Endothelium-dependent vasomotor responses of hypertensive and type 2 diabetic rats: effects of sex, ageing, and therapeutic interventions

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.
I understand that my thesis may be made electronically available to the public.

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Abstract

Impaired endothelial vasomotor function is a hallmark of many chronic disease states, including essential hypertension and type 2 diabetes mellitus. Loss of the homeostatic role of the endothelium in large conduit arteries can contribute to the pathogenesis of cardiovascular conditions in these vessels (e.g. stroke, atherosclerosis). A fundamental understanding of mechanisms controlling endothelial function in hypertension and type 2 diabetes mellitus is required for appropriate clinical strategies targeting the cardiovascular conditions associated with these diseases. The vast majority of basic science studies examining endothelial function in animal models of hypertension and type 2 diabetes have been conducted in males. Studying endothelial function in females is imperative for determining potential sex-specific mechanisms of dysfunction and thus appropriate therapeutic strategies. Thus the global purpose of this thesis is to identify and characterize the pathways controlling impaired vasomotor function in *female* animal models of two chronic disease states: hypertension and type 2 diabetes mellitus.

Chapters 2 and 3 of this thesis examine sex differences in endothelium-dependent vasorelaxation (EDR) and vasocontraction (EDC) of aortic segments isolated from male and female spontaneously hypertensive rats (SHR), a model of essential hypertension, as the animals age between 16 and 30 wk old. All endothelial vasomotor data presented in the Abstract are peak responses to 10^{-5} M acetylcholine. Endothelial vasomotor impairment is represented by lower EDR or by higher EDC. These present data confirmed well-established findings from the literature that 16 wk old male SHR exhibit endothelial vasomotor

impairments (EDR: 77±4 %; EDC: 76±7 %) compared to normotensive Wistar-Kyoto (WKY; EDR: 89±6 %; EDC: 59±8 %; $p<0.05$) controls, and that this impairment worsens with ageing in 30 wk male SHR (EDR: 63±2 %; EDC: 91±3 %; $p<0.05$). The observation that EDR was reduced in 30 wk female SHR (EDR: 76±4 %) compared to 16 wk counterparts (EDR: 101±2 %; $p<0.05$), however, was novel and interesting, as there were previously no reports of vasomotor responses in female SHR older than 19 wk. Moreover, the blunted EDR response of 30 wk female SHR approached the level of impairment exhibited by 30 wk male SHR (but was still slightly greater in females; $p<0.05$). The limited sex difference of the EDR within 30 wk SHR (males –13 % vs. females; $p<0.05$) contrasted that of 16 wk SHR (males –24 % vs. females; $p<0.05$), when the robust and unimpaired relaxation displayed by females was much greater than the significantly blunted response of males. Interestingly, endothelium-dependent contractions in quiescent rings were moderate and similar between 16 wk (EDC: 50±4 %) and 30 wk female SHR (EDC: 59±7 %; p=N/S) as compared to the greater contractions of males that were exacerbated with ageing (see above; $p<0.05$ both sex and ageing comparison).

A major role has been established for the cyclooxygenase (COX)-1-thromboxane A_2/prostaglandin (TP) receptor pathway in the impaired endothelial vasomotor function of male SHR. Indeed, a similar mechanism appears to be responsible for the dysfunction observed in 30 wk female SHR in this thesis since robust endothelial function was restored in these animals with both antagonism of TP receptor (EDR: 111±2 %; EDC: 7±2 %; $p<0.05$) and preferential inhibition of COX-1 (EDR: 112±3 %; EDC: –5±3 %; $p<0.05$). In contrast, preferential inhibition of COX-2 only partially tempered endothelial impairments of 30 wk

female SHR (EDR: 99±5 %; EDC: 27±3 %; $p<0.05$), suggesting that, similar to ageing male SHR, this isoform makes at most a secondary contribution to the dysfunction in 30 wk female SHR. Collectively, these data indicate that ageing female SHR exhibit a mechanism of endothelial impairment that is similar to that of male SHR and that is largely COX-1- and TP receptor-dependent.

Chapter 4 examines the ability of chronic dietary administration of the n-3 polyunsaturated fatty acid (PUFA), docosahexaenoic acid (DHA, 22:6 n-3), to ameliorate endothelial vasomotor function in adult male SHR with established hypertension. The impaired endothelial function of aortic segments isolated from adult male SHR (EDR: 48±6 %) was not improved following 10–12 wk of DHA feeding (EDR: 45±5 %; p=N/S). This finding was unexpected since it has been shown in the literature that feeding other n-3 PUFAs improves vasomotor responses in younger SHR, in which hypertension and its associated consequences are still developing. This is the first report of the effects of n-3 PUFA on endothelial vasomotor responses in adult SHR with established hypertension. These data suggest that dietary DHA do not improve vasomotor function in adult SHR.

