockout or eNOS/COX-1 double-knockout mice wherein endothelium-dependent vasorelaxation, while still impaired, is partially maintained by a compensatory increase in EDHF signaling activity (110). In this way, EDHF-mediated responses, together with PGI₂-mediated signaling activity, are thought to provide a 'vasodilatory reserve' in the face of compromised NO signaling, thereby helping to avoid endothelial dysfunction. In gracilis muscle arterioles of eNOS knockout mice (163), the renal artery of SHR (50), and the forearm vasculature of essential hypertensive patients (350), this compensatory response appears to involve CYP450-derived EETs, which, besides acting as EDHFs, have also been discovered to exert strong anti-inflammatory and anti-mitogenic effects within the vasculature (170), and so, like NO (or PGI₂), may signal to protect against the development of a CVD phenotype.

Notwithstanding the potential importance of either augmented or attenuated EDHF-mediated responses that may occur in states of CVD risk, which mechanistically could involve alterations in the bioavailability of multiple signaling factors (103, 108, 110) including EETs, HETEs, K^+ , MEGJ, or H_2O_2 , among other putative EDHFs (see Section 1.5.1: *Non-NOS/non-COX-derived EDHFs*), the vast majority of studies investigating the pathogenesis of endothelial dysfunction have focused on mechanisms responsible for a reduction in *NO bioavailability* – that is, its level of production, destruction, and VSM sensitivity (308). Through this work, it has become evident that an excess level of vascular oxidative stress, a common condition amid the discerning clinical/experimental features of each CVD risk factor (e.g. months/years of existence in aging, high transmural pressure and neuro-humoral status in states of hypertension, high LDL cholesterol in hyperlipidemia, high glucose and insulin-insensitivity in diabetes), can have multiple adverse effects on NO bioavailability and EDRF signaling activity.

Pivotal Role of NO Bioavailability and Oxidative Stress in the Pathogenesis of Endothelial Dysfunction

Defining Oxidative Stress and ROS

Sies (346) defines *oxidative stress* in biological systems as an imbalance between pro-oxidant and anti-oxidant activities in favor of the former, potentially leading to ROS-induced damage (or dysfunction). This distinction between ‘oxidative stress’ and ‘oxidative damage’ relates to the fact that a chronic or acute accumulation of ROS, and thus increased oxidative stress, per se does not necessarily lead to damage and dysfunction, since biological systems may have the capacity to adapt to, or compensate for, a pro-oxidant environment or challenge. Indeed, ROS, including $O_2^{\cdot-}$, H_2O_2 , and *hydroxyl radical* (OH^{\cdot}) (196, 283, 346), are normally produced and accumulate at low ‘physiological’ levels within the vasculature where through ‘redox’-mediated cell-signaling they make important contributions to the regulation of homeostasis (48, 346). However, while the vasculature does have some potential adaptive/compensatory capacity in regard to accommodating ‘non-physiological’ increases in oxidative stress (see *Consideration of Potential ROS-Mediated Compensatory Adaptations in Attempt to Prevent Endothelial Dysfunction* below in this section), conditions promoting the excess production and/or impaired or outstripped buffering of ROS certainly do lead to a level of vascular oxidative stress that have pathophysiological consequences.

NO Bioavailability and Vascular Oxidative Stress: Original Findings

An appreciation for the impact of oxidative stress on NO bioavailability and endothelial (dys)function first emerged from the discovery that NO produced by endothelial cells can be

destroyed by $O_2^{\cdot-}$ before it is able to diffuse to its target-tissue and stimulate its protective effects – i.e. VSM relaxation signaling in the case of EDRF activity (148, 307) (see Figure 1.4). For example, ACh-stimulated, NO-mediated relaxation of a healthy arterial segment in vitro can be markedly impaired by simultaneous exposure to exogenous $O_2^{\cdot-}$ (via a $O_2^{\cdot-}$ generating system; (148)) or stressors that stimulate endogenous $O_2^{\cdot-}$ generation (e.g. pro-inflammatory cytokines, hormones; (431)), while the presence of exogenous superoxide dismutase (SOD) (307) or previous vascular wall over-expression of endogenous SOD isoforms ((143); e.g. via gene/protein transfer or exercise training) may correct this vasomotor impairment.

The interaction between NO and $O_2^{\cdot-}$ occurs at an extremely fast, near diffusion-limited rate ($6.7 \times 10^9 \cdot M \cdot s^{-1}$; 3 times faster than the dismutation of $O_2^{\cdot-}$ by SOD; (54)), leading to the formation of *peroxynitrite* (*ONOO*), which, in contrast to NO, has many detrimental effects on vascular cell-signaling and function (see below, *Role of Decreased NO Production and Target-Tissue Sensitivity in Endothelial Dysfunction with Links to Oxidative Stress*). Thus, it has been well-established that to allow for the efficient endothelial-to-VSM transfer of an adequate amount of NO when stimulated under a given set of circumstances or conditions, it is essential to maintain an optimal concentration of ROS within the vascular wall, which is in turn accomplished by maintaining optimal balance between vascular pro-oxidants and anti-oxidants, the determinants of which are next briefly defined (see Figure 1.4).

Determinants of Pro-oxidant/Anti-oxidant Balance in the Vascular Wall

Pro-oxidants. In endothelial cells and VSM, pro-oxidant sources of $O_2^{\cdot-}$ may include: *NADPH oxidase*; ‘*uncoupled*’ *eNOS* (explained below in *NO Production and eNOS Activation*); AA metabolizing enzymes – *COX*, *CYP450*, and *LOX*; *xanthine oxidase (XO)*; and the *mitochondrial (Mito) electron transport chain* (54, 89). Among these sources, the multi-subunit enzyme *NADPH oxidase* is typically found to be the chief contributor to vascular $O_2^{\cdot-}$ and H_2O_2 production under both physiological (45, 207) and pathological (147) conditions. In fact, both the endothelium and VSM constitutively express isoforms of *NADPH oxidase* that are localized to the plasma membrane and to various internal membrane compartments (45) where the enzyme has been shown to act as an ‘upstream’ initiator/integrator of ROS production from other pro-oxidant sources, including *eNOS* (204), *xanthine oxidase* (250), and the mitochondria (90), which can contribute significantly to excess vascular oxidative stress under numerous pathological conditions including hypertension and aging (54, 75, 91).

Anti-oxidants. Vascular anti-oxidant capacity to defend against excess ROS accumulation has two main components: *enzymatic* and *chemical*. Chemical anti-oxidants include lipid-soluble

compounds such as *α-tocopherol (vitamin E)*, as well as water-soluble compounds such as *ascorbic acid (vitamin C)* and *low-molecular weight thiols*, most notably *glutathione (GSH)*, the manipulation of which was a major focus of Study III in this Thesis (see *Rationale, Objectives, and Hypotheses of Thesis Studies* in Section 1.7, and Experimental Chapter 4). Beyond directly scavenging $O_2^{\cdot-}$ (among other ROS) and reducing (redox recycling) compounds that have become oxidized, these water-soluble anti-oxidants also act as co-factors for anti-oxidant enzymes (346). Enzymatic anti-oxidants in the vascular wall include three constitutively expressed isoforms of SOD (308), which metabolize $O_2^{\cdot-}$ to H_2O_2 . *Cu,Zn-SOD (SOD-1)* is found predominantly in the cytosol of both endothelial cells and VSM, and *Mn-SOD (SOD-2)* in the mitochondria, while an extracellular version of *Cu,Zn-SOD (SOD-3, or ecSOD)* is found bound to the outer matrix in the vascular extracellular space. The H_2O_2 produced by these SOD isoforms can in turn be metabolized to H_2O by the activity of either *catalase (CAT)* or *GSH peroxidase (GPx)*, both of which are constitutively expressed in the cytosol of endothelial cells and VSM. In addition, H_2O_2 and $O_2^{\cdot-}$ can be metabolized in the presence of *free transition metals (Fe^{2+} or Cu^{2+})*; however this conversion leads to the production of OH^{\cdot} , thus supporting pro-oxidant and not anti-oxidant capacity.

Powerful Role of Oxidative Stress-Mediated NO Destruction in Endothelial Dysfunction.

Under physiological conditions, only a moderate amount of $O_2^{\cdot-}$ is produced in the vascular wall, and its accumulation, and spontaneous interaction with NO, is limited to a 'healthy' level by adequate anti-oxidant defenses, thus preserving endothelial function. In contrast, a large body of evidence suggests that a major mechanism of impaired endothelium-dependent vasorelaxation/dilation responses found in states of CVD and CVD risk is an excess accumulation of ROS leading to NO destruction, reduced NO bioavailability, and under-active EDRF signaling (Figure 1.4). In humans, support for this hypothesis has come from studies demonstrating that endothelial dysfunction of the coronary or arm/forearm conduit or resistance vasculature can be significantly improved, not just by chronic anti-oxidant supplementation, but also by acute exposure to chemical anti-oxidants, such as vitamin C or GSH, in patients with CVD risk factors, including atherosclerosis/hypercholesterolemia (200, 296, 371), diabetes (369, 370), hyperhomocysteinemia (400), as well as essential hypertension (98, 351, 352, 400), and in otherwise healthy individuals aged >60 years (352). Congruent with these findings, ROS measured in the vascular wall in a basal state or upon stimulation with endothelial agonists can be increased several-fold in animal models of these pathological conditions (54, 89, 288), and is often associated with impaired NO-mediated relaxation in isolated arterial segments, which may be corrected, at least in part, not just by chronic in vivo anti-oxidant treatment, but also by acute

in-bath incubation with chemical antioxidants, or with ecSOD, or SOD or SOD+CAT mimetics (44, 54, 308, 309, 346, 376, 411).

Pro-oxidant/Anti-oxidant Balance in States of CVD risk. While a reduction in vascular anti-oxidant capacity – via decreased chemical anti-oxidant concentrations and/or decreased expression or activity of SOD, CAT, and/or GPx enzymes – can certainly contribute to excess ROS accumulation in states of CVD risk ((44, 270, 346); also see *Introduction* to Chapter 4), the oxidative stress-mediated endothelial dysfunction found under these conditions appears to be primarily linked to the marked over-production of $O_2^{\cdot-}$, most notably derived from NADPH oxidase (as introduced above in *Determinants of Pro-oxidant/Anti-oxidant Balance in the Vascular Wall*). In fact, the activity and expression of endothelial and VSM isoforms of NADPH oxidase are regulated by *peptide hormones* (e.g. angiotensin II (AII), endothelin-1), *pro-inflammatory cytokines/chemokines* (e.g. tumor necrosis factor- α , interleukin-1 β , interferon- γ), and *hemodynamic stresses* (i.e. low/oscillatory flow/shear stress; high pressure/circumferential stress) that are known to be involved in the pathogenesis of vascular aging (75), hypertension (206), amid many other CVD risk conditions (54, 147, 196). For example, introduction of AII or pro-inflammatory cytokines into the medium of endothelial or VSM cell cultures results in a sustained increase in NADPH oxidase activity and $O_2^{\cdot-}$ production (80, 146), as does exposure of endothelial cells to low/oscillatory shear stress, which mimicks the ‘pro-atherogenic’ low/oscillatory flow/shear profile that exists at sites of plaque formation (i.e. sharp arterial bifurcations/curvatures), as compared to endothelial cells exposed to laminar unidirectional shear stress, which mimicks a ‘anti-atherogenic’ physiological flow/shear profile (81, 165). Prolonged exposure of vascular cells to these oxidative stress-promoting hormone, cytokine, or shear stress stimuli in turn activates *redox- and shear-sensitive transcription factors*, most notably mitogen-activated protein kinase (MAPK) cascades and nuclear factor (NF)- κ B (75, 363), which can induce the expression of a wide variety of enzymatic and chemical factors mediating CVD phenotypes (71). Likewise, isolated arterial segments exposed to high circumferential stress, which mimicks the intra-vascular pressure exerted on the vascular wall with hypertension, has been shown to elicit increased $O_2^{\cdot-}$ production (214, 389), the expression of a NF- κ B-driven pro-atherogenic (303) or proliferative/fibrotic (214) phenotype, and marked impairment of endothelium-dependent vasorelaxation (389), all of which could be prevented in large part by inhibiting NADPH oxidase subunit assembly and subsequent activation. Moreover, inhibition of NADPH oxidase activity can substantially improve impaired NO-mediated relaxation found in the vasculature of various experimental models of hypertension, including SHR, deoxycorticosterone acetate salt-treated or Dahl salt-sensitive rats, and AII-infused or L-NAME-

infused rats or mice (109, 290, 376, 422), where endothelial and VSM NADPH oxidase subunit expression and $O_2^{\cdot -}$ production may be considerably increased (322).

Role of Decreased NO Production and Target-Tissue Sensitivity in Endothelial Dysfunction with Links to Oxidative Stress

In addition to the powerful acute phenomenon of NO destruction, other mechanisms resulting in a decrease in NO production or decrease in target-tissue sensitivity to NO can reduce NO bioavailability (Figure 1.4) causing under-active EDRF signaling and endothelial dysfunction in states of CVD risk, which in many cases may be linked to chronic exposure to excess vascular oxidative stress.

VSM Sensitivity to NO. In both humans and animals, the dose-dependent dilatory/relaxation response to NO donors (e.g. the intra-arterial infusion or in-bath injection of sodium nitroprusside (SNP)) is typically well preserved even under advanced pathological conditions, suggesting that decreased VSM sensitivity to NO does not play a major role in under-active EDRF signaling and endothelial dysfunction, but it certainly may. For example, it is interesting to note that endothelium-dependent relaxation in the aorta of hypercholesterolemic rabbits has been found to be impaired because of a significant decrease in the sensitivity and maximum amplitude of NO-stimulated relaxation caused by ONOO⁻-mediated tyrosine oxidation-nitration (i.e. nitro-tyrosine residue formation) and subsequent inactivation of VSM SR Ca^{2+} -ATPase, all of which could be reversed by chronic antioxidant treatment (3). In addition, a decrease in VSM sensitivity to NO has been found to contribute to impaired endothelium-dependent relaxation under conditions where sGC expression is significantly down-regulated or when sGC responsiveness to NO is reduced due to oxidation of its heme moiety (via $O_2^{\cdot -}$ or ONOO⁻) (273, 297, 416), thus decreasing cGMP production and subsequent PKG activation in response to the endothelium-derived NO that is able to reach the VSM.

NO Production and eNOS Expression. A decrease in the production of NO can occur via multiple mechanisms affecting either the expression or activation of eNOS, the regulations of which have been studied extensively (20). Expression of eNOS is both redox- and shear-sensitive. Physiological flow/shear stress induces endothelial mRNA and protein expression of eNOS while also stimulating the production of NO (20), which potently inhibits NF- κ B and MAPK activation within the cells of the vascular wall, thus preventing the expression of CVD phenotypes (125, 150, 218). Conversely, prolonged exposure of isolated arterial segments to low/oscillatory flow/shear stress results in reduced eNOS mRNA transcription and protein translation in conjunction with a marked impairment in endothelium-dependent NO-mediated vasorelaxation

(121). While, if found in states of CVD risk, a reduction in eNOS protein content – e.g. via cytokine- or hormone-stimulated post-transcriptional eNOS mRNA de-stabilization (20, 260), may certainly contribute to a decrease in endothelial NO production, it is more often impairment in eNOS activation that can contribute to reduced NO bioavailability and endothelial dysfunction under pathological conditions.

NO Production and eNOS Activation. Proper activation of eNOS in response to physical or chemical stimuli depends not only on Ca^{2+} -dependent CaM binding but also several important post-translational modifications (20, 326). These include eNOS myristoylation/palmitoylation-dependent targeting and localization to endothelial membrane caveolea, protein-protein interactions with inhibitory caveolin or stimulatory heat shock protein 90, and phosphorylation/de-phosphorylation at stimulatory (e.g. serine 1177) or inhibitory (e.g. threonine 495) sites via activation of numerous *kinases*, such as Akt (protein kinase B), PKA, PKC, ERK, ROCK, or AMP-activated protein kinase, and numerous *phosphatases*, such as protein phosphatase-1, -2A, or 2B/calcineurin. Indeed, exposure of cultured endothelial cells to oxidized LDL, but not native LDL, results in a dose-dependent decrease in ACh-stimulated NO production related specifically to membrane caveolea displacement (and thus mis-localization) of eNOS (36), which has also been shown to occur in states of hypertension (133). Moreover, as found under many CVD risk conditions, blunted agonist- or flow-stimulated eNOS serine 1177 phosphorylation, most notably caused by inactivation of Akt via cytokine- or hormone-stimulated cell-signaling (20, 260), directly relates to a decrease in endothelial NO production that can impair endothelium-dependent vasorelaxation (166).

In addition, NO production can be decreased by the endogenous eNOS inhibitor *asymmetric dimethyl-L-arginine (ADMA)*, the plasma concentration of which significantly correlates with most CVD risk factors in humans. Synthesis of this L-arginine analogue depends on rates of protein turnover and the enzymatic degradation of arginine methylated proteins by protein arginine methyltransferase I, while its metabolism depends mostly on the enzymatic activity of dimethylarginine dimethylaminohydrolase, which can be inhibited by ONOO⁻-mediated oxidation-nitration (349).

An important additional mechanism responsible for decreased NO production upon eNOS activation is the bioavailability of its substrate *L-arginine* or its co-factor *BH₄*. If the immediate supply of either L-arginine or BH₄ is inadequate, eNOS becomes ‘uncoupled’, in which case electron transport during enzymatic cycling no longer produces NO but instead O₂⁻ or H₂O₂ (115). In regard to NO bioavailability, the consequence of *eNOS uncoupling* is two-fold since not only is NO production decreased, but NO destruction is increased due to the

spontaneous ONOO⁻-forming reaction of O₂^{-•} with NO. BH₄ is highly susceptible to oxidation-nitration by ONOO⁻ (258), while excess accumulation of H₂O₂, either produced directly or by SOD-mediated dismutation of O₂^{-•}, can down-regulate in endothelial cells the enzyme dihydrofolate reductase responsible for recycling (reducing) oxidized BH₄ (58), which in turn may further enable eNOS uncoupling leading to a ‘vicious cycle’ promoting reduced NO bioavailability and endothelial dysfunction (52, 203, 204). L-arginine supply in endothelial cells may be decreased by ADMA competing for its specific membrane transporter (67) or by an increase in its metabolism through the enzymatic activity of vascular arginases (33, 259, 311, 314), which can be significantly up-regulated with exposure to excess H₂O₂ or OH[•] via conversion (102, 367). In any case, under conditions where eNOS may become a prominent source of vascular ROS, including aging, hypertension, diabetes, and hyperhomocysteinemia, supplementation with BH₄ or L-arginine themselves, or treatments increasing their endothelial bioavailability (e.g. folate), have been shown to improve endothelium-dependent vasodilation/relaxation in both animals and humans (115).

Therefore, a decrease in NO production related in large part to oxidative stress-mediated impairment in eNOS activation can certainly contribute along side NO destruction to the reduced NO bioavailability resulting in under-active EDRF signaling and endothelial dysfunction in states of CVD risk.

Consideration of Potential ROS-Mediated Compensatory Adaptations in Attempt to Prevent Endothelial Dysfunction

As described earlier (in *Cell-Signaling Pathways Responsible for Reduced EDRF Activity Under Pathological Conditions* of this sub-section), EDHF-mediated dilation/relaxation responses, which are thought to be relatively resistant (i.e. less susceptible) to oxidative stress, may become augmented in states of CVD risk to partially compensate for a reduction in NO-mediated signaling, so providing a ‘reserve’ of EDRF signaling activity helping prevent endothelial dysfunction. It is also apparent that acute and chronic compensatory adaptations in the eNOS-NO-sGC signaling axis itself may occur in response to CVD risk conditions, in an attempt to maintain NO bioavailability and endothelial function in the face of increased oxidative stress. Though seemingly paradoxical, these adaptations appear to be mediated through ROS themselves (308), in particular H₂O₂ (42). For instance, under AII-induced pro-oxidant culture conditions, H₂O₂ produced in an NADPH oxidase-dependent manner by aortic endothelium, mediates a substantial increase in the production of NO in what is thought to be a compensatory mechanism for the concomitant production of O₂^{-•} acting to destroy NO (55). In this way, despite increased

oxidative stress, NO bioavailability might be preserved, at least temporarily, by activating eNOS to produce more NO, thus allowing for an adequate amount of NO to reach the VSM undestroyed. In fact, especially during the earlier stages of exposure to CVD risk conditions, agonist-/flow-stimulated NO production can be increased resulting in preserved, or even augmented, endothelium-dependent vasodilation/relaxation, which may be related to both the activation and expression of eNOS by ROS (115).

Indeed, acutely, H₂O₂ exposed to endothelial cells stimulates NO production from eNOS via AKT-dependent stimulatory site phosphorylation as well as inhibitory site de-phosphorylation (97, 368), while exposure of intact vascular segments to H₂O₂ may elicit endothelium-dependent, NOS-mediated vasorelaxation (428, 430). As well, depending on the particular vascular bed or segment, H₂O₂ may elicit endothelium-independent vasorelaxation via direct activation of either VSM sGC (56, 96, 132, 215) or VSM K⁺ channels (238, 334), whereby, in regard to this latter phenomenon, H₂O₂ might act as an EDHF (101, 110). In both these cases of H₂O₂ acutely eliciting endothelium-independent vasorelaxation, uncoupled eNOS or eNOS-SOD coupling have been recognized as potential H₂O₂ sources (96, 336). Chronically, H₂O₂ is a potent stimulus of the expression of eNOS both in vitro and in vivo (42, 53, 97). Thus, under most conditions of CVD risk, eNOS expression is increased (51, 115), which can contribute to enhance NO production, so long as the enzyme does not become pathologically uncoupled via oxidative stress-mediated mechanism(s) as described above (see *NO Production and eNOS Activation*). Concomitant with eNOS, the expression of ecSOD, found to occur in a NO-dependent manner (78, 118), can also be increased under CVD risk conditions, helping to counteract the destruction of NO by O₂^{•-} while further contributing to the production of H₂O₂. Also, sGC expression can be increased in response to CVD risk conditions (25), which may enhance the vasomotor activity of the amount of NO (or H₂O₂) able to reach the VSM, thereby contributing to the preservation of NO bioavailability despite increased oxidative stress.

Therefore, congruent with Sies's definition of oxidative stress in biological systems (see *Defining Oxidative Stress and ROS* at the beginning of this sub-section), the vasculature indeed does have some adaptive/compensatory capacity to accommodate 'potentially non-physiological' increases in oxidative stress, thus preventing oxidative damage and dysfunction. However, upon persistent exposure to an excess level of oxidative stress under CVD risk conditions, the detrimental effects of ROS described throughout this sub-section can certainly overwhelm any compensatory mechanisms, resulting in reduced NO bioavailability and under-active EDRF signaling causing endothelial dysfunction.

In Summary, much work has been done to identify the cell-signaling pathways mediating EDRF activity (Figure 1.4) and to elucidate the pathobiological mechanisms responsible for endothelial dysfunction caused by under-active EDRF signaling in states of CVD risk. While the relative contribution of NO-, PGI₂-, and EDHF-mediated signaling to EDRF activity can vary across vascular bed/territory, macro-/micro-vascular segment, and/or animal/human model, the most prominent and well-characterized EDRF is NO. Commensurate with its established role in protecting against a CVD phenotype and function, a reduction in the bioavailability of NO is often found to be responsible for under-active EDRF signaling and endothelial dysfunction in states of CVD risk, including both hypertension and aging. An imbalance between vascular pro-oxidant and anti-oxidant activities leading to excess ROS accumulation and oxidative stress has been identified as a major mechanism responsible for this reduction in NO bioavailability and EDRF activity, which is predominantly mediated by factors impairing the ability to activate eNOS to produce NO, and by the destruction of NO prior to it stimulating its protective effects in target-tissues.

Background Review #2

Over-Active EDCF Signaling Mechanisms of Endothelial (Dys)function Under CVD Risk Conditions

1.5.3 Over-Active EDCF Signaling and Endothelial Dysfunction

Concomitant with producing EDRFs, the stimulated endothelium may produce EDCF(s), the signaling activity of which can oppose that of the former. Consequently, over-active EDCF signaling relative to EDRF signaling activity can independently contribute to impaired agonist-/flow-stimulated endothelium-dependent vasorelaxation/dilation (Figure 1.3).

While much attention has been given to elucidating the endothelial-VSM cell-signaling mechanisms of EDRF activity and its particular role(s) in causing endothelial dysfunction (as detailed in the Section 1.5.2 Review, *Under-Active EDRF Signaling and Endothelial Dysfunction*; and Figure 1.4), less attention in these regards has been given to EDCF activity (Figure 1.5). However, since EDCF(s) have and continue to be found to play an important role in causing endothelial dysfunction in various states of CVD and CVD risk, the endothelial-VSM cell-signaling mechanisms of EDCF activity, and how that signaling may become over-active under CVD risk conditions, are now being actively explored.

Endothelium-Dependent Contractile Responses: Initial Findings and Characterization of EDCF Cell-Signaling Pathway

In 1982, shortly after Furchgott's discovery of the diffusible ACh-stimulated 'EDRF' ((120); see Section 1.3, *Endothelium-Dependent Vasorelaxation: A Display of Endothelial (Dys)function*), De Mey and Vanhoutte demonstrated in isolated, pre-contracted canine veins that exposure to AA, or the receptor-agonist thrombin, could elicit a dose-dependent increase in tension that was dependent on the presence of the endothelium (83). This represents the first reported observation of an endothelium-dependent contractile response. Following from this were four key findings that have helped to establish a basic characterization of the EDCF cell-signaling pathway. *First*, it was found that endothelium-dependent contractile responses could be elicited not just in veins but also in arteries, and not only by AA and thrombin but also by ACh and other endothelial receptor-agonists, as well as by the receptor-independent Ca^{2+} -mobilizing agent A23187 (185). *Second*, it was found that endothelium-dependent contractile responses could be completely abolished by the non-selective COX inhibitor indomethacin (Indo) (257). *Third*, it was found (using a similar bioassay approach as was used by Furchgott; (120)) that upon stimulation with ACh, an isolated donor strip of artery with endothelium could, via the release of a diffusible 'EDCF' substance(s), elicit contraction in an isometrically-mounted adjacent arterial strip denuded of endothelium, an

effect that was completely abolished when COX was inhibited in the donor strip, but nearly unaffected when COX was inhibited in the isometrically-mounted bioassay strip (424). *And fourth*, it was found that endothelium-dependent contractile responses could be almost abolished by TP receptor antagonists (such as S18886 or SQ29548), either when acutely exposed to isolated intact arterial segments (16), or to the bioassay arterial strips in the experiments described above in Three (424). Thus, the basics of the EDCF cell-signaling pathway appear to be characterized as follows. In response to stimulation, a rise in endothelial $[Ca^{2+}]_i$ likely activates Ca^{2+} -dependent PLA_2 , liberating AA that is next metabolized by COX leading to the synthesis and release of endoperoxides and/or PG(s), which then travel to the VSM, activating the TP receptor to initiate contractile signaling activity (Figure 1.5).

Indeed, subsequent studies have established the pivotal role of COX in endothelium-dependent contractile responses (105, 383), and have identified the TP sub-type of the PG receptor family as being responsible for mediating almost all EDCF signaling activity in endothelium-dependent contractile responses (111). In addition to the four key findings described above, a *fifth* group of findings showed that acute exposure to inhibitors of ROS accumulation (e.g. SOD and/or catalase mimetics or the iron chelator deferoxamine) could, to varying degrees, partially attenuate endothelium-dependent contractile responses in either isolated intact arterial segments (184, 386, 423) or bioassay arterial strips ((424); see Three above). Noteworthy, these findings suggested the possibility of a ROS component to the EDCF cell-signaling pathway (see Figure 1.5), which has led to the exploration of ROS and oxidative stress as contributing factors to the regulation of this pathway, as is further described below (see *Mechanisms of Over-Active EDCF Signaling: Role of PGs, ROS, and Oxidative Stress*).

Contribution of EDCF Responses to Endothelial Dysfunction under CVD Risk Conditions

While endothelium-dependent contractile responses may certainly occur under physiological conditions, EDCF signaling activity is found to be particularly prominent under conditions of CVD risk, where it can result in endothelial dysfunction. In this way, EDCF responses are becoming known as a hallmark of endothelial dysfunction and CVD.

SHR Aorta: The First Reporting of an EDCF Response Impairing Endothelium-Dependent Vasorelaxation

The first observation of endothelium-dependent contractile activity contributing to the pathology of endothelial dysfunction was in the isolated thoracic aorta of SHR (230, 231), and since then, the characteristics of EDCF cell-signaling have been studied most extensively in this conduit vascular segment (105, 112, 360). As in many vascular segments or vascular beds of animals or

humans with CVD risk, endothelium-dependent relaxation/dilation in the aorta of adult SHR was found to be blunted (see account of pioneering studies in Section 1.3, *Endothelium-Dependent Vasorelaxation: A Display of Endothelial (Dys)function*). Hence, of isolated pre-contracted aortic rings, agonist-stimulated dose-dependent relaxation was impaired in SHR compared to normotensive age-matched WKY (199); however, several unique features of this impairment were soon identified. Not only was the predominantly NO-mediated maximal relaxation response to endothelial agonists, including ACh, blunted in SHR aorta, but the vasomotor responses stimulated at higher agonist doses were in many cases no longer that of relaxation but of *overt 're'-contraction* (199, 230, 231). Notably, it was found that this re-contractile response blunting maximal relaxation could be completely reversed in the presence of Indo, resulting in a similarly robust endothelium-dependent L-NAME-sensitive aortic relaxation response between SHR and WKY (230).

Further exploring the apparent EDCF activity in SHR, these pioneering experiments more purposely assessed the endothelium-dependent contractile response to cumulative ACh in SHR aorta from a *quiescent (basal) vasomotor state* (i.e. isolated arterial rings isometrically-mounted and equilibrated but not pre-contracted prior to assessment of agonist-stimulated vasomotor activity). Using this approach, ACh was found to elicit a dose-dependent aortic contractile response that could be prevented by either PLA₂ or COX inhibition (230) or by TP receptor antagonism (16), and could be significantly amplified by either inhibitors of NOS (L-NAME or L-NMMA; (17, 426), scavengers of NO (oxy-hemoglobin or carboxy-PTIO; (17, 426)), or inhibitors of sGC (ODQ or NS2028; (426)).

Thus, the impairment in agonist-stimulated endothelium-dependent vasorelaxation observed in the aorta of adult SHR was not found to be caused by a decrease in NO-mediated EDRF signaling activity, but rather by an increase in COX-mediated EDCF signaling activity that could over-power the EDRF response, thereby causing endothelial dysfunction.

Balancing EDRF and EDCF Signaling Activities to Prevent or Promote Endothelial Dysfunction

The above-described experiments demonstrating that acute inhibition of the eNOS-NO-sGC signaling axis amplifies ACh-stimulated COX- and TP receptor-mediated contractile activity in SHR aorta (17, 426) were important in establishing the concept that concomitantly produced EDRF(s) and EDCF(s) from the stimulated endothelium exert opposing cell-signaling effects on VSM tone and are hence functionally antagonistic of one another (107, 355). Thus, a rise in endothelial [Ca²⁺]_i upon agonist stimulation can result not only in the activation of enzymes/ion channels leading to the production/activation of EDRFs (i.e. NOS-derived NO, COX-derived

PGI₂, or Non-NOS/Non-COX-derived EDHFs; see above Section 1.5.1, *EDRF Cell-Signaling Pathways: Overview*; and Figure 1.4), but also in the activation of COX leading to the production/activation of EDCFs (Figure 1.5), both of which travel (either by free diffusion or through MEGJ) to the VSM to stimulate their respective targets, in turn activating both relaxation and contractile cell-signaling activities (Figure 1.3). In arteries where NO is the predominant EDRF, VSM sGC-mediated relaxation signaling activities (see *EDRF Cell-Signaling Pathways: NOS-derived NO*) must then act against (oppose) the TP receptor-mediated contractile signaling activities stimulated by EDCF(s), and vice versa (i.e. EDCF-stimulated activation of VSM TP receptor-mediated contractile signaling activities must act against sGC-mediated relaxation signaling activities stimulated by NO). It appears that this same functional antagonism between EDRF and EDCF cell-signaling activities operates in arteries/arterioles where EDHF(s) predominantly mediate EDRF responses, since acute inhibition of EDHF signaling activity (via K⁺ channel blockers or the presence of high KCl; see *EDRF Cell-Signaling Pathways: Non-NOS/Non-COX-derived EDHFs*; and Figure 1.4) has been shown also to significantly amplify agonist-stimulated COX- and TP receptor-mediated contractile responses (254).

Therefore, in healthy vasculature, the activity of EDCF(s), if present, can be counter-balanced completely by the activity of EDRFs, resulting in a robust endothelium-dependent relaxation/dilatory response. Whereas, under conditions of CVD risk, endothelium-dependent relaxation/dilation can be significantly impaired in one of three ways: i) an under-activation of EDRF signaling with no EDCF involvement; ii) an imbalance involving over-activation of EDCF signaling with normal EDRF signaling activity (relative to healthy controls; as in the adult SHR aorta, as described above); or iii) worst of all, an imbalance involving both under-active EDRF signaling and over-active EDCF signaling (see below, *COX-Mediated EDCF Responses Contributing to Endothelial Dysfunction in Other SHR Vascular Segments, in Other Animal Models of CVD Risk, and in Humans*).

Identification and Specific Examination of COX-mediated EDCF Responses and Signaling Activities

In order to definitively identify whether an EDCF response may be contributing to impaired agonist-stimulated endothelium-dependent vasorelaxation/dilation in a particular vascular segment or vascular bed, the following sequence of investigation should be employed (which is similar to that employed by the pioneering experiments investigating SHR aortic EDCF signaling activity as outlined above in *SHR Aorta: The First Reportings of an EDCF Response Impairing Endothelium-Dependent Vasorelaxation*).

It must be determined to what extent the blunted dose-dependent relaxation/dilation response (which may or may not be accompanied by overt re-contraction) can be eliminated by NOS or COX inhibition via pharmaco-dissection using L-NAME/L-NMMA and Indo (acute incubation in the case of an isolated vascular segment, or acute infusion in the case of an intact vascular bed such as the human forearm). If the blunted relaxation/dilation can be eliminated completely by L-NAME/L-NMMA, then the likely cause of endothelial dysfunction is solely a decrease in NOS-mediated EDRF signaling activity; whereas, if it cannot be eliminated completely by L-NAME/L-NMMA but can be by Indo, then the likely cause of endothelial dysfunction is solely an increase in COX-mediated EDCF signaling activity. Accordingly, if the blunted relaxation/dilation can only be eliminated completely by both L-NAME/L-NMMA and Indo, then the likely cause of endothelial dysfunction is a combination of decreased NOS-mediated EDRF signaling activity and increased COX-mediated EDCF signaling activity.

To verify and more *specifically examine* the agonist-stimulated COX-mediated EDCF signaling activity apparent in an isolated pre-contracted vascular segment, it has become common practice to assess the dose-dependent contractile response stimulated by the agonist with the vascular segment starting from a quiescent state and incubated with L-NAME ((387); also see thesis studies in experimental chapters). The quiescent state ensures that ample capacity for contraction is provided, while the L-NAME incubation acts to amplify contraction by ensuring that NOS-mediated EDRF signaling does not attenuate the EDCF signaling activity stimulated by the agonist (see above, *Balancing EDRF and EDCF Signaling Activities to Prevent or Promote Endothelial Dysfunction*), which together help to optimize the endothelium-dependent COX-mediated contractile response, thus allowing for a more specific examination and pharmaco-dissection of COX-mediated EDCF signaling and the mechanisms accounting for its over-activation under conditions of CVD risk.

It is worth noting that even to date, some studies investigating endothelial dysfunction in states of CVD risk assume that the impairment in endothelium-dependent vasorelaxation/dilation observed in a vascular segment/bed in response to agonist/flow stimulation was caused only by a decrease in NO-mediated signaling activity, even though L-NAME and Indo were not systematically employed to dissect the response, let alone were experiments designed to specifically examine endothelium-dependent contractile activity (i.e. agonist stimulation of an isolated vascular segment in a quiescent state incubated with L-NAME, as described above in this sub-section). In this way, it is possible that the contribution of COX-mediated EDCF cell-signaling to the pathology of endothelial dysfunction could be unrecognized in those vascular segments/beds in those states of CVD risk.

COX-Mediated EDCF Responses Contributing to Endothelial Dysfunction in Other SHR Vascular Segments, in Other Animal Models of CVD Risk, and in Humans

Since the 1986 reporting of a COX-mediated EDCF response causing endothelial dysfunction in the aorta of SHR ((230, 231); see above, *SHR Aorta: The First Reportings of EDCF Signaling Activity Impairing Endothelium-Dependent Vasorelaxation*), the contribution of EDCF cell-signaling to impaired agonist-/flow-stimulated endothelium-dependent vasorelaxation/dilation responses under CVD risk conditions has not only been further examined in the aorta of SHR, but has also been identified and examined in other SHR vascular segments, in other animal models of hypertension, in other animal models of CVD risk – most notably aging and diabetes (105, 111, 385), and in humans – most notably essential hypertensive patients and otherwise healthy aging individuals (394, 395, 401, 403). In these pathological states, the endothelial dysfunction found often involves both under-active NO-mediated (and/or EDHF-mediated) EDRF signaling and over-active COX-mediated EDCF signaling.

Other SHR Vascular Segments. In addition to the aorta, COX-mediated EDCF responses have been found to contribute to endothelial dysfunction in various other segments of the SHR vasculature. Identification of an endothelium-dependent contractile response impairing ACh-stimulated vasorelaxation in the small branch-order mesenteric arteries/arterioles of SHR provided the first demonstration that COX-mediated EDCF activity was not just a phenomenon of conduit macro-vasculature but also of the resistance micro-vasculature (88, 227). Such COX-mediated EDCF activity has also been identified in resistance arteries/arterioles of other SHR vascular beds/territories, including isolated skeletal muscle cremasteric and gracilis arterioles in response to either agonists (159), flow (162, 198), or pressure (160, 161), and isolated renal arterioles in response to agonists (77, 117, 171). In the macro-vasculature of SHR, COX-mediated EDCF activity contributing to endothelial dysfunction has been clearly identified and specifically examined not just in the isolated conduit aorta but also the intermediate-sized conduit renal artery in response to agonists (180, 228, 254, 255).

In contrast, previous studies of SHR investigating the intermediate-sized conduit CCA ((158, 166, 228); *note: the SHR CCA was the focus of Study I and II of this thesis*) and the smaller downstream basilar resistance artery (244, 339) of the cerebral circulation have found that the impaired endothelium-dependent vasorelaxation observed in these vascular segments appears to involve a decrease in NO-mediated EDRF activity with no indication of a COX-mediated EDCF response. These observations are based on the use of either L-NAME or Indo, but not experiments specifically examining endothelium-dependent contractile activity. Noteworthy, in the foremost EDCF Reviews from 2006 to date (109, 111, 112), the above-indicated studies of the

SHR CCA (158, 166, 228) and cerebral vasculature (244, 339) have been cited as examples to make the point that the existence of COX-mediated EDCF responses is not ubiquitous in the SHR vasculature. Unquestionably, this is an important point to be made, and is almost certainly true not only of SHR but of any other animal model of CVD risk, consistent with the heterogeneity well-known to exist throughout the vasculature regarding both the extent to which CVD risk factor(s) may affect endothelium-dependent vasodilation/relaxation and the varied cell-signaling mechanisms that can account for this endothelial (dys)function (109). However, these Reviews (109, 111, 112), in making this point, call particular attention to the CCA and downstream cerebral circulation as a vascular segment/territory devoid of COX-mediated EDCF signaling activity in SHR, as compared to stroke-prone (SP)-SHR where both the conduit CCA (333) and downstream pial arterioles (246) of the cerebral circulation are identified as possessing COX-mediated EDCF activity that impair endothelium-dependent vasorelaxation. Yet, in addition to the two cited studies investigating the basilar artery of SHR (244, 339), a third uncited study investigating pial arterioles (245) found that the impaired ACh- and ADP-stimulated arteriolar dilation responses observed in SHR vs. WKY could be reversed with acute exposure to the TP receptor antagonist SQ29548, indeed suggesting that COX- (and TP receptor-) mediated EDCF signaling activity contributing to endothelial dysfunction may actually exist in the SHR cerebral vasculature, as it does in SP-SHR. Moreover, since in the cited studies investigating the CCA of SHR (158, 166, 228), L-NAME and Indo were not systematically employed, nor was endothelium-dependent contractile activity specifically examined, the possibility that COX-mediated EDCF signaling activity exists in the CCA of SHR cannot be definitively excluded by these works (for further details, see *Discussion* of Thesis Study I).

Other Animal Models of Hypertension. In addition to SHR and SP-SHR, COX-mediated EDCF responses have been found to contribute to impaired endothelium-dependent vasorelaxation in various macro-vascular and micro-vascular segments of other animal models of hypertension, including the aorta or femoral artery of L-NAME-infused SD rats (298), Wistar rats (292, 323), and WKY (265); the aorta, CCA, or femoral artery of eNOS knockout mice (436); the aorta or small mesenteric arteries of AII-infused Wistar rats (181) or mice (398); the CCA or renal artery of Dahl salt-sensitive rats (433, 434); and the aorta of deoxycorticosterone acetate salt-treated rats (73), among other (trans)genetic or experimental hypertensive models (111). These findings are important as they help confirm that the genetic background of SHR is at least not obligatory for the occurrence of COX-mediated EDCF responses. Although, among animal models of hypertension (and *Other Animal Models of CVD Risk*, as described in the subsequent sub-section), it is apparent that COX-mediated EDCF signaling activity occurs most readily in the

vasculature of SHR, and thus remains the archetypal model for its study ((105); also see below, *Potential Mechanisms of Over-Active EDCF Signaling: Role of PGs, ROS, and Oxidative Stress*).

It was found in SHR aorta that the magnitude of the endothelium-dependent COX-mediated contractile response positively correlated with arterial blood pressure (172), prompting the suggestion that the COX-mediated EDCF activity found in different models of hypertension might be a consequence of chronic exposure of the vascular wall to the high intra-vascular pressure that is common amid the varying neuro-humoral and kidney abnormalities accompanying different models (385). However, not all vascular segments within a particular hypertensive animal are characterized by an augmented COX-mediated EDCF response despite exposure to similar increases in mean arterial pressure. In addition, COX-mediated EDCF activity has been identified in other animal models of CVD risk not associated with high blood pressure, such as normotensive aging and diabetes, as described next.

Other Animal Models of CVD Risk. In addition to models of hypertension, COX-mediated EDCF responses impairing endothelium-dependent vasorelaxation are continuously being identified and specifically examined in the macro-vasculature and micro-vasculature of various animal species (e.g. rodents, rabbits, pigs) in a growing number of CVD risk models (111), for instance: aging (193, 329, 418), type-I (328, 330) and type II (240) diabetes, obesity (27, 141), hyperhomocysteinemia (19), estrogen deprivation (15), hypercholesterolemia/atherosclerosis (293), chronic heart failure (256), and vascular injury/endothelial denudation/regenerated endothelium (155, 291). As in models of hypertension, not all vascular segments within a particular CVD risk animal model are characterized by an augmented COX-mediated EDCF response. Nevertheless, an increase in COX-mediated EDCF signaling activity causing endothelial dysfunction has been identified in aortic, renal, carotid, and femoral conduit arteries and in the mesenteric, skeletal muscle, renal, coronary, and cerebral arterial/arteriolar vascular beds/territories across a range of models of diabetes and related metabolic abnormalities (27, 93, 332), where COX-mediated EDCF responses are often found to be prominent (105, 111).

Notably, it is apparent in the SHR-WKY aortic model that the prominence of COX-mediated EDCF responses may be a characteristic of the aging vascular wall. Indeed, while endothelium-dependent contractile activity is either found to be absent or relatively low (vs. SHR) in the aorta of young adult WKY (~12-20wks), aging into later life (ranging from ~12-24 months) is associated with a progressive blunting of maximal endothelium-dependent relaxation due to an age-related increase in COX-mediated EDCF activity (193, 194). Likewise, aging exacerbates the COX-mediated EDCF activity found in the aorta of adult SHR, resulting in a

further blunting of maximal endothelium-dependent relaxation with age (193, 194). These findings suggest that the earlier manifestation of augmented COX-mediated EDCF activity and endothelial dysfunction found in SHR compared to normotensive WKY may be indicative of a *premature aging* of the vascular wall (105, 385). Indeed, the age-related prominence of COX-mediated EDCF signaling activity has also been identified in the vasculature of other aging but otherwise seemingly healthy animals, including the aorta, femoral artery and small mesenteric arteries of male or female SD rats (329, 345) (note: the CCA of aging male SD rats was the focus of Study III in this Thesis), the aorta and small mesenteric arteries of Wistar rats (153), and the aorta of hamsters (418). In addition, augmented COX-mediated EDCF activity has been found to impair endothelium-dependent vasorelaxation in Wistar rat femoral (155) and porcine coronary (291) arteries with regenerated endothelial cells in response to vascular injury (e.g. balloon angioplasty, photochemical damage). Together, these findings have prompted the suggestion that endothelial dysfunction and augmented EDCF activity may characterize the pre-mature aging of the vasculature brought about by chronic exposure to conditions of CVD risk, such as hypertension or diabetes, which leads to damage to the endothelium resulting in accelerated endothelial cell regeneration and senescence (361).

In Humans. Helping to strengthen the clinical relevance of the above-described findings in experimental animal vasculature, COX-mediated EDCF responses appear also to impair human endothelium-dependent vasorelaxation in various condition of CVD risk (394, 403). This was first discovered in the forearm circulation of adult untreated essential hypertensive patients, wherein the presence of intra-arterially administered Indo was found to partially but significantly improve the impaired dilatory response to cumulative ACh infusion as compared to age-matched normotensive control subjects, wherein a more robust ACh-stimulated dilatory response was unaffected by Indo (354). While improving endothelium-dependent vasodilation in essential hypertensive patients, Indo was found not to be effective in improving endothelial dysfunction in adult patients with secondary forms of hypertension ((354); including primary aldosteronism and reno-vascular hypertension). Of note, these findings are consistent with the more ready occurrence of COX-mediated EDCF responses in the vasculature of SHR as compared to other animal models of hypertension (see above, *Other Animal Models of Hypertension*). In addition to essential hypertension, Indo, or the TP receptor antagonist S18886 has been found to improve the impaired agonist-stimulated endothelium-dependent vasodilation in the forearm of patients with congestive heart failure (187), coronary artery disease (31), women post-ovariectomy/-hysterectomy (402), and otherwise healthy, normotensive individuals aged >60 years (353),

suggesting that COX- (and TP receptor-) mediated EDCF signaling activity helps to define human endothelial dysfunction under CVD risk conditions.

Noteworthy, as in the SHR-WKY aortic model (see above, *Other Animal Models of CVD Risk*), comparisons made between aging essential hypertensive patients and normotensive control subjects also suggest that the endothelial dysfunction and augmented EDCF activity found in essential hypertension may be indicative of pre-mature vascular aging (385, 394, 403). In otherwise healthy, normotensive individuals, the decline in forearm agonist-stimulated endothelium-dependent vasodilation up to age 60 years was found to be largely L-NMMA-/L-arginine-sensitive and essentially Indo-insensitive; conversely, the decline with age beyond 60 years became partly sensitive to Indo (353), suggesting an age-related increase in COX-mediated EDCF signaling activity. In comparison, in essential hypertensive patients, reduced forearm agonist-stimulated endothelium-dependent vasodilation was only Indo-insensitive <30 years of age, after which the decline in this vasodilation became progressively more Indo-sensitive with aging up to and beyond 60 years (353). Thus, it appears in humans as it does in rats that augmented COX-mediated EDCF activity is a characteristic of the aging vascular wall with the conditions of essential hypertension acting to accelerate the workings of this change.

In this thesis, the identity and cell-signaling mechanisms of COX-mediated EDCF activity causing endothelial dysfunction were specifically examined in the CCA conduit vasculature of adult SHR and WKY (Study I and II), and adult and aging SD rats (Study III).

Potential Mechanisms of Over-Active EDCF Signaling: Function of PGs, ROS, and Oxidative Stress

The mechanisms of over-active EDCF signaling could involve changes affecting either the endothelial production of EDCF(s) or the EDCF-mediated activation of VSM contractile pathways.

As is indicated, much of the available evidence on the endothelial-VSM cell-signaling mechanisms of EDCF activity, and its pathological augmentation, pertains to EDCF(s) (over)production, coming in large part from studies in the SHR-WKY vasculature, in particular the isolated aorta.

Mechanisms of EDCF (Over)Production: Critical Role of Regulating the PG Synthesis Pathway

Possible EDCF Candidates. Under pathological conditions in particular, the endothelium can synthesize and release a variety of factors capable of promoting a CVD phenotype in addition to

eliciting vasocontractile activity, most notably ROS and GPCR agonists including not only various PGs but also the peptides AII and endothelin-1 (ET-1), acting, respectively, on VSM PG, AT₁ and ET_A receptors (44, 361). However, neither of these peptides (or other known EDCFs) appears to acutely mediate endothelium-dependent contractile activity stimulated by agonists or flow (385). Conversely, given that agonist-/flow-stimulated endothelium-dependent contractile activity, when found in animal models of CVD risk, can be completely abolished by the non-selective COX inhibitor Indo, and almost eliminated by the TP receptor antagonists S18886 or SQ29548 (see above, *Endothelium-Dependent Contractile Responses: Initial Findings and Characterization of EDCF Cell-Signaling Pathway*), the most likely candidate(s) to act as diffusible EDCF substance(s) are metabolites derived from the activation of COX-1 and/or COX-2, which may include endoperoxides and PGs (see *COX-Derived PGI₂* in Section 1.5.1), as well as ROS (see *Determinants of Pro-oxidant/Anti-oxidant Balance in the Vascular Wall* in Section 1.5.2, and *Role of ROS and Oxidative Stress in EDCF Signaling Activity* below).

As previously introduced for PGI₂ (see *COX-Derived PGI₂* in Section 1.5.1; and Figure 1.4), AA, liberated from membrane phospholipids by phospholipases including Ca²⁺-dependent PLA₂, can be metabolized by either COX-1 or COX-2, with equal potency, to the endoperoxides PGG₂ and PGH₂, which are subsequently metabolized to either PGI₂, PGE₂, PGD₂, PGF_{2α}, or TXA₂ by their respective terminal PGSSs, namely PGI, PGE, PGD, PGF, TX synthase (see Figure 1.5). In addition, PG isomers, notably 8-isoprostane, can be formed either enzymatically via COX, or non-enzymatically via oxidation of membrane polyunsaturated fatty acids including AA (Figure 1.5). But while TXA₂ is the preferential activator of TP receptors, and each other terminal PG for their so-named PG receptor (i.e. PGI₂ of IP receptors, PGE₂ of EP receptors, PGD₂ of PD receptors, and PGF_{2α} of FP receptors; Figure 1.5), it has been established that PGH₂ and all other PGs can also activate VSM TP receptors with varying potencies (from Ref. (139); TXA₂>>8-isoprostane=PGF_{2α}=PGH₂>PGE₂=PGD₂>PGI₂ in the aorta of SHR and WKY). Consequently, if produced in a high enough concentration, it is possible that any one of these endoperoxide-PG metabolites could act as a COX-derived EDCF, provided that the vasocontractile activity activated by TP receptor stimulation was greater than any vasorelaxation activity which might be trans-activated by stimulating PG receptor(s) that are G-protein-coupled to signaling cascades mediating relaxation (e.g. the IP receptor, which is typically known to activate VSM relaxation through Gs-AC-cAMP-PKA signaling activity, unless dysfunctional, like in SHR and aging WKY, as further described below in *Mechanisms of EDCF Activation of VSM Contraction: Role of TP Receptor Signaling Activity*).

Indeed, across studies investigating agonist-/flow-stimulated endothelium-dependent contractile signaling, it has been found that, depending on the stimulus, vascular segment, and particular animal or CVD risk condition under study, a variety of PGs derived from COX-1, and/or COX-2 when expressed, may act as EDCFs, including TXA₂, PGH₂, PGF_{2α}, and 8-isoprostane (105, 111, 394, 403), and, though seemingly paradoxical (considering its well-known role as an EDRF), also PGI₂ (see *Mechanisms of EDCF Activation of VSM Contraction: Role of TP Receptor Signaling Activity* for explanation).

Thus, theoretically, anything changing the acute or chronic regulation parameters of the endothelial cell PG synthesis pathway – i.e. the expression and/or activation of PLA₂, COX-1/COX-2, or PG synthases – could contribute to the cause of augmented EDCF activity, if those changes result in the over-production of PGs capable of eliciting net TP receptor-mediated VSM contraction.

Over-Active EDCF Production via Altered Regulation of the PG Synthesis Pathway. In the SHR-WKY aortic model, key potential causes of augmented EDCF activity relating to EDCF production have been identified as: i) an endothelial cell over-expression of COX and PG synthases, and ii) a greater rise in endothelial [Ca²⁺]_i upon agonist stimulation (360).

Endothelium-derived PGs are over-produced in the aorta of adult SHR and aging WKY (vs. adult WKY), both in a quiescent state and upon agonist stimulation. In response to ACh, PGI₂ (and possibly PGH₂) derived from COX-1 has been found to be the predominant PG produced from the aorta of these animals (from Ref. (139); PGI₂ 10-100-fold >PGF_{2α}>PGE₂>TXA₂>8-isoprostane=PGD₂ in SHR and WKY aorta); accordingly, in SHR and aging WKY, both ACh-stimulated endothelium-dependent aortic PGI₂ production and ACh-stimulated aortic EDCF activity can be completely abolished by Indo or by the preferential COX-1 inhibitor valeryl salicylate (VAS), but only partially attenuated by the preferential COX-2 inhibitor NS398 (139). In contrast, in response to A23187, COX-1-derived TXA₂ in addition to PGI₂ (and possibly PGH₂) appear to act together as EDCFs (from Ref. (140); PGI₂ >TXA₂=PGF_{2α}=PGE₂ in SHR and WKY aorta), as the TX synthase inhibitor dazoxiben was found to completely abolish the A23187-stimulated endothelium-dependent aortic production of TXA₂ and attenuate A23187-stimulated EDCF activity by about half (140). Congruent with these functional-biochemical findings, a significant over-expression of COX-1, PGI synthase, and TX synthase can be found in the aortic endothelium of adult SHR, while both COX-1 and COX-2 and TX synthase are found to be over-expressed in the aortic endothelium of aging WKY (359). Indeed, as in the SHR-WKY aortic model, the augmented EDCF activity found in other SHR vascular segments (399) and in other animal models of CVD risk (240, 298, 398) can also be linked to the

over-production of one or more PGs (TXA₂, PGH₂, PGF_{2α}, 8-isoprostane, PGI₂), which is consistently related to an up-regulation of COX-1 and/or COX-2 expression, while the expression of particular PG synthases has not been well investigated. In agreement also are the few direct studies of EDCF responses in the human vasculature, wherein the ACh-stimulated TP receptor-mediated EDCF response found in quiescent renal artery segments isolated from aging hypertensive-diabetic patients was linked to the renal artery production of PGF_{2α} in response to ACh, which could be abolished by the COX-2 inhibitor celecoxib (418). Moreover, the ACh-stimulated endothelium-dependent relaxation response found in isolated, pre-contracted renal arteries from aging hypertensive patients could be significantly improved by celecoxib, and was coincident with renal artery COX-2 over-expression compared to renal artery segments isolated from age-matched normotensive patients (419).

In addition to chronic expression changes in COX-1 and/or COX-2 and PG synthases, the augmented PG production and EDCF activity, at least in the aorta of adult SHR vs. WKY, has been linked to changes in the acute regulation of endothelial [Ca²⁺]_i homeostasis leading to over-activation of the PG synthesis pathway (360). Indeed, ACh stimulation is found to elicit an increase in endothelial [Ca²⁺]_i that is substantially greater in the aorta of SHR than WKY and associated with augmented EDCF activity (357). Interestingly, in response to A23187, the maximal increase in endothelial [Ca²⁺]_i was comparable between SHR and WKY aorta, while the augmentation in A23187-stimulated aortic contractile response amplitude in SHR vs. WKY, albeit less than if stimulated by ACh, was still apparent (357), most likely explained by the COX-1, PGI synthase, and TX synthase over-expression in the aorta of SHR (360), leading to an augmented production of vasocontractile PGs despite a presumably similar Ca²⁺-triggered release of AA by PLA₂. Whether a similar dysfunction in the acute regulation of endothelial [Ca²⁺]_i contributes to the augmentation of EDCF activity in other SHR vascular beds or in other CVD risk conditions remains to be determined.

Taken together, it certainly appears from the available evidence that the endothelial over-production of EDCF(s) in the form of PGs contributes importantly to the cause of over-active EDCF signaling in states of CVD risk by a mechanism involving the up-regulation, and perhaps over-activation, of the main enzymatic parameters of the PG synthesis pathway, most notably COX-1 or COX-2.

Mechanisms of EDCF Activation of VSM Contraction: Role of TP Receptor Signaling Activity

Compared to mechanisms of EDCF (over)production, much less is known regarding the VSM cell-signaling mechanisms by which EDCFs activate contractile activity, and whether changes in those cell-signaling activities might contribute to over-active EDCF signaling.

PG Receptor Involvement. As has been demonstrated under bioassay conditions, EDCFs, once produced, diffuse to the VSM and cause contraction (see above *Endothelium-Dependent Contractile Responses: Initial Findings and Characterization of EDCF Cell-Signaling Pathway*). While a component of non-TP receptor-mediated EDCF activity has been reported under some conditions, typically involving other PG receptor sub-types such as EP or FP receptors (240, 328, 356), the ability of TP receptor antagonists (S18886 or SQ29548) to nearly or completely abolish agonist-/flow-stimulated endothelium-dependent contractile responses in almost all cases where they have been identified, strongly suggests that this PG receptor sub-type is obligatory in the EDCF-mediated activation of VSM signaling activity (Figure 1.5).

TP-IP Receptor Balance. Considering the well-known role of PGI₂ as an EDRF, its ability to act as a prominent EDCF in SHR and aging WKY is seemingly paradoxical. However, apparent as early as 3 months old in SHR, and progressively apparent after 3 months old in WKY (142), PGI₂ cannot elicit relaxation, but rather elicits TP receptor-mediated contraction in these animals because their VSM IP receptors possess (acquire) a specific dysfunction (i.e. it is not found to be related to its expression or alterations in its downstream Gs-AC-cAMP-PKA signaling axis; (142)). This apparent imbalance between TP and IP receptor activities has also been found in other models of vascular dysfunction. Of note, in mesenteric arteries from aging Long-Evans control and type-II diabetic rats, the impairment found in ACh-stimulated endothelium-dependent vasorelaxation is largely attributed to a COX- and TP receptor-mediated contractile response linked to the over-expression of COX-1 and COX-2 and the ACh-stimulated over-production of PGI₂ as well as TXA₂ and PGE₂ (239, 241), and in these arteries, exogenous PGI₂, like in SHR and aging WKY, is found not to elicit relaxation, but TP receptor-mediated contraction. It is also of note that patients with a mutation rendering their IP receptor dysfunctional demonstrate accelerated atherogenesis and thrombogenesis (14), as do athero-prone apoE knockout mice with their IP receptor gene deleted (138). Accordingly, the COX- and TP receptor-mediated EDCF activity found to impair endothelial function in the aorta of SHR and aging WKY provides an important model example of the consequences of producing/over-producing any endoperoxide or PG under conditions where the signaling activity stimulated by (trans)activating TP receptors (or theoretically any PG receptor G-protein-coupled to a

vasocontractile signaling axis; see below, *Potential VSM GPCR-Mediated Contractile Signaling Pathways*) cannot be successfully offset by the signaling activity stimulated by activating IP receptors (or theoretically any PG receptor G-protein-coupled to a vasorelaxation signaling axis; see above, *EDRF Signaling Pathways / COX-Derived PGI₂* in Section 1.5.1) (refer to both Figure 1.4 and Figure 1.5).

TP Receptor Expression. All PG receptor sub-types were shown to be constitutively expressed in the aortic VSM of SHR and WKY (359), and while an increased expression of VSM DP, EP₃, and EP₄ receptors was found in the aorta of SHR or aging WKY, the expression of all other PG receptors are unaltered, including the expression of both IP and TP receptors (359). Thus, judging from these data, it does not appear unlikely that a change in the expression of TP receptors (or other PG receptors) contributes importantly to the cause of over-active EDCF signaling.

TP Receptor Sensitivity. The dose-dependent contractile response elicited by the synthetic, selective TP receptor agonist U46619 has been found to be no different in SHR and WKY aorta denuded of endothelium (60, 129), suggesting that the sensitivity of VSM TP receptors to agonist stimulation is unaltered between strains. In contrast, while similar results were found for authentic PGF_{2α} as for U46619, contraction in the VSM of SHR aorta was shown to be hyper-sensitive to authentic PGH₂ (129), and in the VSM of aging type-II diabetic rats was shown to be hyper-sensitive to authentic PGE₂ (242). Moreover, in endothelium-denuded femoral artery or aortic segments from adult streptozotocin-induced type-I diabetic or L-NAME-infused hypertensive SD rats, respectively, the VSM contractile response to U46619 was found to be augmented compared to controls SD rats (298, 331). Thus, it appears that a hyper-sensitivity of TP receptors to EDCFs (at least in the form of PGH₂ or PGE₂) might have some contribution to over-active EDCF signaling in states of CVD risk.

EDCF-Stimulated Activation of VSM TP Receptor-Mediated Contractile Signaling Activity. Beyond the knowledge that EDCFs diffuse to the VSM and elicit TP receptor-mediated contractile activity, the exact signaling mechanisms by which this EDCF-stimulated contraction occurs is (was) unknown and has (had) not been investigated (note: until Study I and II of this thesis was performed and published; see below Section 1.7.2 and 1.7.3, *Specific Rationale, Objectives, and Hypotheses of Thesis Study I and Study II*, and Experimental Chapter 2 and 3). Thus, it is (was) unknown whether and what changes in the EDCF-stimulated TP receptor-mediated contractile signaling axis might contribute to over-active EDCF signaling in states of CVD risk.

Potential VSM GPCR-Mediated Contractile Signaling Pathways. Potential mechanisms of contractile signaling upon activation of VSM GPCRs, such as TP receptors, have been identified (see Figure 1.5). As previously introduced (see above Section 1.5.1, *EDRF Cell-Signaling Pathways*), VSM contractile activity is dictated by myosin RLC₂₀ phosphorylation which is in turn controlled by the opposing activities of MLCK and MLCP, the former of which is predominantly activated by Ca²⁺-dependent mechanisms and the latter of which can be activated or inhibited by a number of Ca²⁺-independent mechanisms. GPCRs eliciting VSM contraction are typically coupled to either or both of the G_{q/11} and G_{12/13} class of G-protein(s). Stimulation of G_{q/11} results in the initiation of contraction via a classical pharmaco-mechanical coupling mechanism involving the activation of membrane-bound *phospholipase (PL)C-β* leading to the intra-cellular generation of IP₃ and *diacylglycerol (DAG)*, the former of which binds to SR IP₃ receptors resulting in an increase in VSM [Ca²⁺]_i, the formation of Ca²⁺-CaM complexes, and the subsequent activation of MLCK (348). In contrast, stimulation of G_{12/13} can lead to contraction by the phenomenon of Ca²⁺ sensitization via activation of the RhoA-ROCK signaling axis (342, 348).

RhoA is a molecular switch (341, 342, 348); thus, in a quiescent state, inactivated RhoA-GDP exists in the cytosol where its prenylated hydrophobic tail interacts with GDP dissociation inhibitor (*Rho-GDI*). GPCR-stimulated G_{12/13} activates guanine nucleotide exchange factors (*Rho-GEFs*) resulting in the exchange of GTP for GDP on RhoA and the dissociation of Rho-GDI, after which RhoA-GTP becomes bound to the plasma membrane by its tail. This membrane-bound 'switched-on' (*i.e. activated*) *RhoA-GTP* then interacts with and *activates ROCK*, which can phosphorylate MLCP at multiple inhibitory sites (on the *MYPT1* myosin-targeting subunit), resulting in MLCP inhibition, a greater Ca²⁺-CaM-MLCK:MLCP activity ratio, and a subsequent Ca²⁺-independent increase in contractile activity. To 'switch-off' RhoA-GTP, Rho GTPase activating proteins (*RhoGAPs*) catalyze the hydrolysis of RhoA-bound GTP resulting in the re-association of RhoA-GDP with Rho-GDI in the cytosol. Beyond this well-known role of RhoA-GTP as a ROCK activator, it is noteworthy that both AA (13) and *sphingosylphosphorylcholine (SPC; (335))* have been shown in VSM to activate ROCK in a RhoA-independent manner, and so represent possible alternate mechanisms in the cell-signaling activities involved in GPCR-mediated, ROCK-dependent contractile activity (342, 348). Furthermore, in addition to ROCK, several other protein kinases, most notably Ca²⁺-independent or -dependent, DAG- (and/or AA-) activated *PKC isoforms*, have independently been shown to result in the phosphorylation of MYPT1 and inhibition of VSM MLCP (414), and so represent possible alternate mechanisms in

the cell-signaling activities involved in GPCR-mediated, MLCP-dependent contractile activity (348).

Thus, it appears possible that EDCF-stimulated contractions could be mediated by multiple potential VSM TP GPCR-mediated cell-signaling mechanisms, among which might involve activation of the RhoA-ROCK signaling axis (for further detail, see below *Specific Rationale, Objectives, and Hypotheses of Thesis Study II*, and Experimental Chapter 3). But whether and to what extent any of these cell-signaling mechanisms in fact contributes to EDCF activity or its augmentation in states of CVD risk remained to be determined (also see above, *EDCF-Stimulated Activation of VSM TP Receptor-Mediated Contractile Signaling Activity*).

Role of ROS and Oxidative Stress in EDCF Signaling Activity

Given that excess ROS accumulation and oxidative stress has been identified as a major mechanism responsible for under-active EDRF signaling in states of CVD risk (as detailed in the Section 1.5.2 Review, *Under-Active EDRF Signaling and Endothelial Dysfunction*; and Figure 1.4), it might be expected that ROS and oxidative stress would also contribute to the mechanism(s) responsible for EDCF signaling activity and its augmentation in states of CVD risk, thus providing a common link to the causes of endothelial dysfunction under these pathological conditions.

Continuing from early findings suggesting the possibility of a ROS component in the EDCF cell-signaling pathway (see above, *Endothelium-Dependent Contractile Responses: Initial Findings and Characterization of EDCF Cell-Signaling Pathway*; and Figure 1.5), the role of ROS and oxidative stress in the regulation of EDCF signaling activity has been a focus of investigation in numerous models of CVD risk (note: as it was in both Study II and Study III of this Thesis; see below Section 1.7.3 and 1.7.4, *Specific Rationale, Objectives, and Hypotheses of Thesis Study II and Study III*, and Experimental Chapter 3 and 4).

Acute Role of ROS in EDCF Signaling Activity

The exact role(s) of ROS in acutely regulating EDCF signaling activity has been difficult to establish, perhaps owing to the heterogeneous vasomotor responses found to be elicited by different ROS ($O_2^{\cdot-}$, H_2O_2 , OH^{\cdot}) in different vascular segments from different animals, which, in regard to H_2O_2 for instance, may range from contraction, to relaxation, to sometimes both within the same vascular segment (see above in Section 1.5.2 – *Consideration of Potential ROS-Mediated Compensatory Adaptations in Attempt to Prevent Endothelial Dysfunction*, and below in Experimental Chapter 4). Indeed, across studies, the extent to which a ROS component has been found to acutely mediate the EDCF cell-signaling pathway depends on the stimulus,

vascular segment, and particular animal or CVD risk condition under study (360, 394, 403), and the extent varies greatly, whereby the result of pre-incubating a vascular segment with *ROS inhibitor(s)* has ranged from having no attenuating effect (181, 418) to almost completely eliminating all agonist-/flow-stimulated endothelium-dependent contractile activity (186).

Broadly, there are three potential mechanisms by which ROS could act acutely to augment EDCF responses: i) indirectly, by decreasing EDRF signaling activity; ii) by acting themselves directly as an 'EDCF'; or iii) by directly stimulating VSM (or endothelial) COX thus acting to amplify the COX-PG-TP receptor signaling axis (360, 417).

Potential Indirect Action of ROS in Augmenting EDCF Responses via Decreasing EDRF Activity. Certainly, an accumulation of ROS in the vascular wall can indirectly lead to the augmentation of an (underlying) COX-mediated EDCF response, if that ROS results in decreasing EDRF signaling activity upon agonist/flow stimulation (e.g. via $O_2^{\cdot-}$ destruction of eNOS-derived NO), thereby leaving EDCF-stimulated VSM TP receptor-mediated signaling activity less opposed (also see above, *Balancing EDRF and EDCF Signaling Activities to Prevent or Promote Endothelial Dysfunction*). Nonetheless, considering that ROS inhibitor(s) have been shown to attenuate agonist-/flow-stimulated endothelium-dependent contractile responses both in pre-contracted vascular segments and in vascular segments specifically examined from a quiescent state and pre-incubated with L-NAME (also see above, *Identification and Specific Examination of COX-mediated EDCF Responses and Signaling Activities*), it is apparent ROS can act to augment EDCF signaling activity by a more direct mechanism independent of decreasing EDRF signaling activity.

Potential Direct Actions of ROS in Augmenting EDCF Responses via Modulation of COX-Mediated Activities. In metabolizing AA to endoperoxides, the cyclooxygenation and peroxidation activities of COX can also produce ROS (see above, *Determinant of Pro-oxidants/Anti-oxidant Balance in the Vascular Wall* in Section 1.5.2 Review; and Figure 1.5). Indeed, the ACh- or A23187-stimulated COX- and TP receptor-mediated EDCF responses found in the aorta of adult SHR vs. WKY (357) and in the femoral artery of either aging vs. adult SD rats (329) or adult streptozotocin (STZ)-induced type-I diabetic vs. healthy SD rats (330) have not only been linked to the endothelial COX-1 or COX-2-derived over-production of PGs (see above, *Over-Active EDCF Production via Altered Regulation of the PG Synthesis Pathway*) but also to an Indo-sensitive accumulation of ROS in the endothelium (329, 330, 357), as detected by the fluorescence of dichlorodihydrofluorescein diacetate (DCF), a non-specific intra-cellular ROS dye capable of detecting $O_2^{\cdot-}$, H_2O_2 , OH^{\cdot} , and $ONOO^-$ (89). The fact that pre-incubation with ROS

inhibitor(s) can partially (30-50%) attenuate the EDCF responses found in SHR aorta and SD rat femoral arteries (330, 360, 423), and can also attenuate to varying extents the EDCF responses found in several, but not all ((181, 418); e.g. the aorta of AII-infused Wistar rats), other vascular segments and models of CVD risk (256, 399, 419), certainly suggests that in addition to the requisite COX-PG-TP receptor signaling axis, an agonist-/flow-stimulated COX-derived (or at least COX-mediated) over-production of ROS from the endothelium may play a direct contributing role in mediating EDCF signaling activity in models of CVD risk.

Of note, pre-incubation with standard SOD (which dismutates $O_2^{\cdot-}$ to H_2O_2) and/or CAT (which metabolizes H_2O_2 to H_2O) has been found to be without effect on SHR aortic EDCF activity presumably because of their poor cellular permeability (360), since the cell-permeable $O_2^{\cdot-}$ scavenger Tiron and the cell-permeable SOD inhibitor diethyldithiocarbamic acid (DETCA), alone or in combination, can prevent both ACh- and A23187-stimulated COX-mediated endothelial DCF fluorescence (357) and attenuate by ~30-50% the associated EDCF responses in SHR aorta, the latter of which can also be accomplished by cell-permeable PEG-CAT or deferoxamine (an Fe^{2+} chelator thus preventing the formation of OH^{\cdot} which results from H_2O_2 (usually higher concentrations) reacting with transition metals) (see above, *Determinant of Pro-oxidants/Anti-oxidant Balance in the Vascular Wall* in Section 1.5.2), in addition to inhibitors of MEGJ (which may facilitate transfer of ROS from endothelial cells to VSM) (360) (see Figure 1.5). Together, these findings indicate that the ROS contributing to the EDCF signaling activity in the SHR aorta may include COX-derived $O_2^{\cdot-}$ and in particular its by-products including H_2O_2 and possibly OH^{\cdot} (however, the contribution of other downstream-activated endothelial ROS sources, including NADPH oxidase, cannot be excluded; see Figure 1.5). These same particular ROS appear also to contribute to the EDCF signaling activity found in SD rat femoral arteries, since pre-incubation with Tiron or DETCA alone, but also CAT or deferoxamine alone, or MnTMPyP alone (a cell-permeable SOD+CAT mimetic), can attenuate by ~30-50% the A23187-stimulated EDCF response in the femoral artery of STZ-induced diabetic SD rats (330). In addition to COX-1/COX-2 over-expression, the activity (but not expression) of catalase in both aging and STZ-induced diabetic SD rat femoral arteries is found to be reduced (329, 330). Accordingly, this suggests that in response to agonist stimulation, both an increase in COX-mediated ROS generation and a decrease in ROS buffering capacity may result in the endothelial accumulation and subsequent over-production of H_2O_2 and/or OH^{\cdot} ROS in particular, leading to augmented EDCF signaling activity in states of CVD risk.

Following agonist-/flow-stimulated endothelial COX-derived (over)production of PGs and ROS, it is certainly possible that ROS themselves may act as EDCF(s) 'proper' (i.e. independent

of PGs) to elicit VSM contraction, thus directly mediating EDCF activity. For example, in a detailed study of (apparently healthy) canine basilar arteries (186), it was found that pre-incubation with SOD+CAT could essentially abolish A23187-stimulated endothelium-dependent COX-mediated contractile activity; that an SOD-inhibitable contractile response could be elicited by exposure of endothelium-denuded arteries to exogenous xanthine (X):XO-derived ROS in the presence of CAT; and that the A23187-stimulated arterial production of TXA₂, PGF_{2α}, and PGE₂ could be abolished by removing the endothelium or by Indo but was unaffected by SOD+CAT, altogether indicating that endothelial COX-derived O₂^{•-}, rather than PGs, must be the EDCF stimulating VSM contraction in this particular vascular model. Conversely, in the SHR aorta and STZ-induced diabetic SD rat femoral artery models, where acutely inhibiting O₂^{•-}, H₂O₂, and/or OH[•] production only partially attenuates EDCF activity, the dose-dependent contractile responses of endothelium-denuded arteries to either exogenous X:XO (which can produce a mix of O₂^{•-}, H₂O₂, and/or OH[•]) or H₂O₂ are much more substantial in SHR vs. WKY and STZ-induced diabetic vs. healthy SD rats, and can essentially be abolished by either COX inhibitors or TP receptor antagonists (330, 331, 423). From these findings, it is suggested that the predominant mechanism by which ROS ‘directly’ acts to mediate EDCF activity is most likely by amplifying an already activated [Ca²⁺]_i-PLA₂-AA-COX-PGS-PG-TP receptor-contractile signaling axis through further stimulation of VSM COX (and perhaps also endothelial COX) (see Figure 1.5), thus reconciling the lack of (181, 418) or only partial (256, 330, 399, 419, 423) attenuation of EDCF responses by ROS inhibitor(s) found across models of CVD risk.

Altogether, it appears from the available evidence that the COX-mediated endothelial over-production of O₂^{•-}-derived ROS including H₂O₂ and OH[•], may themselves, independent of potentially decreasing NO-mediated EDRF activity, play an important modulating role in mediating EDCF signaling activity. In this way, a vascular wall pro-oxidant/anti-oxidant balance promoting the accumulation of ROS (see above in Section 1.5.2 Review. – *Pro-oxidant/Anti-oxidant Balance in States of CVD Risk*) could make an important direct contribution to the cause of over-active EDCF signaling in states of CVD risk, in addition to the important contributions made by PG over-production via an up-regulation, and perhaps Ca²⁺-dependent over-activation, of the main enzymatic parameters of the endothelial cell PG synthesis pathway – most notably COX-1/COX-2 (see above, *Mechanisms of EDCF (Over)Production: Critical Role of Regulating the PG Synthesis Pathway*), and perhaps also by VSM TP receptor hyper-sensitivity or loss of VSM IP receptor functionality (see above, *Mechanisms of EDCF Activation of VSM Contraction: Role of TP Receptor Signaling Activity*).

Chronic Role of ROS and Oxidative Stress in EDCF Signaling Activity

Since endothelium-dependent contractile responses can develop in many vascular segments of different animal models of CVD risk (see above, *COX-Mediated EDCF Responses Contributing to Endothelial Dysfunction in Other SHR Vascular Segments, in Other Animal Models of CVD Risk, and in Humans*), it is possible that the excess ROS accumulation and oxidative stress commonly defining these models could play a role in the chronic regulation of EDCF signaling activity. However, compared to the well-investigated role of oxidative stress in chronically regulating EDRF activity, particularly regarding mechanisms of under-active NO-mediated signaling (as detailed in the Section 1.5.2 Review – *Role of Decreased NO Production and Target-Tissue Sensitivity in Endothelial Dysfunction with Links to Oxidative Stress*), not much is known regarding the extent to and mechanism(s) by which oxidative stress chronically regulates EDCF activity and its development under CVD risk conditions.

Potential Role of ROS in Chronically Regulating EDCF Activity via the PG Synthesis Pathway. In SHR, chronic in vivo treatment with the OH[•] scavenger dimethylthiourea was found to reduce by >50% ACh-stimulated COX-1-mediated EDCF activity specifically evaluated in intact aorta, as well as the X:XO-stimulated COX-1 and TP receptor-mediated contractile activity in endothelium-denuded aorta (423). Moreover, in mesenteric arteries of SHR (399), impairment in ACh-stimulated vasorelaxation sensitive to COX-2 inhibition could be restored following chronic in vivo treatment with vitamin C, the NADPH oxidase inhibitor Apocynin, or Atorvastatin (a lipid-lowering drug known to have pleiotropic anti-oxidant effects), which also reversed a related increase in basal intra-vascular ROS content, COX-2 over-expression, and ACh-stimulated COX-2-derived over-production of 8-isoprostane (399). Together, these findings suggest that chronic exposure to the excess ROS accumulation and oxidative stress known to exist in this animal model of hypertension (44, 376) could play a direct role in the regulation of EDCF signaling activity. Suggesting likewise, an excellent most recent study (419) has shown that 12h in vitro static arterial culture in incubation medium containing bone morphogenic protein (BMP)-4, a pro-inflammatory mediator known to be over-expressed in endothelial cells by low/oscillatory ‘pro-atherogenic’ shear stress (343), could induce COX-2 up-regulation and specific COX-2- and TP receptor-mediated EDCF activity able to impair ACh-stimulated vasorelaxation in otherwise healthy mouse aorta, all of which was prevented by co-incubation with Apocynin or the cell-permeable SOD mimetic Tempol, or with the p38 MAPK/transcription factor inhibitor SB202190 (see above in Section 1.5.2. Review – *Pro-oxidant/Anti-oxidant Balance in States of CVD risk*). Further demonstrated from these findings was that the specific COX-2-mediated EDCF signaling activity able to impair an otherwise robust ACh-stimulated

vasorelaxation response of SHR vs. WKY intra-lobar renal arteries, which over-expressed both BMP-4 and COX-2, could in fact be completely reversed by 12h culture in medium containing the BMP-4 inhibitor noggin, which caused marked COX-2 down-regulation. Furthermore, these same results found in SHR renal arteries were also found in renal arteries isolated from hypertensive patients, and like in healthy mouse aorta, 12h BMP-4 incubation of renal arteries isolated from normotensive patients resulted in ROS-mediated COX-2 up-regulation and specific COX-2-mediated contractile activity able to impair ACh-stimulated vasorelaxation.

Congruent with the described SHR findings, chronic *in vivo* AII infusion, a model characterized by increased blood pressure, NADPH oxidase over-expression, and ROS over-production (299), induced both COX-1 and COX-2 up-regulation and specific COX-1-mediated EDCF activity able to impair ACh-stimulated vasorelaxation in the aorta of Wistar rats, while concomitant oral red wine polyphenol anti-oxidant treatment could prevent the over-expression of both COX-1 and COX-2, and the COX-mediated endothelial dysfunction (181). Of note, the EDCF activity found in this study was in fact not attenuated upon acute pre-incubation of the aorta with PEG-CAT and the cell-permeable SOD+CAT mimetic MnTMPyP, thus indicating that ROS may play distinct roles in the acute and chronic regulation of COX-mediated EDRF activity, as has been suggested in other arterial/arteriolar segments from other models of CVD risk (239, 241).

While chronic AII infusion in mice induced impairment of mesenteric artery ACh-stimulated vasorelaxation sensitive to COX-1 inhibition, COX-1 was up-regulated but COX-2 down-regulated (both at the mRNA and protein level), and while concomitant treatment with apocynin prevented the COX-mediated endothelial dysfunction, neither COX-1 nor COX-2 expression was found to be affected (398). In contrast to the described findings in SHR and Wistar rats, the findings from this study indeed suggest that: i) the vascular expression of both COX isoforms may be regulated independent of ROS – i.e. because of the inconsistency found between the COX-1/-2 up-/down-regulation by AII-induced hypertension and oxidative stress, but not down-/up-regulation by preventing oxidative stress; and too ii) there may be important active point(s) of chronic regulation along the EDCF signaling axis other than COX protein content – i.e. because of the inconsistency found between changes in COX-mediated EDCF activity and changes (or not) in COX expression. In fact, these same inconsistencies have been found in recent studies of mesenteric arteries from aging Long-Evans type-II diabetic and control rats (239, 241), wherein chronic *in vivo* treatment with Metformin (241) (a glucose-lowering drug used to treat diabetes) or Losartin (239) (an antagonist of AII (AT₁) receptors) did not affect COX-1 and COX-2 over-expression in type-II diabetic arteries, but did significantly attenuate the increased basal

vascular ROS release, ACh-stimulated COX-derived PGE₂ and TXA₂ over-production, and specific COX-mediated EDCF signaling activity able to impair ACh-stimulated vasorelaxation.

Is it not certain what ROS-dependent and/or -independent chronic regulatory mechanism(s) beyond COX protein expression/content might account for agonist-stimulated over-activation and subsequent PG over-production through the endothelial cell [Ca²⁺]_i-PLA₂-AA-COX-PGS-PG synthesis pathway augmenting EDCF activity under CVD risk conditions. However, in cases under excess oxidative stress where under-active NO-mediated EDRF signaling accompanies over-active EDCF signaling, as was the case in both the described studies of the aging Long-Evans type-II diabetic vasculature (239, 241), one possibility may relate to the inactivation of endothelial PGS synthase by ONOO⁻. In this way, excess ONOO⁻, formed through the interaction of NO with O₂^{-•} (see above in Section 1.5.2, *NO Bioavailability and Vascular Oxidative Stress: Original Findings*), has been shown to cause oxidation-nitration and inactivation of endothelial PGI synthase in particular (317), the inhibition of which has been found to augment EDCF signaling activity in diabetic, SHR, and other states of CVD risk via a build up of COX-derived endoperoxides (not metabolized by PGI synthase) and their subsequent ‘spillover’ through other PGSs to produce potent vasoconstrictive PGs, such as PGF_{2α} and PGE₂, instead of PGI₂ (105, 139, 140). Whether alterations in endothelial intracellular Ca²⁺ handling, the expression/content of PLA₂ or PGSs, or the non-content- and non-substrate-related activation of PLA₂, COX, or other PGSs may be adversely affected by excess oxidative stress under CVD risk conditions remains largely unknown.

Potential Role of ROS in Chronically Regulating EDCF Activity via VSM TP Receptor-Mediated Signaling. Of note, in the described study of aging Long-Evans type-II diabetic vasculature (239), it was also demonstrated that the VSM TP receptor-dependent contractile response to exogenous PGE₂ (but not U46619, PGF_{2α}, or PGI₂) was moderately augmented in diabetic mesenteric arteries, and could be normalized by the chronic in vivo Losartin treatment. Similarly, TP receptor hyper-sensitivity has been found in the femoral artery VSM of STZ-induced type-I diabetic SD rats, and could be normalized by chronic in vivo Apocynin treatment (298), suggesting that chronic exposure to excess ROS and oxidative stress in states of CVD risk could play a role in regulating the EDCF signaling axis by directly affecting VSM TP receptors and/or part(s) of their contractile signaling pathways.

Taken together, the limited evidence available appears to support a possible role for both ROS-mediated and non-ROS-mediated mechanisms in the chronic regulation of the [Ca²⁺]_i-PLA₂-AA-COX-PGS-PG-TP receptor-contractile signaling axis and its over-activation under CVD risk

conditions, by putative control mechanisms both related and unrelated to COX protein expression.

In summary, in contrast to NO-mediated EDRF signaling activity, less is known regarding the endothelial-VSM cell-signaling pathways mediating EDCF activity (Figure 1.5) and the pathobiological mechanisms responsible for endothelial dysfunction caused by over-active EDCF signaling. COX-mediated EDCF activity can become prominent in both the macro-vasculature and micro-vasculature in many states of CVD risk, most notably aging and the 'pre-mature aging' conditions of essential hypertension and diabetes. The factors mediating EDCF signaling activity predominantly include a variety of PGs derived from AA metabolism by either COX-1 or COX-2 isoforms. These PGs selectively activate TP receptors, which beyond eliciting VSM contraction may also result in the promotion of a CVD phenotype and function, thus counter-acting the vaso-protective effects of NO; however, the VSM cell-signaling events responsible for EDCF-stimulated TP receptor-mediated contractile activity have not been investigated. While both the acute and chronic mechanisms remain to be elucidated, vascular COX-1 and/or COX-2 up-regulation, PG over-production, and perhaps also TP receptor hyper-sensitivity have been suggested as possible causes of over-active EDCF signaling, a component of which may involve ROS, thus representing a potential oxidative stress link between reduced NO-mediated EDRF activity and augmented EDCF activity under CVD risk conditions.

1.6 Common Carotid Artery: An Advantageous Model to Study Vascular (Dys)function and the Role of Endothelial Cells

The focus of all Studies in this thesis was on the CCA, wherein the in vitro EDRF and EDCF signaling activities, as well as the in vivo pressure-flow hemodynamics, were investigated in the SHR-WKY hypertension model and an Adult-Aging SD rat model (see above briefing, *Section 1.1. Thesis Briefing*; and below for details, *Section 1.7. Specific Rationale, Objectives, and Hypotheses of Thesis Studies*).

As described in the subsequent sub-sections, the CCA is a blood flow conduit to the cerebral circulation that is of importance both physiologically and clinically and experimentally is a highly accessible and advantageous arterial model to investigate (Figure 1.6), but is understudied particularly regarding EDCF signaling activity and the causes and potential consequences of vascular (dys)function.

1.6.1. Anatomy and Accessibility

CCA Anatomical Relations

There are two essentially identical CCAs. The *left CCA* emerges directly from the top-left side of the aortic arch, while the brachiocephalic artery emerging from the top-right side of the aortic arch, quickly branches in two, giving rise to the *right CCA* (and the right subclavian artery, which continues distally as the brachial artery, then radial and ulnar arteries, etc., supplying blood to the arm, forearm, and hand). The bilateral left and right CCAs, separated by a small interval containing the trachea, travel straight upwards in parallel, more superficial than deep, through the cervical (neck) region giving off no branches until the level of the third cervical vertebrae, where bifurcation of each CCA gives rise to internal and external carotid artery branches. The external branch of the CCA continues upward more superficially (external), giving off multiple branches supplying blood to the neck and facial tissues. The internal branch of the CCA continues deep (internal), giving off no branches before traveling through the carotid canal of the skull (cranium). The intra-cranial internal carotid artery terminates as a trifurcation, giving rise to the middle cerebral artery, the anterior cerebral artery, and the posterior communicating artery. The latter two arteries from each CCA form a major part of the Circle of Willis (along with the posterior cerebral arteries emerging proximally from the basilar artery, which is formed proximally by the joining of the left and right vertebral arteries, which in turn proximally emerge from the left and right subclavian arteries and travel upward through the transverse foramen of each cervical vertebrae, giving off multiple branches, before entering the cranium). Together with the middle cerebral arteries, the Circle of Willis supplies all blood flow to the brain (cerebrum).

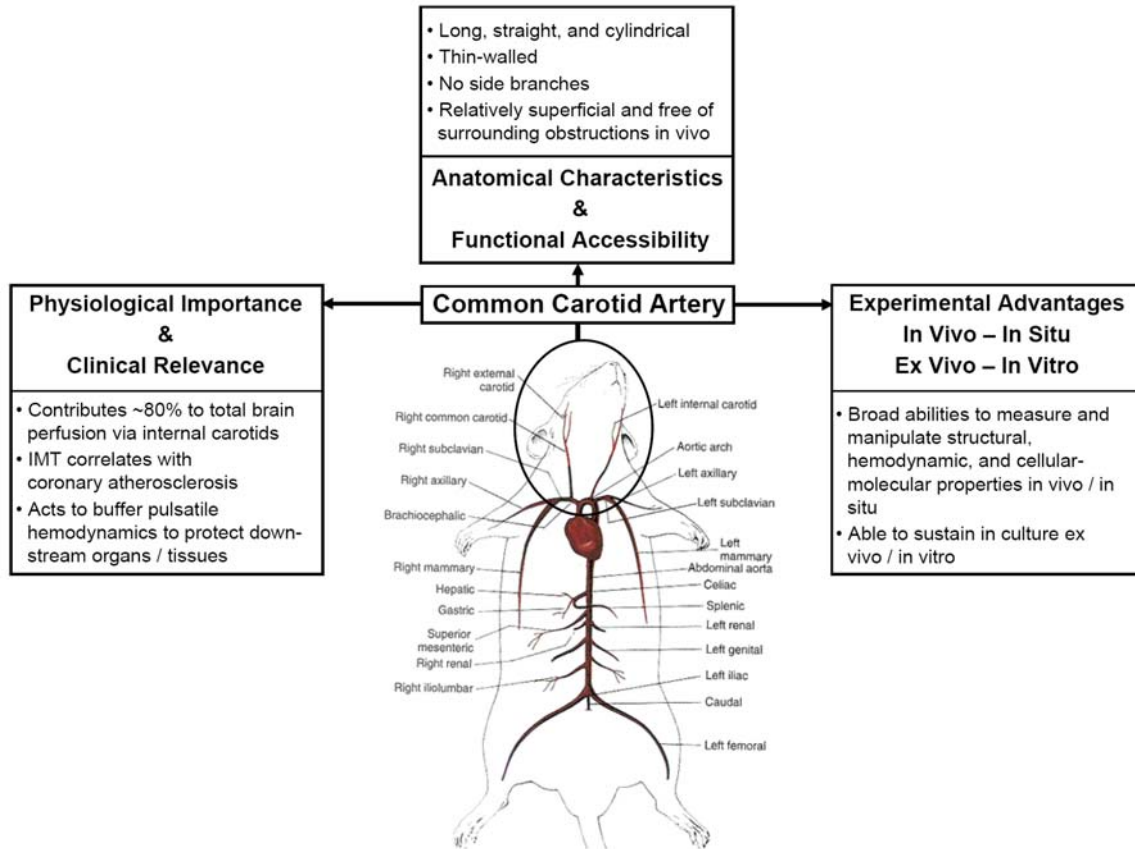


Figure 1.6. Features of the CCA. Refer to Section 1.6 for details. Image of rat arterial circulation from http://www.biologycorner.com/worksheets/rat_circulatory.html (accessed on 09/12/2010).

CCA Macro-Anatomical Characteristics and Functional Accessibility

The CCA is a relatively long, straight, cylindrical, and thin-walled conduit vessel segment with no side branches, and is relatively superficial and free of surrounding obstructions in vivo.

As is highlighted in this section, these characteristics are exceptionally favorable in regard to the ability to measure or manipulate the structural, hemodynamic, and/or cellular-molecular properties of this vessel both in vivo/in situ and ex vivo/in vitro, which has and will continue to allow for a better understanding of cardiovascular function and dysfunction, and the roles played by the endothelium.

1.6.2. Potential Physiological-Pathophysiological Importance and Clinical Relevance

CCA IMT

High-resolution ultrasound may be used to image the CCA in humans, where accurate measurements can be made not only of artery internal diameter changes but also of arterial wall structures, most notably the thickness of the intima-media space (IMT). Increased CCA IMT has been shown to be a strong indicator of coronary atherosclerotic plaque development in various

patient populations (40, 373), demonstrating the clinical utility and potential pathophysiological importance of CCA wall structural alterations. Though in different vascular districts, a significant correlation has been found between CCA IMT and impaired agonist-stimulated vasodilation in the forearm of hypertensive patients (134).

CCA Blood Flow and Pressure: Conduit and Buffering Functions

Large arteries serve two important physiological functions: i) a conduit function, to provide passage of blood from the heart to the tissues; and ii) a buffering (Windkessel) function, to provide efficient dampening of pulsatile hemodynamic fluctuations caused by intermittent ventricular contraction, thus allowing for adequate and constant flow through to the arteriolar and capillary networks of downstream organs/tissues (279, 312).

Conduit Function. Blood flow through the bilateral internal branches of the CCA contributes ~80% to the total perfusion of the brain in both humans and rodents (100). In this way, the CCA is a physiologically important extra-cranial large artery acting as a conduit between the heart and the downstream cerebral micro-vasculature (also see above, *CCA Anatomical Relations*).

Buffering Function. Large artery buffering function is impaired with increased arterial stiffness (i.e. arteriosclerosis; a loss of compliance/distensibility in the elastic macro-vasculature; (284)), most prominent in states of hypertension and advancing age. Arterial stiffness is influenced by i) a change in intra-vascular pressure; and/or ii) alterations to the ‘intrinsic’ visco-elastic mechanical properties of the vascular wall, which are in turn influenced by: a) ‘passive’ structural changes (e.g. relative content of elastin, collagen, and VSM in media extracellular matrix (ECM); ECM geometry/integrity and its state of remodeling; absolute arterial wall mass and state of proliferation; and arterial wall permeability and viscosity); and b) an ‘active’ change in VSM tone (312).

Arterial stiffness can have important pathophysiological consequences (284). Increased arterial stiffness (decreased compliance/distensibility) decreases ventricular-vascular coupling efficiency thus increasing LV after-load and myocardial perfusion (O₂) requirements. As well, flow pulsations are transmitted further into habitually vasodilated organ beds, most notably the brain and kidneys, where the pulsatile energy must be dissipated by more fragile micro-vascular structures causing damage leading to micro-hemorrhaging and micro-infarcts (156, 285, 286). The speed of the peripherally traveling incident pressure wave, characterized as *pulse wave velocity (PWV)*, is greater with increased arterial stiffness, as are the pressure waves reflecting back from distal sites of vascular impedance (i.e. discontinuous regions including branch points,

areas of altered compliance, and high-resistance arterioles), which in turn augments central arterial pressure in systole rather than in diastole, characterized as a *pressure augmentation index (AI)* (279), thus exacerbating LV after-load while decreasing diastolic myocardial perfusion. Together, these consequences may become clinically relevant as complications relating to heart failure, cardiomyopathy, and ischemic heart disease as well as intellectual deterioration and renal failure. Accordingly, measures of arterial stiffness/compliance have been found to predict future CVD events (10, 248).

Clinically, arterial stiffness is routinely evaluated by PWV as well as pressure AI using the CCA (10). Aortic (or carotid-femoral) PWV is assessed by simultaneously measuring CCA and femoral pressure waveforms with surface arterial tonometry (then calculating the estimated difference in measurement site distance over the time-delay of the measured waves), and contour analysis of the CCA pressure waveform allows for assessment of forward and backward traveling reflected waves and AI. Moreover, direct measures of CCA compliance (i.e. change in diameter for a change in pressure) can be assessed using high-resolution ultrasound imaging and arterial tonometry, where decreased CCA compliance has been found to correlate with impaired brachial artery FMD or agonist-stimulated forearm vasodilation (5, 219).

1.6.3. Experimental Animal Investigations: Advantages From In Vivo/In Situ to Ex Vivo/In Vitro

Because of its described favorable anatomical characteristics and accessibility (see above, *CCA Macro-Anatomical Characteristics and Functional Accessibility*), the CCA has been widely employed to investigate a range of candidate CVD risk conditions and the causes and potential consequences of arterial (dys)function. Below, some examples are highlighted.

In Vitro-In Situ Mechanical Properties of Large Arteries

The fact that the CCA is relatively long, straight, and cylindrical with no side branches makes it an ideal vascular model for cannulation and detailed mechanistic examinations of large artery stiffness/compliance and its relationship to the visco-elastic mechanical properties of the vascular wall (312). In vitro, an excised and cannulated CCA segment can be exposed to step-increases or -decreases in pressure, or else to pulsatile pressure, while measuring wall internal diameter changes using very high-frequency ultrasonic echo-tracking, thus allowing for a precise 'dose'-dependent characterization of both static (pressure- and structural/active tone-dependent) and dynamic (also viscosity- and frequency-dependent) compliance properties. Furthermore, given that the CCA is relatively superficial and free of surrounding obstructions in vivo, it can be instrumented to perform similar measures in situ under more physiological conditions of

operating pressure and blood-borne factors (429). Indeed, such approaches (217, 269, 312) have led to the definitive demonstration that VSM tone, the presence of an intact endothelium, and the production and availability of NO may acutely and directly affect large artery stiffness/compliance in both animals and humans (99, 412, 413), before which time only passive distending pressure and inert structural components of the vascular wall were believed to play a role (see above, *Buffering Function*).

In Vivo Manipulations Creating Models of Arterial Remodeling or Endothelial Regeneration

Taking advantage of the two essentially identical, easily accessible, and relatively simple to manipulate CCAs, multiple in vivo large artery response-to-perturbation models have been developed. For example, ligation of both internal and external carotid arteries of one CCA during recovery surgery results in a low/no-flow CCA and a high-flow CCA with no associated pressure changes, thus creating an internally controlled chronic in vivo model of flow-induced arterial remodeling about which to mechanistically investigate in any number of healthy or diseased animal models (168, 169). Alternatively, insertion of a balloon catheter through the external carotid artery of one CCA followed by balloon inflation and luminal agitation during recovery surgery results in endothelial denudation of one but not the other CCA, thus creating an internally controlled chronic in vivo model of endothelial regeneration about which to mechanistically investigate (379). Such models and others' similar (63, 377) have indeed led to the discovery of key roles for NO and numerous other endothelium-derived factors in controlling the vascular wall structural and functional alterations known to be consequential in states of CVD risk (109, 213).

Ex Vivo Arterial Organ Culture and Pro-Atherogenic Mechanisms

Considering together that the bilateral CCAs are straight and cylindrical with no side branches, can be manipulated and characterized under in vivo conditions, and are relatively thin-walled, this arterial segment has become a commonly used model for ex vivo organ culture under specific (patho)physiologically-relevant hemodynamic and biochemical conditions (121, 303, 364). The CCAs may be easily excised, cannulated, and perfused with nutrient-rich medium, one under candidate CVD risk conditions and the other under control physiological conditions, for a period of up to several days. Such approaches have been successful in chronically examining the specific mechanical effects of either flow profiles (i.e. high/normal or low/oscillatory shear stress; (121)), hypertension (i.e. increased intra-vascular pressure; (214, 303)), or reduced vascular wall motion (i.e. decreased compliance/cyclic stretch; (364)) on large artery phenotype and function in isolation from confounding in vivo factors (e.g. neuro-humoral alterations, other CVD risk

factors), and in testing the role of endothelial inflammatory activation on the molecular expression and pro-atherogenic consequences induced within the vascular wall (121, 303).

In Vivo Selective Gene Transfer

For endothelial genes of interest, including eNOS and many others, it has been shown that incubation of the rodent CCA lumen for 15 min with a gene delivery medium, which is infused through the external carotid artery into the CCA space contained by temporary clamping of the proximal CCA and internal carotid artery, results in the selective up-take and expression of the delivered gene only by the one CCA segment incubated and only by the endothelial cell layer of the vascular wall (166). Indeed, this represents a direct and powerful molecular manipulation that may be used in any number of ex vivo organ culture and/or in situ or in vivo animal models to test the specific effect of endothelial cell expressed proteins on vascular (dys)function within the context of an intact vascular wall or animal, respectively.

Thus, the CCA can indeed be an advantageous model to study and understand vascular (dys)function and the roles played by the endothelium.

1.6.4. CCA Under-Studied in Particular Regarding the Causes and Potential Consequences of EDCF Signaling Activity

SHR, WKY, and SD animals are commonly used models to investigate cardiovascular dysfunction under conditions of CVD risk and the roles played by the endothelium. However, while NO-mediated and EDHF-mediated EDRF signaling activities have been a major focus of investigation in the CCA (and most other vascular segments) of these and many other animal models of CVD risk (109), the contribution of COX- and TP receptor-mediated EDCF signaling activity to the CVD phenotype and function that can develop in this vascular segment has not (see above, *COX-Mediated EDCF Responses Contributing to Endothelial Dysfunction in Other SHR Vascular Segments, in Other Animal Models of CVD Risk, and in Humans*). Accordingly, the causes and potential consequences of EDCF signaling activity are largely unknown.

1.7 Specific Rationale, Objectives, and Hypotheses of Thesis Studies

1.7.1. Review and Expansion of Thesis Global Purpose

Global Purpose

As declared in the Thesis Briefing (Section 1.1), the global purpose of this thesis was to gain a better understanding of the cellular-molecular mechanisms accounting for endothelial dysfunction in isolated CCA from animal models known to be characteristic of EDCF signaling activity, in particular essential hypertension and aging.