Chapter 5 examines α_1 adrenergic contraction and EDR of aortic segments isolated from 14 wk old female Zucker diabetic fatty rats (ZDF), a genetic model of high fat diet-induced obesity and type 2 diabetes, and lean non-diabetic female Zucker Lean rats. Additionally, some ZDF received an 8 wk administration of anti-diabetic metformin drug therapy, aerobic exercise training, or a combination of the two. Maximal α_1 adrenergic contractions were over 2-fold higher in high fat-fed ZDF (1.69±0.16 g) compared to Lean (0.71±0.13 g; $p<0.05$). This elevation in ZDF was abolished by exercise training alone

(1.02±0.17 g; $p<0.05$) but was not altered by metformin (1.56±0.19 g; p=N/S). In contrast to the severely impaired endothelial vasomotor function reported in male ZDF in the literature, robust EDR was observed in female ZDF (72±7 %) that was similar to Lean (75±6 %; p=N/S) and that was unaltered by exercise training (76±5 %; p=N/S) or metformin (76±6 %; p=N/S). These results indicate that enhanced α_1 adrenergic contraction is a mechanism of altered vasomotor function in female type 2 diabetic ZDF rats and that it could possibly be addressed by a chronic exercise training intervention.

The main novelty of the thesis is the extension of the current understanding of endothelial vasomotor function to hypertensive and type 2 diabetic *females*. The knowledge gained from examining mechanisms involved in endothelial impairments in ageing hypertensive females and from testing the therapeutic potential of currently used anti-diabetic interventions in the type 2 diabetic female vasculature has interesting potential application. This basic scientific information could help direct clinical therapeutic strategies to target population-specific mechanisms of dysfunction. Understanding female sex-specific endothelial behaviour in patient populations is important for describing cardiovascular complications, defining mechanisms, and applying appropriate therapeutic targets. Findings from this thesis indicate a sex-dependence of the total divergence of endothelial function (e.g. female type 2 diabetic rats vs. male counterparts in the literature) and of the interaction of disease variables (e.g. age) and endothelial vasomotor responses.

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It is at a time like this that I wish I were more proficient at expressing my feelings in writing. But I'll try. I've purposely chosen brevity not out of insincerity but because I simply don't know enough words to do justice to the support and encouragement I have received from so many people.

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AA	arachidonic acid (20:4 n-6 PUFA)
ACh	acetylcholine chloride (muscarinic receptor agonist)
ALA	α-linolenic acid (18:3 n-3 PUFA)
BM	body mass
BP	blood pressure
BSA	bovine serum albumin
COX	cyclooxygenase, a.k.a. PGG_2/PGH_2 synthase
DAG	diacylglycerol
DHA	docosahexaenoic acid (22:6 n-3 PUFA)
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EC_{50}	effective agonist concentration that elicits 50 % of the maximal response
EDCF	endothelium-derived contracting factor
EDHF	endothelium-derived hyperpolarizing factor
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
EPA	eicosapentaenoic acid (20:5 n-3 PUFA)
GLA	γ-linolenic acid (18:3 n-6 PUFA)

IC_{50}	concentration that inhibits 50 % of the maximal enzyme activity
I+L-N	indomethacin and L-NAME co-treatment
Indo	indomethacin (co-inhibitor of COX-1 and -2)
IP receptor	prostaglandin I_2 receptor
L-NAME	N_ω-nitro-L-arginine methyl ester hydrochloride (NOS inhibitor)
LV	left ventricle
LV/BM	left ventricular-to-body mass ratio
MAP	mean arterial pressure
ND	no drug control
NO	nitric oxide
NOS	nitric oxide synthase
NS-398	N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (preferential inhibitor of COX-2)
OLETF	Otsuka-Long-Evans-Tokushima fatty rat
PAGE	polyacrylamide gel electrophoresis
PE	phenylephrine hydrochloride (α_1 adrenergic receptor agonist)
PE-50	polyethylene tubing no. 50
PG	prostaglandin
PGH_2	prostaglandin H_2
PGI_2	prostaglandin I_2, a.k.a. prostacyclin
PK	protein kinase
PUFA	polyunsaturated fatty acid

ROCK	rho kinase
ROS	reactive oxygen species
RV	right ventricle
RV/BM	right ventricular-to-body mass ratio
SBP	systolic blood pressure
SDS	sodium dodecyl sulfate
sGC	soluble guanylate cyclase
SHR	spontaneously hypertensive rat
SNP	sodium nitroprusside dihydrate (NO donor molecule)
SOD	superoxide dismutase
SQ29548	[1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenyl amino)carbonyl]hydrazine]methyl]-7-oxabycyclo[2.2.1]hept-2-yl]-5-heptenoic acid (TP receptor antagonist)
TBS-T	tris-buffered saline with Tween®-20
Tempol	4-hydroxy-TEMPO (SOD mimetic)
TP receptor	thromboxane A_2/prostaglandin receptor
TxA_2	thromboxane A_2
VAS	valeroyl salicylate (preferential inhibitor of COX-1)
VSM	vascular smooth muscle
WKY	Wistar-Kyoto rat
ZDF	Zucker diabetic fatty rat