A further aim was to describe the *in vivo* hemodynamic characteristics of the CCA in each animal model investigated, serving both to identify the pressure-flow environment that the CCA is exposed to *in vivo* and to provide novel assessment of hypertension, aging, and oxidative stress effects on large artery hemodynamics.

Global Objective and Approach Spanning Across Thesis Studies

Under this global purpose, the global objective and approach spanning across the thesis studies was: i) to systematically examine the relative contributions of NOS and COX signaling pathways in mediating the endothelium-dependent relaxation (EDRF) and contractile (EDCF) activities of isometrically-mounted CCA *in vitro*, with particular focus on elucidating the cellular-molecular mechanisms responsible for COX-mediated EDCF signaling activity; and ii) to establish the *in vivo* pulsatile blood pressure and blood flow characteristics of the CCA.

Animal Models Investigated

The animal models investigated under this global purpose and objective included: i) SHR and WKY (in Thesis Study I and II); and ii) Adult and Aging SD rats (in Thesis Study III), both models in which COX-mediated EDCF signaling activity had been previously identified, but not in the CCA. In Adult and Aging SD rats, a chronic GSH anti-oxidant depletion model of oxidative stress was super-imposed.

Preface to Thesis Study I, II, and III

The subsequent sub-sections provide underlying rationale and the main specific objectives and hypotheses for the experiments conducted throughout each thesis study. This is immediately followed by the Experimental Chapters of this thesis, wherein Chapter 2, 3, and 4 presents a manuscript describing Thesis Study I, II, and III, respectively.

1.7.2 Rationale, Objectives, and Hypotheses of Thesis Study I

Described in Chapter 2, Thesis Study I is presented (with permission; see Permission's Pages) as the published article entitled:

Impaired Hemodynamics and Endothelial Vasomotor Function via Endoperoxide-Mediated Vasoconstriction in the Carotid Artery of Spontaneously Hypertensive Rats

found in the April 2009 issue of *American Journal of Physiology: Heart and Circulatory Physiology*.

The **overall objective** of Thesis Study I was, in young adult SHR and WKY, to: i) characterize the in vivo pulsatile blood flow and blood pressure of the CCA across the cardiac cycle; and ii) systematically investigate the relative contribution of the NOS and COX signaling pathways in mediating ACh-stimulated endothelium-dependent CCA relaxation and contractile activities.

Underlying Rationale

Original Focus on CCA. An original major objective of the experimental work in this thesis was to design, construct, and implement an Ex Vivo Arterial Organ Culture System (see above, *Ex Vivo Arterial Organ Culture and Pro-Atherogenic Mechanisms*) to be used to examine the isolated mechanical effects of hypertensive and pro-atherogenic flow/shear hemodynamics on the phenotype and endothelium-dependent vasomotor functions of the CCA from donor SD, WKY, and SHR animals¹. However, upon testing the system that was developed, a number of unforeseen issues became apparent which precluded its reasonable implementation as proposed².

A new direction was taken from this work that took advantage of techniques the Candidate had been establishing, in concert with the Ex Vivo Arterial Organ Culture System, to measure the in vivo pulsatile hemodynamics and in vitro endothelial vasomotor function of WKY, SHR, and SD rat CCA.

Insights from Preliminary CCA Hemodynamic and Vasomotor Function Data. Indeed, there had been very little published data characterizing the pulsatile hemodynamics through the CCA of these animals, and since this knowledge was needed to culture the CCA under

¹ as is detailed in the Candidate's original Doctoral Thesis Proposal, entitled: *Examining the Role of Hypertensive Hemodynamics on Common Carotid Artery Phenotype and Function*.

² Issues with the developed Ex Vivo Arterial Organ Culture System related to both the biochemical and mechanical stability of the culture environment (including pH and pressure drifting), which were associated with comprised functional viability (as assessed by evaluating its in vitro endothelium-dependent vasomotor functions) of CCA segments cultured for 24-36h under 'control' conditions – i.e. at physiological levels of pulsatile pressure and flow/shear in 37°C nutrient-rich culture medium at 0.04 poise viscosity (note: there were also some technical complexities related to controlling pressure and flow independent of pulsatility and rate).

(patho)physiological pressure and flow/shear conditions, methods were developed in the laboratory to accurately quantify the in vivo pulsatile components of pressure and flow through the CCA of WKY and SHR (and SD animals) (for details, see Study I *Methods* and Appendix B: *Supplemental Methods / CCA Hemodynamics / Rationale for Procedural Approach and Quantification of CCA Blood Flow and Pressure*). Similarly, there had been little published data characterizing endothelial vasomotor function in the CCA of these animals because conduit endothelial vasomotor function is typically measured in the aorta. Since endothelial vasomotor function was to be a main outcome measure after CCA culture, methods were adapted from those used in the laboratory to measure aortic in vitro endothelial vasomotor function (114, 143) in order to characterize endothelial vasomotor function in the CCA of WKY and SHR (and SD animals) (for details, see Study I *Methods* and Appendix B: *Supplemental Methods / CCA Vasomotor Function*).

In preliminary hemodynamic testing, it was unexpectedly found that mean blood flow was reduced in the CCA of young adult SHR compared to WKY. Carefully searching the literature, it was discovered that this was consistent with two reports comparing CCA blood flow – either mean blood flow velocity (76) or peak systolic blood flow (166) – in hypertensive (SHR) and normotensive (Wistar or WKY) rodents (as referenced in the *Introduction* to Thesis Study I). However, this occurrence of reduced CCA blood flow in SHR had not been well-characterized. This provided motivation to further investigate and characterize the pulsatile flow and pressure responses across the cardiac cycle in the CCA of SHR and WKY, which therefore became a main objective of Thesis Study I. Preliminary hemodynamic analysis found that SHR CCA blood flow was reduced at peak systole and throughout diastole; however, unexpectedly, flow during late systole was found to be relatively higher in SHR compared to WKY (see *Results* of Thesis Study I for details). It was speculated that this could have resulted from a forward traveling reflected pressure wave acting to prolong the decay of systolic flow (279). Carefully searching the literature, it was discovered that ‘flow augmentation’ in late systole had in fact been observed in the CCA of aged humans and attributed to pressure wave reflection returning from the lower body (and thus a forward traveling wave) in systole to augment CCA pulse pressure ((156); also see above, *CCA Blood Flow and Pressure: Conduit and Buffering Functions*). This provided motivation to assess the inter-relationship between CCA pressure and flow waveforms in SHR and WKY (as described in the *Methods*, *Results*, and *Discussion* of Thesis Study I).

In preliminary vasomotor testing, it was found that endothelium-dependent vasorelaxation of isolated and pre-contracted SHR CCA was impaired compared to WKY. This was consistent with past reports in the literature assessing the vasorelaxation properties of the CCA from

similarly aged (young adult) animals (158, 166, 228), where the dysfunction in SHR CCA was assumed to be the result of impaired NOS-mediated EDRF activity (see above in Section 1.5.3 – *COX-Mediated EDCF Responses Contributing to Endothelial Dysfunction in Other SHR Vascular Segments, in Other Animal Models of CVD Risk, and in Humans*). However, inconsistent with past works, the impaired vasorelaxation was found to be associated with overt re-contraction to higher concentrations of the endothelial cell stimulant ACh, indeed suggesting that the CCA of young adult SHR may possess EDCF activity (see above in Section 1.5.3 – *Endothelium-Dependent Contractile Responses: Initial Findings and Characterization of EDCF Cell-Signaling Pathway*). In the aorta of SHR, EDCF activity had been shown to be mediated by COX signaling (see above in Section 1.5.3 – *SHR Aorta: The First Reporting of an EDCF Response Impairing Endothelium-Dependent Vasorelaxation*). This provided motivation to further investigate and characterize the relative contribution of NOS and COX pathways in mediating endothelium-dependent relaxation in the CCA of SHR and WKY, with a particular focus on characterizing SHR CCA EDCF activity under specific conditions (i.e. from a quiescent state in the presence of L-NAME; see above in Section 1.5.3 – *Identification and Specific Examination of COX-mediated EDCF Responses and Signaling Activities*) and its dependency on COX-1 vs. COX-2 and the TP receptor (see above in Section 1.5.3 – *Endothelium-Dependent Contractile Responses: Initial Findings and Characterization of EDCF Cell-Signaling Pathway*), which therefore became a second main objective of Thesis Study I.

Two past observations (referenced in the *Introduction* to Thesis Study I) provided motivation to consider SHR CCA endothelial vasomotor dysfunction and EDCF activity in conjunction with CCA hemodynamics in Thesis Study I. First, the same study that found reduced peak systolic blood flow in the CCA of SHR (166) also found that ACh-stimulated vasorelaxation was impaired in isolated pre-contracted SHR CCA, and further showed that selective *in vivo* gene transfer of AKT (see above in Section 1.5.2. – *NO Production and eNOS Activation*) to the CCA endothelium could reverse both the endothelial vasomotor dysfunction and reduced blood flow in the SHR CCA, thus suggesting that endothelial vasomotor dysfunction in the CCA might directly affect CCA resistance to flow, presumably by increasing its compliance (decreasing its stiffness) (166). The second observation, from other researchers, was that the diameter and compliance of young adult SHR and WKY CCA, assessed at physiological pressures *in situ* or *in vitro* (see above in Section 1.6.3 – *In Vitro-In Situ Mechanical Properties of Large Arteries*), could be increased with abolition of VSM tone (with potassium cyanide) or with removal of the endothelium (57, 217, 269), suggesting that the mechanical properties of the CCA may be affected by vasomotor activity related to endothelium-derived vasocontractile signal(s). In light of

these observations, the potential that CCA EDCF signaling activity could affect CCA blood flow was considered in Thesis Study I (see *Discussion / Perspectives*), highlighting the prospect that local (CCA) endothelial vasomotor dysfunction might, via altered mechanical properties of the artery wall, be linked directly to CCA hemodynamics.

Main Specific Objectives and Hypotheses

The following provides a descriptive overview of the main specific objectives and hypotheses of each set of experiments conducted throughout Thesis Study I, from the experiments originally conducted to those performed in response to the findings of the original experiments.

Objective A: To establish the in vivo pulsatile blood pressure and blood flow characteristics of the CCA.

Hypothesis A: It was hypothesized (originally) that mean, systolic (maximum), diastolic (minimum), and pulse pressure would be greater in SHR vs. WKY, but that blood flow parameters would be no different between strains.

Objective B: (Given that mean, maximum, and minimum blood flow were reduced in SHR) to examine the blood flow and blood pressure response across the cardiac cycle.

Hypothesis B: It was hypothesized (originally) that SHR blood flow would be persistently reduced across the cardiac cycle compared to WKY.

Objective C: (Given that late systolic blood flow was relatively augmented in SHR) to assess the inter-relationship between pressure and flow waveforms across the cardiac cycle.

Hypothesis C: It was hypothesized that late systolic blood flow augmentation in SHR would accompany systolic pressure augmentation.

Objective D: To establish the dose-dependent ACh-stimulated endothelium-dependent NO-mediated relaxation response in PE-pre-contracted WKY and SHR CCA.

Hypothesis D: It was hypothesized (originally) that both sub-maximal and maximal vasorelaxation would be blunted in SHR vs. WKY and that this would be normalized by L-NAME.

Objective E: (Given that sub-maximal vasorelaxation was not blunted, maximal blunting was associated with overt re-contraction in SHR, and L-NAME did not normalize this difference) to determine the effect of Indo on the ACh-stimulated vasomotor responses.

Hypothesis E: It was hypothesized that Indo might normalize the difference between SHR and WKY.

Objective F: (Given that Indo almost fully restored maximal vasorelaxation in SHR) to specifically examine the dose-dependent ACh-stimulated endothelium-dependent contractile response (EDCF activity) in SHR CCA and its dependency on COX-1 vs. COX-2 and the TP receptor.

Hypothesis F: It was hypothesized that EDCF activity might be eliminated by the preferential COX-1 inhibitor VAS and the TP receptor antagonist SQ29548, but possibly not by the preferential COX-2 inhibitor NS398.

Objective G: To determine the level of eNOS, COX-1, and COX-2 protein expression in the CCA of SHR and WKY.

Hypothesis G: It was hypothesized that eNOS and COX-1, and possibly COX-2 might be over-expressed in the SHR CCA.

1.7.3 Rationale, Objectives, and Hypotheses of Thesis Study II

Described in Chapter 3, Thesis Study II is presented (with permission; see Permission's Pages) as the published article entitled:

RhoA-Rho Kinase Signaling Mediates Endothelium- and Endoperoxide-Dependent Contractile Activities Characteristic of Hypertensive Vascular Dysfunction

found in the May 2010 issue of *American Journal of Physiology: Heart and Circulatory Physiology*.

The **overall objective** of Thesis Study II was to confirm and expand upon the vasomotor findings of Thesis Study I (which established the SHR CCA as an bona fide experimental model to explore the mechanisms of EDCF signaling activity in endothelial dysfunction) by further exploring the cell-signaling mechanisms of EDCF activity in young adult SHR vs. WKY CCA in regard to: i) the role of ROS and COX-derived PG production; and, in particular, ii) the role of the RhoA-ROCK pathway in EDCF-stimulated TP receptor-mediated contraction.

Underlying Rationale

Decision to Focus on CCA EDCF Signaling Activity. In consideration of the hemodynamic and vasomotor findings of Thesis Study I, a decision was made to focus on further exploring the cell-signaling mechanisms of the EDCF activity identified for the first time in the SHR-WKY CCA model. The decision not to actively pursue the novel hemodynamic findings of Thesis Study I [i.e. i) the reduced peak systolic and diastolic CCA blood flow in SHR; and/or ii) the SHR CCA late systolic flow augmentation], or the prospect that COX- and TP receptor-mediated EDCF activity in the CCA of SHR might contribute to shifting its mechanical properties thus affecting

CCA blood flow (see Thesis Study I, *Perspectives*), was partially based on the fact that the technical resources and expertise required to do so definitively (e.g. in the latter case, selective gene transfer to the endothelium in conjunction with in situ/in vivo assessment of stiffness/compliance using high-resolution echo-tracking; see Thesis Study I, *Limitations*) did not at the time make this a reasonable next step in the thesis.

Further Exploration of CCA EDCF Signaling Mechanisms. There were two main motivations for the direction of further exploration of the SHR CCA EDCF signaling activity identified in Thesis Study I. The first related to the possible mechanisms of EDCF production, and the second to the potential mechanism of EDCF activation of VSM contractile activity.

Past works exploring the mechanisms of EDCF production had demonstrated the possible involvement of either COX-1 or COX-2 and the potential for multiple ‘factors’ acutely mediating the EDCF response, including a variety of different PGs as well as a variety of different ROS, depending on the particular endothelial stimulant, vascular segment, or animal model used (see above in Section 1.5.3 – *Mechanisms of EDCF (Over)Production: Critical Role of Regulating the PG Synthesis Pathway* and – *Acute Role of ROS in EDCF Signaling Activity*). Thus, following from the findings of Thesis Study I, it became an objective of Thesis Study II to further elucidate the mechanisms of EDCF production in SHR (and WKY) CCA by testing another endothelial stimulant, confirming the dependency of COX-1 vs. COX-2, exploring which PG(s) are released upon endothelial stimulation, and establishing what role might be played by ROS.

Relating to EDCF activation of VSM contractile activity, the potential mechanism(s) by which this occurs had not previously been investigated in any vascular segment (see above in Section 1.5.3 – *Mechanisms of EDCF Activation of VSM Contraction: Role of TP Receptor Signaling Activity*). It became the major objective of Thesis Study II to test the hypothesis that EDCF-stimulated VSM contractile activity is mediated through activation of RhoA-ROCK signaling (see above in Section 1.5.3 – *Potential VSM GPCR-Mediated Contractile Signaling Pathways* for basic description of RhoA-ROCK signaling pathway). Consideration of this hypothesis was based (directly or indirectly) on several different observations of the previous literature: i) it was known (and confirmed in SHR CCA in Thesis Study I) that TP receptors mediate essentially all EDCF activity in all studies where it has been identified (as described above in Section 1.5.3 – *PG Receptor Involvement*); ii-a) in various cell types, TP receptors, part of the GPCR family, may be coupled to both the G_q class of G-protein, which can activate PLC-IP₃-SR- Ca²⁺ signaling, and the G_{12/13} class of G-protein, which can activate Ca²⁺-independent RhoA-ROCK signaling (as described in the *Introduction* to Thesis Study II, and above in Section 1.5.3 – *Potential VSM GPCR-Mediated Contractile Signaling Pathways*); and ii-b) in VSM in

particular, TP receptors are often preferentially coupled to G_{12/13} (342); iii-a) it had been established in endothelium-denuded and/or membrane permeabilized arterial segments that direct TP receptor PG agonist stimulation could elicit Ca²⁺-independent contraction associated with both membrane-bound RhoA activation and activated-ROCK-mediated MYPT1 phosphorylation ((414); as referenced in the *Introduction* to Thesis Study I); and iii-b) in endothelium-denuded arterial segments that direct exposure to exogenously-produced ROS could elicit contraction associated with RhoA and ROCK activation responses ((177); as referenced in the *Introduction* to Thesis Study I); iv-a) NO-mediated signaling activity can acutely inhibit EDCF activity at the level of the VSM via sGC (as described above in Section 1.5.3 – *Balancing EDRF and EDCF Signaling Activities to Prevent or Promote Endothelial Dysfunction*), and sGC-cGMP-PKG signaling activity has been shown to prevent GPCR-mediated contraction via phosphorylation and inhibition of RhoA (316); and iv-b) conversely, activated RhoA-ROCK has been shown to inhibit NO production via phosphorylation and inhibition of eNOS ((260); referenced also above in Section 1.5.2 – *NO Production and eNOS Activation*); v) activated RhoA and/or ROCK has been found in the conduit artery wall from SHR and other models of CVD risk (324) and is associated with the expression of a pro-atherogenic/proliferative/fibrotic phenotype (320) (see also *Discussion* of Thesis Study II); and vi) acute infusion of ROCK inhibitors is associated with a decrease in blood pressure in SHR (222, 272) and improved vasomotor function in the forearm or coronary circulation of patients with essential hypertension or in other states of CVD risk (192, 236, 237, 261).

Tools and Potential Caveats to Directly Testing this Hypothesis. The main approach to testing the hypothesis that EDCF-stimulated VSM contractile activity is mediated through activated RhoA-ROCK signaling in Thesis Study II was by examining the dose-dependent effect of the commonly used selective ROCK inhibitor Y27632 on the ACh-stimulated contractile response in the CCA of SHR and WKY. However, of this approach were known (a priori) a number of putative concerns that may have precluded direct testing of the hypothesis; accordingly, these concerns were considered experimentally, as briefly reasoned below (and referenced within the *Results* and *Discussion* of Thesis Study II).

First, as is already standard for specifically evaluating EDCF activity, the effect of ROCK inhibition on the ACh-stimulated contractile response was evaluated in the presence of L-NAME, thus excluding any attenuating effect of ROCK inhibition indirectly via changes in NO bioavailability (see iv-a and iv-b above).

Second, even if the selective ROCK inhibitor Y27638 did dose-dependently attenuate EDCF activity, this effect may not have been directly caused by inhibiting activation of VSM

RhoA-ROCK signaling, since Y27638 (especially higher concentrations) has been shown to non-selectively inhibit several isoforms of PKC, which can also become activated and elicit VSM contraction upon GPCR stimulation ((414); also see above in Section 1.5.2 – *Potential VSM GPCR-Mediated Contractile Signaling Pathways*). Accordingly, the effect of a more potent ROCK inhibitor H1152 was also evaluated, as was the effect of a broad-spectrum PKC inhibitor (GF109203X).

Third, even if Y27638 and H1152 did only (specifically) inhibit ROCK in the CCA wall, the attenuating effect of these inhibitors on EDCF activity may not have been directly caused by inhibiting activation of VSM RhoA-ROCK signaling, since RhoA and ROCK are also known to be expressed in endothelial cells (223); thus, it is possible that this attenuating effect may have been caused by reducing EDCF production by inhibiting activation of endothelial RhoA-ROCK signaling. Accordingly, the effect of ROCK inhibition on ACh-stimulated CCA PG production was evaluated.

Forth, the effect of ACh stimulation on CCA RhoA activation itself was evaluated. In this way, it has been shown that VSM ROCK signaling can become activated by factors other than RhoA (including AA or sphingosylphosphorylcholine (342, 348); also see Section 1.5.3 – *Potential VSM GPCR-Mediated Contractile Signaling Pathways*); thus, it appears possible that an attenuating effect of ROCK inhibitors on EDCF activity, even if specific to ROCK and to the VSM, may not necessarily imply involvement of RhoA activation in the response.

Main Specific Objectives and Hypotheses

The following provides a descriptive overview of the main specific objectives and hypotheses of each set of experiments conducted throughout Thesis Study II.

Objective A: To determine the dose-dependent effect of the receptor-independent endothelial stimulant A23187 on CCA contractile activity in SHR and WKY.

Hypothesis A: It was hypothesized that the pattern of A23187-stimulated EDCF activity would be similar to that of ACh in both strains.

Objective B: To determine the effect of the specific COX-1 inhibitor SC560 on ACh-stimulated EDCF activity in SHR and WKY (as compared to the preferential COX-1 inhibitor VAS and the preferential COX-2 inhibitor NS398 used in Thesis Study I).

Hypothesis B: It was hypothesized that SC560 would be effective in eliminating all EDCF activity in both strains.

Objective C: To determine the effect of the cell-permeable agents apocynin (an NADPH oxidase inhibitor), Tiron, (an O₂^{•-} scavenger), Tempol (considered an SOD mimetic), and PEG-CAT (a H₂O₂-metabolizing enzyme) on ACh-stimulated ED CF activity in SHR and WKY.

Hypothesis C: It was hypothesized that Tiron, apocynin, and PEG-CAT might attenuate ED CF activity, while Tempol might augment ED CF activity, in SHR, and possibly also (but to a lesser extent) in WKY.

Objective D: To determine the basal (quiescent) and ACh-stimulated COX-1-derived production of PGI₂ and TXA₂ from the CCA of SHR and WKY.

Hypothesis D: It was hypothesized that: i) the CCA would produce PGI₂ and possibly TXA₂ in a COX-1-dependent manner; ii) both the basal and ACh-stimulated CCA production of these PGs would likely be greater in SHR vs. WKY; and iii) the level of ACh-stimulated CCA PG production might significantly correlate with the concomitant ACh-stimulated contractile response.

Objective E: To determine the effect of (a high-dose of the ROCK inhibitor) H1152 on ACh-stimulated PGI₂ production from the CCA of SHR and WKY.

Hypothesis E: It was hypothesized that H1152 would likely not affect, but if so reduce, ACh-stimulated CCA PGI₂ production in both strains.

Objective F: To determine the basal (quiescent) and ACh-stimulated activation of RhoA in the CCA of SHR and WKY.

Hypothesis F: It was hypothesized that: i) both the basal and ACh-stimulated RhoA activation would likely be greater in SHR vs. WKY; and ii) the level of ACh-stimulated CCA RhoA activation might significantly correlate with the concomitant ACh-stimulated contractile response.

Objective G: To determine the level of RhoA and ROCK-II protein expression in the CCA of SHR and WKY.

Hypothesis G: It was hypothesized that RhoA and ROCK-II would either be similar or over-expressed in the CCA of SHR vs. WKY.

Objective H: To functionally determine the role of ROCK signaling in SHR and WKY CCA contractile activity by assessing the dose-dependent effect of (the ROCK inhibitor) Y27632 on sustained CCA contraction stimulated by either the depolarizing agent KCl (via electro-mechanical coupling) or the GPCR agonists PE (an α₁ receptor-specific agonist) or U46619 (a

synthetic TP receptor agonist) (via pharmaco-mechanical coupling), either in the presence or absence of an intact endothelium, or in the presence an intact endothelium with L-NAME.

Hypothesis H: It was hypothesized that: i) Y27632 would be capable of eliciting vasorelaxation in all cases, but to a greater extent under PE and even more so U46619 contractile conditions; ii) this stimulated vasorelaxation might be mediated in part by endothelium- and NO-dependent signaling; iii) this stimulated vasorelaxation might be augmented in SHR vs. WKY.

Objective I: To determine the dose-dependent effect of (the selective ROCK inhibitor) Y27632 and (the more potent selective ROCK inhibitor) H1152, and high-dose effect of (the broad-spectrum PKC inhibitor) GF109203X, on ACh-stimulated EDCF activity in the CCA of SHR and WKY.

Hypothesis I: It was hypothesized that: i) Y27632 would cause a dose-dependent attenuation of EDCF activity in both strains; ii) GF109203X might attenuate EDCF activity; and iii) H1152 would cause a dose-dependent, but maybe less effective, attenuation of EDCF activity.

Objective J: To determine in SHR and WKY CCA the dose-dependent contractile response stimulated by exogenous H₂O₂, and the effect of (the COX-1 specific inhibitor) SC560, (the preferential COX-2 inhibitor) NS398, (the TP receptor antagonist) SQ29548, (a high-dose of the ROCK inhibitors) Y27638 and H1152, and (the broad-spectrum PKC inhibitor) GF109203X.

Hypothesis J: It was hypothesized that H₂O₂-stimulated contractile activity would be augmented in SHR vs. WKY, and in both cases eliminated by COX-1 (but not COX-2) inhibition and TP receptor antagonism as well as by ROCK inhibition, and possibly attenuated in part by PKC inhibition.

1.7.4 Rationale, Objectives, and Hypotheses of Thesis Study III

Described in Chapter 4, Thesis Study III is presented as the (unpublished) manuscript-article entitled:

Effect of Glutathione Depletion and Aging on Endothelium-Derived Relaxing and Contracting Factor Activity in the Common Carotid Artery of Sprague Dawley Rats

The **overall objective** of Thesis Study III was to: i) characterize the in vivo pulsatile blood flow and blood pressure of the CCA; and ii) investigate the contributions of NOS, COX, and ROS signaling in mediating ACh-stimulated CCA EDRF and EDCF activities, in Adult and Aging SD rats treated with L-buthionine sulfoximine (BSO) to chronically deplete the cellular anti-oxidant GSH.

Underlying Rationale

Decision to Focus on CCA EDRF and EDCF Signaling Activities of Adult and Aging GSH-Depleted SD rats. The decision to focus on exploring CCA EDRF and EDCF signaling activities of Adult and Aging chronically GSH-depleted SD rats in Thesis Study III was led by two main considerations: i) the opportunity to work with this model as part of a larger project undertaken by the laboratory; and ii) the data available in the literature regarding, in particular, EDCF activity, aging, and the potential roles of ROS and oxidative stress.

In regard to opportunity, a major ‘*BSO-Aging*’ study was to be conducted in the laboratory investigating ‘Adult’ (~30 wk old) and ‘Aging’ (~65 wk old) SD rats under ‘CON’ (control) conditions or treated for 10d with BSO, a compound able to deplete cellular GSH – a known chemical anti-oxidant and co-factor for the H₂O₂-metabolizing enzyme GPx (see above in Section 1.5.2 – *Determinants of Pro-oxidant/Anti-oxidant Balance in the Vascular Wall*; and both Figure 1.4 and 1.5). Previously, the laboratory had conducted a chronic BSO treatment study in ~20 wk old SD rats, demonstrating efficacy in depleting liver GSH (a hallmark of the model) and augmenting both liver and conduit vascular (aortic) ROS production, which was associated with two intriguing findings: i) no increase in blood pressure, in contrast to other works demonstrating a marked BSO-induced hypertensive effect in adult SD rats (388); and ii) only modest impairment in isolated aortic NO-mediated EDRF activity, in conjunction with an up-regulated eNOS, SOD, and sGC expression profile suggestive of a possible compensatory adaptation to the elevated oxidative stress (114) (see above in the Section 1.5.2. Review – *Consideration of Potential ROS-Mediated Compensatory Adaptations in Attempt to Prevent Endothelial Dysfunction*). Two subsequent works from separate laboratories found consistent hemodynamic findings in adult BSO SD rats combined with no attenuation (25), or a CAT-inhibitable (i.e. presumably involving H₂O₂) modest augmentation (173), in aortic NO-mediated EDRF activity, also suggesting a compensatory adaptation to elevated oxidative stress in this model. Accordingly, the BSO-Aging study provided an opportunity to further explore the effect of chronic in vivo BSO-induced GSH depletion on CCA hemodynamics and NOS-, COX-, and ROS-mediated EDRF signaling activity in the context of Aging SD rats that might not be as adaptable to oxidative stress, and thus potentially more susceptible to hemodynamic instability and endothelial dysfunction related to reduced NO bioavailability and EDRF activity.

Importantly, the BSO-Aging study also provided a unique opportunity to explore EDCF signaling activity, aging, and the acute and chronic roles of ROS and oxidative stress. Like essential hypertension, aging can be hallmarked by augmented COX-mediated EDCF activity, as has most clearly been demonstrated in both the SHR-WKY aortic model and in the forearm

vasculature of essential hypertensive patients-otherwise healthy individuals, where the endothelial dysfunction found in the hypertensive state appears to be reflective of a ‘pre-mature aging’ of the vascular wall (see above in Section 1.5.3. – *COX-Mediated EDCF Responses Contributing to Endothelial Dysfunction in Other SHR Vascular Segments, in Other Animal Models of CVD Risk, and in Humans*). In SD rat vasculature, EDCF responses are not as readily apparent as in SHR or aging WKY animal models. Yet, in the available literature, augmented COX- and TP receptor-mediated EDCF activity had been identified in the femoral artery of adult animals under chronic STZ-induced type-I diabetic conditions (328, 330) and also in the aorta (183), femoral artery (329), and small mesenteric arteries (345) of aging (50-60 wk old) vs. adult SD rats. Thus, it appeared possible that the CCA of SD rats in the BSO-Aging study might also be established as a model of EDCF signaling activity.

Regarding the role of GSH in EDCF signaling activity, there had been no previous investigations; however, in the available literature, an acute mediating (in particular by H₂O₂) and chronic regulatory role of excess ROS and oxidative stress in EDCF responses had been suggested under various CVD risk conditions in numerous animal vascular models: adult SHR aorta (423) and mesenteric arteries (399); adult AII-infused hypertensive Wistar rat aorta (181) and mouse mesenteric arteries (398); aging Long Evans type-II diabetic rat mesenteric arteries (239, 241); and adult STZ-induced type-I diabetic (330) or aging (329) SD rat femoral arteries (for details see above in Section 1.5.3 – *Acute Role of ROS in EDCF Signaling Activity* and – *Chronic Role of ROS and Oxidative Stress in EDCF Signaling Activity*, respectively). In this context, a proposed unique benefit of exploring the CCA COX- and ROS-mediated EDCF signaling activities of Adult and Aging SD rats under BSO-induced GSH depletion conditions was the possibility of more specifically, than in studies before, evaluating the acute and chronic role of ROS and oxidative stress in EDCF signaling activity. This was proposed since the oxidative stress induced in the BSO model, as compared to the oxidative stress conditions found in the described previous models studied – i.e. SHR, AII infused-hypertension, and diabetic models, was anticipated to be more independent of blood pressure or other overt discerning clinical features of CVD risk (e.g. hyperglycemia/hyperinsulinemia in diabetes) that might, aside from oxidative stress, play a direct role in regulating the EDCF response.

Main Specific Objectives and Hypotheses

The following provides a descriptive overview of the main specific objectives and hypotheses of each set of experiments conducted throughout Thesis Study III, from the experiments originally conducted to those performed in response to the findings of the original experiments.

Objective A: To determine the effect of BSO and Aging on systemic and conduit vascular indicators of treatment efficacy, including: i) GSH content in the liver and the aorta (used as a conduit artery surrogate due to CCA tissue limitations); ii) H₂O₂ content in the liver, plasma, and aorta.

Hypothesis A: It was hypothesized that: i) BSO treatment efficacy would be confirmed by a substantial reduction in liver and aortic GSH and an increase in liver, aortic, and plasma H₂O₂, both of which might be more drastic in Aging vs. Adult; and ii) GSH might be reduced in Aging vs. Adult liver and aorta, and H₂O₂ would be increased in Aging vs. Adult liver, aorta, and plasma.

Objective B: To establish the in vivo pulsatile blood pressure and blood flow characteristics of the CCA under CON and BSO, and Adult and Aging conditions.

Hypothesis B: It was hypothesized that mean, systolic (maximum), diastolic (minimum), and pulse pressure would be: i) not significantly affected in Adult CON and BSO; ii) possibly increased with Aging (in particular systolic and pulse pressures); and iii) possibly increased in Aging BSO vs. CON; and that mean, maximum, and minimum blood flow would be: a) not significantly affected in Adult CON and BSO; b) possibly decreased with Aging; and c) possibly decreased in Aging BSO vs. CON.

Objective C: To establish the dose-dependent ACh-stimulated endothelium-dependent contractile response in quiescent and L-NAME pre-incubated CON and BSO, and Adult and Aging CCA (EDCF activity), and to determine the effect of Indo (to non-selectively inhibit COX), SC560 (to specifically inhibit COX-1), NS398 (to preferentially inhibit COX-2), Tempol (considered a SOD mimetic), and MnTMPyP (considered a SOD+CAT mimetic).

Hypothesis C: It was hypothesized that CCA EDCF activity would be: i) negligible in Adult but augmented in Aging; ii) possibly augmented in BSO in Aging but likely not in Adult (and if at all augmented in Adult, to a lesser extent than in Aging); iii) eliminated with Indo in all cases where found, while SC560 and NS398 might each only partially attenuate the response; and iv) possibly augmented with Tempol and attenuated with MnTMPyP in all cases were found.

Objective D: To establish the dose-dependent ACh-stimulated endothelium-dependent relaxation response in PE-pre-contracted CON and BSO, and Adult and Aging CCA (EDRF activity), and to determine the effect of L-NAME (to inhibit NOS), Indo (to inhibit COX), Tempol (considered a SOD mimetic), and MnTMPyP (considered a SOD+CAT mimetic).

Hypothesis D: It was hypothesized that in CCA: i) maximal vasorelaxation would be blunted in Aging vs. Adult, and this blunting likely eliminated with Indo, possibly attenuated with

MnTMPyP, and possibly augmented with Tempol; ii) maximal vasorelaxation would likely be no different in Adult BSO vs. CON but attenuated in Aging BSO vs. CON; iii) sub-maximal vasorelaxation would be predominantly L-NAME-sensitive, and might be modestly attenuated, no different, or modestly augmented in Adult BSO vs. CON, with this response potentially affected by Tempol or MnTMPyP; iv) sub-maximal vasorelaxation would be predominantly L-NAME-sensitive, and likely attenuated in Aging BSO vs. CON, with this response improved by both Tempol or MnTMPyP.

Objective E: (Given that NO bioavailability and EDRF activity appear to be minimally affected by BSO in adult SD rats) to determine the effect of AII exposure on the dose-dependent ACh-stimulated endothelium-dependent NO-mediated relaxation response in pre-contracted Adult CON and BSO CCA.

Hypothesis E: It was hypothesized that 24h in vitro exposure of CCA to AII: i) might only modestly affect NO-mediated EDRF activity in Adult CON; and ii) could result in significant attenuation of NO-mediated EDRF activity in Adult BSO.

Objective F: (Given the potential role of H₂O₂ in eliciting vasorelaxation or vasocontractile activity) to establish the role of exogenous H₂O₂ in CCA vasomotor activity by: i) determining the dose-dependent H₂O₂-stimulated relaxation response in PE-pre-contracted Adult CON and BSO, and the effect of L-NAME (to inhibit NOS); and ii) determining the dose-dependent H₂O₂-stimulated contractile response in quiescent and L-NAME pre-incubated Adult CON and BSO, and the effect of Indo (to inhibit COX).

Hypothesis F: It was hypothesized that: i) H₂O₂ would likely elicit a substantial vasorelaxation response that was in part L-NAME-sensitive, and might be modestly augmented in Adult BSO vs. CON; and ii) H₂O₂ would likely elicit a substantial vasocontractile response that was predominantly Indo-sensitive, and might be modestly augmented in Adult BSO vs. CON.

Chapter 2

Thesis Study I

Impaired Hemodynamics and Endothelial Vasomotor Function via Endoperoxide-Mediated Vasoconstriction in the Carotid Artery of Spontaneously Hypertensive Rats

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2.1 Preface

This Chapter presents Thesis Study I as a 'Full Text' reproduction of the article *Impaired Hemodynamics and Endothelial Vasomotor Function via Endoperoxide-Mediated Vasoconstriction in the Carotid Artery of Spontaneously Hypertensive Rats* published in 2009 by The American Physiological Society as referenced above.

Written permission to include the reproduced materials in this thesis has been obtained from the publisher and can be found at the end of this thesis in the Permission's Pages (Thesis Study I) immediately prior to the References section.

Included in this Chapter are the Overview (Section 2.2), Introduction (Section 2.3), Materials and Methods (Section 2.4), Results (Section 2.5), and Discussion (Section 2.6) of the article, reproduced as published, except in the format of this thesis.

Figures (Figure 2.1 to 2.4) and the Table (Table 2.1) corresponding to the Results are located in order at the end of Section 2.5.

The Supplemental Material for this article, published as an Online Supplement as referenced above, is reproduced in Appendix A as published, except in the format of this thesis. The Supplemental Material includes Supplemental Methods and Supplemental Results comprised of Supplemental Figure A-1 and A-2.

2.2 Overview

The fact that endothelium removal increases diameter and compliance in the CCA of SHR, and that improving CCA endothelium-dependent vasorelaxation has been shown to normalize a reduced systolic blood flow through the SHR CCA compared to normotensive WKY, suggests that endothelial vasomotor dysfunction may be linked to altered large artery hemodynamics in hypertension. The experiments herein were designed to further investigate WKY and SHR CCA hemodynamics and endothelium-dependent vasomotor functions. It was hypothesized that CCA blood flow and conductance would be reduced throughout the cardiac cycle in SHR, and that endothelium-dependent contractile activity would impair SHR CCA vasorelaxation. We report that mean, maximal systolic, and diastolic blood flow were reduced in SHR vs. WKY CCA, as was vascular conductance. Pressure was augmented in SHR CCA and accompanied by late systolic flow augmentation so that total flow during systole was indeed no different between strains, possibly explained by earlier lower body wave reflection. While ACh stimulation in isolated pre-contracted WKY CCA caused a robust NO-mediated vasorelaxation, endothelium-dependent, COX-mediated contractile activity stimulated by high [ACh] impaired NO- and non-NO/non-COX-mediated vasorelaxation in precontracted SHR CCA. In quiescent CCA, this endothelium-dependent contractile response was COX-1- and TP receptor-mediated, and modulated by the availability of NO. These data collectively suggest that endothelium-dependent, COX-mediated endoperoxide signaling in the CCA of SHR may elicit vasoconstriction, which could shift the mechanical properties of this conduit artery and contribute to reduced CCA blood flow *in vivo*.

2.3 Introduction

The CCAs are of great physiological and clinical importance because their internal carotid artery branches contribute ~80% to the total perfusion of the brain in humans and in rodents (100), and CCA IMT (374) and hemodynamics (294) are strong predictors of arterial atherosclerosis and cardiovascular risk. In humans, CCA blood flow remains relatively similar in early-stage essential hypertensive patients compared to age-matched normotensive subjects, but is significantly reduced in patients with sustained essential hypertension (43, 113, 176, 209). CCA hemodynamics have not been fully investigated in animal models of hypertension, but preliminary evidence indicates that CCA blood flow is reduced in SHR, a model of human essential hypertension. Cunha *et al* found that CCA blood flow velocity was reduced to a greater extent with age in SHR compared to normotensive Wistar rats (76). More recently, Iaccarino *et al* found that CCA systolic blood flow was reduced in a small sample of young adult SHR compared

to WKY, and that AKT gene transfer to the CCA endothelium normalized the blood flow reduction and corrected a blunted response to vasodilator stimulants in SHR (166). These findings suggest that CCA endothelial vasomotor dysfunction in hypertensive humans and animals (109) may be associated with altered CCA hemodynamics.

Interestingly, in the absence of an endothelial lining, CCA diameter and compliance are increased in young adult WKY and SHR (217, 269), suggesting a net vasoconstrictive role for the endothelium; an effect that was found to be greater in SHR (57). Indeed, it has been documented in the aorta of adult SHR that ACh, and other vasoactive compounds that elevate endothelial $[Ca^{2+}]_i$, can stimulate the release of not only EDRF(s) such as NO and EDHF(s), but also of EDCF(s). These EDCF(s) include COX-derived endoperoxides, which elicit a 'net' vasomotor dysfunction by competing with EDRFs (109, 385). However, no studies have systematically investigated this pathway as a potential mediator of vasomotor dysfunction in the CCA of SHR. It is therefore unknown whether impaired endothelium-dependent, endoperoxide-mediated, CCA vasomotor function and reduced CCA blood flow indeed coexist in SHR.

The main objective of the present study was to quantify CCA blood flow and blood pressure across the cardiac cycle in young adult SHR and WKY, and to systematically investigate the relative contributions of the NOS and COX pathways in mediating ACh-stimulated, endothelium-dependent CCA vasomotor function. We hypothesized that CCA blood flow and conductance would be reduced across the cardiac cycle in the CCA of SHR compared to WKY, and that COX and TP receptor-mediated, endothelium-dependent vasoconstriction would impair endothelium-dependent, NO-mediated vasorelaxation in SHR but not WKY CCA.

2.4 Materials and Methods

All experiments were performed using young adult rats aged 16-20wks. WKY and SHR were purchased from Harlan (Indianapolis, IN) at 10wks of age and were housed four per cage in a temperature-controlled facility ($21 \pm 1^\circ\text{C}$) on a reversed 12:12h light:dark cycle, having free access to standard lab chow and tap water. All procedures involving rats were approved by the University of Waterloo Animal Care Committee and were in accordance with the guidelines of the Canadian Council on Animal Care.

An expanded Methods section appears in the Supplemental Material for this article, which is available online at the *American Journal of Physiology-Heart and Circulatory Physiology* website (and in Appendix A).

2.4.1. CCA Hemodynamics

The current study took a number of steps to ensure an accurate quantification of blood flow and pressure in the CCA across the cardiac cycle (see Supplemental Methods in Appendix A for details regarding *Rationale for procedural approach* and *Quantification of CCA blood flow and pressure*). Blood flow through the left CCA of anesthetized rats was measured using a perivascular flow-probe, followed by removal of the flow-probe and insertion of a catheter-tip pressure transducer into the left CCA to measure intra-arterial blood pressure, thereby enabling us to subsequently excise from the same rat the structurally undisturbed right CCA for assessment of vasomotor function. Heart rate during flow and pressure waveform collection remained the same ($p > 0.500$ for differences between cyclic rates from flow waveforms and pressure waveforms; data not shown) signifying a steady cardiovascular state within rats throughout the entire in vivo measurement duration (35-45min). Accordingly, blood flow and pressure data were treated as though they were recorded simultaneously (279), which allowed for analysis of CCA pressure-flow relationships across the cardiac cycle (see Figure 2.1 legend for details).

Pressure augmentation in the large arteries of the upper body is a consequence of ‘backward’ and/or ‘forward’ traveling reflected waves (279). Because in the current study the CCA had to be securely tied around the catheter measuring intra-arterial pressure (see Supplemental Methods in Appendix A), transmission of any reflected waves downstream returning (backward) from sites at or beyond the CCA bifurcation may have been obstructed by the tie and thus not represented in the pressure waveform measured by the catheter-tip transducer; consequently, this pressure waveform presumably included the incident wave and a reflected wave returning (forward) from upstream peripheral sites. The AI (‘augmentation index’) of this resulting CCA blood pressure waveform was defined as $[P_S - P_I] / [P_S - P_D]$ (Figure 2.1, lower panel), wherein P_D is end diastolic pressure, P_I is the pressure at the inflection (i.e. shoulder) point during systole, and P_S is peak systolic pressure (279). As recently described by Hirata et al (156), a blood flow AI in the CCA was similarly defined as $[F_S - F_{LSP}] / [F_S - F_D]$ (Figure 2.1, lower panel), wherein F_D is end diastolic flow, F_S is peak systolic flow occurring at about the same point in the cardiac cycle as P_I , and F_{LSP} is peak flow occurring late in systole normally at about the same point in the cardiac cycle as P_S .

2.4.2. Tissue Preparations

Anesthetized animals were killed by removing the heart. The left ventricle and brain (hemispheres+midbrain+cerebellum) were dissected and weighed. The right CCA was carefully excised, immediately placed in a 4°C Krebs’ bicarbonate buffer solution, and cleaned of fat, surrounding connective tissue, and blood. Some CCA segments were flash-frozen, stored at -

80°C, and later homogenized (see Supplemental Methods in Appendix A). From other CCA segments, arterial rings 2.0mm in axial length were cut in preparation for testing CCA vasomotor function. In a number of cases, arterial rings were mechanically denuded of endothelium (-Endo) by insertion of a 250µm diameter stainless steel wire through the vessel lumen followed by gentle rolling on Whatman paper wet with 4°C buffer. The efficacy of denudation was later confirmed by the absence of a relaxation response to ACh in pre-contracted rings.

2.4.3. CCA Vasomotor Function

CCA vasomotor function experiments were adapted from those previously published by our laboratory using WKY and SHR rat aorta (Ref. (143, 310); see Supplemental Methods in Appendix A for additional details regarding *CCA Vasomotor Function* experiments). Briefly, each arterial ring was mounted between two 127µm diameter wires, one fixed to a glass rod and the other connected to a force transducer, and suspended in a 37°C water-jacketed tissue bath containing 10ml of buffer solution constantly gassed with 95% O₂-5% CO₂. Resting tension on rings was slowly increased over 60min to a final resting tension of 2.75-3.00g, which was found in our preliminary contractile experiments to correspond with the optimal length for drug-stimulated CCA isometric tension production (see Supplemental Figure A-1 in Appendix A).

Rings were pre-incubated for 30min in buffer containing either: no drug (ND), *N*ω-nitro-L-arginine methyl ester (LN; 10⁻⁴M in bath) to inhibit NOS (17, 355, 425-427), Indo (10⁻⁵M) to non-selectively inhibit both the constitutive (COX-1) and inducible (COX-2) isoforms of COX (7, 228, 300, 356), LN+Indo to inhibit both NOS and COX-1/-2, LN+valeryl salicylate (VAS; 3•10⁻³M) to inhibit NOS and COX-1 preferentially (139, 140, 423-425), LN+NS398 (10⁻⁶M) to inhibit NOS and COX-2 preferentially (7, 129, 140, 423), or LN+SQ29548 (10⁻⁶M) to inhibit NOS and to antagonize the TP receptor (7, 127, 300). The type and concentration of inhibitors/antagonists used were based on the indicated previous studies in which efficacy has been demonstrated under similar experimental conditions.

Drug pre-incubated rings were exposed to one of two protocols. In one protocol, cumulative dose-response relationships to ACh followed by the NO donor SNP were measured in rings precontracted with 10⁻⁶M PE, shown in our preliminary experiments to elicit a sustained vasoconstriction with an amplitude 80-90% of maximal PE vasoconstriction (see Supplemental Figure A-1 in Appendix A). In a second protocol, cumulative dose-response relationships to ACh were measured in quiescent (non-pre-contracted) rings. At the beginning of each test of vasomotor function, rings were stimulated to contract twice with exposures to 60mM KCl.

2.4.4. Western Blotting

The relative expression of eNOS, COX-1, and COX-2 enzymes in the CCA of WKY and SHR was determined by Western blotting. Procedures for gel electrophoresis of prepared CCA homogenate samples, protein transfer to PVDF membranes, and immunoblotting and detection of these enzymes were performed as described in the Supplemental Methods in Appendix A.

2.4.5. Drugs, Reagents, and Antibodies

Kreb's bicarbonate buffer solution consisted of (in mM): 131.5 NaCl, 13.5 NaHCO₃, 11.2 glucose, 5.0 KCl, 2.5 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgCl₂, and 0.025 EDTA (prepared fresh daily, pH 7.40). All drugs and reagents were purchased from either Sigma-Aldrich (St. Louis, MO via Oakville, ON, Canada) or BioShop Canada Inc. (Burlington, ON, Canada) unless indicated. LN was reconstituted in distilled water, INDO in DMSO, SQ29548 (Cayman Chemical, Ann Arbor, MI) in ethanol, and VAS (Cayman Chemical) and NS398 (Cayman Chemical) in N₂-purged ethanol. Primary antibodies specific for COX-1 or COX-2 were purchased from Cayman Chemical, and for eNOS from BD (Franklin Lakes, NJ). Appropriate secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA).

2.4.6. Statistics

Data are expressed as mean±SEM; n refers to the number of animals/rings per group or drug condition. Statistical analyses were performed using SAS v9.1 or SAS Enterprise Guide v3.0 software (SAS Institute Inc., Cary NC). Differences were considered statistically significant if p<0.05. Two-tailed, independent-sample Student's t-tests were used for single-point comparisons and one-way or two-way ANOVAs with Bonferroni posttests, when necessary, were used for multiple within and/or between group comparisons.

2.5 Results

2.5.1. Reduced Blood Flow and Conductance in the CCA of SHR

Physical attributes of the WKY and SHR used in these experiments are listed in Table 2.1. Table 2.1 also presents WKY and SHR CCA hemodynamics averaged across 5 min of stable waveforms measured *in vivo* (see Figure 2.1, top panel, for example of measured CCA pressure and flow waveforms). Mean CCA blood flow was significantly reduced (absolute [abs.], ~-25%; relative to body weight [rel.], ~-31%) in SHR compared to WKY, as were maximum (abs., ~-33%; rel., ~-38%) and minimum (abs., ~-63%; rel., ~-65%) flow. Conversely, CCA mean, systolic, diastolic, and pulse pressures were all ~1.5 to 2-fold elevated in SHR compared to WKY, and heart rate was on average ~105 cycles/min higher in SHR. As a result, there was a marked ~65% reduction in mean vascular conductance calculated at the level of the CCA.

In SHR, stenoses of the cervical arteries are not likely to account for reduced CCA blood flow since these rats are devoid of overt atherosclerosis (197). Given that the major organ supplied by the CCA, the brain, was found by others (276) and in the current study to be ~12-14% reduced in normalized weight in young adult SHR vs. WKY (Table 2.1), and that hypometabolism already exists in selected brain regions in these SHR (409), it was speculated that reduced SHR brain weight could have to some degree accounted for the ~25% reduction in mean flow through this conduit artery. However, when this was accounted for in a subset of animals, mean SHR CCA blood flow relative to brain weight remained ~18% reduced compared to WKY (n=6 per strain; data not shown).

2.5.2. CCA Pressure Augmented in SHR and Accompanied by Late Systolic Flow Augmentation

The relationship between CCA pressure and flow across time-normalized cardiac cycles (i.e. as a fraction of a cardiac cycle; 0.000 and 1.000 representing the beginning and end of the cardiac cycle respectively; see Figure 2.1 legend for details) averaged within the 16 WKY and 12 SHR is presented in the lower inset left and middle panels of Figure 2.1. Pressure waveforms analysed from both strains had a systolic inflection in pressure (P_I ; SHR, 174±6 mmHg; WKY, 99±4 mmHg; normalized time; SHR, 0.071±0.005; WKY, 0.113±0.008) prior to peak pulse pressure (P_S ; SHR, 204±9 mmHg; WKY, 102±4 mmHg; normalized time: SHR, 0.275±0.008; WKY, 0.168±0.014), which began earlier and was more prolonged in SHR (all $p<0.001$), resulting in a markedly higher pressure AI in SHR compared to WKY (56.5±3.4% vs. 11.6±1.7%; $p<0.001$).

Peak systolic flow (F_S), which was ~30% lower in SHR vs. WKY (13.21±1.12 vs. 19.19±1.10 ml/min; $p=0.002$), occurred in early systole in both strains just following P_I (normalized time: SHR, 0.115±0.008; WKY, 0.131±0.004). After F_S , although pressure, especially in the case of SHR, continued to rise, flow began to decrease, but much more quickly in WKY than in SHR so that total flow during systole (the 0.000 to 0.390 normalized time interval in both strains) was not statistically different between strains when considering the greater number of cardiac cycles per minute in SHR (SHR, 2.83±0.24 vs. WKY, 3.31±0.26 ml in 1 min; $p=0.197$). Indeed, in all SHR animals there was a secondary peak in flow during late systole (F_{LSP} ; 7.25±0.62 ml/min) just following P_S (normalized time: 0.276±0.015) corresponding to an average flow AI of 50.3±3.7%. In WKY, flow waveforms from only 9 of 16 animals possessed a secondary peak in flow during late systole (F_{LSP} ; 5.55±0.44 ml/min; $p=0.037$ vs. SHR), occurring much after P_S (normalized time: 0.321±0.011), corresponding to a markedly lower average flow AI of 21.1±2.1% ($p<0.001$ vs. SHR). During diastole (0.400 to 1.000 normalized time interval), total flow was ~40% lower in SHR vs. WKY (1.19±0.15 vs. 2.10±0.22

ml in 1 min; $p=0.003$). As a result of these pressure-flow interactions, SHR maintained a markedly lower CCA vascular conductance (Figure 2.1, lower right panel) throughout the entire cardiac cycle compared to WKY (averaged $\sim 55\%$ in systole, $\sim 70\%$ in diastole; $p<0.001$).

2.5.3. Endothelium-Dependent, COX-Mediated Contractile Activity Impairs NOS- and non-NOS/non-COX-mediated Relaxation Responses in the PE-Pre-contracted CCA of SHR

Following measures of left CCA hemodynamics, the right CCA was excised for assessment of the relative contributions of NOS and COX pathways in mediating vasomotor functions in PE-pre-contracted arterial rings pre-incubated with either ND, LN, INDO, or LN+INDO (Figure 2.2). Control experiments confirmed that there were no significant differences in vasomotor functions between CCA harvested from animals following hemodynamic measures and CCA freshly-harvested from age-matched WKY and SHR (see Supplemental Methods in Appendix A). Contractile responses to 10^{-6} M PE were 40-50% lower in SHR than WKY across all drug conditions ($p<0.001$) and this persisted when considered relative to their drug condition-respective KCl-stimulated contractions, which were not different between strains. Incubation with LN and LN+INDO compared to ND and INDO respectively caused a moderate but significant increase in developed tension to KCl in both WKY and SHR (10-15%; $p=0.002$), and a much more significant increase in developed tension to PE (80-95%; $p<0.001$). There was no difference between strains in the PE-LN:PE-ND developed tension ratio, a putative indicator of an NO effect on basal tone (95, 149, 289, 289). A similar pattern of response existed between drug conditions within and between strains in the full PE dose-response relationships evaluated in our preliminary contractile experiments (see Supplemental Figure A-1 in Appendix A).

ACh-Stimulated Responses in PE Pre-contracted WKY and SHR CCA

Control experiments (see Supplemental Methods in Appendix A) confirmed that all ACh-stimulated vasomotor activity in PE-pre-contracted CCA (Figure 2.2, bottom left and right) was endothelium-dependent. ACh elicited a concentration-dependent relaxation in WKY CCA and INDO caused a $\sim 10\%$ increase in both the maximum amplitude (*Max Amp*) and area under the curve (*AUC*) compared to ND (see Figure 2.2, inset data). Incubation with LN or LN+INDO caused an $\sim 90\%$ decrease in both *Max Amp* and *AUC* compared to ND and INDO, respectively. Compared to LN, LN+INDO significantly increased *Max Amp*.

The response to ACh in ND and LN SHR CCA was biphasic: relaxing from a precontracted baseline in a sigmoid-like shape to a *Max Amp* (stimulatory phase; -9.0 to ~ -6.5 LogM) followed by recontraction in a sigmoid-like shape (inhibitory phase; ~ -6.0 to -4.0 LogM).

Thus, these responses were found to be best fit to a bell-shaped dose-response curve that sums the two sigmoidal dose-response relationships wherein $EC50_1$ and $EC50_2$ define the [ACh] resulting in 50% of the theoretical plateau in amplitude for the stimulatory (relaxation) and inhibitory (re-contraction) phases, respectively. In ND SHR CCA, ACh first stimulated relaxation reaching *Max Amp* at -6.1 ± 0.1 LogM after which rings re-contracted through to -4.0 LogM. LN caused a significant blunting of the relaxation phase of the ACh response, inducing an ~75% decrease in *Max Amp* and a lower $EC50_1$. The re-contraction phase was also present in the LN SHR CCA; the extent of re-contraction was ~2.5-fold greater than in ND. Notably, INDO completely abolished the re-contraction phase of both ND and LN SHR CCA, thus markedly increasing both *Max Amp* and *AUC* compared to similar conditions without INDO.

WKY vs. SHR. Peak relaxation (*Max Amp*) to ACh was distinctly lower in ND SHR vs. WKY CCA ($p < 0.001$), moreover a dose-dependent recontraction phase occurred at higher [ACh] (~-6.0 to -4.0 LogM) but only in SHR. In contrast, at lower [ACh] (-9.0 to ~-6.5 LogM), relaxation was indeed augmented in ND SHR vs. WKY CCA ($EC50_1$; $p < 0.001$) resulting in no significant difference in overall *AUC* between strains ($p = 0.130$). This augmented relaxation response in SHR at lower [ACh] persisted in the presence of LN, INDO, or LN+INDO ($p < 0.001$). Similar peak relaxation and *AUC* occurred in INDO SHR compared to INDO WKY CCA, and SHR CCA incubated with LN or LN+INDO had a higher peak relaxation and increased *AUC* compared to responses than both LN and LN+INDO WKY CCA ($p < 0.001$).

SNP, used to assess VSM sensitivity to exogenous NO, elicited comparable sigmoidal-shaped concentration-dependent relaxation responses in all WKY and SHR CCA from all drug conditions (see Supplemental Figure A-2 in Appendix A).

2.5.4. ACh-Stimulated Contraction in Quiescent SHR CCA is Endothelium-Dependent, COX- and TP Receptor-Mediated, and Modulated by the Availability of NO

To further investigate the re-contraction response to ACh > -6.5 LogM in PE-pre-contracted SHR CCA (see Figure 2.2), experiments were performed with freshly harvested, intact or endothelium-denuded (-Endo) quiescent (i.e. not pre-contracted) WKY and SHR CCA pre-incubated with either ND, LN, LN+INDO, LN+VAS, LN+NS398 or LN+SQ29548 (Figure 2.3). The effects of COX inhibitors and the TP receptor antagonist were evaluated only in the presence of LN since this optimizes ACh-stimulated contractile responses (17, 355, 426) and allows for distinguishing effects of these drugs on ACh-stimulated CCA contraction independently of confounding effects of NOS. VAS and NS398 were used at concentrations within the range of their reported selectivity for COX-1 and COX-2, respectively (179, 423). At these concentrations, VAS but not

NS398 abolish ACh-stimulated PG production in SHR aorta (139). [ACh] from -9.0 to -4.0 LogM did not elicit vasomotor activity in any WKY CCA condition. In SHR CCA, [ACh] less than -6.5 Log M did not elicit vasomotor activity, but [ACh] from -6.5 to -4.0 LogM elicited a dose-dependent contraction in ND, LN, LN+NS398 and LN+SQ29548 conditions. An ~3-fold increase in *AUC* and *Max Amp* occurred in the presence of LN compared to ND in SHR CCA, consistent with the effect of LN on ACh-stimulated recontraction in PE-pre-contracted SHR CCA (Figure 2.2) and further confirming that SHR conduit artery contraction in response to ACh is markedly amplified by inhibiting the production of NO (17, 355, 426). This ACh-stimulated contraction was completely abolished by endothelial denudation, INDO (LN+INDO), or the COX-1 preferential inhibitor VAS (LN+VAS), while the COX-2 preferential inhibitor NS398 (LN+NS398) only partially attenuated the response. The TP receptor antagonist SQ29548 (LN+SQ29548) abolished ~90% of the contractile response.

2.5.5. Increased COX-1 and eNOS Protein Expression in the CCA of SHR

Immunoblot analyses revealed significant increases in the protein expression of eNOS (30%) and COX-1 (57%) in CCA of SHR relative to those of WKY (Figure 2.4), whereas no statistically significant difference was detected for COX-2.

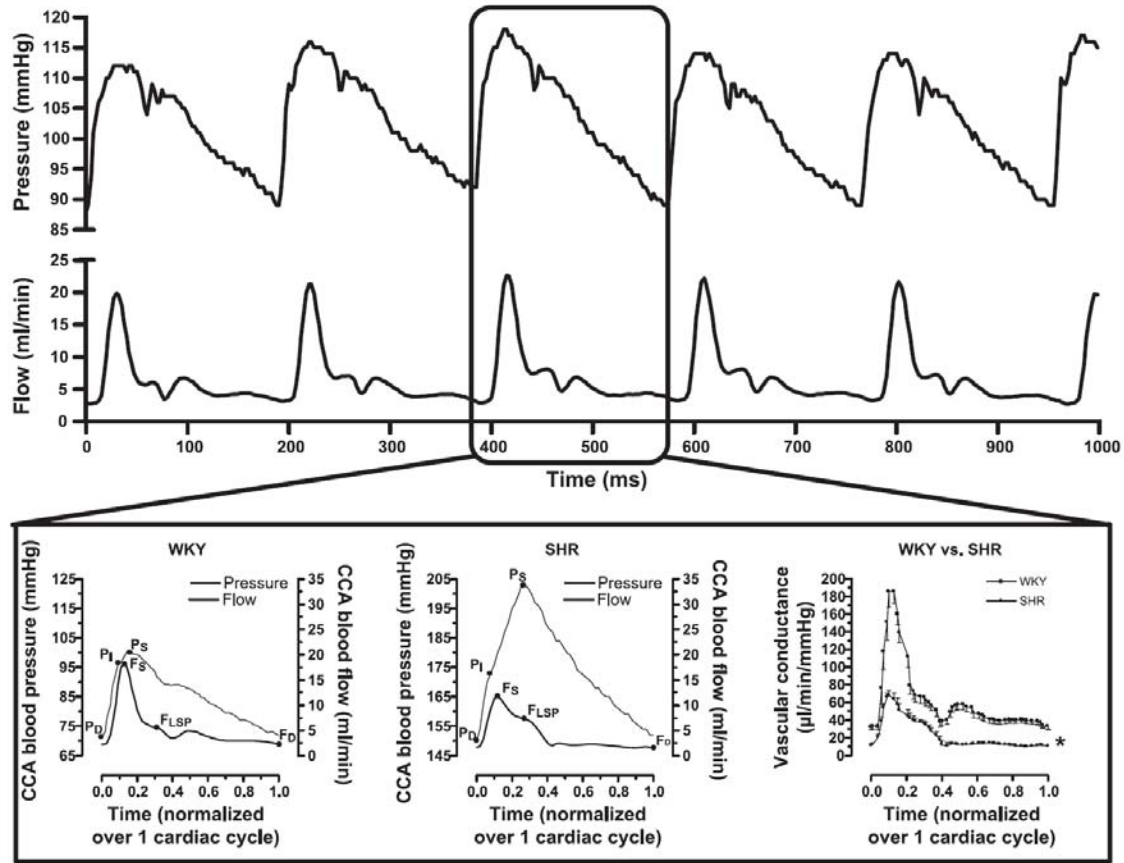


Figure 2.1. Hemodynamics in the CCA of WKY (n=16) and SHR (n=12). The top panel shows a raw 1s CCA blood pressure and blood flow waveform measured in a WKY animal. The lower panel shows the relationship between pressure and flow in the CCA of WKY (left) and SHR (middle) over 1 cardiac cycle (data represent means; error bars were omitted for clarity). CCA blood flow and pressure waveforms for each rat were extracted from the same phase of the respiratory cycle as depicted (beginning and end of each waveform identified by the diastolic nadir of the previous waveform and the waveform to be extracted respectively using Chart™ software algorithms). Because of differences in cardiac cycle times between WKY (170-260ms) and SHR (130-155ms), these extracted waveforms were next individually (i.e. per waveform, per rat) time-normalized across the cardiac cycle (0.000 depicting the beginning, and 1.000 the end, of the cardiac cycle) then averaged within a strain so that the hemodynamic waveforms synchronized with the cardiac cycle. Vascular conductance over 1 cardiac cycle in the CCA of WKY vs. SHR (right) was calculated from these individual time-normalized flow and pressure values then averaged within WKY and SHR (data represent means±SEM; *p<0.01 vs. WKY at each normalized time point). $[P_S - P_I] / [P_S - P_D]$ and $[F_S - F_{LSP}] / [F_S - F_D]$ were used to calculate the augmentation index of the pressure and flow waveforms respectively (see *Methods* for details, and *Results* for statistical comparisons).

Table 2.1 Physical attributes and CCA hemodynamics of young adult WKY and SHR

	WKY	SHR	p
<i>Age, wks</i>	17.2±0.2	17.2±0.2	0.948
<i>Weight</i>			
Whole body, g	305±4	334±4	<0.001
Left ventricle, mg (or /g body)	685±9 (2.24±0.02)	850±14 (2.54±0.03)	<0.001
Brain, mg (or /g body)	1832±3 (5.78±0.08)	1730±2 (5.00±0.08)	<0.001
<i>Heart Rate, bpm</i>	303±9	408±7	<0.001
<i>CCA Blood Flow, ml/min (or /100g body)</i>			
Maximum	19.20±1.00 (6.47±0.34)	12.84±0.98 (3.99±0.34)	<0.001
Minimum	1.57±0.35 (0.53±0.12)	0.58±0.26 (0.18±0.08)	<0.030
Mean	5.10±0.38 (1.72±0.14)	3.84±0.32 (1.19±0.11)	<0.020
<i>CCA Blood Pressure, mmHg</i>			
Systolic	101±4	202±8	<0.001
Diastolic	69±4	147±5	<0.001
Pulse	31±1	55±4	<0.001
Mean	84±4	172±6	<0.001
<i>Mean Vascular Conductance, μl/min/mmHg (or /100g body)</i>	62.3±5.4 (21.1±1.9)	22.4±1.7 (6.9±0.6)	<0.001

Data represent means±SE of stable hemodynamic waveforms measured in the left CCA of anesthetized WKY (n=16) and SHR (n=12). Due to differences in whole body weight between strains, data were reported in both absolute terms and relative to 100 g of whole body weight (/100 g body) where appropriate.

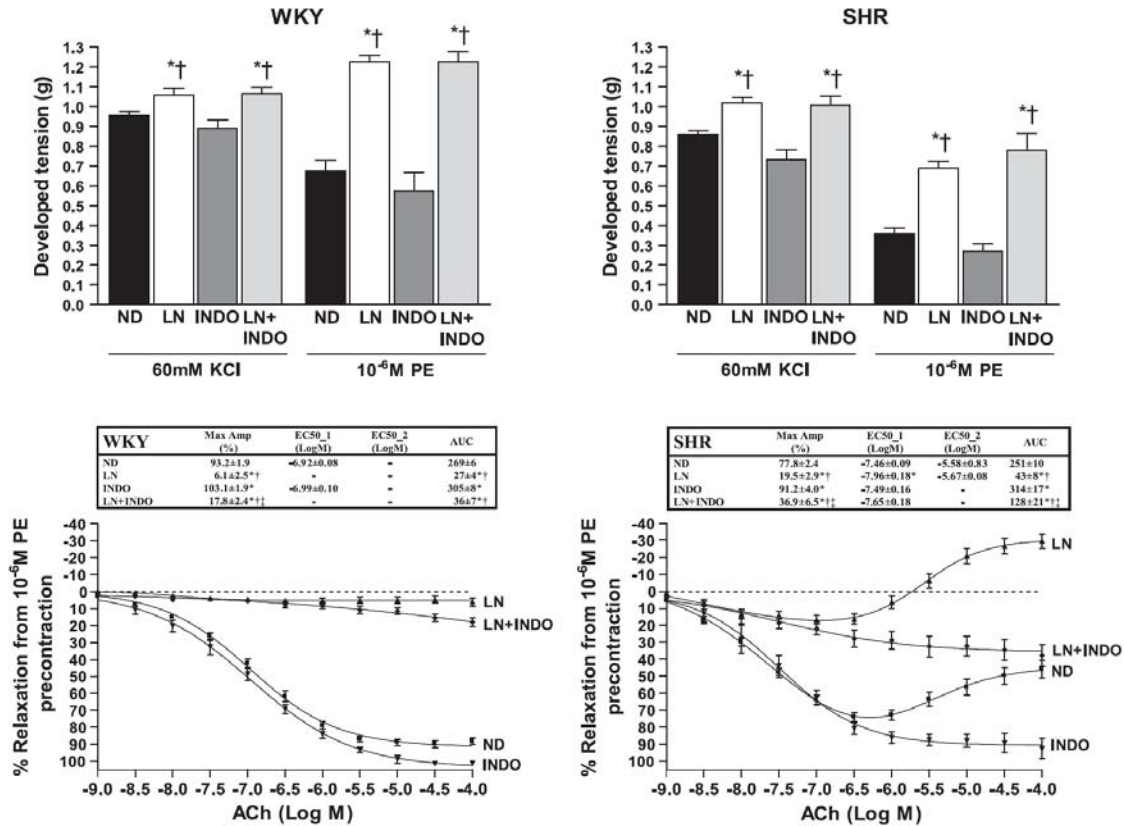


Figure 2.2. Vasomotor function in WKY and SHR CCA rings incubated with either no drug (ND; n=27), *N* ω -nitro-L-arginine methyl ester (LN; n=21), Indo (INDO; n=9), or LN+INDO (n=12). Arterial rings were stimulated to contract with KCl then washed and pre-contracted with PE (top left and right), after which rings were exposed to cumulative ACh concentrations (bottom left and right). Data represent means \pm SEM and are expressed relative to PE pre-contraction. $p < 0.05$; * vs. ND, † vs. INDO, ‡ vs. LN within strain. See *Results* for *WKY* vs. *SHR* statistical comparisons. *Max Amp*, maximum amplitude of the curve; *EC50_1* and *EC50_2*, ACh dose resulting in 50% of the plateau in amplitude of the relaxation and re-contraction phases of the curve, respectively; *AUC*, area under the curve.

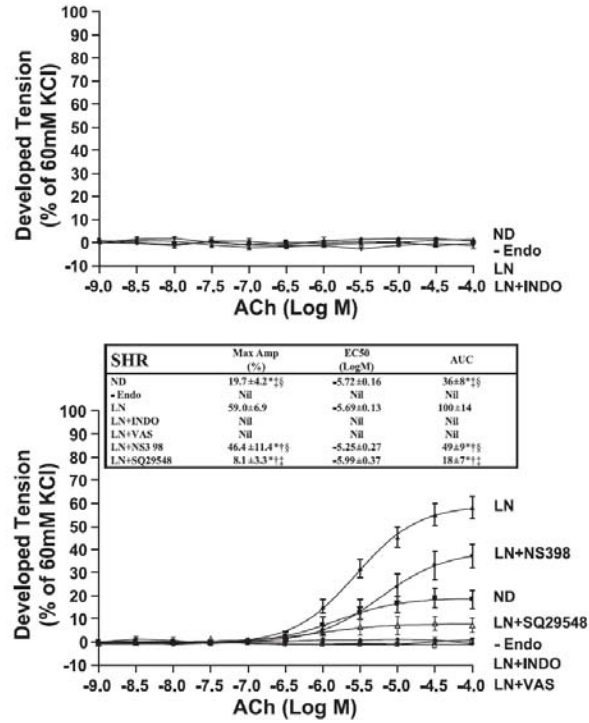


Figure 2.3. ACh-stimulated contraction in quiescent WKY and SHR CCA rings. Endothelium-denuded arterial rings (-Endo; n=3 per strain) were incubated with no drug (ND) and intact rings with either ND (n=3-5 per strain), *N* ω -nitro-L-arginine methyl ester (LN; n=3-11 per strain), LN+Indo (LN+INDO; n=3-4 per strain), LN+valeryl salicylate (LN+VAS; n=5), LN+NS398 (n=5), or LN+SQ29548 (n=4) prior to exposure to cumulative ACh concentrations. Data represent means \pm SEM and are expressed relative to contraction stimulated by KCl. $p < 0.05$; * vs. LN, † vs. ND, ‡ vs. LN+NS398, § vs. LN+SQ29548 within strain. *Max Amp*, maximum amplitude of the curve; *EC50*, ACh dose resulting in 50% of the plateau in amplitude of the curve; *AUC*, area under the curve.

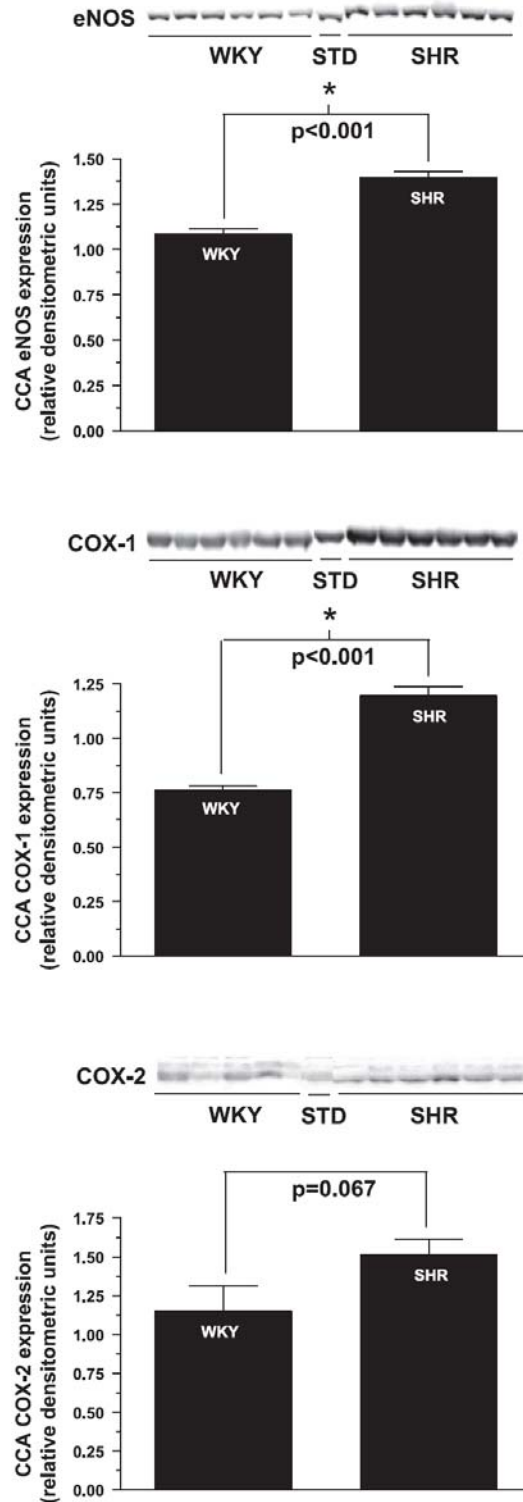


Figure 2.4. Expression of eNOS, COX-1, and COX-2 protein in the CCA of WKY and SHR by Western blot. Shown are representative immunoblots of 1 standard (STD), 6 SHR, and 6 WKY CCA samples. Bar graph data represent means±SEM of protein expression in 12 SHR and 12 WKY CCA assayed in duplicate, quantified by densitometry, and expressed relative to STD.

2.6 Discussion

The present study provides evidence that blood flow through the CCA of young adult SHR is reduced at peak systole and throughout diastole compared to WKY, whereas MAP and PP are much higher throughout the entire cardiac cycle, resulting in a persistently decreased vascular conductance in SHR. Furthermore, this study demonstrates that co-existent with the altered CCA hemodynamics, ACh-stimulated vasorelaxation in CCA excised from SHR is blunted as a result of a competitive, COX- and TP receptor-mediated vasocontractile response that is exacerbated by inhibiting the production of NO. Together, these findings support the working hypothesis that CCA vasomotor dysfunction, via endothelium-dependent, COX- and TP receptor-mediated contractile activity, could be associated with altered CCA hemodynamics in SHR.

2.6.1. CCA Hemodynamics Across the Cardiac Cycle in WKY and SHR

Our findings that mean, maximum systolic, and minimum diastolic CCA flow and conductance were significantly reduced in young adult SHR compared to age-matched WKY are congruent with previous reports of reduced CCA blood flow in sustained essential hypertensive patients (43, 113, 176, 209) and in young adult SHR (76, 166). To our knowledge though, the present study is the first in the WKY-SHR model of hypertension to have analysed CCA pressure and flow across the cardiac cycle and to have investigated their interrelationships, an approach which produced a number of novel findings. A markedly reduced total flow during diastole accounted for the greatest part of the ~25% reduction in mean CCA blood flow in SHR. Although peak systolic flow was also significantly reduced, total systolic flow per minute of cardiac cycles was no different between strains because of a higher heart rate in SHR vs. WKY and a prolonged post-systolic peak decay in SHR CCA flow that may have been the result of a much higher pressure augmentation during this same time (i.e. post-peak-to-late systole). CCA pressure augmentation measured in our study presumably represents a reflected waveform returning early from upstream peripheral sites – e.g. due to aortic degeneration and increased pulse wave velocity (74, 156). This forward reflected waveform in systole would have accelerated (or prolonged the decay of) forward CCA flow, thus augmenting total systolic flow. Late systolic flow augmentation, attributed to pressure wave reflection returning early from the lower body, has recently been reported in the aging human CCA, and was implicated in cerebral microvascular damage (156). In the case of the young adult SHR in the present study wherein total CCA blood flow was reduced predominantly because of a reduction in flow during diastole, it could be speculated that the late systolic flow augmentation may indeed assist in preventing hypo-perfusion of downstream brain tissues.

2.6.2. CCA Vasomotor Function in WKY and SHR

In young adult SHR conduit arteries, α_1 adrenergic receptor-stimulated contractile responses have previously been found to be either similar to WKY (7, 8, 166, 228, 254, 356, 396) or lower (131, 137, 143, 228) as in the current study. Since there was no difference between our WKY and SHR CCA in KCl-stimulated contractile responses or in the PE-LN:PE-ND developed tension ratio, neither a general impairment of contractile function (131) nor a NO-mediated suppression of contractile activity (8, 94, 95), respectively, likely accounts for the differences in PE responsiveness. Adaptations in CCA α_1 -adrenergic receptors and their intracellular signaling mechanisms themselves may provide an explanation (131, 397). Importantly, there is no apparent reason to suspect that these differing PE responses corrupted our evaluation of WKY and SHR CCA ACh-stimulated vasomotor functions and the elucidation of the COX-EDCF-TP receptor mechanism.

The current study demonstrates that whereas ACh initiates a robust endothelium-dependent, NOS-mediated relaxation response in the PE precontracted CCA of WKY, ACh initiates two distinct competing responses in the CCA of SHR: an endothelium-dependent relaxation response to lower [ACh] mediated by both NOS and non-NOS/non-COX signaling, and an endothelium-dependent contractile response to higher [ACh] mediated by COX. Previous studies have shown impaired ACh-stimulated vasorelaxation but no overt re-contraction in CCA from SHR vs. WKY aged 12-24wks (158, 166, 228), which is presumed to be NO-dependent (109) even though NOS inhibitors were not systematically used. Indo has elicited robust vasorelaxation in SHR or SP-SHR CCA in some (61, 333) but not other (228) studies. There does not appear to be a single compelling potential explanation for the discrepancies between the past and the current study of the SHR CCA, but important factors might include: the source and/or exact age of the animals investigated (158, 166, 228); the pre-contractile agonist used (158, 228); and the range of [ACh] evaluated, especially the exclusion of higher [ACh] in past works (158, 166). The current study was designed to provide a more definitive assessment by including the higher [ACh] that elicit the recontraction phase, systematically evaluating NOS and COX blockade effects, and elucidating the involvement of the COX-EDCF-TP receptor axis.

Our results demonstrate that impaired endothelium-dependent relaxation in SHR CCA can be caused exclusively by competitive COX-mediated vasocontraction. Data from the NOS inhibition, eNOS expression, and sensitivity to SNP experiments do not support a major role for altered NO bioavailability in causing the SHR CCA ACh-stimulated vasomotor dysfunction. This interpretation is consistent with reports from SHR aorta (109, 385). Conversely, non-specific COX inhibition completely abolished the re-contraction response to higher [ACh] in pre-

contracted SHR CCA resulting in a similar peak relaxation to that observed for WKY CCA. The endothelium-dependent contractile response to cumulative [ACh] in quiescent SHR CCA was also completely abolished by non-selective COX inhibition, and by preferential inhibition of COX-1 but not of COX-2. Likewise, this ACh-stimulated contraction in quiescent SHR CCA was almost completely abolished by TP receptor antagonism. Supporting these vasomotor findings, we further showed that COX-1, but not COX-2, was significantly over-expressed in the CCA tissue of SHR compared to WKY, consistent with previous aortic studies (129, 359). Thus, it is likely that a TP receptor-binding endoperoxide product(s) of AA metabolism by COX-1 was responsible for the ACh-stimulated CCA contractile activity. In SHR aorta, COX-1-derived EDCF(s) generated in response to ACh have been identified as PGH₂ and PGI₂, which activate VSM TP receptors (109, 385). It should be noted that very recent evidence in SD rat femoral arteries suggests that VAS at the dosage used herein might have TP receptor antagonist properties in addition to its COX-1 inhibition effects (331). The possibility that this also occurs in the current study does not change the overall interpretation, however, since even if the VAS experiment is ignored, the combination of the INDO and NS398 results suggests that COX-1 is the source of the EDCF(s), and the SQ29548 results suggest that the TP receptor is the EDCF target.

The robust ACh-stimulated WKY CCA relaxation in the current study was almost exclusively mediated by NO signaling since this response was blunted ~90% by LN and negligibly affected by INDO. In contrast, the SHR CCA relaxation phase stimulated by lower [ACh], which was augmented compared to WKY over the same dose range, again was negligibly affected by INDO but interestingly was blunted only ~70-80% by LN or LN+INDO. Together these data suggest that this augmented ACh-stimulated response in SHR CCA was mediated by a non-NOS/non-COX-dependent signaling mechanism, traditionally considered to involve an EDHF pathway (108, 110). Others have found augmented relaxation to lower [ACh] in the aorta and/or CCA from SHR or SP-SHR aged 8-20wks (95, 143, 289, 372), speculated to be an age-dependent compensation to hypertension development (95, 109, 289). However, there have been no attempts to systematically investigate the mechanism responsible; notably, none have determined the effect of blocking Ca²⁺-activated K⁺ channels which are ubiquitously involved in EDHF signaling (110). In SHR vasculature, peak VSM hyper-polarization (92) and ACh-stimulated EDHF-mediated relaxation is generally found to be impaired (108, 110), but not in all cases (50, 313) which may be related to age and/or the vascular wall remodeling state (108). Although our results might suggest that an EDHF may in fact be responsible for the augmented

low [ACh]-stimulated SHR CCA relaxation, there are no additional data directly substantiating this supposition.

2.6.3. Perspective

Congruent with the working hypothesis that CCA endothelial vasomotor dysfunction could be associated with altered CCA hemodynamics in hypertension, Iaccarino et al found that improving vasomotor dysfunction with AKT gene transfer to the CCA endothelium corrected a ~30% reduction in peak systolic CCA flow in SHR. The authors suggested this improvement was a result of increased vessel wall compliance thus reducing CCA resistance to flow (166). This is consistent with findings by Levy and associates (57, 217, 269) that the endothelium can alter CCA diameter and compliance in SHR, which might be attributed to the disappearance of a contracting factor produced in the SHR endothelium (269). Though it is impossible with our methodological approach to directly link the in vitro vasomotor function responses with the in vivo hemodynamics, in light of the Iaccarino et al and Levy and associates findings (57, 166, 217, 269), it is intriguing to speculate from our data that endothelium-dependent, endoperoxide-mediated signaling may elicit vasoconstriction in the CCA of SHR and contribute to the ~25% reduced mean blood flow through this artery in vivo. This would likely contribute most to the ~33% reduced peak systolic flow we observed, whereas the ~63% reduced total flow we found during diastole would likely be more dependent on elevated downstream arterial/arteriolar resistance in SHR due to luminal narrowing, rarefaction, and endothelial vasomotor dysfunction (28). This overall interpretation highlights that the vascular system is a continuum (347), with both conduit and resistance vessel endothelial vasomotor dysfunction contributing to altered CCA hemodynamics in hypertension.

2.6.4. Limitations

No in vivo experiments to manipulate CCA NO or endoperoxide signaling were performed in the current study to establish whether these vasomotor pathways indeed do affect blood flow through the CCA of SHR. So this study cannot conclude with causality that the overactive endoperoxide signaling and enhanced ability of the SHR CCA to contract played a physiologically important role in reducing CCA blood flow in vivo. Rather, this *Perspective* is put forth as a working hypothesis to be further investigated. In such an investigation, it will be necessary to ensure that experiments directly manipulating CCA endoperoxide or NO signaling in vivo, as was accomplished by Iaccarino et al via selective gene transfer to the CCA endothelium (166), avoid concomitant effects on downstream cerebrovascular tone and/or uncontrollable systemic

compensations, which may occur with acute or chronic infusions or topical application of drugs, that consequently would corrupt interpretation of CCA flow changes.

2.6.5. Clinical Relevance

Because the CCAs contribute ~80% to the total perfusion of the brain (100), and normal brain function is highly reliant on CCA blood flow (287), a 25% reduction in this parameter could result in significant hypo-perfusion that may contribute to the predisposition of SHR to localized neurological deficits (9, 266). Future investigation of the SHR CCA circulation will provide insight into the roles of reflected pressure waveforms in altering CCA blood flow (37, 195), and in this way may help elucidate mechanisms that govern CCA and cerebral blood flow in human essential hypertension (43, 156, 208). Future investigation of the COX- and TP receptor-mediated vasocontractile characteristics of the SHR CCA will provide insight into the possible role of over-active endoperoxide signaling in shifting the mechanical properties of the CCA (i.e. stiffness/compliance) affecting blood flow, which could have both downstream (cerebral perfusion) and upstream (left ventricular after-load) consequences. Accordingly, this signaling pathway may prove to be an important therapeutic target in the prevention and treatment of hypertensive complications related to ventricular-vascular coupling (412).

Chapter 3

Thesis Study II

RhoA-Rho Kinase Signaling Mediates Endothelium- and Endoperoxide-Dependent Contractile Activities Characteristic of Hypertensive Vascular Dysfunction

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3.1 Preface

This Chapter presents Thesis Study II as a 'Full Text' reproduction of the article *RhoA-Rho Kinase Signaling Mediates Endothelium- and Endoperoxide-Dependent Contractile Activities Characteristic of Hypertensive Vascular Dysfunction* published in 2010 by The American Physiological Society as referenced above.

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Included in this Chapter are the Overview (Section 3.2), Introduction (Section 3.3), Materials and Methods (Section 3.4), Results (Section 3.5), and Discussion (Section 3.6) of the article, reproduced as published, except in the format of this thesis.

Figures (Figure 3.1 to 3.7) corresponding to the Results are located in order at the end of Section 2.5. The Figure (Figure 3.8; Schematic of potential mechanism) corresponding to the Discussion is located at the end of Section 3.6.

The Supplemental Material for this article, published as an Online Supplement as referenced above, is reproduced in Appendix B as published, except in the format of this thesis. The Supplemental Material includes Supplemental Methods and Supplemental Results comprised of Supplemental Table B-1 and Supplemental Figure B-1 to B-6.

3.2 Overview

Hypertensive vasomotor dysfunction is defined by endothelium-dependent contractions involving PGs and ROS. Since both TP receptor signaling and ROS activate RhoA-ROCK in VSM preparations, we hypothesized that enhanced endothelium-dependent contraction in the CCA of SHR is ROCK-mediated. ACh-stimulated contractions were ~2-fold greater in SHR vs. normotensive WKY, abolished by endothelial denudation or COX-1 inhibition, and nearly eliminated by TP receptor blockade. RhoA but not ROCK-II protein expression was increased (~50%) in SHR CCA. Inhibition of ROCK, but not PKC, caused a dose-dependent reduction in endothelium-dependent contractions to ACh across strains, at the highest dose mirroring the effect of high-dose TP receptor antagonism. Conversely, ROCK inhibition caused dose-dependent and endothelium- and NO-independent relaxation in CCA pre-contracted with the TP receptor agonist U46619. PGI₂ was the predominant PG produced by ACh-stimulated CCA with >2-fold more released from SHR vs. WKY, and its production was unaffected by ROCK inhibition. RhoA activation was ~2-fold higher in quiescent SHR CCA compared to WKY, and significantly increased by ACh stimulation. Augmenting chemical superoxide-quenching with tiron or inhibiting the NADPH oxidase-derived superoxide-producing pathway with apocynin reduced ACh-stimulated contractile activity in SHR more than in WKY, whereas the superoxide dismutase mimetic Tempol amplified the response. CCA exposure to exogenous H₂O₂ caused contractions, similar to ACh stimulation, that were: greater in SHR than WKY, abolished by COX-1 inhibition, and highly attenuated by TP receptor blockade or ROCK inhibition. These results indicate that RhoA-ROCK may act as a molecular switch transducing signals from endothelium-derived PG(s) and ROS, which are over-produced in SHR CCA, to 'turn on' VSM contractile pathways, thus mediating the enhanced endothelium- and endoperoxide-dependent vascular contractions characteristic of hypertension, among other CVD states such as diabetes and aging.

3.3 Introduction

Endothelium-dependent vasomotor (dys)function is dictated by a balance between EDRFs and EDCFs (109). Though much is known regarding VSM relaxation mechanisms of EDRFs (212, 320), this is not the case for EDCFs. Whether EDCFs activate the well-characterized RhoA-ROCK contractile pathway in systemic arteries is uncertain. RhoA activation by GPCR stimulation of G_{12/13} results in [Ca²⁺]_i sensitization via ROCK-mediated phosphorylation of the myosin targeting subunit MYPT1 of MLCP, leading to its inhibition (342). This in turn causes sustained myosin RLC₂₀ phosphorylation, and hence contraction, through the classic [Ca²⁺]_i-

dependent CaM-MLCK pathway initiated by GPCR stimulation of $G_{q/11}$ -linked PLC β . However, since these mechanisms have largely been studied under conditions of vascular endothelial denudation and/or membrane permeabilization, there is little direct evidence demonstrating how the endothelium or EDCFs may activate/modulate RhoA-ROCK-mediated vasocontractile activity.

Several lines of evidence suggest that vascular RhoA-ROCK signaling plays a pathophysiological role in CVD states (223, 320), including both human and animal models of hypertension (212). EDCF(s) impair endothelium-dependent vasorelaxation, causing vascular dysfunction in SHR compared to normotensive WKY (109, 385). The factors responsible for this endothelium-dependent contractile activity appear to be COX-derived endoperoxides and/or PG(s) and ROS (360, 385). Importantly, EDCF activity is nearly abolished by antagonism of VSM TP receptors, part of the GPCR family (275). These findings, together with the fact that rodent VSM contraction in response to ROS (177) or agonist stimulation of TP receptors (414) is mediated predominantly by RhoA activation and ROCK-mediated MYPT1 phosphorylation, gave us compelling reason to hypothesize that activation of RhoA-ROCK signaling may be a VSM mediator of EDCF actions, and enhanced in arteries from hypertensive animals. Therefore, the purpose of the current study was to test this hypothesis by investigating the effect of ROCK inhibition on EDCF activity in the CCA of SHR, a model in which we recently found marked endothelium-dependent, COX- and TP receptor-mediated contractile activity ((85); i.e. Thesis Study I).

3.4 Materials and Methods

3.4.1. Materials

See text below for details. Unless otherwise indicated, all reagents and drugs were purchased from Sigma-Aldrich (St. Louis, MO via Oakville, ON, Canada) or BioShop Canada Inc. (Burlington, ON, Canada).

3.4.2. Animals and Vascular Tissue Preparations

All experiments were performed using young adult SHR and WKY aged 18-24wks (purchased at age 7-10wks from Harlan Laboratories, Indianapolis, IN, or Charles River Laboratories, Wilmington, MA) housed in a reversed 12h light:12h dark cycle, temperature-controlled facility ($21\pm 1^\circ\text{C}$) with free access to standard laboratory chow and tap water. All procedures involving rats were approved by the University of Waterloo Animal Care Committee and were in accordance with the guidelines of the Canadian Council on Animal Care.

We chose to study CCA from young adult SHR and WKY to further develop our past findings (85). Young adult animals were used so that early established alterations in vascular function could be investigated in response to sustained essential hypertension, since the rise in SHR blood pressure with age reaches a relatively stable plateau by 12-20wks (e.g. see Refs. (119) and (437)). The CCA segment of the systemic vasculature was investigated because of its importance both physiologically (CCA contributes ~80% to total brain perfusion; (100)) and clinically (CCA IMT (374) and hemodynamics (294) are strong predictors of atherosclerosis and CVD risk), and because of its high degree of accessibility.

Animals were anesthetized via a 60-70mg/kg body weight intra-peritoneal injection of sodium pentobarbital (Vetoquinol N.-A. Inc., Lavaltrie, QC, Canada). In some cases, CCA hemodynamics were measured using a Millar[®] catheter-tip pressure transducer and Transonic[®] perivascular flow probe as we previously described in detail (85). Animals were then killed by removal of the heart. The left and right ventricles were dissected and weighed. The right and left CCA were excised and cleaned of surrounding connective tissue in 4°C buffer solution containing, in mM, 131.5 NaCl, 13.5 NaHCO₃, 11.2 glucose, 5.0 KCl, 2.5 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgCl₂, and 0.025 EDTA, prepared fresh daily in ultra-pure water at pH 7.40. Some CCA segments were blot-dried, flash-frozen, and stored at -80°C for later homogenization as detailed in the Supplemental Methods section of the Supplemental Material for this article, which is available online at the *Am J Physiol Heart Circ Physiol* website (and in Appendix B). Other CCA segments were immediately prepared into arterial rings; those cut 2.0mm in axial length used for testing CCA vasomotor functions, and those cut 3.0mm in axial length used for measuring CCA PG production and RhoA activation, as explained below. Digital images of CCA rings were captured through a dissecting microscope's calibrated eyepiece and processed using ImageJ software (v1.37, standard toolbar, Rasband WS., NIH, Bethesda MD) to quantify their dimensions. Care was taken not to damage the endothelium upon CCA excision and processing as evidenced by a robust endothelium-dependent vasorelaxation response to the muscarinic receptor agonist ACh (maximal WKY CCA relaxation, >90% of pre-contraction to the α_1 -adrenergic receptor agonist PE; see Supplemental Figure B-1 in Appendix B). To test the vasomotor functions of CCA without an intact endothelium (-Endo), arterial rings were first denuded mechanically as described previously (85). We found that, compared to endothelium-intact rings, this consistently resulted in the loss of ACh-stimulated CCA relaxation, and a >80% reduction in CCA eNOS protein expression assessed via Western blotting (data not shown).

3.4.3. Vascular Mechanical and Molecular Functions

Vasomotor activities, PG production, and RhoA activation were examined to characterize CCA function. Each CCA ring was mounted and equilibrated at an optimal length for drug-stimulated isometric tension production in a 37°C tissue bath containing 5ml of 95% O₂-5% CO₂-gassed buffer solution, as we have previously described in detail (85). Next, rings were stimulated to contract twice with exposures to 60mM KCl and washed thoroughly, after which rings were pre-incubated for 30-90min in buffer without or with enzyme/protein inhibitor(s), mimetic(s), or receptor antagonist drugs as described in the *Results*, then exposed to one of four protocols.

In one protocol, *Y27632-stimulated relaxation responses* were examined, wherein dose-dependent relaxation to the selective ROCK inhibitor Y27632 (Calbiochem, San Diego CA), and then to a maximal dose of the NO donor SNP, was measured in CCA that had been stimulated to contract near-maximally with one of three distinct agents: KCl, PE, or the TP receptor agonist U46619 (Cayman Chemical, Ann Arbor, MI). In a second protocol, *ACh-, Ca²⁺ ionophore A23187-, and H₂O₂-stimulated contractile responses* were examined, wherein dose-dependent contraction to these vasomotor stimulants was measured in quiescent (basal, non-pre-contracted) CCA. And in a third and fourth protocol, *ACh-stimulated PG production* and *RhoA activation*, respectively, were examined, wherein a maximal ACh dose (determined to be 10⁻⁴ M) was used to stimulate quiescent CCA. In the third protocol, the bathing buffer was collected at the time of peak contraction (4-5 minutes following ACh stimulation), snap-frozen in liquid N₂, stored at -80°C, and later used to assess PG production by measuring the stable metabolites of TXA₂ and/or PGI₂ using competitive EIA kits according to the manufacturer's instructions (Cayman). PG concentrations were normalized to CCA protein content measured in homogenized rings via a standard BCA protein assay (see Supplemental Methods online). In the fourth protocol, CCA rings were snap-frozen in liquid N₂ at the time of peak contraction, quickly homogenized (~5 minutes) and stored at -80°C, then used within 12-18 hours to assess RhoA activation by measuring GTP-bound RhoA using an absorbance-based G-LISA™ kit according to the manufacturer's instructions (Cytoskeleton Inc., Denver CO). Rings were homogenized in the kit's lysis buffer and protease inhibitor cocktail, in addition to 1X Halt™ Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford IL), after which homogenate supernatant protein concentration was immediately determined using the kit's Precision Red™ protein assay, and equalized to 0.5 µg/µl protein. RhoA activation was measured using 30 µg of protein homogenate loaded in equal volume. Prolonged (>24 hours) storage of either CCA rings or ring homogenates at -80°C was found to significantly degrade the RhoA activation signal (data not shown).

3.4.4. Vascular Protein Expression

Homogenization and Western blotting as previously described (85) was used to quantify the relative expression of RhoA and ROCK-II protein in WKY and SHR CCA. See Supplemental Methods in Appendix B for details. The ROCK-II (i.e. ROCK α) isoform of the protein was investigated because it is most associated with MYPT1 phosphorylation and VSM Ca²⁺ sensitization (282).

3.4.5. Data Analyses and Statistics

Data are expressed as means \pm SE. For details of data collection and curve fitting analysis, see Supplemental Methods in Appendix B. Statistical analyses were performed using Statistical Analysis Software v9.1 (SAS Institute, Cary, NC). Differences were considered statistically significant if $P < 0.05$. Two-tailed, independent-sample Student's t -tests were used for single-point comparisons, and one- or two-way ANOVAs with Bonferroni posttests, when necessary, were used for multiple within and/or between group comparisons.

3.5 Results

3.5.1. Rat Physical Characteristics and CCA Hemodynamics

As indicated in Supplemental Table B-1 in Appendix B, compared to WKY, aged-matched young adult SHR exhibited a markedly increased CCA blood pressure as well as greater LV mass and CCA wall cross-sectional area, indicative of cardiovascular hypertrophy. Furthermore, we confirmed our previous findings (85) that CCA blood flow and conductance are significantly reduced in SHR vs. WKY and that CCA pressure augmentation accompanied by late systolic flow augmentation, which are emerging markers of cardio- and cerebro-vascular damage and risk (156), were already much more exaggerated in this young adult hypertensive state (data not shown).

3.5.2. ROCK inhibition with Y27632 Can Reverse SHR and WKY CCA Contractions Independent of NO- and Endothelium-Mediated signaling

Y27632-Stimulated Vasomotor Activity in Pre-Contracted SHR and WKY CCA

We first assessed the ability of Y27632 to reverse tonic contractions (i.e. developed to a plateau over 30-45min; see Supplemental Figure B-2 in Appendix B) stimulated by either electro-mechanical coupling via KCl, thought to be more dependent on Ca²⁺-dependent CaM-MLCK signaling (46), or pharmaco-mechanical coupling via the α_1 -adrenergic receptor agonist PE (Sigma-Aldrich) or the TP receptor agonist U46619 (Cayman), thought to be more dependent on Ca²⁺ sensitization via G_{12/13}-RhoA-ROCK-mediated MYPT1 phosphorylation (378). Y27632 elicited a robust dose-dependent relaxation under all three pre-contractile conditions in both

strains (Figure 3.1), indicating that RhoA-ROCK activity contributes significantly to both GPCR- and membrane depolarization-stimulated SHR and WKY CCA tonic contraction, as it does in other vascular beds/animals (46). This Y27632-stimulated vasorelaxation effect was least potent under KCl pre-contraction compared to either GPCR-mediated contractile state and most potent under PE pre-contraction (Figure 3.1).

There were no strain-dependent differences found in Y27632-stimulated vasorelaxation under KCl pre-contraction, while there were a number of small differences under GPCR-stimulated contractile states: SHR CCA under PE pre-contraction was slightly more sensitive to Y27632 ($p=0.028$), and under U46619 pre-contraction had a slightly blunted peak magnitude of relaxation ($p=0.015$), compared to WKY (Figure 3.1). However, collectively these differences were relatively modest, suggesting that there is not a striking distinction between young adult SHR and WKY regarding the contribution of RhoA-ROCK activity to tonic CCA contraction in response to a given level of membrane-depolarization or concentration of α_1 adrenergic receptor or TP receptor agonist.

Effect of NOS Inhibition, Endothelium Removal, and SNP Exposure

Because ROCK isoforms are expressed not just in VSM but also in endothelial cells (223), and because RhoA-ROCK activation and NO production/signaling pathways may be inter-connected (320), we further assessed whether EDRFs could be mediating the observed Y27632-stimulated reversal of SHR and WKY CCA tonic contraction. This was accomplished by examining conditions wherein NOS was inhibited with L-NAME or the CCA endothelium was removed (-Endo). There were no differences found between No Drug and L-NAME or -Endo groups in Y27632-stimulated vasorelaxation under KCl pre-contraction, while there were again a number of small differences under GPCR-stimulated contractile states: L-NAME or -Endo decreased the sensitivity to Y27632 under PE pre-contraction and reduced the magnitude of relaxation to Y27632 under U46619 pre-contraction (Figure 3.1). Collectively, these data suggest that ‘unmasked’ EDRF activity, and in particular NO, could in fact be mediating a small part of the reversal of tonic contraction stimulated by Y27632, as has previously been suggested (212); however, this vasorelaxation response stimulated by ROCK inhibition is predominantly independent of NO- and endothelium-mediated signaling, consistent with previous findings in intact aorta from SHR and eNOS knockout mice (222).