Chapter 1

Introduction

1.1 Overview

The chronic conditions of essential hypertension and type 2 diabetes are becoming increasingly prevalent worldwide and are associated with an elevated risk of cardiovascular morbidities and mortality. Hypertensive and type 2 diabetic patients often present with certain cardiovascular conditions (e.g. atherosclerosis, coronary artery disease, peripheral arterial disease, aneurysm, stroke) that are specific to large conduit arteries (e.g. aorta, carotid arteries, coronary arteries). In many chronic diseases, including hypertension and type 2 diabetes, the vascular endothelium becomes dysfunctional and is not able to maintain adequate control of its many homeostatic functions in the vascular wall (e.g. vascular tone, vascular permeability, cell proliferation, thrombosis, inflammation, cellular adhesion, platelet function). A central role exists for this impaired endothelial function in the macrovascular complications listed above that are associated with hypertension and type 2 diabetes. Endothelial dysfunction can be experimentally detected and characterized in many ways, including altered vasomotor responses of isolated arterial segments. Vasomotor dysfunction is commonly reported as blunted vasorelaxation and/or heightened vasocontraction to known physiological stimuli. The experiments comprising this thesis assess vascular function in hypertension and in type 2 diabetes mellitus by examining vasomotor responses of aortic segments isolated from animal models of these disease states. The first two experimental

chapters (Chapters 2 and 3) examine potential mechanisms underlying the vasomotor dysfunction specifically in ageing hypertensive female rats and how these responses compare to those of male counterparts. The third experimental chapter (Chapter 4) assesses the therapeutic potential of chronic dietary supplementation with docosahexaenoic acid, an n-3 polyunsaturated fatty acid found in fatty fish, on vasomotor responses and blood pressure of adult male hypertensive rats. The fourth experimental chapter (Chapter 5) characterizes vasomotor responses in type 2 diabetic female rats and how administration of the anti-diabetic drug metformin and chronic aerobic exercise training, alone and in combination, affects these responses.

1.2 Background

Regulation of vascular tone by endothelial integration and transmission of vasokinetic signals

Vascular 'tone' is the contractile state of a blood vessel and can be adjusted by active contraction and relaxation of vascular smooth muscle (VSM) cells oriented circumferentially in the vascular wall. The resultant physiological response is decreased and increased radius of the vessel lumen, termed vasoconstriction and vasodilation, respectively.

Vascular tone is normally tightly controlled through multiple and redundant pathways. At any given time the net balance of many vasodilatory and vasoconstrictory signals converging on the VSM determines vascular tone. The single layer of endothelial

cells lining the vessel lumen contributes to the regulation of this phenomenon by integration and appropriate transmission of vasokinetic signals to the VSM. The signals can be mechanical (e.g. shear, pressure), neural/humoral (e.g. agonists acting on a receptor protein), or ionic (e.g. non-receptor mediated alteration of electrochemical gradient across a cell membrane) in nature. Additionally, these signals can be endocrine (i.e. originating in distant tissues), paracrine (i.e. originating in neighbouring cells in the vascular lumen, the vascular wall, or the underlying non-vascular tissue), or autocrine (i.e. endothelial cells stimulating themselves). Conduction of these signals from the endothelium to the VSM occurs through the release of various endothelium-derived vasokinetic paracrine factors which then initiate VSM relaxation or contraction (31).

Endothelium-dependent, NO-mediated vasorelaxation as a marker of overall endothelial function

The three main endothelium-dependent vasodilatory effector molecules known to exist are: 1) nitric oxide (NO); 2) prostaglandin (PG) I_2 (a.k.a. prostacyclin); and 3) endothelium-derived hyperpolarizing factor (EDHF)(Figure 1-1). The mechanisms of NO- and PGI_2-mediated dilation in healthy arteries are well characterized, however the mode(s) of action and specific effector molecule(s) of EDHF-mediated dilation remain elusive. Of these three vasodilatory molecules, NO coordinates the large majority of conduit artery relaxation. Due to the secondary importance of PGI_2 and EDHF in macrovascular relaxation, their roles in this phenomenon will only be discussed peripherally. In addition to its potent vasodilatory

action, NO plays many other critical roles in the maintenance of vascular homeostasis through regulation of: coagulation and thrombosis (9); adherence and transmigration of leukocytes into the vascular wall (24); VSM proliferation (39); and oxidative stress and oxidation of low-density lipoprotein (39). All of these NO-inhibitable processes contribute to the development and progression of atherosclerosis (80), supporting an important anti-atherogenic role for NO in large arteries. Endothelium-dependent NO-mediated vasomotor function is often used experimentally as a surrogate marker of overall NO function, and thus of other aspects of endothelial health and function mediated by NO (121).

The biochemical pathway of NO-mediated vasodilation is well established (Figure 1-2). Endothelial NO production is initiated when certain physical (e.g. shear stress) and chemical (e.g. muscarinic activation) stimuli raise the endothelial cytosolic calcium concentration. This leads to activation of endothelial NO synthase (eNOS), the enzyme that catalyses the conversion of *L*-arginine and molecular oxygen to *L*-citrulline and NO. By virtue of its small size and neutral charge, NO can diffuse freely across membranes and within cellular compartments to stimulate many different targets. In the case of NO-mediated vasodilation, NO diffuses into the cytosol of underlying VSM cells and activates soluble guanylate cyclase (sGC). Conversion of GTP to cGMP by activated sGC initiates protein kinase (PK) G-mediated phosphorylation of various calcium regulatory proteins. The result is relaxation of the VSM actin-myosin complexes and vasodilation (31).