SNP was able to elicit an additional relaxation response, beyond that caused by the maximal Y27632 dose, in both strains under all experimental conditions (Figure 3.1).

3.5.3. Endothelium-Dependent, Endoperoxide-Mediated CCA Contractions are Greater in SHR and Markedly Attenuated by ROCK Inhibition but not by PKC Inhibition

ACh-Stimulated Vasomotor Activity in Pre-contracted SHR and WKY CCA

We confirmed our previous findings (85) that ACh, while stimulating a robust endothelium- and NO-mediated vasorelaxation response in PE pre-contracted WKY CCA, in fact stimulates an endothelium-dependent, COX-mediated ‘re-contraction’ response at ≥ -6.5 LogM ACh that significantly impairs an otherwise robust, predominantly NO-mediated vasorelaxation response in SHR CCA (see Supplemental Figure B-1 in Appendix B).

ACh-Stimulated Contractile Activity in Quiescent SHR and WKY CCA: Effect of NOS Inhibition and Endothelium Removal

We further confirmed our previous findings (85) that contraction stimulated by ACh concentrations ≥ -6.5 LogM in quiescent SHR CCA was completely abolished by –Endo compared to No Drug, whereas contraction was markedly increased (>5 -fold) in the presence of L-NAME (Figure 3.2A) indicating the endothelium dependence of this effect and that NO strongly inhibits EDCF activity consistent with findings in the conduit vasculature of SHR and eNOS knockout mice (85, 109, 385, 426). In quiescent WKY CCA, whereas no contractile activity was stimulated by cumulative ACh in the No Drug condition, there was an ACh-stimulated, endothelium-dependent contractile response unmasked in the L-NAME condition (Figure 3.2A) that was about half the magnitude of the corresponding SHR response ($p < 0.001$), again consistent with previous works (109, 385).

Effect of Selective COX-1 and COX-2 Inhibition, and TP Receptor Antagonism

The primary objective of the current study was to investigate the effect of ROCK inhibition on SHR and WKY EDCF activity. Therefore, considering that NO inhibition of EDCF activity in SHR aorta is mediated to a large extent by sGC (426), and that cGMP-stimulated PKG-I signaling can prevent VSM contraction via phosphorylation of RhoA thereby inhibiting RhoA activation (316), the effects of all enzyme/protein inhibitor(s), mimetic(s), and receptor antagonist drugs were evaluated only while co-incubated with L-NAME. This approach is consistent with previous works in this area (85, 109, 357).

We confirmed our previous findings (85) that the non-selective COX inhibitor Indo and the preferential COX-1 inhibitor VAS (Cayman) completely abolish ACh-stimulated CCA contractions, whereas the preferential COX-2 inhibitor NS398 (Cayman) partly blunts the responses (Figure 3.2B), this latter effect possibly due to a non-selective inhibition of COX-1 as previously discussed (85). VAS was recently reported to cause mild non-specific TP receptor

antagonism (331). Thus, we further clarified the COX-1 dependency of endothelium-dependent contractile activity, which is consistent with findings in the conduit vasculature of SHR and COX-1 knockout mice (85, 109, 385), by using the more selective and potent inhibitor SC560 (Cayman) (338). This compound indeed completely abolished ACh-stimulated CCA contraction (Figure 3.2B) but did not affect KCl- or U46619-stimulated contractions (Supplemental Figure B-3 in Appendix B) thus confirming its specificity.

The TPr antagonist SQ29548 (Cayman), which highly attenuated contraction stimulated by U46619 but did not affect KCl-stimulated contraction (Supplemental Figure B-3 in Appendix B), abolished ~90% of the ACh-stimulated contractile response in SHR and completely abolished this response in WKY (Figure 3.2B).

Effect of Selective ROCK Inhibitors and PKC-Specific Inhibition

The selective ROCK inhibitor Y27632 caused a dose-dependent attenuation of ACh-stimulated contraction in both SHR and WKY (Figure 3.2C) that at the highest dose (-5.0 LogM) mirrored the degree of abolishment caused by SQ29548, consistent with our hypothesis that the vasocontractile signal following COX-1-derived EDCF stimulation of VSM TP receptor is transduced by RhoA-ROCK activation. Within the dose range used in these experiments, Y27632 has previously been shown not only to inhibit ROCK but possibly also certain PKC isoforms (414), which if activated can phosphorylate CPI-17 (PKC-potentiated inhibitor protein of 17kDa) leading to Ca²⁺ sensitization via inhibition of MLCP at its PP1c (protein phosphatase type 1c) catalytic site (342). Therefore, to further elucidate the potential roles of these kinases in the EDCF response, we also assessed the effects of H1152 (Calbiochem), a more potent and structurally distinct ROCK inhibitor (compared to Y27632) which has little effect on PKCs (315), and of GF109203X (Calbiochem), a highly-selective broad-spectrum PKC inhibitor (375). H1152 caused a dose-dependent attenuation of ACh-stimulated contraction (highest dose, -6.0 LogM) very similar to Y27632, but with greater potency, in both SHR and WKY (Figure 3.2C). In contrast, ACh-stimulated contraction was unaffected by GF109203X across strains (Figure 3.2D). The dose of GF109203X used for these experiments (-5.5 LogM) was chosen because of its effectiveness in completely eliminating SHR and WKY CCA contraction to the potent PKC activator phorbol-12,13-dibutyrate (PDBu; Calbiochem; Supplemental Figure B-4 in Appendix B). Conversely, we found that neither high dose Y27632 nor H1152 altered PDBu-stimulated contraction (Supplemental Figure B-4 in Appendix B). Moreover, GF109203X did not affect U46619- or KCl-stimulated contraction, whereas both high dose Y27632 and H1152 caused a >50% reduction in U46619-stimulated contraction without affecting contraction to KCl (Supplemental Figure B-3 in Appendix B).

Altogether these data strongly indicate that endothelium-, COX-1-, and TP receptor-dependent CCA contractions in response to ACh are mediated by ROCK, without PKC involvement.

A23187-Stimulated Contractile Activity in Quiescent SHR and WKY CCA: Effect of COX-1/-2, TP Receptor, and ROCK Inhibition

To confirm that the above findings were not exclusively related to endothelial muscarinic receptor signaling via ACh, a known endothelial Ca²⁺ mobilizer (357), we also assessed the effects of Indo, SC560, NS398, SQ29548, Y27632, and H1152 on endothelium-dependent, A23187-stimulated contraction in quiescent CCA (Supplemental Figure B-5 in Appendix B). These experiments yielded a similar pattern of results to our ACh-stimulated contractile data, signifying that the common initiating element required to trigger CCA EDCF signaling is a rise in endothelial intra-cellular Ca²⁺ (357).

3.5.4. COX-1-Dependent CCA PG Production is Greater in SHR, Positively Correlated with ACh-Stimulated Contraction, and Unaffected by ROCK inhibition

PGI₂ and TXA₂ Production in Quiescent and ACh-Stimulated SHR and WKY CCA

COX-derived endoperoxides and/or PGs appear to be EDCF(s) (360). In response to high-dose ACh stimulation, PGI₂ has been found to be the main PG produced from the aorta of SHR and WKY, with TXA₂ produced at a much lower concentration (360). In agreement with these previous works, although we could detect the production of TXA₂ in quiescent SHR CCA, we did not detect a statistically significant increase in this PG upon stimulation with -4.0 LogM ACh, nor was its production affected by the preferential COX-1 inhibitor VAS (Supplemental Figure B-6 in Appendix B). In contrast, a ~2-fold higher production of PGI₂ was evident in quiescent SHR vs. WKY CCA (Figure 3.3A; p<0.001), and a >3-fold increased production of this PG from SHR CCA occurred in response to ACh-stimulated contraction (Figure 3.3A and 3.3B, respectively).

Effect of Selective COX-1 Inhibitors

As expected, COX-1 inhibition with either VAS (data not shown) or SC560 completely abolished ACh-stimulated contraction in SHR CCA (Figure 3.3B) and reduced PGI₂ production below the quiescent state level; however, SC560 was significantly more effective in doing so, causing a >60% reduction (Figure 3.3A; vs. VAS, ~20% reduction; p<0.001), thus confirming the relatively greater potency of this inhibitor. SC560 was even more effective in reducing PGI₂ production in ACh-stimulated WKY CCA (Figure 3.3A), suggesting a complete reliance on COX-1 for its generation in these vessels.

Effect of Selective ROCK Inhibition

Because both endothelial and VSM cells were present in our experimental vascular preparations, we wanted to confirm that the attenuation of endothelium-dependent CCA contractions by ROCK inhibition was not caused by a possible ROCK-dependent decrease in EDCF production. We found that although ACh-stimulated contraction in SHR and WKY CCA was almost completely eliminated by high-dose H1152 (Figure 3.3B; corroborating Figure 3.2C), PGI₂ production was unaffected (Figure 3.3A), thus implying that the effect of ROCK inhibition on COX-1-and TP receptor-mediated EDCF signaling is VSM specific, and not dependent on decreased EDCF production.

Correlation Between ACh-Stimulated SHR and WKY CCA PGI₂ Production and Associated Contraction

In WKY CCA, ACh-stimulated contraction was about half the magnitude observed in SHR (Figure 3.3B, $p < 0.001$; corroborating Figure 3.2A) and associated with a less pronounced (~2-fold) increase in PGI₂ production (Figure 3.3A; SHR vs. WKY, $p < 0.001$). Indeed, within each strain, there was a significant positive correlation between CCA PGI₂ production and contraction (Figure 3.3C), which we were able to demonstrate directly for the first time because of our unique experimental approach which provided for contemporaneous assessment of both variables.

3.5.5. RhoA but not ROCK-II Protein is Over-Expressed in the CCA of Young Adult SHR

We confirmed our past findings (85) that CCA eNOS and COX-1 proteins are over-expressed in young adult SHR vs. WKY (data not shown), consistent with the CCA functional data reported herein and previously (85). Indeed, there is considerable variability surrounding the expression of RhoA-ROCK signaling components in the vasculature of young adult SHR and WKY (222, 267, 321, 324). So we used immunoblot analysis to establish the relative CCA expression of RhoA and ROCK-II protein in our SHR and WKY (Figure 3.4A and 3.4B), revealing that only RhoA was significantly over-expressed in SHR CCA (54% vs. WKY, per μg CCA protein).

3.5.6. RhoA Activation is Greater in the CCA of Young Adult SHR and Increased by ACh Stimulation

RhoA Activation in Quiescent SHR and WKY CCA

The function of RhoA depends on its molecular switch from an 'inactive' GDP-bound state to an 'active' GTP-bound state (342). Compared to normotensive controls, a more than 2-fold higher GTP-bound RhoA-to-total RhoA ratio has been found in freshly harvested intact aorta and isolated VSM cultured from SHR and SP-SHR, among other hypertensive models (267, 321, 324). Congruent with these works, we found that RhoA activation was ~2-fold higher per μg of

protein in quiescent SHR CCA compared to WKY (Figure 3.5A; $p=0.033$). Notably, this effect could not have been caused by a difference in NO bioavailability between strains (thus inhibiting RhoA activation by cGMP-PKG-I-dependent RhoA phosphorylation (316)) since these measures were made on CCA incubated with L-NAME. Moreover, the higher level of quiescent CCA RhoA activation measured in SHR was markedly greater than the increase in SHR RhoA protein expression per μg of CCA protein (see Figure 3.4A), suggesting that total RhoA over-expression could not solely account for the over-activation.

Effect of ACh Stimulation on SHR and WKY CCA RhoA Activation

In mounted de-endothelialized aortas isolated from SD rats, stimulation with the TP receptor agonist U46619 increases both contraction and RhoA activation (325). Moreover, increased RhoA activation has been stimulated by thrombin in cultured VSM isolated from SHR and WKY aorta (321). However, to our knowledge, no study has investigated the effect of agonist stimulation on RhoA activation in intact arteries from SHR. We found that CCA RhoA activation was significantly increased in SHR ($83\pm 16\%$; $p=0.001$) and to a similar extent in WKY ($81\pm 26\%$; $p=0.032$) in response to ACh stimulation (Figure 3.5A), which elicited a >2 -fold greater contraction in SHR than in WKY (Figure 3.5B, $p<0.001$; corroborating Figure 3.3B and Figure 3.2A).

In contrast to the quiescent PG production-RhoA activation status, wherein each were ~ 2 -fold higher in SHR (see Figure 3.3A and Figure 3.5A, respectively), the $\sim 80\%$ increase in ACh-stimulated RhoA activation found in both SHR and WKY (Figure 3.5A) did not mirror the differential increase in PGI_2 production in response to ACh (Figure 3.3A), which was ~ 2 -fold higher in SHR vs. WKY. Moreover, no significant correlation could be detected between ACh-stimulated RhoA activation and contraction, as was found for ACh-stimulated PG production and contraction (Figure 3.3C), possibly related to the number of animals/rings used.

3.5.7. ROCK Inhibition but not PKC Inhibition Highly Attenuates Endothelium- and NO-Independent, COX-1- and TP Receptor-Dependent CCA Contraction Stimulated by Exogenous H_2O_2

In addition to PGs, ROS may play a role in EDCF signaling (357, 360). In SD rats, VSM contraction stimulated by exogenous X:XO (which can produce a mix of $\text{O}_2^{\cdot-}$, H_2O_2 , and OH^{\cdot}) has been associated with RhoA activation and ROCK-mediated MYPT1 phosphorylation (177). Moreover, exposure of conduit vascular segments from various rodent models to exogenous X:XO, or H_2O_2 itself, stimulates contractile activity that is sensitive to COX inhibition and TP receptor antagonism (360). We confirmed and extended upon these findings by assessing the

effect of COX-1, TP receptor, ROCK, and PKC inhibition on exogenous H₂O₂-stimulated contraction of SHR and WKY CCA.

H₂O₂-Stimulated Contractile Activity in Quiescent SHR and WKY CCA: Effect of NOS Inhibition and Endothelium Removal

Consistent with previous works (360), both –Endo and L-NAME caused a significant augmentation of CCA contraction to exogenous H₂O₂ in both strains (data not shown). Therefore, similar to the rationale for the approach to assessing ACh-stimulated contractions, we evaluated the effects of all enzyme inhibitors and receptor antagonist drugs using CCA with intact endothelium only while co-incubated with L-NAME.

Effect of COX-1, TP Receptor, ROCK, and PKC Inhibition

Exogenous H₂O₂-stimulated contraction was almost 2-fold greater in SHR vs. WKY CCA (Figure 3.6; p<0.001). In both strains, the selective COX-1 inhibitor SC560 completely abolished contraction to H₂O₂, as did the TP receptor antagonist SQ29548 and the selective ROCK inhibitor H1152 in WKY; whereas in SHR, SQ29548 and H1152 similarly abolished >90% of the H₂O₂-stimulated contractile response (Figure 3.6), as they did in the ACh-stimulated contractile responses described above (Figure 3.2B and 3.2C, respectively). Conversely, the selective PKC inhibitor GF109203X had a minimal effect on H₂O₂-stimulated contraction in either strain (Figure 3.6).

Effect of Catalase Treatment

Both PEG-CAT (500 U/ml; from bovine liver) and un-conjugated CAT (1200 U/ml; from bovine liver) abolished all contractile activity to exogenous H₂O₂ (data not shown), confirming the specificity of the H₂O₂ contractile effects.

Together these data suggest that SHR and WKY CCA contractions in response to the potential EDCF H₂O₂ are dependent on COX-1 and mediated by TP receptor and RhoA-ROCK signaling.

3.5.8. Augmentation of ACh-Stimulated CCA Contraction by Tempol and Attenuation by Tiron and Apocynin Suggest that Endogenous H₂O₂ Acts as an EDCF

Since herein we found that ACh- and H₂O₂-stimulated CCA contractions are each greater in SHR vs. WKY, and each reliant on a signaling mechanism including COX-1, TP receptor, and RhoA-ROCK to elicit these contractions, we further explored the effect of modulating ROS generating or ROS quenching pathways on ACh-stimulated SHR and WKY CCA contraction.

ACh-Stimulated Contractile Activity in Quiescent SHR and WKY CCA: Effect of Tiron and Apocynin

ROS could affect EDCF signaling either directly by stimulating VSM contraction, or indirectly by decreasing NO bioavailability (360). In the presence of L-NAME, thus excluding the latter possibility, both the cell-permeable $O_2^{\cdot-}$ scavenger Tiron (Figure 3.7A) and the cell-permeable NADPH oxidase inhibitor Apocynin (Figure 3.7B) moderately reduced the sensitivity and maximal amplitude of ACh-stimulated contraction in SHR CCA, whereas in WKY CCA there was only a small, non-significant reduction in sensitivity, consistent with work demonstrating a greater burst of endothelial ROS production in SHR vs. WKY aorta following ACh stimulation (357).

Notably, at the commonly used concentrations employed in our experiments, there is evidence suggesting that Tiron can bind Ca^{2+} thereby affecting contraction (135), and Apocynin may elicit vasorelaxation by a mechanism possibly involving ROCK inhibition (318). Importantly, we found that neither Tiron nor Apocynin affected contraction stimulated by KCl (a Ca^{2+} -dependent mechanism; see Ref. (46)) or U46619 (a ROCK-dependent mechanism; see Ref. (378) and Supplemental Figure B-3 in Appendix B) thereby discrediting these potential non-specific effects in our SHR and WKY CCA preparations.

Effect of Tempol and PEG-catalase

Tempol is a cell-permeable nitroxide compound which can metabolize $O_2^{\cdot-}$ to H_2O_2 , and hence is considered a SOD mimetic (411). In both SHR and WKY CCA, Tempol significantly augmented ACh-stimulated contraction (Figure 3.7A) without exerting non-specific effects on KCl- or U46619-stimulated contractions (Supplemental Figure B-3 in Appendix B).

Taken altogether, these data suggest that endogenous H_2O_2 , possibly derived from an elevated NADPH oxidase-derived $O_2^{\cdot-}$ production and made more available by the SOD mimetic properties of Tempol, may act as an EDCF in the CCA of SHR and WKY, congruent with our exogenous H_2O_2 responses (Figure 3.6) and work in other vascular beds and/or animal models using various other inhibitors (360). As such, it was further hypothesized that CAT, which completely eliminated contraction to exogenous H_2O_2 (described above in *Results*), might inhibit ACh-stimulated contraction at least to the same extent as Tiron and Apocynin. We exposed SHR and WKY CCA to a 500 U/ml concentration of PEG-CAT for at least 3hrs prior to assessing ACh-stimulated contraction as to ensure its penetration into the cells of the vascular wall (411), an unmitigated concern when using non-conjugated catalase (358, 360). However, contrary to the hypothesis, PEG-CAT actually augmented contraction stimulated by ACh in both strains to the same extent as did Tempol (Figure 3.7A). As addressed in the *Discussion*, this last finding

potentially raises some controversy regarding the precise vasomotor action of endogenous H_2O_2 that may be produced in response to ACh in the current model.

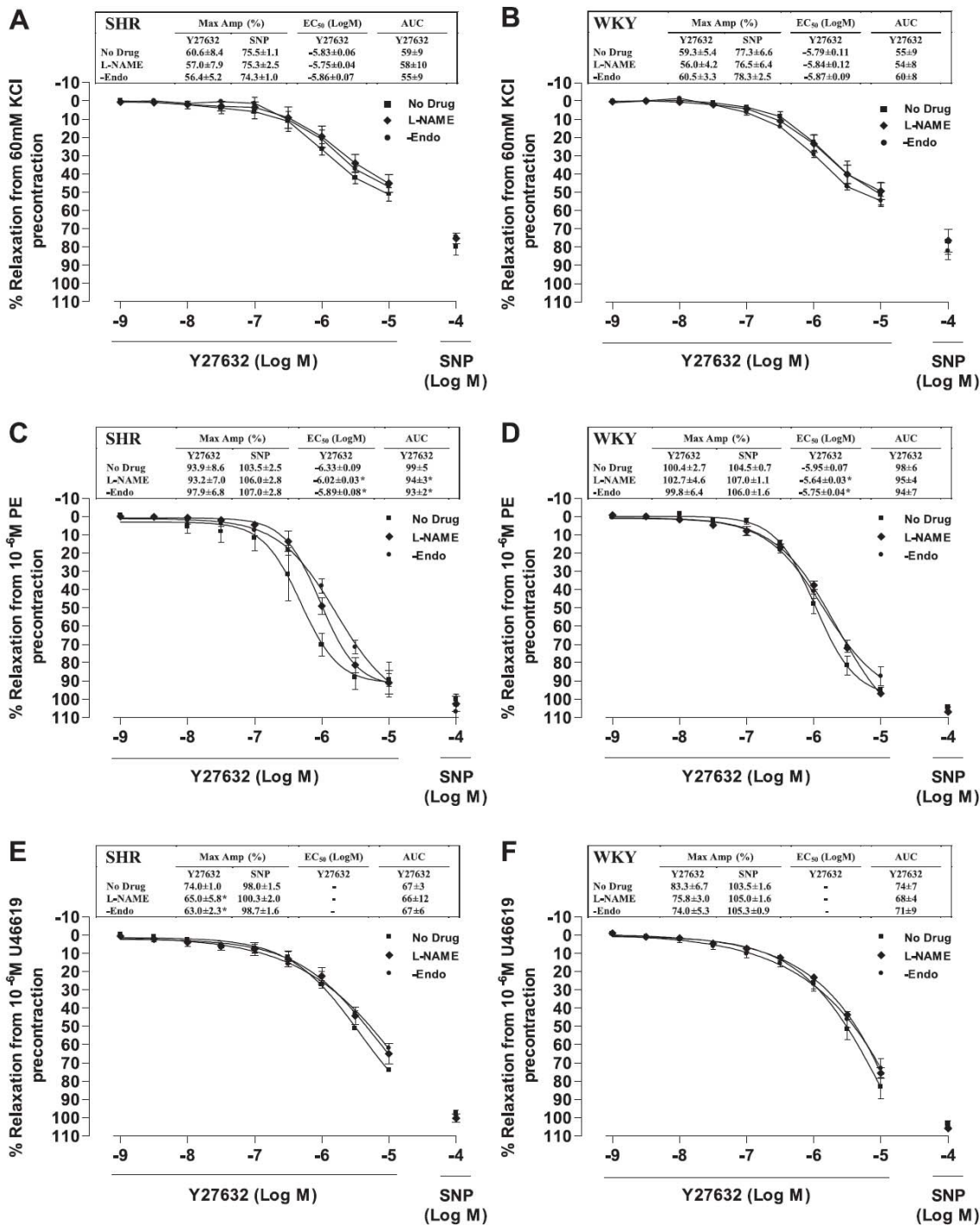
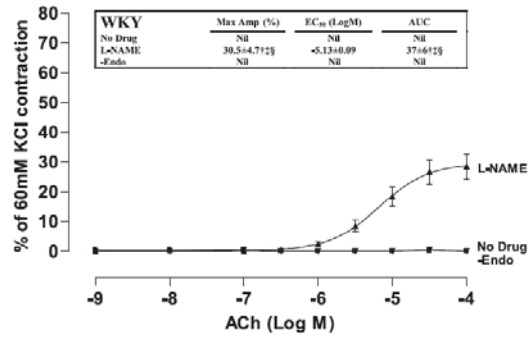
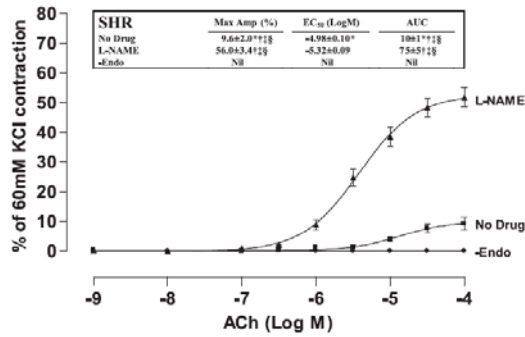


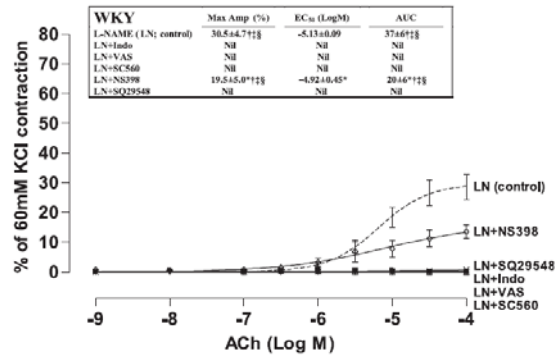
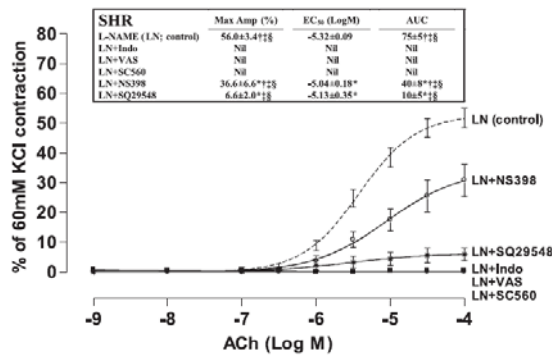
Figure 3.1. For Figure Legend, see next page.

Figure 3.1. Vasorelaxation stimulated by Y27632 in denuded (-Endo, n=4) or intact SHR and WKY CCA rings incubated with either No Drug (n=4) or *N*^o-nitro-L-arginine methyl ester (L-NAME, 10^{-4.0} M, n=4). Rings were stimulated to contract with a near-maximal dose of either KCl (A,B), PE (C,D), or U46619 (E,F), then exposed to cumulative Y27632 doses followed by a maximal dose of SNP. Pre-contraction magnitude with KCl and U46619 was similar between strains and unaffected by NOS inhibition or endothelium removal whereas contraction to PE was lower in SHR compared to WKY but augmented in both strains by NOS inhibition or endothelium removal (see Supplemental Figure B-2 in Appendix B), as we have previously reported and discussed (85). Data represent means±SEM, expressed relative to sustained pre-contraction. p<0.05; * vs. No Drug within strain. See *Results* for SHR vs. WKY comparisons. *Max Amp*, maximum curve amplitude; *EC*₅₀, dose resulting in 50% of *Max Amp*; *AUC*, area under the curve. Because Y27632-stimulated relaxation in U46619 pre-contracted rings did not begin to plateau, no reasonable *EC*₅₀ could be computed.

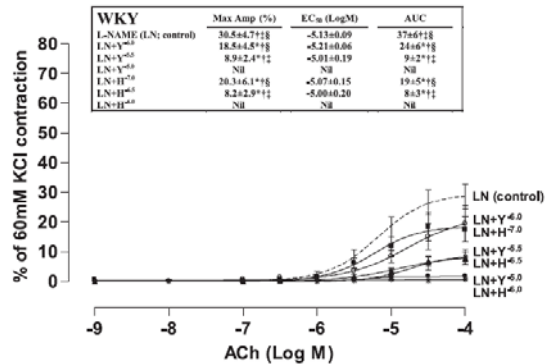
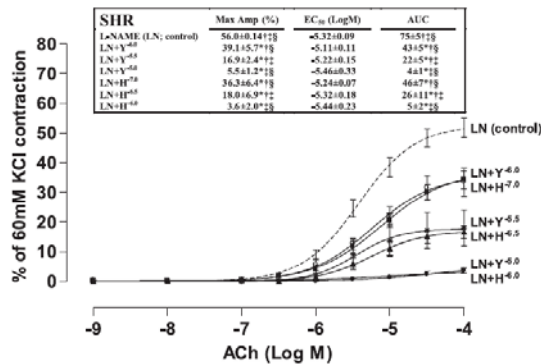
A Effect of NOS inhibition and endothelium denudation



B Effect of COX inhibition TPr antagonism



C Effect of dose-dependent ROCK inhibition



D Effect of high-dose PKC inhibition

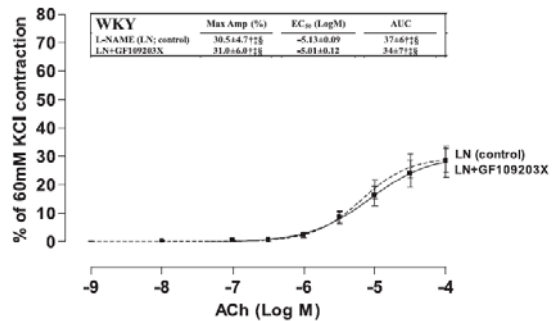
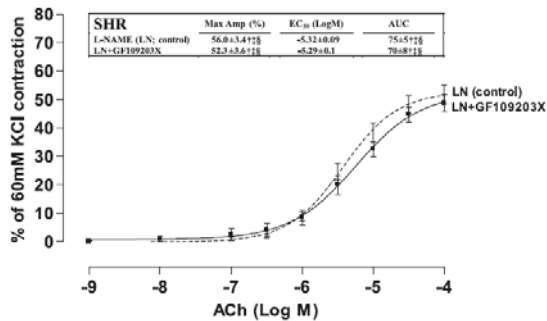


Figure 3.2. For Figure Legend, see next page.

Figure 3.2. Contraction stimulated by ACh in denuded (-Endo, n=3-4) or intact quiescent SHR and WKY CCA rings incubated with either No Drug (n=4-6), *N*⁰-nitro-L-arginine methyl ester (L-NAME or LN, 10^{-4.0} M, n=17-18), which acted as a Control for all other drug conditions (see *Results*), or L-NAME co-incubated with Indo (LN+Indo, 10^{-5.0} M, n=3-4), valeryl salicylate (LN+VAS, 3*10^{-3.0} M, n=4-5), SC560 (LN+SC560, 3*10^{-7.0} M, n=7-8), NS398 (LN+NS398, 10^{-6.0} M, n=8-9), SQ29548 (LN+SQ29548, 10^{-6.0} M, n=6-8), Y27632 (Y) at 10^{-6.0}, 10^{-5.5}, 10^{-5.0} M (LN+Y^{-6.0}, n=7-8; LN+Y^{-5.5}, n=8-9; LN+Y^{-5.0}, n=5-7), H1152 (H) at 10^{-7.0}, 10^{-6.5}, 10^{-6.0} M (LN+H^{-7.0}, n=7-8; LN+H^{-6.5}, n=8-9; LN+H^{-6.0}, n=5-7), or GF109203X (LN+GF109203X, 10^{-5.5} M, n=8-9). For clarity, the curve with curve parameters representing the contraction response in LN control rings from SHR (A, top left) and WKY (A, top right) was duplicated as a dashed line in B), C), and D) in their respective graphs. Data represent means±SEM, expressed relative to previous contraction stimulated by KCl. p<0.05; * vs. LN, † vs. LN+SQ29548, ‡ vs. LN+ Y^{-6.0} or LN+H^{-7.0}, § vs. LN+Y^{-5.5} or LN+H^{-6.5} within strain. PKC, protein kinase C; *Max Amp*, maximum curve amplitude; *EC*₅₀, dose resulting in 50% of *Max Amp*; *AUC*, area under the curve.

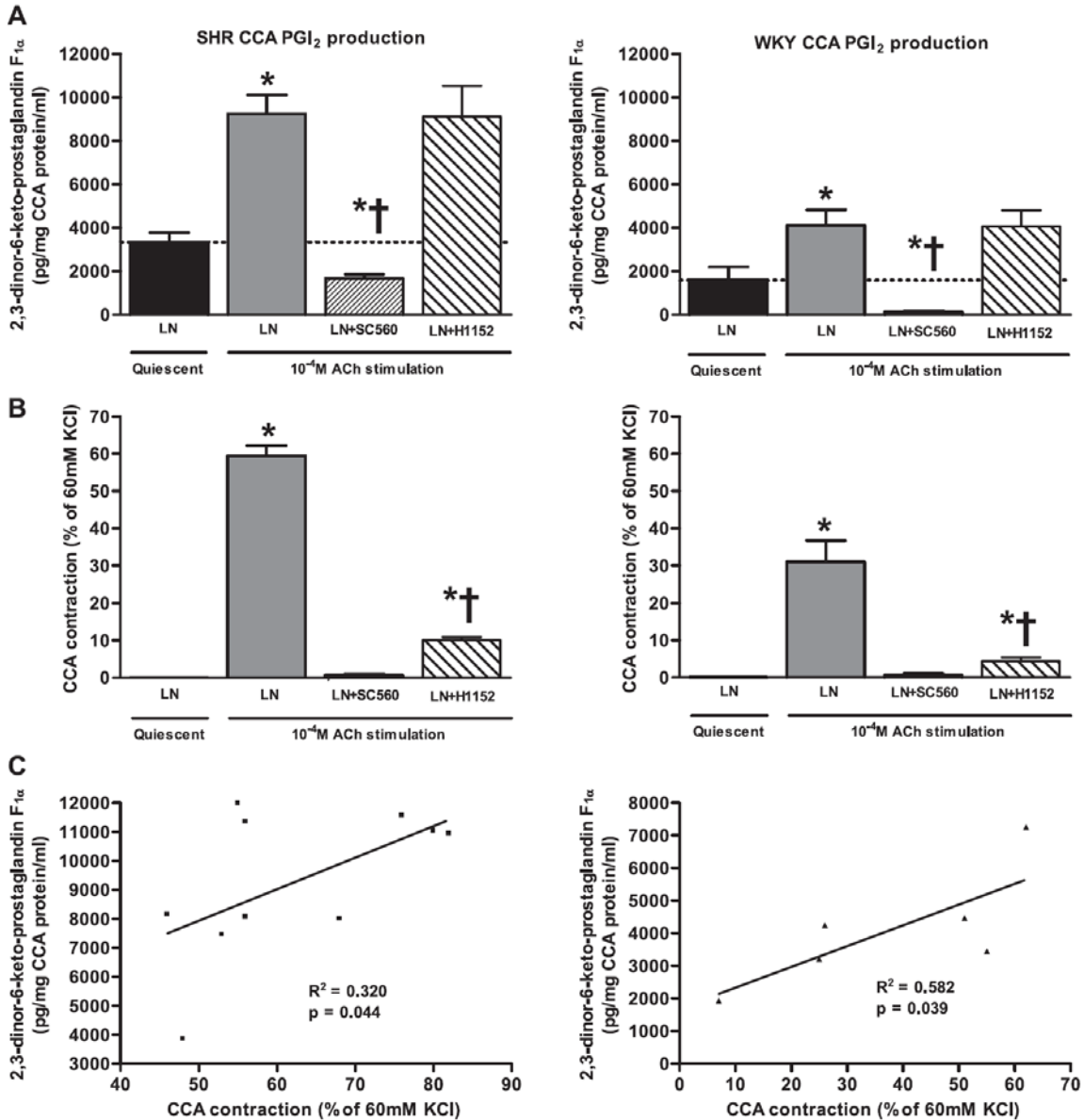


Figure 3.3. Production of PGI₂ (A) from quiescent or ACh-stimulated CCA rings isolated from SHR and WKY and incubated with N⁰-nitro-L-arginine methyl ester (L-NAME, or LN, 10^{-4.0} M, n=6-7) or LN co-incubated with SC560 (LN+SC560, 3*10^{-7.0} M, n=6-7), or H1152 (LN+H1152, 10^{-6.0} M, n=6-7). Rings were stimulated to contract with 60mM KCl then washed. In some cases, rings were incubated in a quiescent state for 45min before bathing buffer was collected. In other cases, after 40min of quiescent incubation, rings were exposed to a maximal dose of ACh eliciting peak contraction within 4-5 minutes (B; expressed relative to previous KCl-stimulated contraction), at which time bathing buffer was collected. The concentration of 2,3-dinor-6-keto-PGF_{1α} in the collected buffer was used as a marker of CCA PGI₂ production, expressed relative to CCA ring protein per ml of bathing buffer. C) represents the relationship between individual ring PGI₂ production in A) and its corresponding ACh-stimulated tension development in B). Data represent means±SEM. P<0.05; * vs. quiescent LN, † vs. ACh-stimulated LN within strain. See Results for SHR vs. WKY comparisons.

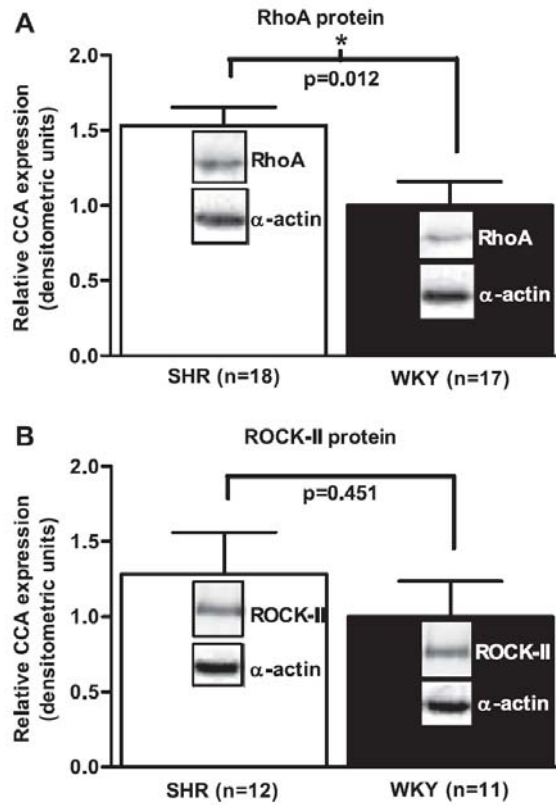


Figure 3.4. Expression of RhoA (A) and ROCK-II (B) protein in the CCA of SHR and WKY by Western blot. Inset the bar graphs for RhoA or ROCK-II protein are representative immunoblots of a SHR and WKY CCA sample run on the same gel. Bar graph data represent means \pm SEM of protein expression assayed in at least duplicate, quantified by densitometry, normalized to α -actin, and expressed first relative to a Standard run on each gel, then to WKY. Equal protein loading (30 μ g per sample) and transfer efficiency were confirmed with ponceau red dye. *denotes statistical significance.

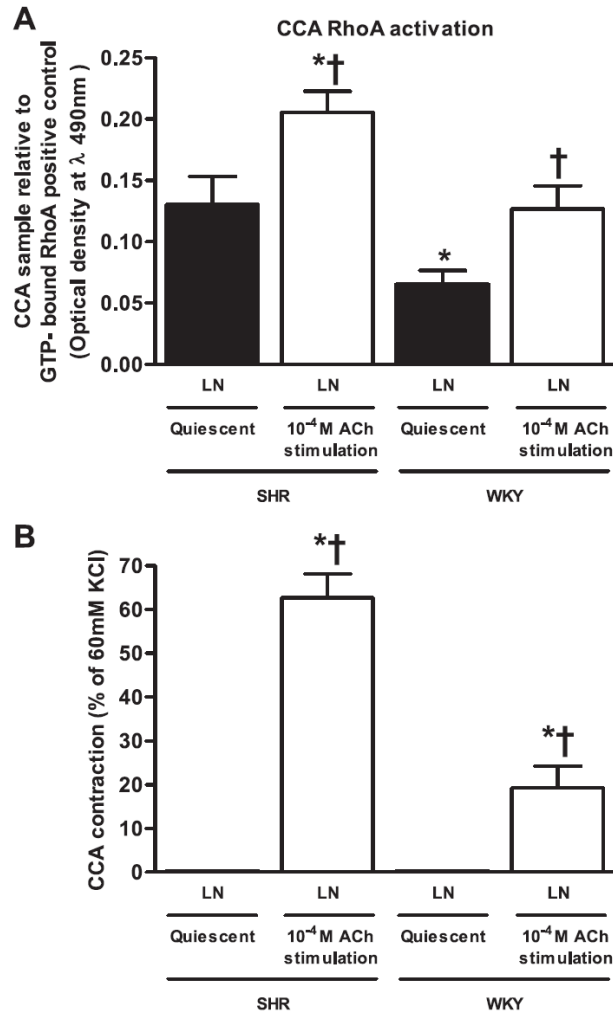


Figure 3.5. Activation of RhoA (A) in quiescent or ACh-stimulated CCA rings isolated from SHR and WKY and incubated with *N*^o-nitro-L-arginine methyl ester (L-NAME, or LN, 10^{-4.0} M, n=5-6). Rings were stimulated to contract with 60mM KCl then washed. In some cases, rings were snap-frozen after being incubated in a quiescent state for 45min. In other cases, after 40min of quiescent incubation, rings were exposed to a maximal dose of ACh eliciting peak contraction within 4-5 minutes (B; expressed relative to previous KCl-stimulated contraction), then snap-frozen. The optical density corresponded to the amount of 'active' GTP-bound RhoA in each sample, expressed relative to the optical density of a constitutively active RhoA protein standard acting as a positive control. Data represent means±SEM. P<0.05; * vs. SHR quiescent LN, † vs. WKY quiescent LN.

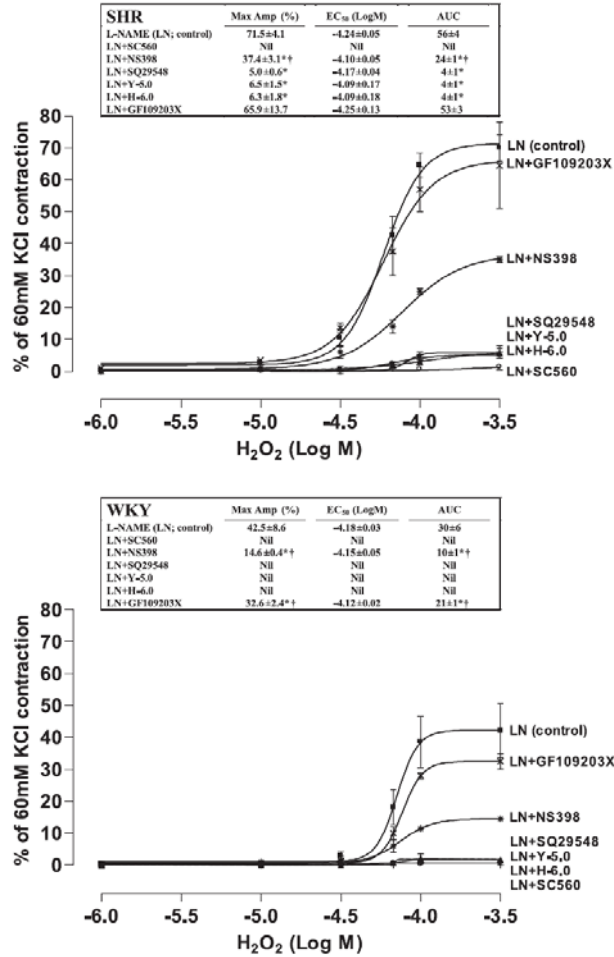


Figure 3.6. Contraction stimulated by H₂O₂ in intact quiescent SHR (*top*) and WKY (*bottom*) CCA rings incubated with either N⁰-nitro-L-arginine methyl ester (L-NAME or LN, 10^{-4.0} M, n=8-10), which acted as a Control for all other drug conditions (see *Results*), or L-NAME co-incubated with SC560 (LN+SC560, 3*10^{-7.0} M, n=5-6), NS398 (LN+NS398, 10^{-6.0} M, n=5-6), SQ29548 (LN+SQ29548, 10^{-6.0} M, n=5-6), Y27632 (or Y; LN+Y^{-5.0}, n=5-6), H1152 (or H; LN+H^{-6.0}, n=5-6), or GF109203X (LN+GF109203X, 10^{-5.5} M, n=6-7). Rings were stimulated to contract with 60mM KCl then washed, after which rings were exposed to cumulative H₂O₂. Data represent means±SEM, expressed relative to previous KCl-stimulated contraction. p<0.05; * vs. LN, † vs. LN+SQ29548, within strain. See *Results* for SHR vs. WKY comparisons. *Max Amp*, maximum curve amplitude; *EC*₅₀, dose resulting in 50% of *Max Amp*; *AUC*, area under the curve.

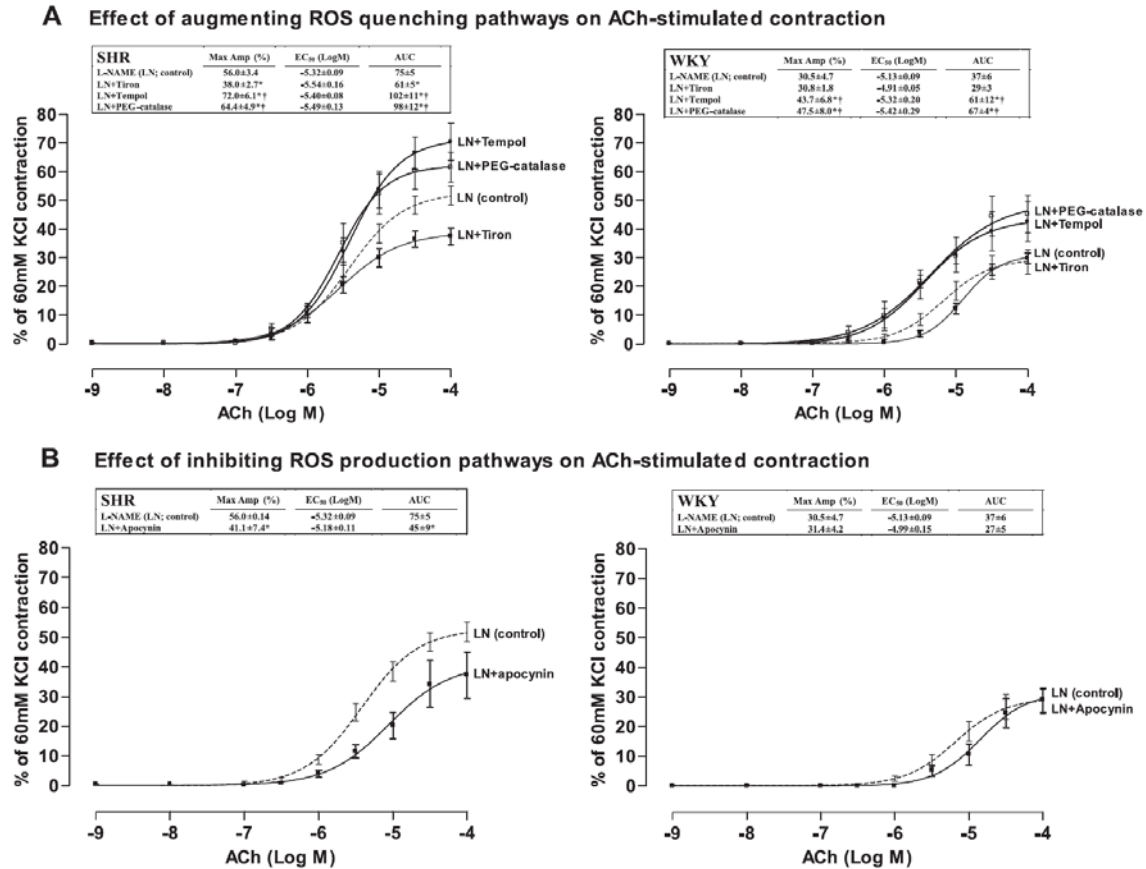


Figure 3.7. Contraction stimulated by ACh in intact quiescent SHR and WKY CCA rings incubated with either *N*⁰-nitro-L-arginine methyl ester (L-NAME or LN, 10^{-4.0} M, n=8-10), which acted as a Control for all other drug conditions (see *Results*), or L-NAME co-incubated with Tiron (LN+Tiron, 10^{-2.0} M, n=6-7), Tempol (LN+Tempol, 10^{-3.0} M, n=6-7), PEG-Catalase (LN+PEG-catalase, 500 U/ml, n=6-7), or Apocynin (LN+Apocynin, 10^{-3.0} M, n=6-7). Rings were stimulated to contract with 60mM KCl then washed, after which rings were exposed to cumulative ACh (A and B). For clarity, the curve with curve parameters representing the ACh-stimulated contractile response in LN control rings from SHR (Figure 3.2A, *left*) and WKY (Figure 3.2A, *right*) was duplicated as a dashed line in A) and B) in their respective graphs. Data represent means±SEM, expressed relative to previous KCl-stimulated contraction. p<0.05; * vs. LN, † vs. LN+Tiron, within strain. See *Results* for SHR vs. WKY comparisons. *Max Amp*, maximum curve amplitude; *EC*₅₀, dose resulting in 50% of *Max Amp*; *AUC*, area under the curve.

3.6 Discussion

The data presented provide important new information related to vascular EDCF signaling and support our hypothesis that EDCF(s) trigger RhoA-ROCK signaling in the arterial wall, particularly in SHR. Thus, RhoA-ROCK may act as a molecular switch transducing signals from EDCF(s) to 'turn on' VSM pathways related to contractile activity, uniquely implicating RhoA-ROCK in the multi-faceted disorder of endothelium-dependent vascular dysfunction hallmarking hypertension. This work contributes to a growing body of evidence elucidating the mechanisms of RhoA-ROCK signaling in VSM contraction (342, 414) and its over-activation and function in CVD states including hypertension (212, 223, 320).

3.6.1. Methodological Considerations

Vascular endothelial denudation and/or membrane permeabilization preparations have provided much insight into the regulation of RhoA-ROCK in VSM contraction. However, as recently highlighted by Neppl et al (277), such preparations by their nature cannot be employed to investigate whether endothelial cell signaling affects RhoA-ROCK-mediated VSM activation and function, which they showed can occur at a molecular level in important ways. Studying intact vasculature, though, carries with it the inherent difficulty of accurately interpreting such findings because of the presence of both endothelial and VSM cells, each expressing RhoA and ROCK (223), and thus the possibility of ROCK inhibition having cell-specific effects. Importantly, it is apparent in the current study that the prevention of endothelium-dependent, COX-1- and TP receptor-mediated CCA contractions can be attributed to the action of ROCK inhibition on the VSM exclusive of the endothelium. Indeed, alterations in EDRF production/activity can be excluded because TP receptor-stimulated contraction could be reversed by ROCK inhibition in a manner independent of an intact endothelium or the EDRF NO, and because ROCK inhibition in the presence of L-NAME could prevent TP receptor-stimulated contraction, together with the fact that L-NAME was co-incubated while testing the inhibitory effect of ROCK inhibition on ACh and H₂O₂ stimulation. Moreover, alterations in EDCF production can be excluded because ACh-stimulated PG production was not affected by ROCK inhibition.

The specificity of ROCK inhibitors, with respect to their activity toward PKC isoform(s) (342), presents another difficulty in studying the role of RhoA-ROCK in vascular functions. However, by demonstrating herein that contraction stimulated by PKC activation was unaffected by the two structurally distinct ROCK inhibitors used, and that broad-spectrum PKC inhibition did not affect ACh- or H₂O₂-stimulated contraction, it is apparent that the effect of the ROCK

inhibitors on endothelium-dependent CCA contractions cannot be attributed to their previously reported non-specific effects (414).

3.6.2. Evidence for a Direct Role of EDCF in Activating Vascular RhoA-ROCK Signaling

Some reports in the pulmonary vasculature suggest that EDCF(s) may play a significant role in mediating hypoxia-stimulated pulmonary vasoconstriction by activating VSM RhoA-ROCK signaling, although the exact identity of the potential EDCF(s) remains elusive (304, 305). While major inherent differences exist between the pulmonary and systemic vasculature, the current study's data may help elucidate the mechanism(s) by which EDCF(s) activate RhoA-ROCK in the pulmonary vasculature, and possibly provide important insight into the potential mechanisms of established and emerging therapeutic interventions used to treat pulmonary hypertension (1, 26), including PGI₂ analogs (e.g. epoprostenol, iloprost) and ROCK inhibitors (e.g. Fasudil).

As the current manuscript was in preparation for submission, Chan et al published a study that evaluated the effect of the ROCK inhibitors Y27632 and HA1077 (Fasudil) on contractions in the aorta of SHR and WKY (60). The data from this independently conceived and executed work, though generated using a distinct segment of the systemic conduit arterial system from differently aged animals, eloquently supports our findings that high-dose ROCK inhibitors can almost abolish ACh- and A23187-stimulated contractile activity, in addition to corroborating our findings that this effect must be mainly due to a direct inhibition of VSM ROCK. Notably, the current study both strengthens and extends the work by Chan et al, and other studies evaluating effects of ROCK inhibitors on vascular function, in a number of important regards. Our well-controlled functional experiments establish that inhibition of endothelium-dependent contractions was indeed concentration-dependent, and maximally effective in both strains at the typically used high-dose of Y27632, as well as H1152, a more potent and specific ROCK inhibitor than either Y27632 or HA1077. Moreover, our control experiments discredit the possibility of any PKC-related non-specific effects previously reported of Y27632, thus solidifying the interpretation of vascular function data sets relying on the effect of ROCK inhibitors.

As an obvious major extension of the study by Chan et al, we demonstrate not only that quiescent RhoA activation is elevated in SHR CCA compared to WKY, but also show for the first time that RhoA activation is stimulated by ACh, thus providing molecular support of the functional ACh-stimulated findings. Furthermore, beyond confirming with different pharmacological tools previous works suggesting that ROS contributes to conduit vascular endothelium-dependent contractions (360), we establish for the first time that H₂O₂, which is speculated to be an EDCF (360), elicits a COX-1- and TP receptor-dependent contraction in both SHR and WKY CCA that is indeed mediated by ROCK. Thus, our data provide strong evidence

that the two most likely candidate species for EDCFs, COX-1-derived PGs and ROS, both trigger VSM contraction by TP receptor-dependent RhoA-ROCK signaling.

Noteworthy, by using Tempol and PEG-CAT for the first time in ACh-stimulated SHR and WKY vascular preparations, we found that these two pharmacological tools in fact *both* augmented ACh-stimulated CCA contraction. A potential explanation to reconcile these unexpected and seemingly paradoxical results could emerge from considering that H₂O₂ has also been shown to elicit relaxation, acting presumably as an EDHF endogenously, in many vascular beds and animal models including intermediate-sized arteries from SHR and WKY (50, 85, 128), and that Tempol can act also as a catalase mimetic (411). Thus, it appears possible that Tempol, like CAT, augmented ACh-stimulated contractile activity not by enhancing (as conventionally suggested) but by diminishing the bioavailability of H₂O₂ (411), which endogenously may have in fact been acting not as an EDCF (as suggested by our exogenous H₂O₂ data) but as an EDRF thus curtailing contraction, akin to the effect of NO demonstrated herein and by others (85, 109, 385, 426). Because these findings were contrary to our hypothesis of how these pharmacological tools (in particular PEG-CAT) would act, we have no additional data to directly substantiate this possible caveat put forth. Therefore, it is clear that further functional-biochemical and pharmacological exploration is required to definitively support or refute this controversial hypothesis.

3.6.3. Proposed Mechanism: Over-production of EDCF(s) Cause Over-Active RhoA-ROCK Signaling in Intact SHR Vasculature

Although it is well established that both endothelium-dependent contractions (85, 109, 360, 385) and RhoA-ROCK activation (212, 267, 272, 320, 321, 324, 407) are greater in hypertensive vasculature, the important root cause(s) of these pathological characteristics have not been fully elucidated. Reconciling findings from the current study with previous literature, a novel mechanism accounting for conduit artery endothelium-dependent RhoA-ROCK activation and contractile activity can be hypothesized to better understand what may account for triggering the over-activation of this signaling pathway in hypertension (Figure 3.8). We propose that over-production of EDCFs could be a main cause of over-active RhoA-ROCK signaling in the intact vascular wall of SHR.

To our knowledge, it is currently unknown whether RhoA, ROCK, and their regulatory proteins are differentially expressed in SHR endothelium. We herein, and others (321), have found greater RhoA protein in intact CCA or aorta, and cultured aortic VSM, from young adult SHR or SP-SHR vs. WKY, whereas others found no expression difference (267, 324). Similarly, we herein, and others (60, 222, 267, 321, 324), have found no difference in ROCK-II protein or

mRNA, whereas others have found greater ROCK-II or ROCK-I expression (6, 272), in intact, denuded, or aged CCA or aorta, or cultured aortic VSM, from SHR or SP-SHR. Likewise, expression of Rho-GDI α , a RhoA-ROCK regulatory factor, has been found to be no different (267, 321) or greater (34) in SHR or SP-SHR aorta. From these conflicting results, it is not clear whether an over-expression of VSM RhoA and ROCK associated proteins per se could itself be a significant cause of greater membrane-bound RhoA-GTP (i.e. activated RhoA) (267, 321, 324) and/or ROCK-mediated MYPT1 phosphorylation (i.e. activated ROCK) (267, 272, 407) found in intact conduit arteries or cultured VSM from hypertensive models (324). Certainly it could play a role (272, 321), as may be reflected in reports of SHR VSM contractile hyper-responsiveness to agonists such as PGH₂ (130); however, in the current study and in past works (267, 321, 324), the level of RhoA expression does not appear to account for the increase in SHR RhoA activation, nor an increase in MYPT1 phosphorylation (267).

Alternatively, the main cause of over-active RhoA-ROCK signaling in SHR vasculature may be an over-production of GPCR agonists. As we propose for the first time, those agonist(s) could include EDCFs. Endothelium-derived PGs (360) and ROS (357) are both produced in greater amounts in SHR vs. WKY conduit vasculature. This may be best explained by a greater rise in endothelial intra-cellular Ca²⁺ upon agonist stimulation (357), coupled with an over-expression of endothelial cell COX-1 and PGI₂ and TXA₂ synthases (359) as well as subunits of NADPH oxidase (143). Indeed, ACh elicits a large endothelium-dependent increase in SHR and WKY vascular PGI₂ production, which the current study established has a strong direct correlation with contraction magnitude; conversely, the endothelial Ca²⁺ mobilizers A23187 and ADP elicit a large increase in TXA₂ production (360). COX-1 activation appears to be the source of these endoperoxide derivatives in SHR and WKY; whereas, in other vessel beds (399) and/or animal models and in humans (418), various PGs derived from either over-expressed COX-1- or COX-2-specific activation, including PGF_{2 α} and 8-isoprostane, have been reported to be associated with endothelium-dependent contractions. In response to endothelial Ca²⁺ mobilizers, COX (357), and NADPH oxidase as indicated for the first time in the current study, may be sources of ROS which might possess EDCF action.

Stimulation of VSM TP receptors is essential in eliciting endothelium-dependent, endoperoxide-mediated contractions (85, 109, 360, 385, 399, 418). As clearly demonstrated in the current findings, ROCK signaling activated via TP receptor stimulation by PGs, and possibly ROS, plays an obligatory role in transducing these endothelium-derived signals within the VSM. Certainly, future investigation is necessary to elucidate exactly how EDCFs promote the RhoA-GTP membrane association shown in the current study, in turn activating ROCK, and to establish

exactly what effect this has on MYPT1 and RLC₂₀ phosphorylation status (177, 277, 325, 342, 414). In addition, it will be important to establish at this molecular level how the production of EDRF(s), including NO (212, 316, 320), and also EDHF(s) (e.g. via K⁺ channels; (178)), counteract these EDCF-triggered RhoA-ROCK-mediated signaling events, and vice versa.

In SHR VSM, the IP receptor, which typically transduces a vasorelaxation signal from PGI₂, is equally expressed (359) but dysfunctional (142) compared to WKY VSM. So instead, PGI₂ is transduced, like TXA₂ and PGF_{2α}, as a vasocontractile signal via the TP receptor which is also equally expressed across strains (359). Accordingly, in contrast to the evidence suggesting hyper-responsiveness to PGH₂ (130), we herein, and others (60), have shown that SHR VSM contraction to the ROCK-dependent TP receptor agonist U46619 is no different from WKY. Therefore, taken altogether, it appears that both endothelium-dependent contractions and RhoA-ROCK activation in SHR conduit arteries may be best explained by EDCF over-production. Moreover, the SHR VSM contractile hyper-responsiveness to exogenous H₂O₂ shown herein and by others (360) also may best be explained not necessarily by an over-expression of VSM RhoA and ROCK associated proteins, but by H₂O₂ stimulation of VSM COX-1 (201), which is over-expressed in SHR VSM (359), thus resulting in PG over-production that, via VSM TP receptors, elicits RhoA activation and ROCK-mediated MYPT1 phosphorylation (177).

3.6.4. Pathophysiological and Clinical Significance

Impaired vasorelaxation caused by endothelium-dependent contractions occurs not only in SHR but also various other hypertensive animal models and in human essential hypertension (385), as well as in aging (418) and diabetic (328) models. Our current data collectively signify that this vasomotor dysfunction characteristic may be a biomarker of over-active vascular RhoA-ROCK signaling, supported by the fact that bio-molecular evidence exists for vascular RhoA-ROCK over-activation in all of these risk factor cohorts (220, 253, 324, 420). Beyond its contractile effects, over-active RhoA-ROCK stimulates cytoskeletal re-organization, proliferation/differentiation, migration, inflammation, and apoptosis (223), all cellular processes implicit in CVD states including hypertension. Thus, small artery/arteriolar EDCF activity, recently shown in SHR to be prevented by statin treatment (399), which is known to have a pleiotropic RhoA-ROCK inhibition-related effect (220), may be responsible for both active-functional (endothelium-dependent vasomotor tone) and passive-structural (vascular remodeling and rarefaction) resistances, thereby directly contributing to elevated blood pressure. Furthermore, since EDCF activity in conduit vasculature, in which the current experiments were performed, beyond potentially exerting a mechanical consequence (85), may also impart a biological consequence (e.g. inflammatory mediators, VSM migration/activation), this could help

explain at a cellular-molecular level why hypertension conveys such a high risk of developing large vessel diseases related to RhoA-ROCK over-activation such as atherogenesis (234) and aortic aneurism (408), which also occur with aging and diabetes. Accordingly, a better understanding of the mechanisms governing endothelium-dependent contraction and RhoA-ROCK signaling in both conduit and resistance vasculature should uncover new prevention and/or treatment strategies, beyond COX or TP receptor blockade (319), to combat vascular pathologies characteristic of hypertension and other CVD disorders.

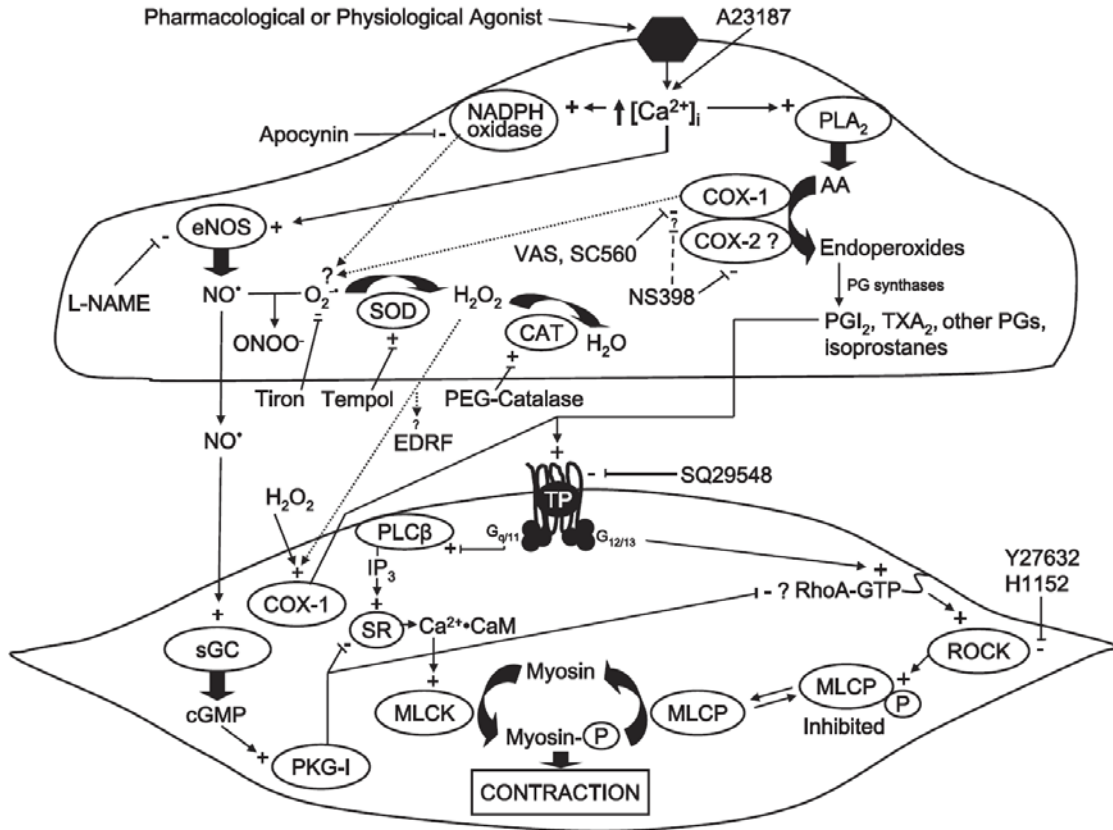


Figure 3.8. Proposed mechanism accounting for endothelium-dependent contractile activity prominent in the CCA of SHR. See *Discussion* for details.

Chapter 4
Thesis Study III

**Effect of Glutathione Depletion and Aging on Endothelium-Derived Relaxing
and Contracting Factor Activity in the Common Carotid Artery of Sprague
Dawley Rats**

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4.1 Preface

This Chapter presents Thesis Study III as the manuscript *Effect of Glutathione Depletion and Aging on Endothelium-Derived Relaxing and Contracting Factor Activity in the Common Carotid Artery of Sprague Dawley Rats* written in journal article style.

Included in this Chapter are the Overview (Section 4.2), Introduction (Section 4.3), Materials and Methods (Section 4.4), Results (Section 4.5), and Discussion (Section 4.6) of the manuscript in the format of this thesis.

Figures (Figure 4.1 to 4.4) and Tables (4.1 to 4.3) corresponding to the Results are located in order at the end of Section 2.5.

4.2 Overview

The role of the anti-oxidant GSH in mediating vascular (dys)function, and how that role may depend on age, is unclear. The main purpose of the current study was to investigate the effect of chronic in vivo BSO-induced GSH depletion (30mM BSO in drinking water for 10d) on EDRF and EDCF activity in the isolated CCA of Adult (25-36wk old) and Aging (60-75wk old) SD rats. Confirming treatment efficacy, BSO induced depletion of GSH in both the liver (~55%; $p < 0.01$) and conduit vasculature (~30% in aorta; $p < 0.01$), and elevated indices of oxidative stress, including plasma (~40%; $p < 0.01$), liver (~65%; $p < 0.01$), and aortic (~45%; $p = 0.09$) H_2O_2 content, as compared to control rats (CON) consuming untreated drinking water. There were no significant differences ($p > 0.05$) in liver or aortic GSH or H_2O_2 content between Adult and Aging rats. Moreover, blood pressure and blood flow measured in the CCA were unaffected by Aging, or BSO. EDRF activity was assessed in excised, pre-contracted CCA as endothelium-dependent relaxation to cumulative ACh (-9.0 to -4.0 LogM). In both Adult and Aging CCA, ACh stimulated a robust NOS-mediated relaxation response, which in Aging CCA was blunted moderately (~14% at maximum amplitude; $p < 0.01$) at higher ACh concentrations (-6.0 to -4.0 LogM), an effect abolished by COX inhibition, but not by NOS inhibition or by the ROS inhibitors Tempol or MnTMPyP. Assessing ACh-stimulated EDCF activity specifically in quiescent NOS-inhibited CCA confirmed that a dose-dependent contractile response could be elicited at ACh concentrations between -6.0 to -4.0 LogM, which was ~3.5-fold greater in Aging vs. Adult CCA ($p < 0.01$) and abolished completely by COX-1-specific inhibition, but unaffected by ROS inhibitors. In response to BSO treatment, ACh-stimulated relaxation in Adult pre-contracted CCA was not significantly affected, even under the stress of 24h exposure to AII. In Aging pre-contracted CCA, maximal ACh-stimulated relaxation was also unaffected by BSO, whereas the relaxation sensitivity to ACh was actually augmented modestly (~0.40 LogM leftward shift in EC_{50} ; $p = 0.01$), an effect found to be eliminated by either COX ($p < 0.01$) or ROS ($p < 0.04$) inhibitors. ACh-stimulated contractile responses in both Adult and Aging quiescent CCA were unaffected by BSO. Likewise, BSO had no effect on the vasomotor responsiveness to exogenous H_2O_2 , which stimulated a dose-dependent and partly NOS-mediated relaxation response in pre-contracted Adult CCA, and a dose-dependent and predominantly COX-mediated contractile response in quiescent Adult CCA. In conclusion, the data herein demonstrate that NOS-mediated EDRF signaling activity is well-preserved, and Age-related COX-mediated EDCF signaling activity is unaffected, in the CCA of Adult and Aging SD rats following BSO treatment, suggesting that, regardless of age, chronic GSH depletion itself does not play an obligatory role in causing endothelium-dependent vasomotor dysfunction.

4.3 Introduction

GSH (L- γ -glutamyl-L-cysteinyl-glycine), the most abundant low-molecular weight thiol in vivo, has been found to play a multi-faceted role in anti-oxidant defense against the accumulation of ROS including $O_2^{\cdot-}$, H_2O_2 , and OH^{\cdot} (152). Aging (18, 151, 243, 268, 295, 337) and age-associated CVD risk factors such as hypertension (123, 388, 435), hyperlipidemia (2, 205, 233), and hyperglycemia/diabetes (191, 331), are characterized by increased oxidative stress and have been linked to a decline in GSH content and related anti-oxidant defenses in a variety of tissues including the heart and vasculature. However, the specific role of GSH in mediating endothelial function, and how that role may depend on age, is unclear.

Impaired endothelium-dependent vasorelaxation is a putative biomarker of endothelial dysfunction and a hallmark of overt CVD and its associated risk factors (410). Vasorelaxation is mediated by EDRFs, the most predominant of which is eNOS-derived NO (107, 109). A major cause of impaired endothelium-dependent vasorelaxation relates to reduced NO bioavailability, largely attributed to the destruction of NO by ROS, particularly by $O_2^{\cdot-}$ (54). Accordingly, work investigating the role of GSH in endothelial (dys)function has focused on its potential to affect NOS-mediated vasorelaxation. Early studies found that acute augmentation of GSH in CVD states (2, 200, 296, 404) or acute depletion of GSH in healthy states (2, 123, 210) could elicit, respectively, an improvement or impairment of endothelium-dependent relaxation in the conduit or peripheral vasculature. Furthermore, chronic depletion of GSH in vivo was initially reported to be associated with a pronounced increase in blood pressure linked to biomarkers of elevated ROS and reduced NO bioavailability (123, 388, 435). However, subsequent reports from our laboratory (114) and others' (25, 173) examining the direct effect of chronic in vivo GSH depletion on vascular function have found varied responses, with NO-mediated relaxation either undergoing a modest impairment (114), no change (25), or modest augmentation (173), and with little (25) or no (114, 173) increase in blood pressure. Thus, the pathophysiological consequence of chronic GSH depletion on endothelial vasomotor function remains controversial. Moreover, no investigation of GSH and endothelial vasomotor function has focused on the potential interactive effect of GSH depletion with age.

In addition to NO, other signaling mediators may act as EDRFs, including certain COX-derived PGs, and, under some circumstances, H_2O_2 (109). It is also becoming increasingly evident that endothelial cells can produce contracting factors, EDCFs, which competitively inhibit vasorelaxation (109, 385). These EDCFs have been identified as COX-derived PGs (105, 360, 385), with a possible regulatory influence of ROS (332, 360). Hence, impaired vasorelaxation may be multi-factorial, dictated by an imbalance between EDRF and EDCF activity driven by

cascades of NOS-, COX-, and ROS-mediated signaling (109). In fact, conduit vasomotor dysfunction can be caused not only by under-active EDRF signaling but in particular by over-active EDCF signaling in various states of oxidative stress and CVD risk including advancing age (142, 144, 153, 329, 359, 395, 403, 418).

It was therefore hypothesized that chronic depletion of GSH would alter endothelium-dependent vasorelaxation by affecting NOS-, COX-, and ROS-mediated signaling and that aging would cause a shift toward impaired EDRF activity and augmented EDCF activity, resulting in vasomotor dysfunction that would be expected to be exacerbated by GSH depletion. The main purpose of the current study was to test this hypothesis by systematically investigating endothelium-dependent vasomotor functions in conduit arteries isolated from adult and aging rats treated chronically with BSO, an irreversible γ -glutamyl-cysteine synthase inhibitor effective in depleting cellular GSH (251).

4.4 Methods

4.4.1 Animals

Experiments were performed using male SD rats obtained from a colony maintained at the University of Waterloo, established using breeders purchased from Harlan Laboratories (Indianapolis IN). Animals were housed in a reversed 12h light:12h dark cycle, temperature-controlled facility ($21\pm 1^\circ\text{C}$) with free access to standard laboratory chow and tap water. All procedures involving these animals were in accordance with the guidelines of the Canadian Council on Animal Care and approved by the University of Waterloo Animal Care Committee.

A 60-70mg/kg body weight intra-peritoneal injection of sodium pentobarbital was used to anesthetize each animal prior to hemodynamic measures and/or excision of blood and tissues.

4.4.2 Aging and BSO treatment: Experimental design

Animals were investigated at either 6-9 months (25-36wks) or 15-18 months (60-75wks) of age. Herein, these younger and older animal cohorts are defined as 'Adult' and 'Aging', respectively (see Results for cohort characteristics). SD rats were chosen for use in the current study because they represent a commonly employed, otherwise healthy model to study the effects of GSH depletion (21-25, 29, 114, 122-124, 173, 388, 435) and elevated oxidative stress (211, 262, 299).

Ten days prior to testing, animals from the Adult and Aging cohorts were randomly selected to continue receiving untreated tap water, defined as CON, or tap water containing 30 mM (67g/100ml) BSO. This water was prepared fresh for drinking every other day. The mode (drinking water), concentration (30mM), and duration (10 days) of BSO treatment was chosen

based on our previous work demonstrating efficacy in depleting tissue GSH and augmenting tissue ROS in adult SD rats (114).

4.4.3 Hemodynamic Measures

Arterial hemodynamics were measured in the CCA of a subset of anesthetized animals from each group using a high-fidelity catheter-tip pressure transducer (Millar Instruments, Inc., Houston TX) and transit-time perivascular flow-probe (Transonic Systems Inc., Ithaca NY) as we have previously described in detail (85). HR was determined by a frequency/time count of the pressure-flow waveforms.

4.4.4 Tissue Preparations

Anesthetized animals were killed by excision of the heart. Blood was collected into a heparinized syringe and then centrifuged (at 4°C) to yield plasma. Liver, CCA (left and right), and descending aortic tissues were excised and cleaned of surrounding connective material in 4°C buffer solution containing, in mM, 131.5 NaCl, 13.5 NaHCO₃, 11.2 glucose, 5.0 KCl, 2.5 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgCl₂, and 0.025 EDTA, prepared fresh daily in ultra-pure water at pH 7.40. Care was taken not to damage the endothelium upon excision and processing of the vascular segments.

Sections of liver, aorta, and CCA tissues were blot-dried and, together with plasma, flash-frozen and stored at -80°C for later biochemical analysis (as described below). To test vasomotor function, freshly-harvested CCA were immediately prepared into arterial rings cut 2.0mm in axial length. Rings either remained completely intact or were mechanically denuded of endothelial cells (-Endo) as we have previously described and validated (84, 85).

4.4.5 Measurement of Vascular Function

Vasomotor responses were examined in the CCA primarily because of our recent work establishing that over-active COX-mediated contractile signaling was related to vasomotor dysfunction and impaired hemodynamics in the CCA of other rodent strains ((84, 85); i.e. in Thesis Study I and II). Furthermore, beyond its high degree of accessibility, this conduit vascular segment is attractive to study because of its significance both physiologically (owing to the major contribution of the CCA to total brain perfusion (100)) and clinically (owing to the strong correlation of CCA IMT (374) and CCA hemodynamics (294) to atherosclerosis and CVD risk).

CCA vasomotor responses were examined by measuring isometric tension in rings mounted between wires and equilibrated in a 37°C tissue bath containing 5ml of 95% O₂-5% CO₂-gassed buffer solution, as we have previously described in detail (84, 85). The resting tension on each ring was set to 2.75g, which was shown in our preliminary experiments to correspond with the optimal length for agonist-stimulated isometric tension production in CCA

rings from SD rats (data not show). As a reference, each ring was first stimulated to contract twice with exposure to maximal (60mM) KCl and then washed thoroughly. Rings were incubated for 45min in buffer without (No Drug) or with drugs known to manipulate NOS, COX, or ROS signaling, as described in the *Results* and corresponding Figures. From this quiescent state, or following near-maximal sustained agonist-stimulated pre-contraction, rings were cumulatively exposed to a specific agonist to assess vasocontractile or vasorelaxation responses, respectively. These experimental protocols were primarily designed to examine EDCF and EDRF activities, respectively, as we have previously explained in detail (84, 85).

4.4.6 Measurement of Tissue GSH and GSSG

To characterize the impact of BSO treatment and aging on GSH and its oxidized disulfide, GSSG, we used an HPLC protocol to quantify GSH and GSSG content in the liver and conduit vasculature, where aortic tissue was used as a surrogate due to CCA sample volume limitations. According to procedures we have previously described (114, 301), these tissues were homogenized and extracted, and then injected through a Mircosorb 5 μ m 25cm x 4.5cm column and detected spectrophotometrically at a wavelength of 350nm. Values from tissue samples were normalized to their wet weight.

4.4.7 Measurement of Plasma and Tissue ROS

To characterize the impact of BSO treatment and Aging on oxidative stress, we quantified ROS in plasma, liver, and aorta using a 10-acetyl-3,7-dihydroxyphenoxazine (Amplex[®] Red)-horseradish peroxidase (HRP) assay which detects H₂O₂ (89). Procedures for this assay were slightly modified from what we have previously described (114, 310). Briefly, tissue sample homogenates (ground in ice-cold PBS) or plasma (diluted in PBS) were added to a 10 μ M Amplex[®] Red-0.2U/ml HRP solution (89) in a microplate well (1:1; 100 μ l total volume) and incubated for 30min in the dark at room temperature, after which fluorescence from resorufin, an oxidation product of Amplex[®] Red, was measured every 15min for 1h using 530/590nm excitation/emission wavelengths. A standard curve ($R^2=0.998$) was used to calculate sample H₂O₂ concentration. Values from tissue samples were normalized to protein content determined via a standard bicinchoninic acid assay.

Liver and aortic ROS were also assessed by measuring the fluorescence of dichlorofluorescein, an oxidation product of 2',7'-dichlorodihydrofluorescein-diacetate (DCFH-DA; considered a general ROS detector (89)), according to procedures we have previously described (114, 310)).

4.4.8 Exposure to Angiotensin II

Some CCA from Adult CON and BSO SD rats were cultured for 24h with AII or vehicle, after which their vasomotor responses were examined. CCA were excised and prepared into arterial rings as described above in the *Methods*, but in 4°C sterile media comprised of DMEM with 100U/ml penicillin, 100µg/ml streptomycin, and 0.25µg/ml amphotericin B. Using a protocol adapted from others' past works (66, 87, 431), 2mm rings were placed in sterile dishes (BD Falcon™; 60x15 mm) containing 6ml of media without or with 1µM AII and cultured for 24h in a standard cell culture incubation environment (sterile-filtered room air plus 5% CO₂ at 37°C and 100% humidity).

Vasomotor responses were also examined in 2mm CCA rings excised from Adult SD rats that had been infused for 8d with AII (or vehicle) using a protocol adapted from previous studies (299), as follows. Animals were anesthetized (with isoflurane) before an osmotic mini-pump (Alzet Model 2002; DURECT Corporation, Cupertino CA) was implanted subcutaneously in the nape of the neck. Prior to implantation, mini-pumps were pre-filled with either AII reconstituted in sterile saline or saline alone. The mini-pumps delivered an average of 0.47mg/kg/d AII.

4.4.9 Drugs, Chemicals, and Solutions

All reagents were purchased from either Sigma-Aldrich (St. Louis, MO via Oakville, ON, Canada) or BioShop Canada (Burlington, ON, Canada) unless otherwise indicated.

Stock solutions of PE, KCl, ACh, SNP, H₂O₂, L-NAME, Tempol, MnTMPyP (Calbiochem, EMB Biosciences, La Jolla, CA), CAT (from bovine liver), and AII were prepared in ultra-pure water; Indo in DMSO; and U46619 (Cayman Chemical, Ann Arbor, MI), SC560 (Cayman Chemical), and NS398 (Cayman Chemical) in N₂-purged ethanol; all at a 1000-fold higher concentration than the desired in-bath/culture concentration, and then diluted 1:1000 with buffer/media. The effect of DMSO or ethanol vehicles on vasomotor function at this 1000X dilution was found to be negligible in pilot experiments. Stock solution(s) of each compound used were either prepared fresh daily or stored at -20°C, depending on its reconstituted stability as reported by the manufacturer. BSO was prepared directly in tap water, as described above in the *Methods*.

Amplex Red[®]-HRP assay kits and DCFH-DA (Molecular Probes[®] Inc., Eugene, OR) were purchased from Invitrogen[™]. Stock solutions for these assays were prepared and stored as directed by the manufacturer.

DMEM (HyClone[®] SH30021 medium) and penicillin-streptomycin-amphotericin B solutions were purchased from Thermo Fisher Scientific Inc. Stock solutions of culture media were prepared and stored under sterile conditions.

Sodium pentobarbital was purchased from Vetoquinol N.-A. Inc. (Lavaltrie, QC, Canada) and isoflurane from Abbott Laboratories Ltd. (Saint-Laurent, QC).

4.4.10 Data Analysis and Statistics

Data are expressed as means \pm SE. Curve fitting and statistical comparisons were performed using GraphPad Prism v4.03 (La Jolla, CA). Differences were considered statistically significant if $P < 0.05$. n refers to the number of rats or ring responses per condition. Ring cumulative tension responses to a vasomotor stimulus were individually best fit by non-linear regression to a sigmoidal dose-response curve, generating maximum amplitude (Max Amp), dose resulting in 50% of Max Amp (EC50), and area under the curve (AUC) parameters, which were then averaged within their respective condition. Student's t -tests were used for single-point comparisons, and one- or two-way ANOVA with Bonferroni posttests, when necessary, were used for multiple within and/or between group comparisons.

4.5 Results

4.5.1 General Characteristics of SD rats with Age and BSO treatment

Data and statistics on animal age, body weight, and food and water intake are presented in Table 4.1. Aging animals were initially (before treatment) \sim 15% heavier than Adult animals. Body weight in Adult CON animals was stable over the 10 day treatment period, whereas body weight in Aging CON animals decreased despite a similar intake of food and water. In both age cohorts, BSO was associated with a decrease in body weight, likely explained by a corresponding decrease in food intake.

Within our rat colony, the mortality rate of the Aging (60-75 week old) cohort was \sim 9%, consistent with survival rate data reported of SD rats commercially available from Harlan (personal communication with Harlan Laboratories).

4.5.2 Liver and Aortic GSH Decreased with BSO Treatment but not Age

Data and statistics on liver and aortic GSH content are presented in Table 4.2. GSH content in both the liver and aorta was not different between Adult and Aging animals. Within each age cohort, BSO treatment was effective in decreasing GSH in both the liver (\sim 55%) and aorta (\sim 30%). There was no Age interaction with BSO treatment.

4.5.3 Indices of Oxidative Stress were Increased with BSO Treatment but not Age

Data and statistics on plasma, liver, and aortic H_2O_2 content are presented in Table 4.2. H_2O_2 levels were not different between Adult and Aging animals in the liver or aorta while in the

plasma there was actually a decrease in H₂O₂ with aging. Within each age cohort, BSO was effective at increasing H₂O₂ in the plasma (~40%), liver (~65%), and aorta (~45%), though the increase in aortic H₂O₂ did not reach statistical significance. There was no significant Age interaction with BSO treatment. DCF fluorescence showed similar trends, with increased liver and vascular ROS in response to BSO but not aging (data not shown).

Data and statistics on liver and aortic GSSG (oxidized GSH) and the GSH:GSSG (redox) ratio are presented in Table 4.2. GSSG was increased significantly with aging in aorta (~45%) but not in liver, whereas GSH:GSSG was decreased with aging in both tissues (liver, ~15%; aorta, ~25%), perhaps suggesting some age-dependent redox abnormality. Within each age cohort, GSH:GSSG was significantly decreased by BSO in the liver (~30%) but not the aorta, as a result of a less pronounced decrease in aortic (~20%) compared to liver (~40%) GSSG. There were no significant Age interactions with BSO treatment.

4.5.4 CCA Hemodynamics were Unaffected by Age and BSO treatment

Data and statistics on HR, blood pressure, and blood flow in the CCA are presented in Table 4.3. Adult CON animals were normotensive, and there were no significant effects or interactions of Age and/or BSO treatment on the hemodynamic variables measured, indicating that neither aging nor BSO-induced GSH depletion resulted in altered CCA hemodynamics.

4.5.5 BSO-Induced GSH Depletion did not Impair EDRF Activity nor Augment Aging-Related EDCF Activity

ACh-Stimulated Responses in Pre-Contracted CCA (Assessment of EDRF Activity): Effect of Endothelial and NOS Signaling Pathways

ACh is a commonly used agonist to test endothelium-dependent vasomotor functions (25, 84, 85, 114, 329). Under the No Drug condition (Figure 4.1A), cumulative ACh elicited a robust vasorelaxation response in Adult CCA (97.4±1.2% Max Amp; -6.64±0.14 LogM EC₅₀; 260±12 AUC). Aging was associated with a ~14% decrease in the Max Amp of ACh-stimulated vasorelaxation (83.9±2.7%; p<0.01) while EC₅₀ (p=0.12) and AUC (p=0.18) were not significantly affected by aging. In response to BSO treatment, ACh-stimulated vasorelaxation parameters were unaffected in Adult CCA (p>0.50), whereas in Aging CCA, BSO caused a ~0.40 LogM leftward shift in EC₅₀ (-7.06±0.08; p=0.01) while Max Amp (p=0.61) and AUC (p=0.06) were not significantly affected. These data indicate that aging resulted in a moderate blunting of the maximal vasorelaxation response to ACh, and that BSO-induced GSH depletion resulted in no change in adults, and with aging actually caused a modest augmentation, in vasorelaxation sensitivity.

Eliminating endothelial signaling under the –Endo condition (represented in Figure 4.1B) completely abolished vasomotor activity to cumulative ACh in Adult and Aging, CON and BSO PE-pre-contracted CCA, thus confirming that ACh-stimulated relaxation in fact represents EDRF activity in these animals/conditions.

Inhibiting NOS signaling with L-NAME (10^{-4} M; (84, 85, 114, 329, 330)) (Figure 4.1B) caused a >85% reduction in Adult CCA relaxation to cumulative ACh ($13.7 \pm 2.7\%$ Max Amp; 24 ± 6 AUC; $p < 0.01$ vs. No Drug), indicating that endothelium-dependent vasorelaxation in the CCA from SD rats is almost exclusively mediated by the EDRF NO. In Aging CCA, the response to ACh was also reduced >85% but was biphasic: causing a small amount of vasorelaxation from -9.0 to -6.5 LogM (Max Amp, $3.1 \pm 1.1\%$ at -6.5 LogM) followed by re-contraction through to -4.0 LogM (Max Amp, $-8.9 \pm 4.8\%$ at -4.0 LogM) (Figure 4.1B), suggesting that CCA from Aging SD rats in particular may possess vasocontractile (i.e. EDCF) activity in response to high-dose (i.e. -6.0 to -4.0 LogM) ACh that could be responsible for the age-related blunting of maximal vasorelaxation. There was no BSO treatment, or Age-BSO interaction, effect within these responses ($p > 0.45$), suggesting that NO was likely mediating the observed increase in low-dose (i.e. -9.0 to -6.0 LogM) ACh-stimulated vasorelaxation sensitivity induced by BSO.

ACh-Stimulated Responses in Quiescent CCA (Assessment of EDCF Activity): Effect of Endothelial and NOS Signaling Pathways

ACh-stimulated contraction, which can blunt maximal relaxation causing net vasomotor dysfunction (85, 109), is best assessed in a quiescent vasomotor state, classically in the presence of L-NAME to inhibit competitive NOS signaling activity, thus amplifying the response (84, 85, 385). Cumulative ACh did not elicit contraction in No Drug quiescent Adult CON or BSO CCA, whereas a modest dose-dependent contractile response was elicited in Aging CON and BSO CCA from -6.0 to -4.0 LogM ACh (Max Amp, $3.8 \pm 1.6\%$ at -4.0 LogM; data not shown). In the presence of L-NAME (Figure 4.1C), a modest contractile response to ACh was uncovered from -6.0 to -4.0 LogM in Adult CCA ($6.1 \pm 0.9\%$ Max Amp; -5.08 ± 0.16 LogM EC_{50} ; 6 ± 1 AUC), while the ACh-stimulated contractile response in Aging CCA was amplified >3.5-fold ($24.5 \pm 4.8\%$ Max Amp; -5.09 ± 0.17 LogM EC_{50} ; 22 ± 4 AUC; $p < 0.01$ vs. No Drug). These data confirm that ACh-stimulated EDCF activity exists in the CCA of SD rats and that this activity is greater in Aging vs. Adult animals (3.5- to 4-fold under the L-NAME condition; $p < 0.01$). The contractile parameters from these ACh-stimulated responses were no different between CON and BSO treatment within both Adult and Aging CCA ($p > 0.50$), suggesting that EDCF activity is unaffected by BSO-induced GSH depletion. There was no Age-BSO interaction effect on these ACh-stimulated responses ($p > 0.55$). The average threshold for eliciting ACh-stimulated

vasocontractile activity was \sim -6.0 LogM across Age, BSO treatment, and L-NAME, and these factors had no effect on EC₅₀ (p>0.40).

Eliminating endothelial signaling with -Endo (represented in Figure 4.1C) abolished all vasomotor activity to cumulative ACh in Adult and Aging, CON and BSO CCA, thus confirming that ACh-stimulated contraction actually represents EDCF activity in quiescent CCA from these animals/conditions.

SNP-Stimulated Responses in Pre-Contracted CCA: Effect of Endothelial and NOS Signaling Pathways

SNP is a NO donor and is commonly used to test the sensitivity of VSM to endothelium-independent NO-mediated relaxation (25, 85, 114). Cumulative SNP elicited a robust vasorelaxation response in Adult CCA (100.1 \pm 0.4% Max Amp; -8.34 \pm 0.04 LogM EC₅₀; 332 \pm 4 AUC) that was unaffected by Age, BSO treatment, or the Age-BSO interaction (Figure 4.1D; p>0.25), indicating that neither aging nor BSO-induced GSH depletion affected VSM sensitivity to NO. These SNP-stimulated responses in Adult and Aging, CON and BSO CCA were unaffected by -Endo or L-NAME (Figure 4.1D; p>0.35), or by any other pre-incubation drug, or drug combination, condition used in the current study (p>0.20; data not shown).

PE-Stimulated Responses in Quiescent CCA: Effect of Endothelial and NOS Signaling Pathways

PE is a specific agonist of α_1 adrenergic receptors located on the plasma membrane of VSM, as well as endothelial cells (85, 182, 252), and is commonly used to test pharmaco-mechanical contractile coupling (84). Under the No Drug condition (Figure 4.1E), cumulative PE elicited a robust vasocontractile response in Adult CCA (104.0 \pm 4.1% Max Amp; -6.82 \pm 0.06 LogM EC₅₀; 189 \pm 12 AUC). Aging was associated with a \sim 40-45% decrease in PE-stimulated vasocontraction (p<0.01), characterized by a 65.1 \pm 4.9% Max Amp and 100 \pm 10 AUC, and a \sim 0.35 LogM rightward shift in EC₅₀ (-6.48 \pm 0.07; p=0.01), whereas there was no BSO treatment or Age-BSO interaction effect on the PE-stimulated responses (p>0.35). These data indicate that α_1 adrenergic receptor-mediated contractile signaling was markedly impaired by aging, but unaffected by BSO-induced GSH depletion.

Inhibiting NOS signaling with L-NAME (Figure 4.1F) augmented PE-stimulated contraction in both Adult and Aging CCA (p<0.01 vs. No Drug), characterized, respectively, by a \sim 25% and \sim 55% increase in Max Amp (128.4 \pm 3.6% and 122.6 \pm 4.7%), a \sim 60% and \sim 70% increase in AUC (302 \pm 12 and 233 \pm 17), and a \sim 0.55 and \sim 0.45 leftward shift in EC₅₀ (-7.37 \pm 0.07 and -6.91 \pm 0.12 LogM). L-NAME completely eliminated the difference between the Max Amp of

PE-stimulated contraction in Aging vs. Adult CCA, and the differences in AUC and EC₅₀, while still apparent ($p < 0.01$), were markedly attenuated. Eliminating endothelial signaling with –Endo had similar effects as L-NAME ($p < 0.01$ vs. No Drug; data not shown), together indicating that the age-related impairment in α_1 adrenergic receptor-mediated contractile signaling may have at least in part been caused by augmented endothelial NO signaling in Aging CCA. As in the No Drug conditions, there was no BSO treatment or Age-BSO interaction effect within the L-NAME condition responses ($p > 0.35$). Besides L-NAME, no pre-incubation drug (and drug combination) condition used in the current study had an affect on PE-stimulated CCA contraction (in Adult or Aging, CON or BSO; data not shown), with the exception of MnTMPyP, which in all cases modestly attenuated both contractile sensitivity and magnitude ($p < 0.01$; data not shown).

KCl-Stimulated CCA Responses

KCl is a depolarizing agent and is commonly used to test electromechanical contractile coupling (84). Maximal (60mM) KCl reference contractions in quiescent CCA, ~1.0g in amplitude, were not significantly affected by Age, BSO treatment, or the Age-BSO interaction ($p > 0.30$; data not shown), or by any other pre-incubation drug, or drug combination, condition used in the current study ($p > 0.30$; data not shown).

Effect of COX and ROS Inhibitors on ACh-stimulated Responses in Pre-Contracted and Quiescent CCA

In pre-contracted Adult CON and BSO CCA (Figure 4.2A and 4.2C, respectively), neither the non-selective COX-1/2 inhibitor Indo (10^{-5} M; (84, 85, 329, 330)), the nitroxide ROS scavenger Tempol (10^{-4} M; (62, 84, 274, 411)), nor the manganese porphyrin ROS scavenger MnTMPyP (10^{-4} M; (79, 274, 330)) significantly affected ACh-stimulated relaxation parameters ($p > 0.16$). In pre-contracted Aging CON CCA (Figure 4.2B), ACh-stimulated relaxation parameters were also unaffected by Tempol or MnTMPyP ($p > 0.25$), whereas Indo increased Max Amp ($98.3 \pm 2.5\%$; $p = 0.04$) so that the difference between Adult and Aging CON CCA relaxation (see Figure 4.1A) was eliminated. Indo also increased Max Amp in pre-contracted Aging BSO CCA ($96.4 \pm 2.0\%$; $p < 0.01$), as well as right-shifted the EC₅₀ of ACh-stimulated relaxation (-6.64 ± 0.11 LogM; $p < 0.01$) (Figure 4.2D), so that the difference between Adult and Aging BSO CCA relaxation (see Figure 4.1A) was eliminated. Both Tempol and MnTMPyP, while not affecting Max Amp ($p > 0.65$), also right-shifted the EC₅₀ (-6.72 ± 0.10 and -6.62 ± 0.17 LogM, respectively; $p < 0.04$), as well as decreased the AUC (211 ± 20 and 205 ± 9 , respectively; $p < 0.01$), of ACh-stimulated relaxation in pre-contracted Aging BSO CCA (Figure 4.2D) so that the difference between Aging CON and BSO CCA relaxation (see Figure 4.1A) was eliminated. Together these data indicate

that the age-related blunting of maximal ACh-stimulated CCA relaxation was mediated solely by COX signaling, and suggest that the BSO-induced augmentation in the sensitivity of ACh-stimulated NO-dependent CCA relaxation with aging might have involved COX and ROS signaling.

The effect of the COX and ROS inhibitors on quiescent CCA stimulated with ACh was assessed only in the presence of L-NAME, which amplifies endothelium-dependent contractile responses (as confirmed herein; see above in *Results*) thus improving the ability to distinguish if, and the extent to which, a particular inhibitor affects ACh-stimulated EDCF activity (84, 85, 329, 330). In quiescent Adult CON and BSO CCA (Figure 4.2E), Indo eliminated the contractile activity stimulated by ACh, as did the COX-1 specific inhibitor SC560 ($3 \cdot 10^{-7}$ M; (84, 331, 338)) and the preferential COX-2 inhibitor NS398 (10^{-6} M; (84, 85, 139, 329, 423)), whereas Tempol had no effect. In quiescent Aging CON and BSO CCA (Figure 4.2F), both Indo and SC560 eliminated all contractile activity stimulated by cumulative ACh, while NS398 partially attenuated this activity ($9.4 \pm 2.3\%$ Max Amp; -4.87 ± 0.16 LogM EC_{50} ; 9 ± 3 AUC; $p < 0.01$ vs. L-NAME), perhaps related to non-selective COX-1 inhibition even at its supposedly 'COX-2 preferential' concentration used in the current study and previously (85, 423). Neither Tempol nor MnTMPyP had a significant effect on the ACh-stimulated contractile response in Aging CON or BSO CCA (Figure 4.2F; $p > 0.09$). Together these data suggest that the ACh-stimulated contractile activity in quiescent CCA of Adult and Aging animals was mediated by COX-1-specific signaling but apparently not by ROS signaling, consistent with the maximal ACh-stimulated relaxation response findings in pre-contracted CCA (see Figure 4.2A-D).

Exogenous H₂O₂-Stimulated Responses in Pre-contracted or Quiescent CCA: Effect of NOS and COX Signaling Pathways

Since GSH is a co-factor for the H₂O₂-metabolizing enzyme GPx (152), limiting GSH bioavailability can result in the accumulation of H₂O₂ (114), as confirmed herein by our biochemical findings (see Table 4.2). H₂O₂ can elicit vasomotor activity; however, depending on the species, vascular territory, and/or phenotype of the animal studied, H₂O₂ has been proposed to act as either an EDRF (101, 110) or an EDCF (360), and exogenous H₂O₂ stimulation has been shown to elicit either vasorelaxation (128, 365, 428) or vasoconstriction (84, 306, 327, 344, 365, 366), or both (126, 365). To determine the vasomotor responsiveness to H₂O₂ in the CCA of our SD rats, and whether those responses are affected by BSO-induced GSH depletion, we examined exogenous H₂O₂-stimulated relaxation and contraction in Adult CON and BSO CCA.

In pre-contracted CCA (Figure 4.3A), H₂O₂ elicited a small contractile response (in some but not all cases) from -6.0 and -4.17 LogM (on average, $-10.8 \pm 3.2\%$ Max Amp at -4.17 LogM),

followed by a robust dose-dependent relaxation response through to -3.0 LogM ($69.0 \pm 2.4\% \text{ Max Amp}$; $-4.01 \pm 0.03 \text{ LogM EC}_{50}$; $66 \pm 3 \text{ AUC}$) that was no different between CON and BSO ($p > 0.55$). L-NAME right-shifted the EC_{50} ($-3.61 \pm 0.03 \text{ LogM}$; $p < 0.01$) and decreased the AUC (45 ± 2 ; $p < 0.01$) of relaxation stimulated by H_2O_2 in both CON and BSO CCA, while Max Amp was unaffected ($p > 0.55$). Eliminating endothelial signaling with $-\text{Endo}$ had a similar effect as L-NAME on the H_2O_2 -stimulated relaxation response parameters ($p < 0.01$ vs. No Drug; data not shown). Together, these data indicate that H_2O_2 could indeed stimulate relaxation in the CCA of our SD rats, which was in part mediated by endothelial NO signaling, and that these H_2O_2 -stimulated relaxation responses were unaffected by BSO-induced GSH depletion.

A maximal dose of SNP (-4.0 LogM) following the H_2O_2 dose-relaxation response (which elicited $\sim 70\%$ relaxation at $-3.0 \text{ LogM H}_2\text{O}_2$; see Figure 4.3A) resulted in near complete relaxation ($90\text{-}100\%$; data not shown) in both CON and BSO CCA, thus confirming robust VSM responsiveness to NO after cumulative (high-dose) H_2O_2 exposure.

CAT (1200 U/ml ; $(84, 330, 423)$) abolished all vasomotor activity to cumulative H_2O_2 in pre-contracted CCA, thus confirming the specificity of the H_2O_2 -stimulated response; conversely, Tempol did not affect the response ($p > 0.25$; data not shown).

H_2O_2 -stimulated contractile activity (Figure 4.3B) was specifically assessed in quiescent CCA (co)incubated with L-NAME ($84, 330, 423$), similar to the approach used to optimally assess ACh-stimulated contractile activity (see above in *Results*). Under these conditions, H_2O_2 elicited a contractile response from -6.0 to -3.5 LogM ($20.1 \pm 1.9\% \text{ Max Amp}$; $-4.30 \pm 0.05 \text{ LogM EC}_{50}$; $16 \pm 2 \text{ AUC}$) that was no different between CON and BSO CCA. Indo caused a $\sim 90\%$ reduction in CCA contraction to cumulative H_2O_2 ($4.0 \pm 0.8\% \text{ Max Amp}$; $2 \pm 1 \text{ AUC}$; $p < 0.01$ vs. L-NAME) in both CON and BSO. Together these data indicate that H_2O_2 could indeed stimulate contraction in the CCA of our SD rats, which was predominantly mediated by COX signaling, and that these H_2O_2 -stimulated contractile responses were unaffected by BSO-induced GSH depletion.