Determinants of NO bioavailability

All functions mediated by NO are governed by its 'bioavailability'. This is defined as the degree of endpoint biological effect produced by a stimulus that acts via a NO-mediated signaling pathway. In general, the three main determinants of NO bioavailability are: 1) the rate of NO production; 2) the rate of NO destruction; and 3) the responsiveness of the target tissue to NO (Figure 1-2)(121). This third determinant is generally not greatly altered in hypertension or type 2 diabetes with the exception that VSM responsiveness to NO is reduced in senescent hypertensive animals compared to normotensive counterparts (10). Thus NO bioavailability in these disease states is largely determined by factors controlling NO production and destruction and will be further discussed.

The level of eNOS activation and the amount of eNOS protein expression both determine the rate of NO production. A number of events contribute to acute regulation (activation and inhibition) of NO production by eNOS, including: 1) phosphorylation and dephosphorylation of eNOS at several sites (i.e. Ser114, Ser615, Ser633, Ser1177, Thr495) by several kinases (i.e. 5'-AMP-activated protein kinase, calmodulin kinase II, PKA, Akt/PKB, PKC, PKG) and phosphatases (i.e. protein phosphatase-1, -2A, -2B/calcineurin), respectively; 2) protein-protein interaction between eNOS and calmodulin, heat shock protein-90, caveolin, and certain G-protein coupled receptors; and 3) acylation (i.e. myristoylation and plamitoylation) by specific saturated fatty acids (44; 100). Additionally, sufficient levels of co-factors (i.e. flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin, calcium) and substrates (i.e. molecular oxygen, *L*-arginine, NADPH) for eNOS are required for NO production (44). On the other hand, eNOS becomes 'uncoupled'

when levels of its cofactors and/or substrates are inadequate. In this situation, eNOS begins generating superoxide anions instead of NO (22; 37; 45). The consequences of eNOS uncoupling with respect to NO bioavailability are two-fold since NO production by eNOS is decreased and NO destruction by eNOS-derived superoxide anion (discussed below) is increased. Chronic regulation of eNOS expression (protein content) and post-transcriptional modification are controlled by various physiological stimuli (e.g. shear stress, chronic exercise, hypoxia, cytokines) acting through several transcription factors (e.g. activator protein-1 and -2, nuclear factor-κB)(44). Thus a complex interaction of many biochemical events must be coordinated for efficient and adequate production of NO by eNOS. Dysregulation of one or more of these processes could result in reduced NO production, therefore contributing to impaired NO bioavailability.

Elevated NO destruction is the second determinant contributing to the reduced NO bioavailability implicated in hypertension and type 2 diabetes. NO is a free radical and can readily donate its unpaired electron to many biological molecules, rendering it inactivated. A primary scavenger of NO is superoxide anion, a reactive oxygen species (ROS) produced by several pro-oxidant enzymes in the vascular wall (e.g. NAD(P)H oxidase, eNOS, mitochondrial electron transport chain, xanthine oxidase). Interaction of superoxide anion and NO leads to the formation of peroxynitrite and thus NO destruction and reduced NO bioavailability (Figure 1-2)(84; 121). In healthy arteries, superoxide anion is quenched to hydrogen peroxide by superoxide dismutase (SOD) and further metabolized to water by either catalase or glutathione peroxidase, preventing its interaction with and destruction of NO (31; 84). However, vascular overproduction of ROS (i.e. oxidative stress) is a

fundamental disruption of vascular homeostasis in many cardiovascular and metabolic diseases, including hypertension and type 2 diabetes (121). The vascular consequences of reduced NO bioavailability due to impaired NO production and/or enhanced ROS-dependent NO destruction in these diseases are as great and varied as the plethora of homeostatic roles of NO in this tissue.

Prostanoid-mediated vasoconstriction

In addition to eNOS activation, an agonist-stimulated elevation of endothelial intracellular calcium concentration stimulates phospholipase A_2 to hydrolyse phospholipids and to release arachidonic acid (AA, Figure 1-3). Cyclooxygenase (COX, a.k.a. PGG_2/PGH_2 synthase) becomes activated and metabolizes AA to PGG_2 and PGH_2. One fate for PGH_2 is that it can be released from the endothelial cell to stimulate cell membrane thromboxane A_2/prostaglandin (TP) receptors on VSM, thereby initiating calcium influx into VSM and subsequent vasoconstriction (15). A second outcome for PGH_2 is that it can be enzymatically converted to PGI_2 by PGI_2 synthase, or to several other prostanoids, including thromboxane A_2 (TxA_2) and $PGF_{2\alpha}$. Like its precursor, PGI_2 can also be released from the endothelium to stimulate the PGI_2 receptor (IP receptor) on the VSM cell membrane. This action initiates VSM relaxation through a cAMP-mediated second messenger cascade and is one of the three main endothelium-dependent vasodilatory mechanisms of healthy arteries (Figure 1-1). Therefore, the vasoconstrictory and vasodilatory actions of PGH_2 and PGI_2, respectively, normally oppose each other. However, in some chronic disease states, such as