Effect of AII Exposure on ACh-Stimulated Responses in Pre-Contracted CCA

ACh-stimulated relaxation has been shown to be preserved in CCA isolated from adult heterozygous GPx-1-deficient mice, whereas, in contrast to wild-type CCA, heterozygous GPx-1-deficient CCA were not able to defend against 24h exposure to AII in culture, which induced substantial oxidative stress-mediated endothelial dysfunction (66). Since BSO-induced GSH depletion in the current study did not significantly impair endothelium-dependent CCA vasomotor functions, we examined ACh-stimulated relaxation responses in Adult CON and BSO CCA after the additional stress of 24h culture with AII.

PE-stimulated contraction in cultured CCA was markedly diminished and would not establish a sustained pre-contractile state; hence, the pre-contraction prior to cumulative ACh exposure in cultured CCA was established by exposure to a near-maximal dose of the thromboxane-prostanoid receptor agonist U46619 ($10^{-6.5}$ M; (84, 331)). U46619 was found to stimulate a robust dose-contractile response in SD CCA ($157.8 \pm 6.5\%$ Max Amp relative to 60mM KCl contraction; -7.40 ± 0.05 LogM EC_{50} ; 143 ± 8 AUC) that was no different between CON and BSO, nor between No Drug and AII culture conditions ($p > 0.45$) (data not shown). Furthermore, similar to PE-stimulated contractile responses (see Figure 4.1E and F), U46619-stimulated CCA contractile responses were augmented by L-NAME ($188.7 \pm 10.5\%$ Max Amp; -7.68 ± 0.04 LogM EC_{50} ; 218 ± 9 AUC; $p < 0.01$), while unaffected by Indo ($p > 0.65$) (data not shown).

ACh-stimulated relaxation responses were no different between CON and BSO in U46619 pre-contracted CCA either freshly-harvested ($69.9 \pm 3.8\%$ Max Amp; -6.01 ± 0.10 LogM EC_{50} ; 146 ± 10 AUC) or cultured under control (No Drug) conditions ($45.2 \pm 3.1\%$ Max Amp; -5.71 ± 0.13 LogM EC_{50} ; 78 ± 5 AUC; $p > 0.15$) (Figure 4.4A; consistent with the relaxation responses to cumulative ACh found in freshly-harvested Adult CON vs. BSO CCA pre-contracted with PE shown in Figure 4.1A). In both freshly-harvested and cultured CCA, L-NAME almost completely eliminated relaxation to cumulative ACh ($8.0 \pm 1.0\%$ Max Amp; AUC 18 ± 1 ; $p < 0.01$ vs. No Drug; Figure 4.4A), while –Endo abolished all vasomotor activity (data not shown), thus confirming that ACh-stimulated relaxation from the U46619-stimulated pre-contractile state was predominantly mediated by NO. Accordingly, the blunted vasorelaxation response to ACh observed following arterial culture (Figure 4.4A) was likely caused by prolonged exposure of the CCA to an environment free of endothelial shear stress, which is known to promote robust NO bioavailability (136).

In response to culture with AII (Figure 4.4B and 4.4C), the Max Amp ($36.7 \pm 2.6\%$) and AUC (63 ± 5) of ACh-stimulated relaxation were modestly decreased in both CON and BSO CCA, however these effects were not significant ($p > 0.09$). Furthermore, AII culture did not affect CON or BSO CCA relaxation responses to cumulative SNP ($p > 0.45$; data not shown). These data indicate that CCA isolated from Adult SD rats were able to defend against the stress of AII exposure even following BSO-induced GSH depletion. Likewise, we found that an 8d infusion of AII (vs. saline vehicle) in Adult CON SD rats, while inducing severe hypertension (177 ± 5 vs. 137 ± 4 mmHg mean CCA pressure; $p < 0.01$; data not shown), also did not significantly affect ACh-stimulated CCA relaxation response parameters ($p > 0.35$; data not shown).

Table 4.1. General characteristics of animals in relation to BSO treatment and Age.

	Adult		Aging		Effect		
	CON	BSO	CON	BSO	Age p value	BSO p value	Age x BSO p value
Age, months	8.2 ± 0.2	8.5 ± 0.1	16.7± 0.3	17.0± 0.2	--	--	--
Body weight Initial, g	499± 8	506± 5	571± 11	580± 9	<0.01	--	--
Change after 10d treatment, %	0.3± 0.4	-4.2± 0.5	-2.5± 0.5	-8.8± 1.3	<0.01	<0.01	0.02
Food consumption, g • d⁻¹	23.1± 0.5	19.8± 0.5	23.4± 0.8	16.8± 1.2	NS (0.06)	<0.01	0.03
Water consumption, ml • d⁻¹	47± 2	55± 2	56± 4	47± 3	NS (0.99)	NS (0.90)	<0.01

Adult and Aging SD rats were administered either vehicle (tap water; CON) or 30mM BSO for 10d in the drinking water. Data represent means±SEM; n=26-31 per condition. A p<0.05 was considered statistically significant; NS, not significant. Based on water consumption, the estimated dose of BSO received by the Adult and Aging rats was 2.5-3.5 mmol • kg⁻¹ • d⁻¹.

Table 4.2. Biochemical Characteristics of Animals in Relation to BSO treatment and Age.

	Adult		Aging		Effect		
	CON	BSO	CON	BSO	Age	BSO	Age x BSO
					p value	p value	p value
Tissue GSH and GSSG, nmol • mg wet weight⁻¹							
Liver							
GSH	7.020± 0.256	3.655± 0.409	7.171± 0.319	2.863± 0.265	NS (0.39)	<0.01	NS (0.21)
GSSG	0.774± 0.070	0.541± 0.040	0.896± 0.038	0.456± 0.061	NS (0.73)	<0.01	NS (0.06)
GSH:GSSG ratio	9.87± 0.84	6.82± 0.60	8.08± 0.32	6.09± 0.22	0.02	<0.01	NS (0.33)
Aorta							
GSH	0.393± 0.020	0.295± 0.023	0.444± 0.035	0.291± 0.022	NS (0.37)	<0.01	NS (0.29)
GSSG	0.028± 0.003	0.021± 0.002	0.038± 0.006	0.032± 0.004	0.03	0.03	NS (0.58)
GSH:GSSG ratio	15.37± 1.92	14.35± 0.86	12.62± 1.17	10.09± 1.17	0.01	NS (0.20)	NS (0.58)
Plasma H₂O₂, μmol • l⁻¹	1.446± 0.129	1.886± 0.150	1.020± 0.155	1.585± 0.146	0.02	<0.01	NS (0.67)
Tissue H₂O₂, nmol • mg protein⁻¹							
Liver	2.844± 0.344	4.173± 0.358	2.479± 0.229	4.478± 0.593	NS (0.94)	<0.01	NS (0.42)
Aorta	3.530± 0.453	4.920± 1.127	3.670± 0.710	5.334± 1.041	NS (0.76)	NS (0.09)	NS (0.88)

Reduced GSH, oxidized GSH disulfide (GSSG), and H₂O₂ concentrations in plasma and/or tissues from Adult and Aging SD rats following administration of either vehicle (tap water; CON) or 30mM BSO for 10d. Data represent means±SEM; n=8-14 per condition. A p<0.05 was considered statistically significant; NS, not statistically significant. Aortic tissue was used as a conduit vascular surrogate due to CCA sample volume limitations (see *Methods*).

Table 4.3. Hemodynamic Characteristics of Animals in Relation to BSO treatment and Age.

					Effect		
	Adult		Aging		Age	BSO	Age x BSO
	CON	BSO	CON	BSO	p value	p value	p value
Heart rate, cycles • min⁻¹	345± 9	337± 11	322± 6	327± 9	NS (0.07)	NS (0.89)	NS (0.46)
Carotid blood pressure, mmHg							
Systolic	139± 5	142± 3	133± 3	141± 5	NS (0.54)	NS (0.18)	NS (0.51)
Diastolic	106± 4	107± 2	97± 2	103± 3	NS (0.12)	NS (0.18)	NS (0.29)
Pulse	33± 2	36± 1	37± 1	38± 2	NS (0.15)	NS (0.33)	NS (0.76)
Mean	122± 5	123± 2	117± 2	121± 4	NS (0.43)	NS (0.33)	NS (0.46)
Carotid blood flow, ml • min⁻¹							
Maximum (systolic)	30.0± 1.9	27.8± 1.2	27.2± 1.2	27.7± 1.8	NS (0.35)	NS (0.64)	NS (0.49)
Minimum (diastolic)	5.0± 0.5	5.3± 0.5	5.10± 0.4	5.8± 0.2	NS (0.51)	NS (0.21)	NS (0.73)
Mean	10.1± 0.6	10.1± 0.6	10.3± 0.7	11.1± 0.4	NS (0.23)	NS (0.49)	NS (0.53)
Carotid conductance, Mean, $\mu\text{l} \cdot \text{min}^{-1} \cdot \text{mmHg}$	83± 5	82± 4	89± 6	91± 4	NS (0.18)	NS (0.92)	NS (0.78)

CCA blood pressure and blood flow in Adult and Aging SD rats following administration of either vehicle (tap water; CON) or 30mM BSO for 10d. Data represent means±SEM; n=5-7 per condition. A p<0.05 was considered statistically significant; NS, not statistically significant.

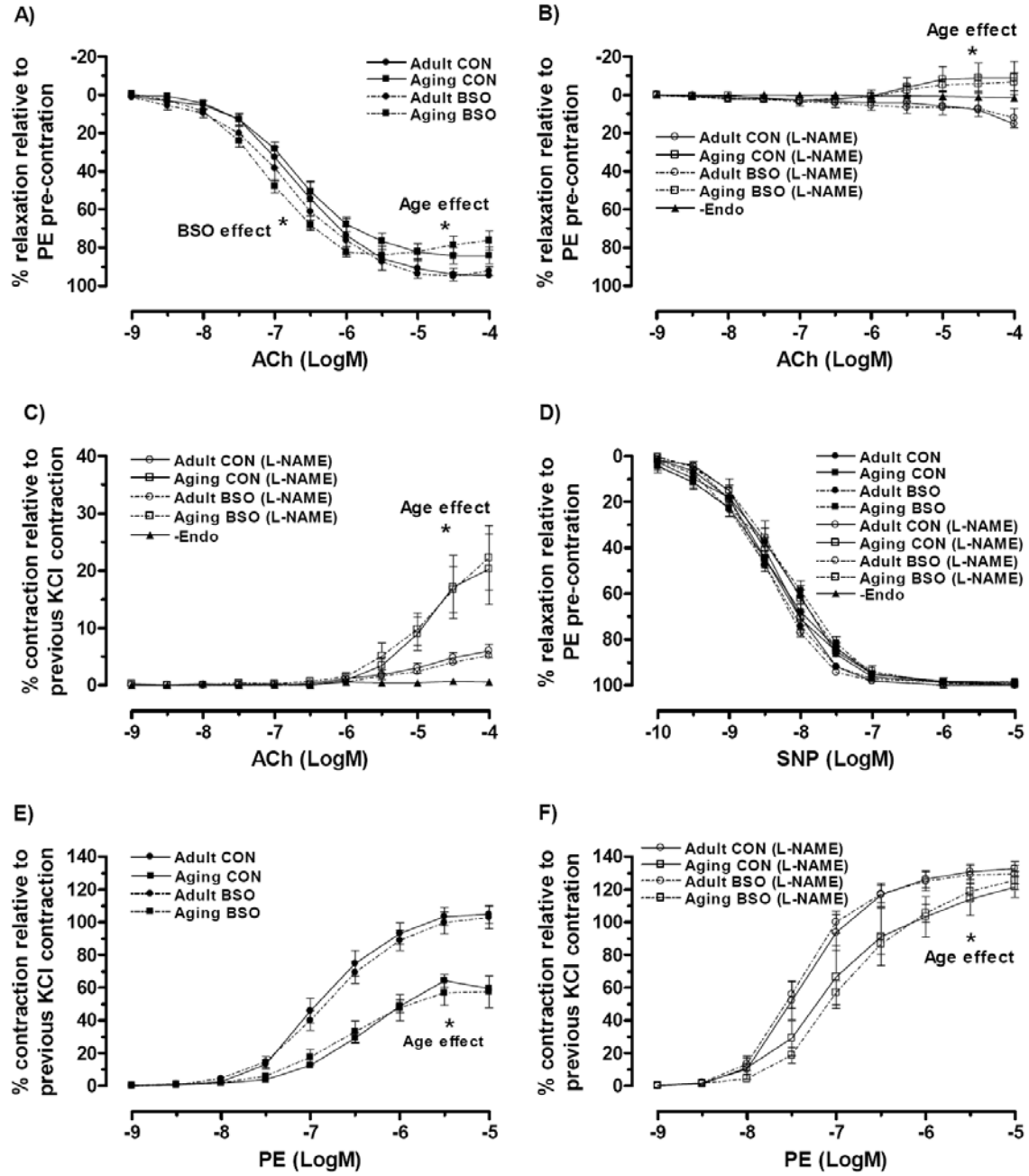


Figure 4.1. For Figure Legend, see next page.

Figure 4.1. Endothelium- and NO-dependent vasomotor functions of the CCA excised from Adult and Aging SD rats treated for 10d with either vehicle (CON) or BSO (30mM), a GSH depleting agent (see Table 4.2 for liver and conduit vascular GSH data). To assess EDRF activity, arterial rings were first stimulated with the depolarizing agent KCl (60mM), then washed thoroughly and pre-contracted with a near-maximal dose of the α_1 adrenergic receptor agonist PE (10^{-6} M), after which rings were exposed to the muscarinic receptor agonist ACh (panel A and B). To assess endothelium-independent vasorelaxation, rings were exposed to the NO donor SNP (panel D). To assess EDCF activity, arterial rings were first stimulated with KCl (60mM) then washed thoroughly, after which rings (in a quiescent state) were exposed to ACh (panel C). To assess endothelium-independent vasoconstriction, rings were exposed to PE (panel E and F). Data were collected using intact rings pre-incubated with either no drug (n=5-13) or the NOS inhibitor N^G -nitro-L-arginine methyl ester (L-NAME; $10^{-4.0}$ M; n=5-14), or in endothelium-denuded rings (-Endo; n=3-4); see *Results* for additional details. Data represent means \pm SEM, expressed relative to PE pre-contraction or previous KCl contraction, as indicated. $p < 0.05$ was considered statistically significant. * denotes a significant main effect from ANOVA; see *Results* for statistical comparisons of curve-fit parameters. Absolute KCl contraction was no different across Age or BSO treatment and unaffected by drug pre-incubation (net average: 1.00 ± 0.03 g).

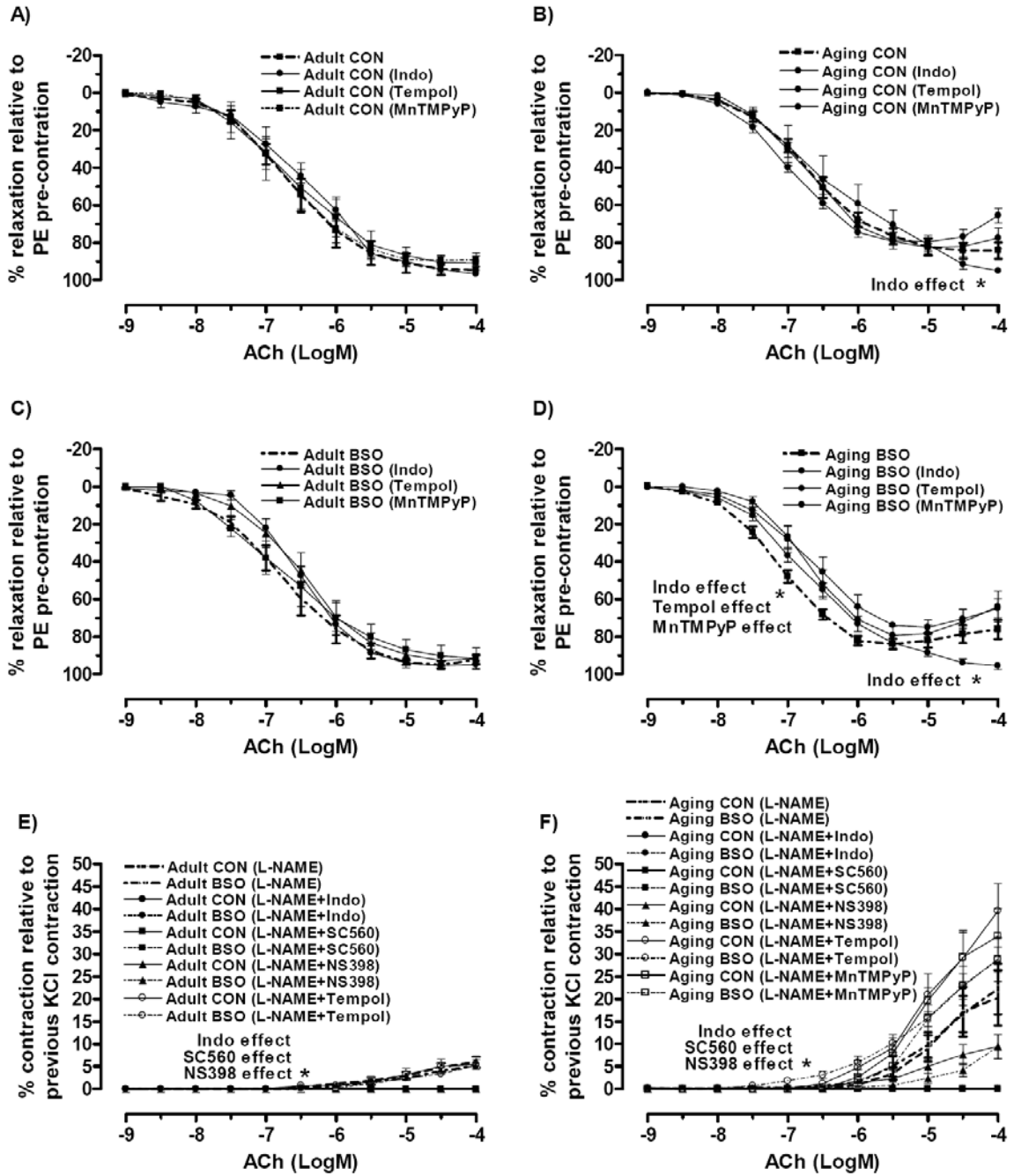


Figure 4.2. For Figure Legend, see next page.

Figure 4.2. EDRF and EDCF activity stimulated by ACh in intact CCA rings, excised from Adult and Aging SD rats treated for 10d with either vehicle (CON) or the glutathione-depleting agent BSO, and pre-incubated with either the non-selective COX inhibitor Indo ($10^{-5.0}$ M; n=4-12), the COX-1-specific inhibitor SC560 ($3 \cdot 10^{-7.0}$ M; n=3-4), the preferential COX-2 inhibitor NS398 ($10^{-6.0}$ M; n=3-4), the nitroxide ROS scavenger Tempol ($10^{-4.0}$ M; n=3-7), or the manganese porphyrin ROS scavenger MnTMPyP ($10^{-4.0}$ M; n=3-5), in the absence (for assessment of EDRF activity; panels A-D) or co-presence (for assessment of EDCF activity; panels E and F) of the NOS inhibitor *N*⁰-nitro-L-arginine methyl ester (L-NAME+; $10^{-4.0}$ M); see *Results* for additional details. EDRF and EDCF activities were assessed as described in the Figure 4.1 legend. For clarity, the curves in Figure 4.1A representing the ‘Control’ no drug conditions of ACh-stimulated vasomotor activity in PE ($10^{-6.0}$ M)-pre-contracted rings (EDRF activity), and the curves in Figure 4.1C representing the ‘Control’ L-NAME conditions of ACh-stimulated vasomotor activity in quiescent rings (EDCF activity), were duplicated as thick dashed lines herein the appropriate graphs. Data represent means \pm SEM, expressed relative to PE pre-contraction or previous KCl contraction, as indicated. $p < 0.05$ was considered statistically significant. * denotes a significant main effect from ANOVA; see *Results* for statistical comparisons of curve-fit parameters.

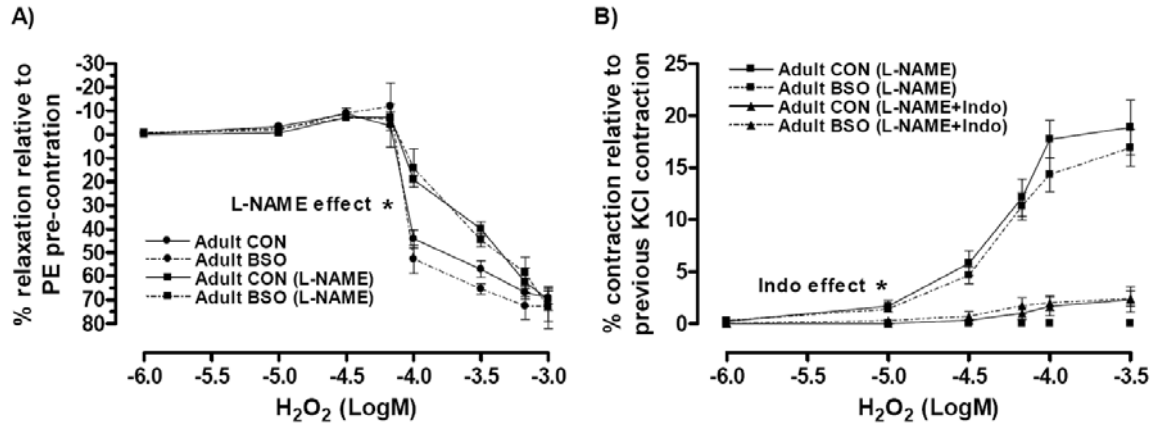


Figure 4.3. Vasomotor functions stimulated by H_2O_2 in intact CCA rings, excised from Adult SD rats treated for 10d with either vehicle (CON) or the glutathione-depleting agent BSO, and pre-incubated with either no drug ($n=5-10$), the NOS inhibitor *N*^o-nitro-L-arginine methyl ester (L-NAME; $10^{-4.0}$ M; $n=4-16$), and/or the non-selective COX inhibitor Indo ($10^{-5.0}$ M; $n=3-7$); see *Results* for additional details. To assess the vasorelaxation effect of H_2O_2 (panel A), arterial rings were first stimulated with the depolarizing agent KCl (60mM), then washed thoroughly and pre-contracted with a near-maximal dose of the α_1 adrenergic receptor agonist PE (10^{-6} M). To specifically assess the vasoconstrictile effect of H_2O_2 (panel B), arterial rings were first stimulated with the depolarizing agent KCl (60mM), and then washed thoroughly to establish a quiescent state. Data represent means \pm SEM, expressed relative to PE pre-contraction or previous KCl contraction, as indicated. $p < 0.05$ was considered statistically significant. * denotes a significant main effect from ANOVA; see *Results* for statistical comparisons of curve-fit parameters.

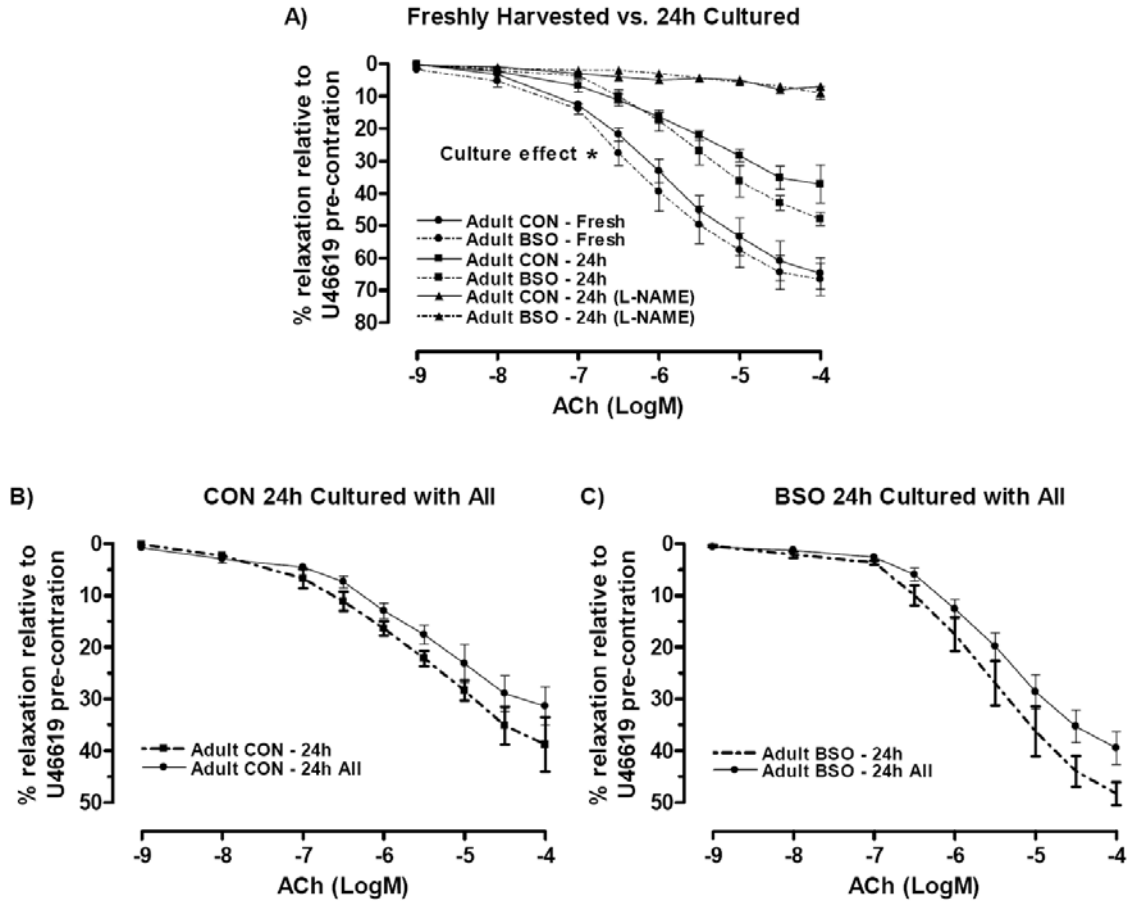


Figure 4.4. Vasomotor functions of the intact CCA rings, excised from Adult SD rats treated for 10d with either vehicle (CON) or the glutathione-depleting agent BSO, cultured for 24h without or with AII (10^{-6} M), and pre-incubated with either no drug ($n=5-6$) or the NOS inhibitor *N*^o-nitro-L-arginine methyl ester (L-NAME; $10^{-4.0}$ M; $n=2-3$); see *Results* for additional details. To assess endothelium-dependent vasorelaxation, arterial rings freshly-harvested (Fresh; $n=5-6$), 24h-cultured (24h; $n=5-6$), and 24h-cultured with AII (24h AII; $n=5-6$) were first stimulated with the depolarizing agent KCl (60mM), then washed thoroughly and pre-contracted with a near-maximal dose of the TP receptor agonist U46619 ($10^{-6.5}$ M), after which rings were exposed to the muscarinic receptor agonist ACh. For clarity, the curves in Figure 4.4A representing the ‘Control’ no drug conditions of ACh-stimulated vasomotor activity in U46619 ($10^{-6.5}$ M)-pre-contracted CON and BSO rings cultured for 24h, were duplicated as thick dashed lines in Figure 4.4B and 4.4C, respectively. Data represent means \pm SEM, expressed relative to U46619-pre-contraction or previous KCl contraction, as indicated. $p<0.05$ was considered statistically significant. * denotes a significant main effect from ANOVA; see *Results* for statistical comparisons of curve-fit parameters.

4.6 Discussion

The experiments herein were primarily designed to investigate the effect of GSH depletion and Aging on the endothelium-dependent signaling pathways affecting vasomotor activity in the conduit vasculature. Novel aspects of this work are the systematic approach used to examine endothelium-dependent vasomotor function in response to the GSH-depleting drug BSO, which unlike past approaches accounted for the possibility that the depletion of GSH might affect either EDRF or EDCF signaling activities, and might exert differential effects with age, or in response to AII exposure. The major findings of the current study provide evidence that chronic BSO treatment, while effective in significantly depleting GSH and elevating ROS, neither: 1) induced vasomotor dysfunction in Adult animals, where NO-mediated EDRF activity was robust, even under the stress of AII; nor 2) worsened age-related vasomotor dysfunction, wherein a similarly robust maximal NO-mediated EDRF activity was moderately impaired by competitive COX-mediated EDCF activity. The data presented help to elucidate past findings from our laboratory (114) and others (25, 173) signifying little-to-no direct pathological effect of chronic GSH depletion in vivo on vascular function linked to EDRF signaling activity, and demonstrate for the first time that chronic GSH depletion does not overtly affect EDCF signaling activity, a hallmark of endothelium-dependent vasomotor dysfunction in aging and other states of CVD risk.

4.6.1 GSH and Vascular Hemodynamic Function

The role of GSH in maintaining hemodynamic homeostasis is controversial. Experimental GSH depletion via chronic BSO treatment in adult SD rats has been reported to cause arterial blood pressure to increase either: markedly (by 40-80 mmHg; (122, 388, 435)); mild-to-moderately (by 10-25 mmHg; (21-23, 25, 29, 124)); or not at all (24, 114, 173). Herein, we confirm and extend upon our previous pressure-related hemodynamic findings by showing that BSO treatment had no effect on either blood pressure or blood flow in the CCA of Adult SD rats. We anticipated that BSO treatment might alter CCA hemodynamics with aging, which can be predisposing to arterial hypertension (49, 278, 340) and cerebral hypo-perfusion (157). However, under both CON and BSO treatment conditions in the current study, neither blood pressure nor blood flow was affected in the CCA of our Aging SD rats.

In our previous work (114) we speculated that the divergent reports of BSO-induced blood pressure effects in the literature may be due to an interaction between the tail cuff plethysmography measurement procedure and a central BSO effect predisposing animals to stress-induced blood pressure elevation. To date, all studies reporting no blood pressure change (24, 114, 173) and 4 of 6 studies reporting mild-to-moderate hypertension (21-23, 25) in response

to chronic BSO treatment have used indwelling catheters, while all studies reporting marked hypertension (122, 388, 435) have used tail cuff plethysmography. Thus, the available data maintain support for our previous speculation (114). More importantly, these data together support the conclusion that chronic GSH depletion has little impact on conduit artery hemodynamic homeostasis, even in the context of Aging, as established herein.

4.6.2 GSH and EDRF Activity

The current study found that ACh-stimulated EDRF activity in the CCA from both Adult and Aging animals could almost exclusively be accounted for by NOS-mediated signaling, and that this EDRF signaling activity could temper contractions stimulated by PE, U46619, or ACh. The main experiments herein clearly demonstrate that BSO treatment did not cause impairment of NO-mediated EDRF signaling activities or the endothelium-independent NO-mediated relaxation activity stimulated by SNP, despite the fact that our BSO treatment, consistent with past studies, was effective in depleting tissue GSH (liver by >50% (114, 173, 388, 435), and aortic by >30% (25)), provoking a redox abnormality characterized by a decreased tissue GSH:GSSH ratio (123) and elevated markers of oxidative stress (21-23, 25, 122, 124, 388, 435), in particular an increase in both systemic and vascular ROS, including H₂O₂, by 40-65% (29, 114, 123).

Our laboratory was first to investigate the direct effect of chronic GSH depletion on isolated vascular function (114). This built on studies solely focused on the acute effects of GSH manipulations in vivo (123, 200, 210, 296, 404) or in vitro (2) suggesting a pathological role of reduced GSH related to impaired NO-mediated vasomotor activity. While our previous work did find that chronic BSO-induced GSH depletion and ROS elevation in vivo enhanced PE-stimulated contraction, and blunted ACh-stimulated NO-mediated relaxation in the conduit aortic vasculature excised from adult SD rats (114), these effects were relatively modest as compared to studies showing substantial impairment of endothelium-dependent conduit artery relaxation linked to oxidative stress in animal models of CVD risk (143, 271), or in healthy adult SD rats chronically exposed to oxidative stress-promoting agents such as AII (211, 299). Cell culture studies using harvested aortic endothelial cells have found little effect of BSO-induced GSH depletion on NO bioactivity (164, 270), supporting the view that GSH depletion has little effect on endothelial function. An over-expression of eNOS, SOD-1/-2, and the VSM NO target effector sGC in the vascular wall was proposed to explain our previous findings (114). Indeed, subsequent work from other laboratories have confirmed over-expression of these proteins in the conduit aortic vasculature of adult SD rats in response to chronic BSO-induced GSH depletion (25, 173, 432). Furthermore, other results from these studies are consistent with the current study, including the demonstration of no impairment in ACh-stimulated NO-mediated vasorelaxation and little-or-

no impairment in SNP-stimulated vasorelaxation despite increased markers of oxidative stress such as nitro-tyrosine, a biological footprint for NO destruction by $O_2^{\cdot-}$ (25, 388, 435). Thus, the data presented herein, in concert with past findings (25, 114, 173), indicate that the conduit arterial vasculature of adult SD rats is able to accommodate for a chronic GSH depletion and prevent any substantial reduction in NO-mediated EDRF activity.

Our previous work (114) proposed that a physiological or pharmacological stressor in addition to GSH depletion might elicit substantial vascular dysfunction in BSO-treated animals by exceeding their apparent compensatory buffering capacity. In the current study, we established that the ability of our SD rats to defend against BSO-induced impairment in NO-mediated EDRF activity was in fact well preserved under the anticipated stress of either aging (4, 154, 224, 340, 421) or 24h AII exposure (66, 86, 87, 431). These data add new perspective on the capacity of the vasculature to accommodate for GSH depletion, while providing further support to the available evidence, as described above (25, 114, 173), indicating no direct apparent pathological effect of chronic GSH depletion on conduit artery EDRF signaling activity.

The cell-signaling mechanism(s) responsible for these apparent vascular compensatory adaptations to chronic *in vivo* GSH depletion and increased oxidative stress induced by BSO remain to be determined. It is tempting to speculate that a BSO-induced vasorelaxation mechanism involving H_2O_2 might be operating in the conduit arterial vasculature of SD rats. In this regard, Iwata et al (173) reported that chronic BSO treatment augmented the sensitivity to ACh-stimulated NO-mediated relaxation in the aorta of adult SD rats, an effect which was eliminated by the H_2O_2 -metabolizing agent CAT. Somewhat unexpectedly, we found in the current study a similar augmentation in ACh-stimulated NO-mediated relaxation in Aging but not Adult BSO CCA, an effect which was eliminated by either the COX inhibitor Indo or by the ROS inhibitors Tempol, considered a SOD mimetic (62, 274, 411), or MnTMPyP, considered a SOD+CAT mimetic (79, 274, 330). A potential explanation for these findings could be that ROS produced in a COX-dependent manner stimulates NO-mediated vasorelaxation. Certainly, COX, which can be over-expressed in SD rat conduit vasculature in aging and other pathophysiological states (329), has been identified as a source of elevated vascular ROS in response to endothelial agonists including ACh (328, 329, 357), and herein we demonstrate that H_2O_2 , the accumulation of which was elevated by BSO treatment, could exogenously elicit CCA relaxation at least in part by an endothelium- and NO-dependent manner. However, we also demonstrate herein that exogenous H_2O_2 could elicit COX-mediated CCA contraction, thus signifying a potentially multifaceted role of H_2O_2 in conduit artery vasomotor activity, which appears to depend on both the concentration of H_2O_2 (126, 128) and the prevailing level of contractile tone (365). To what

extent these exogenous H₂O₂-stimulated vasomotor findings may reflect the vasomotor activity of endogenously produced H₂O₂ is uncertain. For that matter, whether ACh stimulation at any level of pre-contractile tone can in fact increase the endogenous concentration of conduit vascular H₂O₂ high enough to elicit any vasomotor activity (35, 367), and whether there are differences in these effects between BSO and CON, is also uncertain. Accordingly, while data presented by Iwata et al, and by the current study, may indirectly suggest that BSO-treated vasculature could preserve NO-mediated EDRF activity in the face of elevated ROS via vasorelaxant properties of H₂O₂, it is clear that further investigation is necessary to directly assess this as a probable mechanism.

4.6.3 GSH, EDCF Activity, and Aging

The Aging animal cohort in this study was not associated with hypertension, a decline in hepatic or vascular GSH, or a substantial change in hepatic or vascular redox environment. However, maximal ACh-stimulated CCA relaxation was found to be moderately blunted compared to the younger Adult animal cohort. This impaired response was likely not caused by a reduction in the bioavailability of the EDRF NO (4, 154, 224, 340, 421), as evidenced by a preserved ability of SNP to elicit relaxation in Aging CCA, and by a preserved ability of L-NAME to attenuate ACh-stimulated relaxation, augment ACh-stimulated contraction, and augment PE-stimulated contraction in Aging CCA, the latter of which was actually enhanced with Aging – i.e. the PE-L-NAME-to-PE-No Drug ratio, a putative biomarker of the effect of NO on basal tone (85, 95). Instead, this Age-associated vasomotor impairment was related exclusively to a competitive endothelium-dependent contractile response that could be eliminated by COX-1-specific inhibition. Hence, the results herein confirm that endothelial dysfunction in the CCA of our Aging animals can be attributable to enhanced EDCF activity mediated by COX-dependent signaling (142, 153, 329, 359, 418).

To the best of our knowledge, the current study is the first to examine the effect of manipulating GSH bioavailability on COX-mediated vasomotor function. It is well established from animal models of essential hypertension, diabetes, and aging that the EDCF signaling axis involves activation of COX-1 and/or COX-2 which are typically over-expressed in the vascular wall of these animals, leading to the production of PGs in response to endothelial agonists (e.g. -6.0 to -4.0 LogM ACh), in turn stimulating VSM TP receptors that initiate contractile activity (105, 360, 385). In addition, a number of studies suggest that the production of ROS may amplify this EDCF signaling axis (332, 360, 417). We hypothesized that COX-mediated CCA EDCF signaling activity might be exacerbated by BSO treatment, since reduced GSH bioavailability induced by BSO is known to elevate vascular oxidative stress (114), and to affect PG production both in endothelial cell cultures (38, 47) and in rodent tissues including the aortic wall and plasma

of SD rats (29, 41, 122-124, 235, 247). The data herein, however, clearly demonstrate that the ACh-stimulated contractile responses found in the CCA of both Adult and Aging animals were unaffected by BSO treatment. These findings suggest that regardless of Age, chronic GSH depletion induced by BSO does not augment COX-mediated EDCF activity in the conduit vasculature of SD rats.

Shi et al has found in SD rats that aging (12 month vs. 20 weeks old; (329)) or adult (20 weeks old) STZ-induced diabetes (328, 330) impaired conduit femoral artery vasomotor function via endothelium-dependent COX- and TP receptor-mediated contractile activity. This dysfunction was partially attenuated by CAT or MnTMPyP incubation (330), and related to increased endothelial ROS production from over-expressed COX (328, 329), and to decreased vascular wall catalase activity but not GSH content (329, 330). Furthermore, exogenous H₂O₂ stimulated COX- and TP receptor-dependent contractile activity in endothelium-denuded vasculature of the STZ-treated animals (330, 331). Collectively, it is suggested from these findings that a redox abnormality promoting H₂O₂ accumulation does contribute significantly to COX-mediated EDCF activity in SD rat conduit vasculature (332, 360).

In view of this work by Shi et al, it is somewhat surprising that the BSO treatment in the current study did not augment endothelium-dependent CCA contractile activity despite significantly lowering vascular GSH content and evidently causing accumulation of H₂O₂. It is possible that the systemic and vascular redox abnormalities caused by the BSO-induced GSH depletion were not marked enough in magnitude or duration to provoke a significant vascular wall over-expression of COX (329-331), or other proteins involved in the endothelial production and/or VSM activity of vasocontractile PGs (360). Furthermore, it is possible that the BSO-induced GSH depletion and redox abnormalities in the current study did not affect endothelium-dependent contractile activity because of the particular vascular model used, which appears to be characterized by COX-mediated EDCF activity wherein ROS does not contribute to the cell-signaling axis. Indeed, the COX-mediated CCA EDCF activity found in the CCA model used herein was augmented with age despite no detectable age-related change in vascular GSH content or oxidative stress. While the CCA was certainly capable of eliciting COX-mediated contractile activity in response to (exogenous) H₂O₂, the agonist-stimulated endothelium-dependent contractile responses found in our CCA preparations were not significantly affected by incubation with ROS inhibitors (Tempol or MnTMPyP), as they were in Shi et al's femoral artery preparations (330), implying that any ROS species produced upon endothelial stimulation were likely not contributing to CCA EDCF signaling activity. For this reason, it is likely that any redox abnormalities caused by BSO-induced GSH depletion would not have augmented COX-mediated

EDCF activity in our CCA, as the aging- or diabetes-related redox abnormalities did in Shi et al's femoral arteries (328-330). In this way, it is conceivable that GSH depletion subsequent to BSO treatment might augment endothelium-dependent contractile activity and impair endothelial vasomotor function in an animal/vascular model where ROS contributes significantly to the development of COX-mediated EDCF signaling; however, this remains to be determined.

4.6.4 Study Conclusion and Pathophysiological Perspective

It is concluded from the current study that chronic *in vivo* depletion of GSH induced by 10d BSO treatment, although increasing oxidative stress, does not impair endothelium-dependent NOS-mediated relaxation activity or augment age-exacerbated endothelium-dependent COX-mediated contractile activity in the conduit CCA vasculature of 25-36wk old Adult or 60-75wk old Aging SD rats.

The findings herein may have implications regarding the specific role of GSH in the pathogenesis of vascular dysfunction in response to aging or other age-associated CVD risk conditions, since it is suggested from these findings that chronic GSH depletion itself does not play an obligatory role in causing endothelium-dependent vasomotor dysfunction, relating either to under-active NOS-mediated EDRF signaling or to over-active COX-mediated signaling, regardless of age, at least to the point in life of our Aging cohort (which may be considered 'upper middle-aged' compared to 100+ wk old 'survivor' SD rats (4, 18, 188, 189, 224, 278, 421)). Identifying the time-dependent molecular, biochemical, and functional responses to BSO-induced GSH depletion in the CCA of the commonly used SD rat model, and in the context of other vascular and/or animal models that may perhaps be more susceptible/less adaptable to oxidative stress and aging (381), will certainly provide additional insight into the ROS-mediated compensatory adaptations that might occur with chronic GSH depletion, and help to discern the potential circumstances under which GSH may or may not act as an effective target and/or biomarker for the prevention or treatment of age-associated vascular pathologies.

Chapter 5

General Summary and Perspectives

5.1 Summary of Key Findings, Conclusions, and Novel Contributions of Thesis Studies

It was the global purpose of this thesis to gain a better understanding of the cellular-molecular mechanisms accounting for endothelial dysfunction in the CCA of animal models known to be characteristic of EDCF signaling activity. Consistent with this purpose, the studies of this thesis systematically examined the relative contributions of NOS and COX signaling pathways in mediating the endothelium-dependent relaxation (EDRF) and contractile (EDCF) activities of the CCA isolated from young adult WKY and SHR (Study I and II) and from Adult and Aging SD rats (Study III), with particular focus on elucidating the cellular-molecular mechanisms responsible for COX-mediated EDCF signaling activity.

The individual vasomotor findings of each of these studies have been thoroughly discussed in their respective Chapters (see Sections 2.6, 3.6, and 4.6). Section 5.1.1 and 5.1.2 below briefly summarizes the key findings, conclusions, and novel and/or important contributions of these vasomotor works, after which Section 5.1.3 briefly summarizes the novel and/or important contributions of the hemodynamic findings of the thesis studies.

This is followed (in Section 5.2 and 5.3) by a brief perspective on the general conclusions of the thesis studies and future direction of the research area.

5.1.1. Young Adult WKY-SHR CCA Model (Study I and II)

Key Findings and Conclusions

- 1)
 - ACh-stimulated endothelium-dependent and largely L-NAME-sensitive CCA relaxation was no different-to-modestly augmented sub-maximally in SHR vs. WKY; whereas maximal relaxation in SHR CCA was significantly blunted by an overt re-contractile response, which was completely eliminated by COX inhibition.

Conclusion:

- *An endothelium-dependent COX-mediated contractile response impairs endothelium-dependent vasorelaxation in the CCA of young adult SHR, while NO-mediated EDRF signaling activity remains well-preserved.*
- 2)
 - Examination of vasomotor activity in quiescent CCA found an ACh-stimulated EDCF response in SHR, but none in WKY; whereas L-NAME uncovered the

existence of EDCF activity in WKY, while amplifying >5-fold (vs. no drug) the EDCF activity in SHR.

Conclusion:

- ***NO-mediated EDRF activity can completely suppress any COX-mediated EDCF activity found in WKY CCA, while the NO-mediated EDRF activity in SHR, which is at least as robust as in WKY (as per Conclusion 1), cannot fully suppress the augmented COX-mediated CCA EDCF activity.***

- 3)
- Specific EDCF response examination in quiescent L-NAME pre-incubated CCA, revealed that ACh-stimulated contractile activity was >2-fold augmented in SHR vs. WKY, completely eliminated by COX-1-selective, but not COX-2-preferential inhibition, and nearly abolished by either selective TP receptor antagonism or selective ROCK inhibition, but unaffected by broad-spectrum PKC inhibition.
 - CCA contractile activity stimulated by the ROCK-dependent TP receptor agonist U46619 was no different between strains under the specific vasomotor examination conditions, or in no drug or endothelium-denuded conditions.
 - Specific vasomotor examination also revealed that while exogenous H₂O₂-stimulated CCA contractile activity, similar to ACh stimulation, was ~2-fold greater in SHR vs. WKY and nearly abolished by COX-1, TP receptor, and ROCK inhibition (but not PKC inhibition), the effects of cell-permeable ROS quenching/metabolizing compounds on ACh-stimulated contractile activity were only found to be modest, and were inconsistent, in both strains.
 - eNOS, COX-1, and RhoA, but not COX-2 or ROCK, proteins were found to be over-expressed in the vascular wall of SHR vs. WKY CCA.
 - Under the specific conditions examined, both basal and ACh-stimulated CCA PGI₂ production were increased >2-fold in SHR vs. WKY, which was sensitive to COX-1 inhibition but not ROCK inhibition.
 - RhoA activation was ~2-fold greater in basal SHR vs. WKY CCA under the specific conditions examined, and increased 1.5-2-fold in both strains upon ACh stimulation.

Conclusions:

- ***EDCF activity in the young adult SHR-WKY CCA model appears to be characterized by a COX-1-PG-TP receptor-RhoA-ROCK contractile signaling axis, which might be subject to modest amplification via ROS-mediated COX-1 activation.***

- *Augmented EDCF activity in the CCA of SHR appears to be predominantly related to COX-1 over-expression and EDCF over-production in the form of PGI₂, which results in TP receptor-mediated over-activation of RhoA-ROCK signaling activity, leading to contraction.*

Novel and/or Important Contributions

- First published studies to specifically evaluate both endothelium-dependent relaxation and contractile activities in the CCA of SHR and WKY.
- As a result, establishes (Study I) with confirmation (Study II) the SHR-WKY CCA as a bona fide experimental model to examine EDCF activity – a conduit vascular model more assessable and conducive to ex vivo/in situ/in vivo experimental investigations than previously identified SHR-WKY EDCF vascular models.
- Furthermore, establishes the CCA of young adult SHR-WKY – representing an ‘early-into-pathology’ essential hypertensive state – as a vascular model of endothelial vasomotor dysfunction solely caused by over-active COX-mediated EDCF signaling and not by reduced NO bioavailability and under-active EDRF signaling.
- Provides assessment of previously identified cellular-molecular-biochemical characteristics of the EDCF signaling axis; in doing so, establishing the particular EDCF signaling axis characteristics of the young adult SHR-WKY CCA, while offering comparative validation and contrast to particular findings from other vascular and animal models of EDCF activity – most notably the well-examined archetypal SHR-WKY aortic model.
- Provides the first examination among published studies of the mechanism by which EDCF(s) may stimulate VSM TP receptor-mediated contractile activity.
- As a result, presents through carefully-controlled experiments, direct evidence that activation of VSM RhoA-ROCK signaling plays an obligatory role in EDCF activity, and thus, the proposal that augmented EDCF activity may be a biomarker of over-active vascular RhoA-ROCK signaling.

5.1.2. Adult-Aging SD Rat CCA Model + Super-Imposed Chronic In Vivo BSO-Induced GSH Depletion Model of Oxidative Stress (Study III)

Key Findings and Conclusions

- 1)
 - No detectable differences in hepatic or vascular GSH or ROS content could be found in ‘Aging’ 60-75wk old vs. ‘Adult’ 25-36wk old SD rats, while, in both Age cohorts, 10d BSO treatment induced depletion of both hepatic (~55%) and vascular (~30%) GSH content, together with an ~40-65% increase in hepatic, plasma, and vascular ROS content.

Conclusions:

- *GSH depletion and elevated ROS accumulation suggests treatment efficacy within the BSO model of oxidative stress employed.*
 - *In the Adult-Aging SD rat cohort investigated, systemic and vascular GSH bioavailability and oxidative stress appear to be well-maintained with Age (up to 75wks), and equally responsive to GSH manipulation.*
- 2)
 - ACh-stimulated endothelium-dependent and largely L-NAME-sensitive CCA relaxation was no different sub-maximally in Aging vs. Adult; whereas maximal relaxation in Aging CCA was moderately blunted, which was completely eliminated by COX inhibition but unaffected by cell-permeable ROS metabolizing compounds (ROS inhibitors).
 - BSO-induced GSH depletion, while resulting in no difference-to-modest augmentation in ACh-stimulated CCA sub-maximal relaxation, did not affect maximal relaxation in either age cohort.
 - Exogenous H₂O₂-stimulated, partially L-NAME-sensitive CCA relaxation was no different with BSO treatment.
 - BSO-induced GSH depletion did not significantly affect ACh-stimulated endothelium-dependent and largely L-NAME-sensitive CCA relaxation following 24h in vitro static arterial culture with exposure to AII.

Conclusion:

- *An endothelium-dependent COX-mediated ROS-independent BSO-insensitive contractile response impairs endothelium-dependent vasorelaxation in the CCA of Aging SD rats, while NO-mediated EDRF signaling activity remains well-preserved, even following 24h chronic AII exposure.*

- 3) • Examination of vasomotor activity in quiescent CCA found an ACh-stimulated EDCF response in Aging, but none in Adult; whereas L-NAME uncovered the existence of EDCF activity in Adult, while amplifying >3.5-fold (vs. no drug) the EDCF activity in Aging.

Conclusion:

- ***NO-mediated EDRF activity can completely suppress any COX-mediated EDCF activity found in Adult CCA, while the NO-mediated EDRF activity in Aging SD rats, which is as least as robust as in Adult SD rats (as per Conclusion 2), cannot fully suppress the augmented COX-mediated CCA EDCF activity.***

- 4) • Specific EDCF response examination – in quiescent L-NAME-pre-incubated CCA – revealed that ACh-stimulated contractile activity was >3.5-fold augmented in Aging vs. Adult, completely eliminated by COX-1-selective, but not COX-2-preferential inhibition, and unaffected by ROS inhibitors.
- Specific vasomotor examination also revealed that BSO-induced GSH depletion had no effect on this ACh-stimulated CCA contractile activity in either Age cohort, or on exogenous H₂O₂-stimulated, predominantly COX-mediated CCA contractile activity.