hypertension, certain arteries undergo a paradoxical shift from PGI_2-*IP* receptor-mediated vasodilation to PGI_2-*TP* receptor-mediated vasocontraction (43). For this reason PGH_2 and, in some cases, PGI_2 have been identified as endothelium-derived contracting factors (EDCF)(43; 147). Thus the expression and activities of COX, PGI_2 synthase, TP receptors, and IP receptors will collectively determine whether the overall influence of COX-derived prostanoids on vasomotor tone will be one that is dilatory or constrictory in nature. The complexity of prostanoid-mediated vasocontraction is compounded by the fact that TP and IP receptors can be coupled to several sub-types of G-proteins, each of which is associated with a different second messenger pathway and which can lead to opposite vasomotor outcomes (15). Furthermore, an additional vasoconstrictory prostanoid, TxA_2, has been implicated in the endothelium-dependent vasocontraction observed in hypertension. However, TxA_2 contributes very little, if at all, to these responses in aorta of spontaneously hypertensive rats (SHR), the animal model of essential hypertension that is used in the present thesis (6; 43; 52; 68; 75; 85; 128; 144).

The importance of tight control of endothelial function

Several different endothelial processes must be well balanced in order for the endothelium to function properly: 1) NO production by NOS balanced with NO destruction by interaction with various biological molecules; 2) ROS production by pro-oxidant enzymes balanced with ROS quenching by anti-oxidant mechanisms; and 3) release and vasodilatory action of NO (and other dilatory molecules) balanced with release and vasoconstrictory

action of EDCF (and other constrictory molecules). If appropriately tight control of these processes is maintained then endothelial function, including vasodilation, will remain intact. On the other hand, imbalance of one or more of these processes can negatively influence endothelial function in chronic diseases like hypertension and type 2 diabetes mellitus and thus contribute fundamentally to morbidity and mortality in these patients. Understanding the basic underlying mechanisms responsible for endothelial impairment is crucial for the development and application of efficacious therapies aimed at the *causes*, and not merely the *symptoms*, of these specific disease populations.

1.3 Global purpose of the thesis

The global purpose of this thesis is to identify and characterize the pathways controlling impaired vasomotor function in *female* animal models of two chronic disease states: hypertension and type 2 diabetes mellitus. In patients with either of these diseases, reduced endothelial function has a major detrimental impact on morbidity and mortality because it contributes to secondary clinical vascular complications such as atherosclerosis (104). However, there is currently a very limited understanding of endothelial function in *females* with these chronic diseases since the majority of studies have only examined males. It is important for basic science to establish if and how endothelial impairment occurs in hypertensive and in type 2 diabetic females. This understanding of the fundamental sex

differences in the progression of these two diseases will allow for more specific clinical treatment.

1.4 Animal models of disease used in the thesis

The SHR strain is a widely used genetic model of human essential (a.k.a. primary) hypertension. This model was developed in the 1950's from the inbreeding of Wistar rats that were selected for higher blood pressure (BP). The normotensive control strain that resulted is the Wistar-Kyoto rat (WKY) (153). As the name suggests, SHR spontaneously develop elevated BP compared to WKY over the first 12 wk of life (the difference is detectable as early as 2 wk of age) (54) and this elevation persists into adulthood and senescence (10; 40).

The Zucker diabetic fatty rat (ZDF, specifically termed: ZDF/Gmi^{TM}-*fa*) is a genetic model of type 2 diabetes that was developed by inbreeding Zucker obese rats that were homozygous recessive for a mutated *fa* gene (115). This mutation produces a shortened and ineffective leptin receptor (146), thus preventing leptin from signaling satiety and leading to overeating. ZDF (i.e. *fa/fa*) exhibit obesity, hyperglycemia, hypertriglyceridemia, insulin resistance, and initial hyperinsulinemia followed by eventual hypoinsulinemia (42; 115). The Zucker lean control strain are either heterozygous (+/*fa*) or homozygous dominant (+/+) and remain lean and non-diabetic. Male ZDF develop these type 2 diabetes-like symptoms spontaneously when fed a normal fat-content diet (16.7 % kcal fat), whereas female ZDF

require a high fat-content diet (47.9 % kcal fat) for these symptoms to develop (34). One advantage to this high fat induction of type 2 diabetes-like symptoms in female ZDF is that this model provides a control group for hyperglycemia, since female ZDF that are fed a normal fat-content diet ad libitum become obese and hyperinsulinemic but do not develop overt hyperglycemia (34).

1.5 In vitro vascular myography for assessment of endothelium-dependent vasomotor responses

Vascular myography involves the measurement of development of positive (gain) and negative (loss) isometric tension in a ring segment isolated from the blood vessel of interest. The intact blood vessel is harvested from the donor, submersed in cold (4 ^{o}C) physiologic buffer (pH ~7.4), and blood is cleared from the lumen and adhering connective tissue is gently removed from the outside of the vessel. Once cleaned, the vessel is transferred to fresh cold buffer and visualized under a calibrated dissecting microscope (8x magnification) for additional connective tissue removal. The vessel is aligned with a ruler and cut into sequential transverse ring segments of known length using a single-edged razor blade. A triangular wire tissue support (#2, Figure 1-4A) is passed through the lumen of one ring (#3, Figure 1-4A).