Conclusion:

- ***EDCF activity in the Adult-Aging SD rat CCA model appears to be characterized by a COX-1-dependent contractile signaling axis, which is not subject to ROS-mediated amplification and is unaffected by BSO-induced GSH depletion.***

Novel and/or Important Contributions

- First among published studies to specifically evaluate both endothelium-dependent relaxation and contractile activities in the CCA of Adult and Aging SD rats.
- As a result, establishes that the Adult-Aging SD rat CCA can be used as an experimental model to examine EDCF activity – a conduit vascular model more assessable and conducive to ex vivo/in situ/in vivo experimental investigations than previously identified SD rat EDCF vascular models.
- Furthermore, establishes the CCA of Adult-Aging SD rats – likely representing an ‘early-into-pathological aging’ state – as a vascular model of endothelial vasomotor dysfunction solely caused by over-active COX-mediated EDCF signaling and not by reduced NO bioavailability and under-active EDRF signaling.

- Confirms in the CCA, previous findings in adult SD rat aorta that chronic BSO-induced GSH depletion does not impair NO-mediated EDRF signaling activity, and further establishes that the ability to accommodate this perturbation is preserved under conditions of Aging or AII exposure.
- Provides the first examination among published studies of the effect of manipulating GSH bioavailability on COX-mediated vasomotor function.
- As a result, provides evidence that chronic in vivo GSH depletion and increased oxidative stress in the apparent absence of CVD risk factors in Adult, or in Aging, does not augment COX-mediated EDCF signaling activity.

5.1.3. CCA Hemodynamic Characteristics in Young Adult WKY-SHR and Adult-Aging + BSO SD Rat Models

An additional aim of this Thesis was to describe the in vivo hemodynamic characteristics of the CCA in each animal model investigated, which served two main purposes: to identify the pressure-flow environment that the CCA was exposed to in vivo, and to provide an assessment of large artery hemodynamics in the model states of spontaneous hypertension (Thesis Study I), aging (Thesis Study III), and BSO-induced GSH depletion (Thesis Study III).

The individual hemodynamic findings in each animal model investigated have been discussed in detail in their respective Chapters (see Sections 2.6 and 4.6).

The following briefly summarizes the novel and/or important contributions of these hemodynamic findings.

Novel and/or Important Contributions of Findings

- In WKY, SHR, and SD rats – three animal types commonly used to investigate cardiovascular pathologies – provides (or will provide pending publication of Study III) previously unavailable data quantifying the in vivo pulsatile (mean, maximum, and minimum) blood pressure and blood flow characteristics, and the corresponding agonist-stimulated endothelial vasomotor functions, of the CCA – a vascular model commonly used in vitro/ex vivo/in situ/in vivo to investigate the relationship between hemodynamic forces and vascular pathogenesis.
- Provides the first examination of SHR-WKY CCA hemodynamics across the cardiac cycle; in doing so, providing compelling and conclusive evidence supporting past preliminary and incidental findings suggesting a reduction in mean and maximal CCA blood flow in young adult SHR vs. WKY, and also providing evidence for late systolic flow augmentation in the SHR CCA.

- Confirms past findings that BSO-induced GSH depletion has no effect on CCA blood pressure in adult SD rats, while further establishing that BSO has no effect on either CCA blood flow in adult SD rats, or on CCA blood pressure or blood flow in Aging SD rats, and that there are no apparent changes in CCA hemodynamics in a likely ‘early-into-pathological aging’ state in SD rats.

5.2 General Conclusions of Thesis Studies

Regarding the global purpose of this thesis – to gain a better understanding of the cellular-molecular mechanisms accounting for endothelial dysfunction – the findings of the thesis studies presented herein, as summarized in Sections 5.1.1 and 5.1.2 above, support the following general conclusions:

- COX- and TP receptor-mediated EDCF signaling activity can be a prominent characteristic of the CCA vascular wall.
- Endothelial dysfunction at the early stages of either spontaneous hypertension or normotensive aging may be related to the development of over-active EDCF signaling which is unrelated to reduced NO bioavailability and under-active EDRF signaling.
- ROS, oxidative stress, and GSH status do not play obligatory roles in over-active EDCF signaling activity.
- EDCF activity can be characterized by the activation of vascular RhoA-ROCK signaling.

5.3. Global Perspective and Future Directions: Of Cause and Consequence

5.3.1. Causes of Endothelial Vasomotor Dysfunction Related to Over-Active EDCF Signaling

Despite a relatively firm grasp of the basic cellular-molecular components and cell-signaling axis mediating and modulating EDCF signaling activity (see Figure 3.8 depiction of proposed mechanism in Thesis Study II), the underlying causes of over-active EDCF signaling under CVD risk conditions remain to be elucidated. As concluded in the most recent 2010 published articles reviewing endothelium-dependent COX-mediated contractile activity:

“The mechanisms that regulate the balance between endothelium-derived relaxing and contracting factors and the processes transforming the endothelium from a protective organ to a source of vasoconstrictor, pro-aggregatory, and pro-mitogenic mediators such as COX-dependent EDCFs, remains to be determined.” (direct quote from *Human Endothelial Dysfunction: EDCFs* by A. Viridis, L. Ghiadoni, and S. Taddei; (403)).

“It is likely that the prominence of endothelium-dependent contractions observed in arteries of aging and diseased (essential hypertension, diabetes) animals and humans reflects the progressive inability of the endothelial cells to generate enough NO to curtail the production of EDCF. Shifting from the normal release of NO (and EDHF) to that of EDCF likely plays an important role in the development of vascular disease” (direct quote from *COX-Mediated Endothelium-Dependent Contractions: From the Past to Recent Discoveries* by M. Wong and PM Vanhoutte; (417)).

As detailed in the Section 1.5.2 Review (*Under-Active EDRF Signaling and Endothelial Dysfunction*), reduced NO bioavailability and under-active EDRF signaling has been identified as a major cause of endothelial vasomotor dysfunction under CVD risk conditions by mechanisms relating to the development of a pro-oxidant / anti-oxidant imbalance leading to excess ROS accumulation and oxidative stress resulting in increased NO destruction and multiple impairments in the ability to activate eNOS to produce enough NO to stimulate robust VSM relaxation. Accordingly, it is natural to suspect that both NO and ROS might be contributing factors to the cause(s) of over-active EDCF signaling under CVD risk conditions.

Indeed, previous studies and the studies of this thesis have established that both NO and ROS, independently, or in concert (through ROS-mediated NO destruction), can acutely act, respectively, to negatively and positively modulate the defined $[Ca^{2+}]_i$ -PLA₂-AA-COX-1/-2-PGS-PG-TP receptor-RhoA-ROCK contractile signaling axis mediating EDCF activity: NO via competitive VSM relaxation signaling activity, and ROS via activation of endothelial or VSM COX-1/-2 (as detailed in the Section 1.5.3 Review – *Over-Active EDCF Activity and Endothelial Dysfunction*, and throughout the Experimental Thesis Chapters; and depicted in Figure 3.8). Thus, the increased ROS and oxidative stress and under-active NO-mediated EDRF signaling often accompanying states of CVD risk certainly can be contributing factors to the acute cause of EDRF / EDCF imbalance and endothelial vasomotor dysfunction related to over-active EDCF signaling. However, it is important to consider that these factors do not necessarily mediate the chronic underlying molecular cause of the prominent existence of EDCF signaling activity under CVD risk conditions.

In this regard, the collective findings of the studies in this thesis, by demonstrating in early-stage spontaneous hypertension and aging that the prominent existence of EDCF signaling activity is principally disconnected from changes in NO bioavailability or oxidative stress, suggest that over-active EDCF signaling may develop under CVD risk conditions by a cause independent of decreased NO bioavailability or increased ROS accumulation and oxidative stress. Beyond what little is known about ROS-/NO-dependent molecular signaling mechanisms and the chronic up-regulation of the EDCF pathway (as detailed in the Section 1.5.3 Review / *Potential Mechanisms of Over-Active EDCF Signaling: Function of PGs, ROS, and Oxidative Stress*), what might be the molecular signaling mechanism(s) responsible for a ROS-/NO-independent chronic up-regulation of the EDCF pathway remain(s) to be elucidated. Therefore, ROS-/NO-independent and -dependent regulatory mechanisms should be a focus of future studies in the EDCF research arena. One such study focus might include examination of the potential involvement of a ROS-/NO-independently-activated pro-inflammatory cell-signaling axis resulting in the over-expression and/or the ability to over-activate multiple components of the endothelial PG synthesis pathway (PLA₂, COX-1/-2, and/or PGSs) under CVD risk conditions.

Future Application of the CCA Model to Study EDCF Signaling Causes. Noteworthy, the CCA, of which the EDRF / EDCF vasomotor and corresponding hemodynamic characteristics were quantified in SHR, WKY, and SD rats in the studies of this thesis (summarized in Section 5.1.3), could be a particularly advantageous future model in which to explore both the acute and chronic regulatory mechanisms causing EDRF / EDCF imbalance and over-active EDCF signaling resulting in endothelial vasomotor dysfunction in states of CVD risk. These advantages (generally introduced in Section 1.6; depicted in Figure 1.6) especially include the fact that: i) the CCA can be cultured for prolonged periods (i.e. long enough to establish chronic molecular changes) under specific hemodynamic conditions (i.e. high vs. normal pressure; low/oscillatory vs. normal flow) and biochemical conditions (e.g. high/normal glucose; high/normal pro-/anti-oxidants; high/normal pro-inflammatory cytokine(s)) known or speculated in vivo to be associated with EDCF activity; and ii) selective CCA endothelium gene transfer and protein expression is possible even in live animals, thus allowing for specific manipulation of endothelial proteins that are candidates for affecting EDCF signaling activity (e.g. COX-1/-2, PGSs, PLA₂, NADPH oxidase, TP or IP receptors, RhoA-ROCK).

5.3.2. Consequences of Endothelial Dysfunction Related to Over-Active EDCF Signaling: Reaching Beyond VSM Activation

As outlined to begin this thesis, endothelial dysfunction measured as impaired agonist-/flow-stimulated endothelium-dependent vasorelaxation/dilation indeed predicts future CVD-related clinical events and correlates significantly with multiple indicators of cardiovascular dysfunction and overt pathologies, including indices of atherosclerosis (coronary plaque, CCA IMT, circulating inflammatory biomarkers), arterial stiffness (PWV, CCA AI and distensibility), and major target organ damage (LV hypertrophy, reduced renal function, cerebral decline/white matter hyper-intensities) (as referenced in Section 1.3; depicted in Figure 1.1). This is believed to reflect the ability of the endothelium to interact not only with itself and with the VSM but also with inflammatory/immune cells and platelets/clotting factors, in doing so serving a pivotal function in vascular homeostasis by regulating a tight balance not only of relaxation and contraction but also the prevention or promotion of VSM proliferation/migration and collagen-elastin matrix remodeling, inflammation and adhesion, and blood fluidity and coagulation (as referenced in Section 1.2; depicted in Figure 1.1). While the ability and mechanisms by which NO-mediated EDRF signaling in particular may prevent these cellular-molecular processes and their acute and chronic mechanical and biological consequences has been well-established (as detailed in the Section 1.5.2 Review), investigations of the role of over-active EDCF signaling in these processes and related cardiovascular consequences is indeed just beginning and has focused on the TP receptor, found to be expressed in platelets, endothelial cells, and inflammatory/immune cells (104, 111).

In this regard, the findings in Study II of this thesis demonstrating that RhoA-ROCK is an mediator of over-active COX- and TP receptor-dependent EDCF signaling represents another mechanistic clue as to how the dysfunctional endothelium might promote a CVD phenotype and function. Indeed, the activation of RhoA-ROCK signaling has previously been shown in multiple cell-types to stimulate not only contraction but also cytoskeleton re-organization, proliferation/differentiation, migration, inflammation, and apoptosis; moreover, its inhibition has been found to significantly delay atherogenesis and aortic aneurism formation (as referenced in the *Pathophysiological and Clinical Significance* Discussion of Thesis Study II). Therefore, future studies should focus not only on further elucidating the molecular mechanisms by which RhoA-ROCK may contribute to the EDRF / EDCF imbalance and endothelial vasomotor dysfunction related to over-active EDCF signaling under CVD risk conditions, but also on the direct relationship between that over-active EDCF-TP receptor-RhoA-ROCK signaling and its mechanical and biological consequences.

Future Application of the CCA Model to Study EDCF Signaling Consequences.

Noteworthy, the CCA (of which, as stated above, the EDRF / EDCF vasomotor and corresponding hemodynamic characteristics were quantified in SHR, WKY, and SD rats in the studies of this thesis; see Section 5.1.3) could be a particularly advantageous future model in which to explore the possible relationships between over-active COX-mediated EDCF-TP receptor-RhoA-ROCK signaling and mechanical and biological consequences in states of CVD risk. These advantages (generally introduced in Section 1.6; depicted in Figure 1.6) especially include the fact that: i) the CCA is readily accessible for measuring (stiffness/compliance) or manipulating (e.g. high/normal or low/oscillatory flow/shear) its endothelium-dependent mechanical or biological characteristics in situ/in vivo, after which a detailed pharmacodissection of its vasomotor characteristics may be performed; and ii) the CCA can be cultured for prolonged periods (i.e. long enough to establish chronic biological changes) under specific hemodynamic and biological/biochemical conditions that mimic pro-atherogenic conditions (for instance (303), with circulating monocyte/macrophage + a pro-inflammatory cytokine stimulant or pro-atherogenic flow/pressure profile), after which the extent of atherogenic consequence can be measured (for instance, adhesion molecule expression and macrophage uptake into the sub-intimal space) and a detailed pharmacodissection of its vasomotor characteristics performed.

5.4. Closing Remarks

It is the hope that gaining a better understanding of the causes and consequences of endothelial vasomotor signaling may lead to the identification of new or more effective targets or indices to be used in prevention and treatment strategies aimed at countering the development and clinical complications of CVD conditions. This thesis mechanistically and methodologically adds to an emerging body of evidence suggesting that EDCF signaling activity, in addition to under-active NO-mediated EDRF signaling, contributes importantly to the endothelial vasomotor dysfunction that hallmarks both experimental animals and humans in states of CVD risk. As the areas of vascular research and medicine/health move forward, it will be exciting to see how the factors identified as mediating over-active EDCF signaling may be used as primary or adjunct targets or indices in the prevention and treatment of CVD in regard to both pharmacological interventions and diet and exercise lifestyle regimes.

Appendices

Appendix A

Supplemental Material for Thesis Study I in Chapter 2

Supplemental Methods

CCA Hemodynamics

Rationale for Procedural Approach. Previous works investigating the hemodynamic and/or mechanical properties of the rat CCA have concurrently measured blood pressure in one of the bilateral CCAs, which requires total occlusion of the artery, and internal diameter and/or flow velocity in the other CCA (76, 362, 429). Although this approach has the advantage of quantifying CCA blood pressure and flow simultaneously, it has the major disadvantage of potentially altering the normal physiological hemodynamics of the CCA and downstream cerebrovascular beds due to contralateral CCA occlusion, given that acute unilateral CCA occlusion in rats results in a ~50% increase in contralateral CCA blood flow (169), and a distinct reduction in total blood flow to both cerebral hemispheres (82). Therefore, in the current study, blood flow through the left CCA of anesthetized rats was measured using a perivascular flow-probe, followed by removal of the flow-probe and insertion of a catheter-tip pressure transducer into the left CCA to measure intra-arterial blood pressure, thus allowing us to obtain an uninterrupted measure of CCA blood flow followed by a direct measure of CCA blood pressure within the same arterial segment.

Quantification of CCA Blood Flow and Pressure. Blood flow through the left CCA of WKY and SHR rats was measured using a TS420 flow-meter module and a MA1PRB transit-time perivascular flow-probe (ultrasound frequency, 7.2MHz; bidirectional flow scale setting, 1V=20ml/min; maximum flow range, 100ml/min; interfaced with flow-meter module using 1.25m extension cable; Transonic Systems Inc., Ithaca, NY) chosen so that the in vivo pressurized CCA from each strain of rat would fill >75% and <100% of the flow-probe's acoustic window volume (1.1x2.0x1.5mm) in order to avoid probe positional sensitivity and arterial lumen impingement. The output voltage signal from the flow-meter is low-pass filtered (3-pole Butterworth) to exclude high-frequency non-flow related noise, with factory bandwidth output filter settings of 0.1, 10, 40, and 160Hz. In each animal, it was determined that only filtering at 40 or 160Hz could fully resolve the pulsatile contours of the CCA flow signal (4.6-7.1Hz fundamental harmonic [i.e. heart rate, 275-425bpm] + higher harmonics), but that the 160Hz filter also allowed a considerable amount of noise into the output signal (data not shown). Therefore, only data from the 40Hz output voltage signal filter was used in the present study to quantify both

the steady and pulsatile components of the left CCA blood flow waveform in WKY and SHR, which should have allowed for resolution of the first harmonic (i.e. fundamental 'beat') and 5-9 higher harmonics (i.e. contours relating to cardiac events and/or arterial properties) in the signal (see Ref. (279); also see TS400 Series Flow-meter Operator's Manual, Technical Note 95).

Prior to surgery on the day of experiments, rats were anesthetized via an intra-peritoneal injection of pentobarbital sodium (60-70mg/kg body weight) and placed lying supine on a heating pad adjusted so that the temperature between the animal and the pad was $38\pm 1^{\circ}\text{C}$. Through a small incision in the neck, the left CCA was carefully dissected free from the carotid sheath and a $\sim 1.5\text{cm}$ arterial segment cleaned of fat and surrounding connective tissue was exposed. Using blunted micro-forceps the exposed arterial segment was gently maneuvered into the flow-probe's acoustic window, which was pre-filled with surgical lubricant (Surgilube[®], Fourgera & Co., Melville, NY), and re-positioned to its anatomical location using a custom probe-positioning device. The entire incision area was next filled with lubricant and covered with Parafilm[®] to promote good acoustical coupling (i.e. a signal strength $>60\%$) and prevent dehydration and heat loss. A stable signal of pulsatile blood flow in the left CCA was collected for at least 5min.

Immediately following blood flow data collection, the flow-probe was gently removed and the lubricant cleared from the incision area which was then rinsed well with sterile 37°C saline. With flow interrupted, a small incision was made at the cephalic end of the exposed left CCA segment for the intra-arterial insertion of a pressure catheter, which was secured in the artery with a removable tie (4-0 surgical suture) adjacent to the incision. In each animal, a comparison was made between two intra-arterial catheter-transducer systems: a fluid-filled catheter (PE50 tubing catheter $\sim 30\text{cm}$ long filled with heparinized saline to ensure no air bubbles/clots) coupled via a 23-gauge needle tip and 3-way stopcock to a domed membrane pressure transducer (BS4 72-4496, Harvard Apparatus, South Natick, MA), versus a high-fidelity catheter-tip transducer (Model SPR-320, 2F Mikro-Tip[®] Pressure Transducer Catheter, Millar Instruments, Inc., Houston, TX). The order of the catheter measurements was counter-balanced, where in half of the animals from each strain the fluid-filled catheter was inserted into the exposed left CCA segment first and a stable intra-arterial pressure signal was collected for at least 5min, followed by removal of the fluid-filled catheter and the immediate insertion of the catheter-tip transducer for an additional $>5\text{min}$ collection of a stable intra-arterial pressure signal, and vice versa for the other half of the animals. It was demonstrated from these measurements that using the catheter-tip pressure transducer system was far superior in quantifying the pulsatile, contoured components of CCA blood pressure as compared to the commonly employed (202, 382) fluid-filled catheter-transducer system, which we found was able to accurately quantify MAP but only

registered pressure oscillations of 5-10 mmHg and did not capture any waveform contours relating to cardiac events and arterial properties (data not shown). In addition, this fluid-filled catheter-transducer system did not distinguish the well-established difference in CCA pulse pressure between WKY and SHR (59, 74). For this reason, only data from the catheter-tip pressure transducer system was used in the present study to quantify both the steady and pulsatile, contoured components of the left CCA blood pressure waveform in WKY and SHR. It should be noted that, in contrast to our findings described above, rodent aortic pressure waveforms with very similar mean and pulsatile, contoured components have been recorded from fluid-filled and Mikro-Tip[®] catheter transducer systems in previous studies from other laboratories (74, 280), perhaps owed to more favorable catheter dimensions (e.g. PE50+PE10 tubing fused to tip) which may have provided an improved dynamic frequency response with minimal damping in their fluid-filled systems (202, 382).

The total time from injection of anesthesia into the animal to completion of stable blood flow and blood pressure measurements was in each case between 35-45min. Each pressure catheter, which had to be inserted into the lumen of the left CCA and tied thus totally occluding the arterial segment, only remained in the lumen for 10-12min.

Blood flow and blood pressure signals were calibrated daily and recorded at a 1000Hz sampling rate to a PowerLab[®] data acquisition and analysis system (4SP channel unit, Chart[™] v5.5.1, ADInstruments, Colorado Springs CO). The Transonic flow-meter and Harvard pressure transducer were interfaced directly to the PowerLab[®] unit, whereas the Millar Mikro-Tip[®] pressure transducer catheter was interfaced to the PowerLab[®] unit via a ML110 ADInstruments Bridge Amp set to DC high-pass and 100Hz low-pass filtering.

CCA Vasomotor Function

Vasomotor Function of Freshly-Harvested Pre-contracted CCA. To exclude the possibility that the measures of right CCA vasomotor function were somehow adversely affected by first investigating left CCA hemodynamics before right CCA excision (e.g. animal under anesthesia for ~40min, transient disturbances of the CCA circulation during hemodynamic measures), the same measures of vasomotor function across drug conditions were also performed using right CCA freshly harvested from age-matched WKY (n=8) and SHR (n=8) upon which no hemodynamic measures were made before vessel excision. Indeed, no differences in the amplitude of KCl- or PE-stimulated contraction or *Max Amp*, *EC50*, or *AUC* indices of ACh- or SNP-stimulated vasomotor function were found between arterial rings harvested from animals following hemodynamic measures compared to freshly-harvested rings (all $p > 0.200$; data not shown).

Effect of Endothelial Denudation on the Vasomotor Function of Pre-contracted CCA. In the absence of a functional endothelium, cumulative ACh elicited neither vasorelaxation nor vasoconstriction in pre-contracted CCA freshly harvested from age-matched WKY and SHR, whereas SNP elicited complete vasorelaxation (n=3-5 CCA rings from each strain; data not shown).

Each transducer (Model MLT0201/D, ADInstruments) used to measure isometric force production in mounted CCA (Vascular Myography unit, Radnoti Glass Technology Inc., Monrovia, CA) was interfaced to an 8SP PowerLab[®] system via a ADInstruments Bridge Amp (DC high pass filter; 100Hz low-pass filter) and was calibrated daily. Isometric tension signals in g were recorded at a 100Hz sampling rate throughout the entire experimental protocol. The total time from right CCA dissection and excision to completion of each of the in vitro vasomotor function protocols was between 5-6h.

When achievable, the cumulative response to ACh or to SNP for a given CCA ring were individually fit by non-linear regression to dose-response curves (either sigmoidal or bell-shaped; GraphPad Prism[®] v4.03 software; San Diego CA) and then averaged within their respective drug treatment and strain. In the LN and LN+INDO WKY CCA conditions, ACh-stimulated responses did not fit well to any model dose-response curve-fitting equation; therefore no accurate *EC50* could be found, so the *Max Amp* and *AUC* computed from the measured data points were reported (see Figure 2.2, inset data).

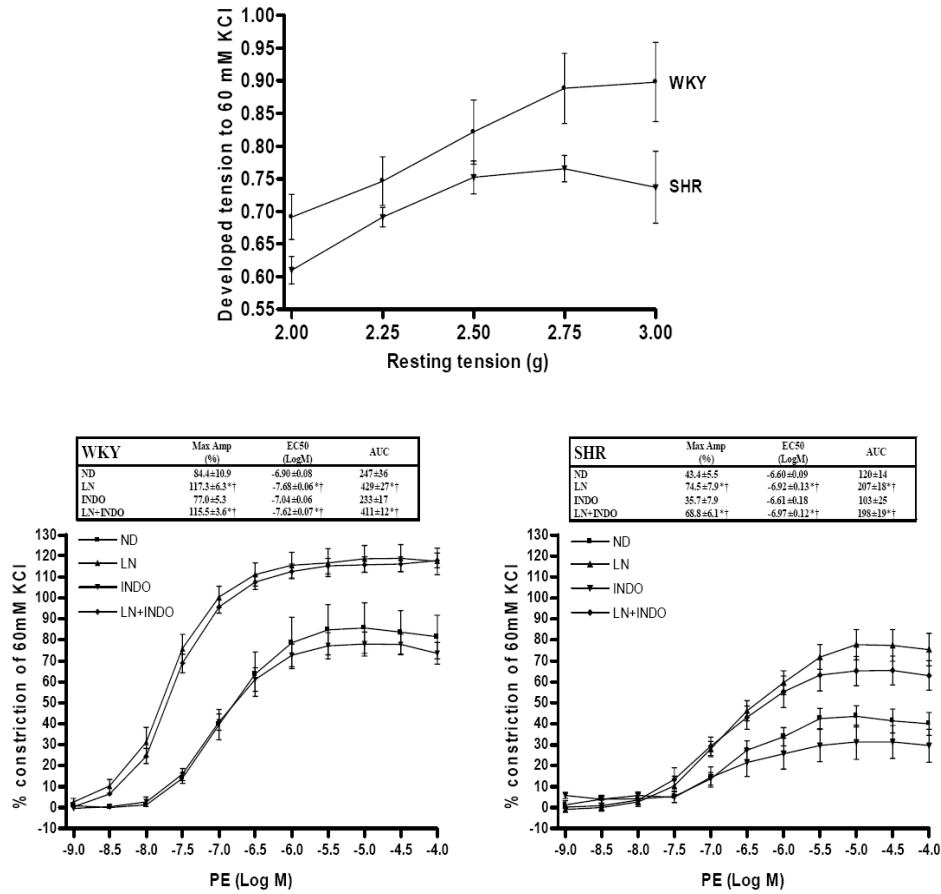
Western Blotting

Tissue preparation and immunoblotting was performed as previously described (114, 143, 310). Frozen CCA segments were hand-homogenized in 200µl of ice-cold extraction buffer (10mM NaH₂PO₄, 1% SDS, 6M urea at pH 7.4), incubated at 60°C for 3h with recurrent vortexing, and centrifuged (15min, 10000rpm), after which the supernatant samples were retrieved and a bicinchonic acid (BCA) protein assay was performed, and samples were prepared for Western blotting by diluting them to 1.0µg/µl in gel loading buffer containing 150mM dithiothreitol (DTT).

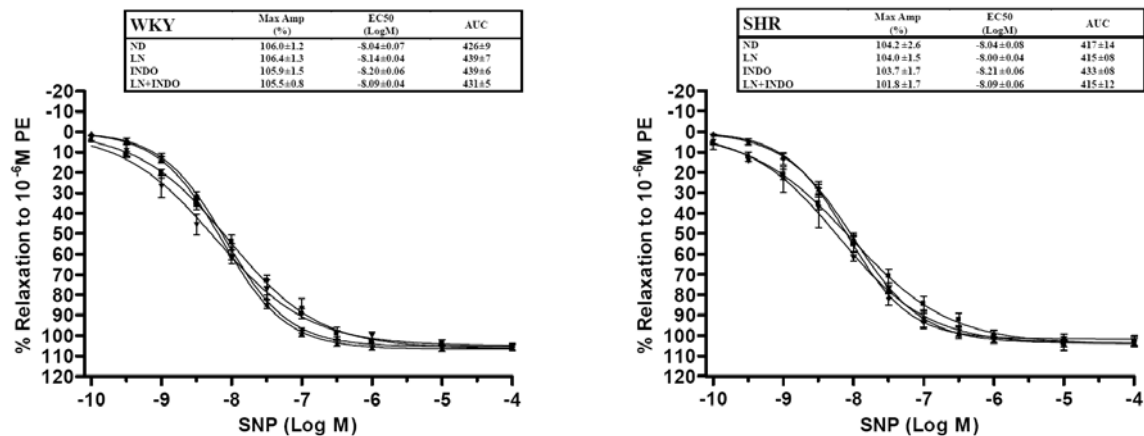
Immediately prior to electrophoresis (175V for ~50-60min), prepared CCA samples were denatured for 5min at 95°C and loaded at 30µg/lane onto sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gels (5-10%). Proteins separated in the gels were transferred (25V for 40-45min) onto microporous PVDF membranes (Roche Diagnostics, Mannheim, Germany) and prior to immunoblotting were stained with ponceau red dye to confirm equal total protein loading of samples in lanes. Immunoblotting and detection of individual proteins was performed by first blocking the membranes for 1h at room temperature, then incubating for 1h with primary

antibodies specific for either eNOS (1:750), COX-1 (1:200), or COX-2 (1:200), followed by incubation with HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (1:2000-4000) for 1h before the protein-immunocomplexes were detected and their signals quantified using enhanced HRP-luminol chemiluminescence reagents (Amersham, Little Chalfont, UK) and a Syngene system (Syngene, Cambridge, UK). All blocking and antibody solutions were made using Tris (10mM)-buffered saline (100mM) with 0.1% Tween-20 (TBS-T) containing either 5% bovine serum albumin (BSA) or 10% non-fat milk powder, and thorough washes after blocking and antibody incubation periods were done with TBS-T alone.

Supplemental Results



Supplemental Figure A-1. Preliminary contractile experiments in CCA rings excised from 16-20wk old WKY and SHR. Beginning at a resting tension of 2.00g, 2.0mm arterial rings were stimulated to contract with KCl at 0.25g resting tension increments until the magnitude of developed isometric tension reached a plateau (top), after which rings were washed every 15min for 1h, incubated with either no drug (ND; n=6 per strain), *N* ω -nitro-L-arginine methyl ester (LN; n=6 strain), Indo (INDO; n=6 per strain), or LN+INDO (n=6 per strain) for 30min, then exposed to cumulative PE concentrations (bottom left and right; data expressed relative to contraction stimulated by KCl). Data represent means \pm SEM. $p < 0.05$; * vs. ND, † vs. INDO, within strain. *Max Amp*, maximum amplitude of the curve; *EC50*, PE dose resulting in 50% of the plateau in amplitude of the curve; *AUC*, area under the curve.



Supplemental Figure A-2. Vasomotor function in WKY and SHR CCA rings incubated with either ND (n=27), *N*ω-nitro-L-arginine methyl ester (LN; n=21), INDO (n=9), or LN+INDO (n=12). Arterial rings were pre-contracted with PE then exposed to cumulative concentrations of the NO donor SNP. Prior to this, rings had been stimulated to contract with KCl then washed and pre-constricted with PE, after which rings were exposed to cumulative ACh concentrations (see *Methods* and Figure 2.2). Between ACh and SNP dose-response curves, rings were washed every 15min for 1h. Data represent means±SEM and are expressed relative to PE precontraction. *Max Amp*, maximum amplitude of the curve; *EC50*, SNP dose resulting in 50% of the plateau in amplitude of the curve; *AUC*, area under the curve.

Appendix B

Supplemental Material for Thesis Study II in Chapter 3

Supplemental Methods

Homogenization and Western Blotting

Frozen CCA segments were individually hand-homogenized in 80-100 μ l of ice-cold lysis buffer (20mM HEPES, 10mM NaCl, 1.5mM MgCl, 1mM DTT, 20% Glycerol, and 0.1% Triton X-100; Sigma-Aldrich or BioShop) at pH 7.40 with Complete Protease Inhibitor Cocktail (1X; Roche Diagnostics, Laval QC, Canada) and Halt Phosphatase Inhibitor Cocktail (1X; Thermo Scientific, Rockford IL), after which the supernatant was isolated via centrifugation (15min, 10000rpm), its protein concentration determined via a standard BCA protein assay, and the sample diluted to 1.0 μ g/ μ l in gel loading buffer containing 150mM DTT.

Prepared samples were denatured (5 min, 95°C), loaded at 30 μ g/lane onto SDS-PAGE gels (5-12%), and exposed to electrophoresis (50-60min at 175V). Proteins separated in the gels were transferred (40-45min at 25V) onto microporous PVDF membranes (Roche Diagnostics, Mannheim, Germany).

Prior to immunoblotting, membranes were stained with Ponceau Red dye to confirm equal total protein loading and transfer of samples.

Membranes were blocked in solution for 1h at room temperature, then incubated with either mouse anti-RhoA-specific monoclonal antibody solution (1:1000; Cytoskeleton Inc., Denver CO) or mouse anti-ROCK-II (i.e. ROCK α)-specific monoclonal antibody solution (1:200; BD Biosciences, Franklin Lakes, NJ) for at least 1h, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody solution (1:1000-2000; Santa Cruz, Santa Cruz, CA) for 1h before the protein-immunocomplexes were detected and their signals quantified using enhanced HRP-luminol chemiluminescence (reagents from Amersham, Little Chalfont, UK) and a Syngene system (Syngene, Cambridge, UK). Each membrane was re-probed for α -actin (primary antibody dilution, 1:300000, Sigma-Aldrich, St. Louis, MO) and quantified.

All blocking and antibody solutions used were prepared using TBS-T containing either 5% BSA or 10% nonfat milk powder. Between blocking, primary antibody, secondary antibody, and chemiluminescent reagent incubations, membranes were washed thoroughly with TBS-T alone.

Data Analyses

To minimize ring-to-ring variability, duplicate CCA rings from the same animal were always used for each drug condition and then their responses averaged. *n* refers to the number of

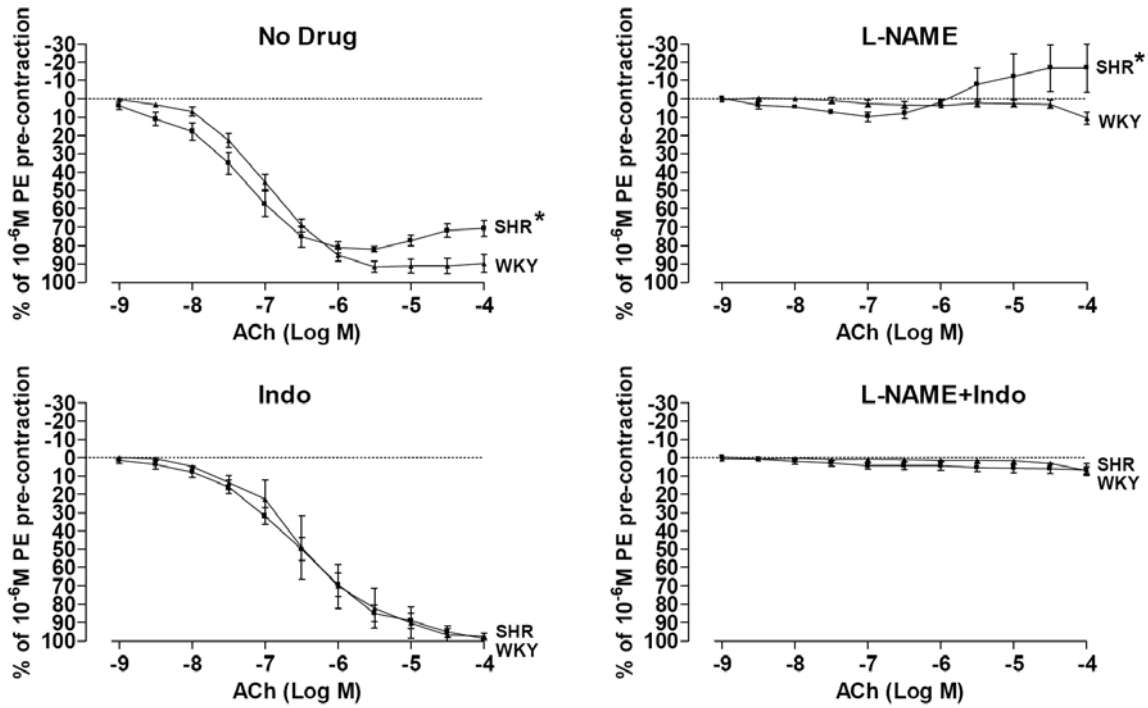
averaged ring responses per drug condition/strain. When achievable, each averaged ring's cumulative tension response to a vasomotor stimulus was best fit by non-linear regression to a sigmoidal dose-response curve using GraphPad Prism[®] v4.03 (San Diego CA), generating maximum amplitude (*Max Amp*), dose resulting in 50% of *Max Amp* (*EC₅₀*), and area under the curve (*AUC*) parameters that were then averaged within their respective drug condition/strain.

Supplemental Results

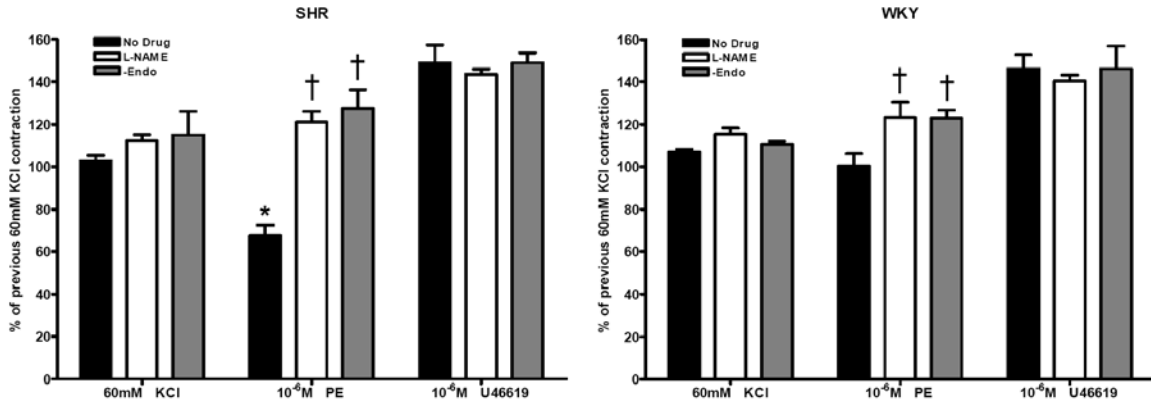
Supplemental Table B-1. Physical attributes of young adult SHR and WKY

	SHR	WKY	p
<i>Age, wks</i>	22.9±0.4	22.8±0.2	0.286
<i>Weight</i>			
Whole body, g	359±4	355±3	0.445
Left ventricle, mg	1.069±0.030	0.843±0.024	<0.001
Right ventricle, mg	0.200±0.009	0.212±0.010	0.378
<i>CCA dimensions (passive)</i>			
Outer diameter, mm	1.061±0.003	0.912±0.006	<0.001
Inner (lumen) diameter, mm	0.609±0.003	0.498±0.003	<0.001
Wall thickness, mm	0.226±0.002	0.201±0.001	0.003
Lumen cross-sectional area, mm ²	0.291±0.003	0.198±0.002	<0.001
Wall cross-sectional area, mm ²	0.588±0.006	0.462±0.006	<0.001

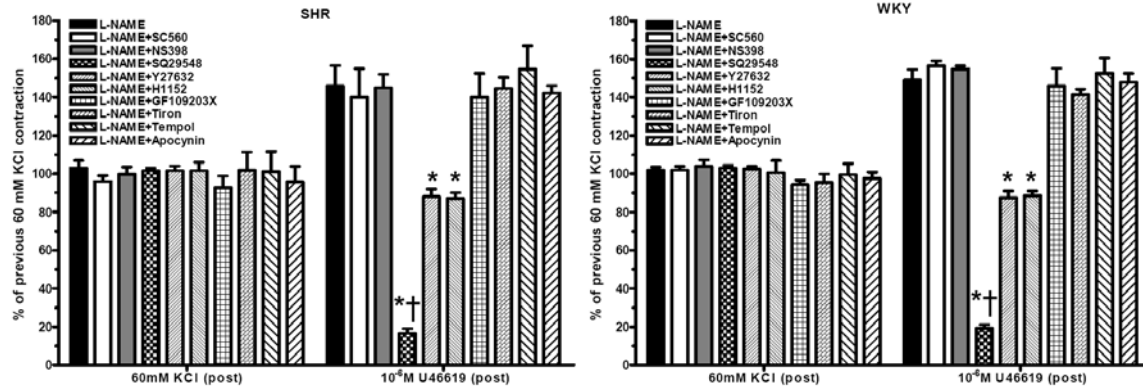
Data represent means±SEM. SHR and WKY ranged in age from 18 to 24wks and in weight from 300 to 400g. There was <30g weight-gain within animals across this age range. CCA dimensions were measured in a non-pressured, non-stretched (i.e. passive) state as described in the *Methods*.



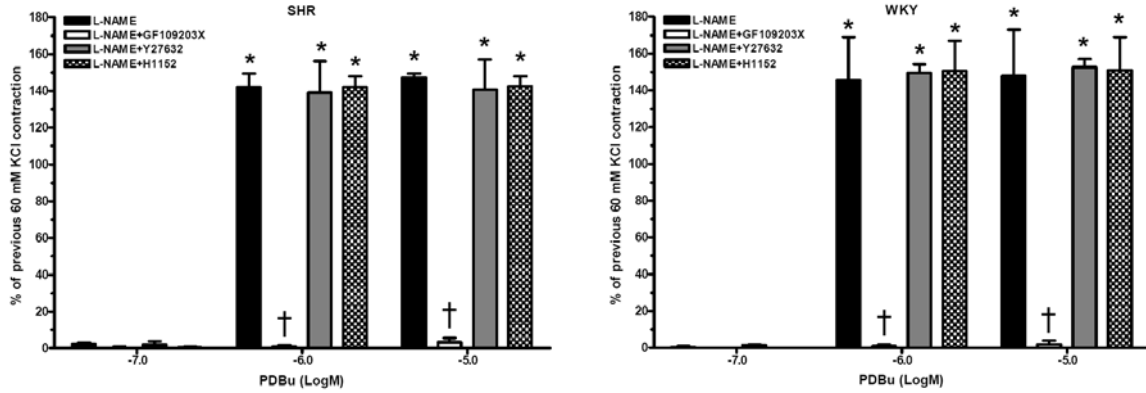
Supplemental Figure B-1. ACh-stimulated vasomotor function in PE-pre-contracted CCA rings isolated from SHR and WKY and incubated with either No Drug (n=6), the NOS inhibitor N^o-nitro-L-arginine methyl ester (L-NAME, 10^{-4.0} M, n=6), the COX inhibitor Indo (10^{-5.0} M, n=6), or L-NAME+Indo (n=6). Arterial rings were stimulated to contract with 60mM KCl then washed and pre-contracted with a near-maximal dose of PE, after which rings were exposed to cumulative ACh doses. Data represent means±SEM and are expressed relative to PE pre-contraction. *P<0.05 vs. WKY.



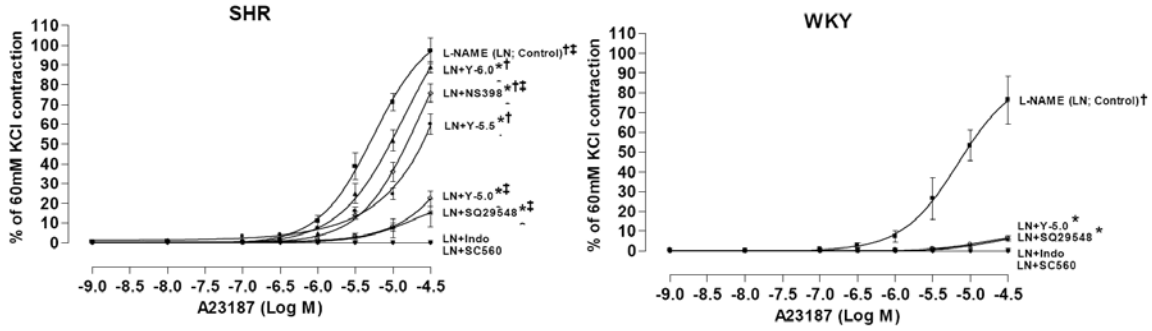
Supplemental Figure B-2. Magnitude of contraction prior to cumulative Y27632-stimulated relaxation (see Figure 3.1) in CCA rings isolated from SHR and WKY and incubated with either No Drug (n=4) or the NOS inhibitor *N*^o-nitro-L-arginine methyl ester (L-NAME, 10^{-4.0} M, n=4), or denuded of endothelium (-Endo; n=4). Arterial rings were stimulated to contract with 60mM KCl then washed, after which rings were pre-contracted with either KCl, PE, or U46619. Contractions were allowed to develop to a near-maximal plateau over 30-45min, and control experiments confirmed contractile sustainability over the entire protocol (~120min). Data represent means±SEM and are expressed relative to previous KCl contraction. **p*<0.05 vs. WKY, † vs. No Drug.



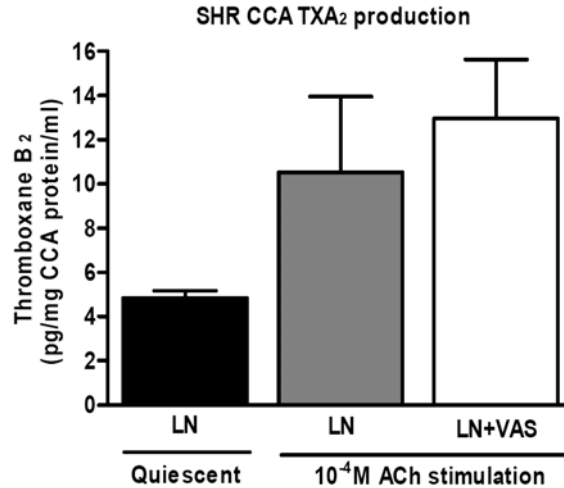
Supplemental Figure B-3. Magnitude of KCl- and U46619-stimulated contraction following cumulative ACh-stimulated contraction in CCA rings isolated from SHR and WKY and incubated with either the NOS inhibitor *N*^o-nitro-L-arginine methyl ester (L-NAME, $10^{-4.0}$ M, n=8), which acted as a Control for all other drug conditions (see *Results* for details), or L-NAME co-incubated with the selective COX-1 inhibitor SC560 (L-NAME+SC560; $3 \times 10^{-7.0}$ M, n=5), the preferential COX-2 inhibitor NS398 (L-NAME+NS398; $10^{-6.0}$ M, n=5), the TP receptor antagonist SQ29548 (L-NAME+SQ29548; $10^{-6.0}$ M, n=5), the ROCK inhibitors Y27632 (L-NAME+Y27632; $10^{-5.0}$ M, n=6) or H1152 (L-NAME+H1152; $10^{-6.0}$ M, n=6), the PKC inhibitor GF109203X (L-NAME+GF109203X; $10^{-5.5}$ M, n=5), the superoxide anion scavenger Tiron (L-NAME+Tiron; $10^{-2.0}$ M, n=6), the SOD mimetic Tempol (L-NAME+Tempol; $10^{-3.0}$ M, n=6), or the NADPH oxidase inhibitor Apocynin (L-NAME+APO; $10^{-3.5}$ M, n=6). To test the specificity of these inhibitors, mimetics, and/or antagonists for affecting CCA contraction, arterial rings were stimulated to contract with 60mM KCl then washed and exposed to cumulative ACh to stimulate contraction (see Figure 3.2 and 3.7), after which rings were washed thoroughly and stimulated to contract with either 60mM KCl (post) or $10^{-6.0}$ M U46619 (post). These contractions were tonic – i.e. allowed to develop over 30-45min. Data represent means±SEM and are expressed relative to initial KCl contraction. * $p < 0.05$ vs. L-NAME rings, † vs. L-NAME+Y27632 or L-NAME+H1152, pre-contracted with $10^{-6.0}$ M U46619.



Supplemental Figure B-4. Contraction stimulated by the potent PKC activator phorbol-12,13-dibutyrate (PDBu) in CCA rings isolated from SHR and WKY and incubated with either the NOS inhibitor *N*^ω-nitro-L-arginine methyl ester (L-NAME, $10^{-4.0}$ M, n=4), which acted as a Control for all other drug conditions (see *Results* for details), or L-NAME co-incubated with the broad-spectrum PKC inhibitor GF109203X (L-NAME+GF109203X; $10^{-5.5}$ M, n=4) or the ROCK inhibitors Y27632 (L-NAME+Y27632; 10^{-5} M, n=4) or H1152 (L-NAME+H1152; 10^{-6} M, n=4). Arterial rings were stimulated to contract with 60mM KCl then washed, after which rings were exposed to PDBu doses cumulatively. Data represent means±SEM and are expressed relative to KCl contraction. * $p < 0.05$ vs. -7.0 LogM PDBu within drug condition, † vs. L-NAME, L-NAME+Y27632, or L-NAME+H1152 within PDBu concentration.



Supplemental Figure B-5. Contraction stimulated by the calcium ionophore A23187 in quiescent SHR and WKY CCA rings incubated with the NOS inhibitor *N*^o-nitro-L-arginine methyl ester (L-NAME, $10^{-4.0}$ M, n=8), which acted as a Control for all other drug conditions (see *Results* for details), or L-NAME co-incubated with the non-selective COX inhibitor Indo (LN+Indo, $10^{-5.0}$ M, n=4), the selective COX-1 inhibitor SC560 (LN+SC560, $3 \times 10^{-7.0}$ M, n=7), the preferential COX-2 inhibitor NS398 (LN+NS398, $10^{-6.0}$ M, n=7), the TP receptor antagonist SQ29548 (L-NAME+SQ29548; $10^{-6.0}$ M, n=5), or the ROCK inhibitor Y27632 at $10^{-6.0}$, $10^{-5.5}$, $10^{-5.0}$ M concentrations (L-NAME+Y^{-6.0}, n=6; L-NAME+Y^{-5.5}, n=7; L-NAME+Y^{-5.0}, n=5). Arterial rings were stimulated to contract with 60mM KCl then washed, after which rings were exposed to cumulative A23187. Data represent means \pm SEM and are expressed relative to previous contraction stimulated by KCl. $p < 0.05$; * vs. LN, † vs. SQ29548, ‡ vs. LN+Y^{-6.0}, § vs. LN+Y^{-5.5} within strain.



Supplemental Figure B-6. Production of TXA₂ from quiescent or ACh-stimulated CCA rings isolated from SHR and incubated with the NOS inhibitor *N*⁰-nitro-L-arginine methyl ester (L-NAME, or LN, 10^{-4.0} M, n=5) or L-NAME co-incubated with the preferential COX-1 inhibitor valeryl salicylate (LN+VAS, 3*10^{-3.0} M, n=5). Arterial rings were stimulated to contract with 60mM KCl then washed. In some cases, rings were incubated in a quiescent state for 45 minutes before the bathing buffer was collected. In other cases, after 40 minutes of quiescent incubation, rings were exposed to a maximal dose of ACh eliciting peak contraction within 4-5 minutes, at which time the bathing buffer was collected. The concentration of TXB₂ in the collected buffer was used as a marker of CCA TXA₂ production. For detection within the linear range of the assay, collected buffer had to be systematically concentrated (6-fold), which was performed according to the kit's instructions using commercially available C18 columns. Data represent means±SEM and are expressed relative to CCA ring protein per ml of bathing buffer.

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