The ring is loaded onto the myography unit by suspending the tissue support from a calibrated isometric force transducer using a length of silk suture (#1, Figure 1-4A). The

wire of an immobile glass support foot (#4, Figure 1-4A) of the myography unit is then passed through the lumen of the ring and secured in place. While handling and loading rings onto the myography unit, care is taken to minimize disruption of the tissue, in particular the inner surface of the vessel (i.e. the endothelium). Once the ring is suspended between the centres of the two wire tissue supports, a small amount of passive tension (~0.25–0.30 g) is applied to the ring by raising the isometric force transducer, and thus the upper wire, relative to the lower fixed wire in a controlled manner using a treaded mechanism. A pump-perfused, water-jacketed isolated tissue bath (#5, Figure 1-4A) containing warm (37 ^{o}C) and continuously aerated (95:5 % O_2:CO_2 gas mixture) physiologic buffer (pH ~7.4) is raised so that the ring is submersed in buffer (#6, Figure 1-4A). The buffer in the tissue bath is replaced periodically (every ~15–30 min) throughout the entire myography protocol.

Passive tension is applied to the ring at timed intervals (e.g. +0.5 g every 5 min) until a pre-determined level of optimal passive "resting" tension is attained. This group-specific "optimal resting tension" will have been determined in pilot testing as the level of passive resting tension that allows for maximal active tension development by the ring in response to potassium chloride (KCl)-induced depolarization. The ring is equilibrated for 15–30 min at optimal passive resting tension, during which time tension levels are adjusted as necessary to maintain a constant level. Passive resting tension of the ring is not adjusted after the equilibration period. Following equilibration, the ring is stimulated to contract with KCl (60 mM) and, once a steady-state level of tension has been attained and recorded, the buffer is replaced and the ring fully relaxes back to baseline (i.e. optimal resting tension). The KCl-induced contraction is then repeated a second time, followed by buffer replacement and

relaxation to baseline. The tension development to this second KCl stimulus is recorded and used as a 'reference contraction' for normalization of contraction dose-responses (see below). The ring is then incubated (30 min) in the absence (i.e. No Drug control) or presence of an inhibitor of choice (for isolation of specific pathways of interest). If a relaxation dose-response (Figure 1-4B) will be measured, then the ring is pre-contracted with a sub-maximal dose of phenylephrine hydrochloride (PE, 10^{-7} M). Once a steady pre-contraction has been achieved, cumulative doses of the vasodilatory agonist of choice are added in addition to the PE in a serial manner. If a contraction dose-response (Figure 1-4C) will be measured, no PE pre-contraction is performed and cumulative doses of the vasoconstrictory agonist of choice are simply added in a serial manner on top of the passive resting tension of the ring (i.e. a 'quiescent' ring preparation). Regardless of whether the dose-response being measured is one of relaxation or contraction, a steady-state level of tension is allowed to develop to each cumulative agonist dose before the response to that particular dose is recorded.

Two endothelium-dependent dose-responses used throughout the thesis are: 1) endothelium-dependent relaxation to acetylcholine chloride (ACh); and 2) endothelium-dependent contraction to ACh. The two paragraphs that follow describe the similarities and differences between these two dose-responses as well as what each specific response represents.

Endothelium-dependent relaxation to ACh is assessed in rings that have been pre-contracted with sub-maximal PE. This PE stimulation causes the ring to develop some level of active positive tension (i.e. contraction) against which ACh-mediated relaxation can be detected. The loss of tension to serial ACh doses is expressed as a percentage of PE-

stimulated pre-contraction on a ring-by-ring basis. The ACh relaxation dose-response represents the net vasomotor response of the various endothelium-dependent vasodilatory and vasoconstrictory molecules being released due to an ACh dose-dependent rise in the endothelial intracellular calcium concentration. In the rat aorta (the vessel studied in the thesis) these molecules are primarily NO (dilatory) and prostaglandins (constrictory). Some diseased arteries exhibit an initial relaxation (loss of tension) to lower (i.e. $<10^{-7}$ M) ACh doses followed by contraction (gain of tension) to higher (i.e. $>10^{-7}$ M) ACh doses. This contraction that follows a previous relaxation response is termed 're-contraction'. Thus the ACh relaxation response mimics the in vivo environment in two important ways: some sub-maximal level of 'tone' is present, and the two major vasomotor pathways that are sensitive to elevated endothelial calcium levels are functional.

Endothelium-dependent contraction to ACh is assessed in rings that have not been pre-contracted with PE. This contractile response is detected as positive tension development from the baseline level of passive resting tension occurring in the ring prior to addition of the first ACh dose. It is standard operating procedure to perform the ACh contraction dose-response in the presence of NOS inhibition with Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME). This eliminates the major dilatory contribution of NO to the vasomotor response of rat aorta. Additionally, any small amounts of endothelium-derived relaxation signals that are still produced in the presence of NOS inhibition will not be detected since the ring has not been stimulated to produce any active tone prior to initiating the ACh contraction dose-response. The tension developed to serial ACh doses is expressed on a ring-by-ring basis as a percentage of the contractile response to the second dose of KCl

initiated at the beginning of the protocol (i.e. the 'reference KCl contraction'). The ACh contraction dose-response thus represents the isolated vasocontractile response to endothelium-dependent, COX-derived EDCF molecules being released due to an ACh dose-dependent rise in the endothelial intracellular calcium concentration. The experimental procedure followed for the ACh contraction dose-response yields valuable information about the nature of the COX-EDCF pathway, albeit in a pharmacologically-isolated manner (i.e. NOS inhibition) which, when considered alone, is less physiologically relevant than the ACh relaxation dose-response.

1.6 Rationale for the thesis topic

Oxidative stress and COX-derived EDCF mediate endothelial dysfunction in hypertension

Impaired aortic vasomotor function of male SHR compared to their male normotensive WKY counterparts is characterized by reduced endothelium-dependent relaxation (59; 63; 68; 70; 76; 77; 85; 127; 144) and enhanced endothelium-dependent contraction (49; 77; 85) to muscarinic receptor simulation by ACh. Additionally, while both male WKY (59; 63; 74; 75; 77; 127) and SHR (63; 74; 75; 119; 127) exhibit declining endothelial function with ageing, this effect is exacerbated in the latter. This phenomenon has been described as "premature ageing" of male SHR aortic endothelium (52; 147). A shift towards enhanced PG-mediated contraction appears to provide the fundamental basis for the endothelium-dependent dysfunction observed in male SHR and with ageing in male SHR and

WKY (43; 147). Indeed, robust endothelial relaxation is restored (59; 62; 63; 68; 70; 75; 77; 85; 144) and endothelial contraction is abolished (6; 48; 52; 62; 63; 77; 85; 119; 154) in these animals with inhibition of COX or TP receptor. Furthermore, aortic relaxation to ACh in this animal model is almost entirely dependent on the NO pathway (62; 63). The fact that this response is fully restored with COX or TP receptor inhibition suggests that aortic NO production per se is similar between male WKY and SHR and with ageing in both of these strains (43). Collectively these data support the conclusion that endothelial vasomotor function is reduced in a COX- and TP receptor-dependent manner in male SHR (vs. WKY) and with ageing.

It would be an oversimplification to state that aortic vasomotor tone of male SHR is controlled purely by a balance of EDCF-mediated vasoconstriction and NO-mediated vasodilation and that these two pathways are regulated totally independently of one another. Elevated vascular oxidative stress, most likely in the form of enhanced production of superoxide anion, occurs in aorta of male SHR (86; 161) and plays a pivotal role in both NO bioavailability (see *Background*, Section 1.2 above) and generation of EDCF by COX in the vascular wall (43; 147). Aortic contractions to exogenous superoxide anion are elevated in male SHR compared to WKY and are blunted with inhibition of COX or TP receptor (5; 154). Additionally, endothelium-dependent contractions to ACh that are mediated through the COX-ECDF-TP receptor pathway in SHR are enhanced in the presence of excess superoxide anion (155; 156) and blunted in the presence of anti-oxidant compounds (154; 156). It has been suggested that stimulation of SHR aorta with ACh results in the production of NO and superoxide anion, which partially scavenge and inactivate each other. However,

some of the NO is able to stimulate VSM relaxation and some of the superoxide anion is able to activate or potentiate COX-1-derived EDCF production, leading to TP receptor-dependent VSM contraction (43; 147). Thus, elevated generation of superoxide anion appears to provide a link between the NO pathway and the COX-EDCF pathway and their control of endothelium-dependent vasomotor function in male SHR.

These data characterizing mechanisms of impairment in aorta of male SHR parallel findings in the forearm circulation of patients with essential hypertension. Reduced endothelial relaxation to ACh occurs in hypertensive patients compared to age-matched normotensive counterparts (81; 114; 137-141) and these impairments are oxidative stress- (138) and COX-dependent (137; 138; 140). Additionally, the relaxation responses of both normotensive and hypertensive patients decline with ageing, and with an earlier onset and to a greater degree in the latter (139-141). The observation of apparently accelerated deterioration of endothelial function in hypertensive patients has been postulated to be due to "premature ageing" of the vascular endothelium in this population (140). Thus, elevated production of COX-derived EDCF and oxidative stress both contribute to the impaired endothelium-dependent relaxation of male SHR and human hypertensive patients compared to normotensive counterparts and this impairment worsens with ageing in both SHR and patients. The striking similarities between findings in hypertensive humans and in SHR strengthen the argument for the use of SHR as a model of endothelial vasomotor disturbances in essential human hypertension and with ageing in this disease.

The influence of n-3 PUFAs on vasomotor function and BP in established hypertension is understudied

It was observed in the 1970's that plasma lipid levels and the incidence of cardiovascular disease are remarkably low in Greenland natives despite consumption of a high-fat diet (7). It was postulated that the high content of n-3 (a.k.a. omega-3 or ω-3) polyunsaturated fatty acids (PUFAs) in the traditional Inuit diet (derived mostly from marine animals) was a major contributing factor and much research has since focused on the potential benefits of n-3 PUFAs in cardiovascular health. n-3 PUFAs are long-chain PUFAs with the first carbon-carbon double bond occurring in the third position from the methyl, or N-terminal, end of the molecule (i.e. between carbons 3 and 4). The nomenclature used to describe the structure of PUFA molecules is as follows: *X*:*Y* n-*Z*, where *X* is the number of carbon atoms in the chain, *Y* is the number of double bonds in the chain, and *Z* is the position (from the N-terminal) of the first double bond. For example, docosahexaenoic acid (DHA) is described as "22:6 n-3", meaning it is a 22-carbon chain containing 6 double bonds, the first of which occurs in the third position (i.e. between carbons 3 and 4).

Consumption of n-3 PUFAs, either from natural sources (e.g. fatty fish) or supplementation with concentrated oil capsules (e.g. 'fish oil'), has been associated with reduced risk of various cardiovascular diseases (117). Additionally, n-3 PUFA intake appears to alter several processes that may directly influence large vessel-specific cardiovascular pathologies such as atherosclerosis (reviewed in: (23)). These n-3 PUFA effects include: improved endothelial function; improved blood lipid profile; reduced BP; reduced platelet aggregation; reduced vascular inflammation; reduced thrombosis; reduced

cardiac arrhythmia; reduced vascular release of chemoattractants/growth factors; and reduced vascular expression of adhesion molecules. In particular, the reported endothelioprotective (13; 158; 159) and hypotensive (12; 13; 25; 41; 47; 65; 66; 78; 88; 92; 124; 126; 129; 158) effects of n-3 PUFAs, such as DHA and eicosapentaenoic acid (EPA, 20:5 n-3) may make them good therapeutic candidates for the treatment of hypertension.

Several studies have reported enhanced aortic ACh relaxation (13; 158; 159) and reduced BP (12; 13; 25; 41; 47; 65; 66; 78; 88; 92; 124; 126; 129; 158) following n-3 PUFA supplementation in "younger" (i.e. <12 wk old) male SHR. n-3 PUFA supplementation restored robust endothelial function in these SHR (13; 158; 159), such that the response was similar to WKY counterparts in the one study that included this WKY control group (13). The hypotensive effects of various n-3 PUFAs show a range of effectiveness in these younger SHR, reducing systolic BP (SBP) by 9 to 40 mm Hg (12; 13; 25; 41; 47; 65; 66; 78; 88; 92; 124; 126; 129; 158). It is noteworthy, however, that supplementation in all of these reports began before full establishment of endothelial impairments (e.g. re-contraction) and elevated BP. Thus the therapeutic effects of n-3 PUFAs in these animals actually represent *abrogation of development* of endothelial dysfunction and hypertension. Unfortunately, there are no reports of endothelial function following supplementation that was commenced after establishment of hypertension – neither in "adult" (i.e. >12 wk old) SHR nor human hypertensive patients with no other confounding cardiovascular diseases. In contrast to consistent BP-lowering effects in younger male SHR, dietary n-3 PUFAs do not appear to induce such an obvious hypotensive response in adult counterparts. There have been reports of both reductions (–14 to –21 mm Hg) (12; 78) and no changes (92; 103) in SBP of adult

SHR following n-3 PUFA feeding that was begun after 12 wk of age (i.e. after reaching a relatively steady elevation in BP). There are also mixed reports in human hypertensive patients that n-3 PUFAs either slightly lower (8; 17; 73; 105; 118) or do not alter BP (38; 82; 99). A recent meta-analysis of randomized trials concluded that n-3 PUFA supplementation slightly but significantly reduced BP in hypertensive humans (51). Collectively these BP data indicate that n-3 PUFAs (e.g. DHA, EPA), administered as a component of the diet, do not consistently alter BP in adult male SHR with established hypertension but may offer small reductions in BP to human hypertensive patients. There is a lack of reports examining endothelial vasomotor responses following n-3 PUFA supplementation in adult SHR or hypertensive patients, both of which exhibit established hypertension. Understanding the basic vascular biology effects of n-3 PUFAs requires that the endothelium-dependent responses be characterized following supplementation with these compounds. This knowledge will help realize the full therapeutic potential of n-3 PUFAs. It is possible that dietary manipulation with n-3 PUFA-rich foods or supplementation with purified n-3 PUFAs may compliment or even reduce the patient's reliance on current anti-hypertensive therapies.

Hyperglycemia-induced oxidative stress mediates endothelial dysfunction in diabetes

The current theory on the etiology of diabetic vascular complications places hyperglycemia-induced damage squarely at the foundation. Hyperglycemia-induced elevation of superoxide anions derived from the mitochondrial electron transport chain has been proposed (19) as a common mechanism causing upregulation of the four major

pathways responsible for endothelial impairment in type 2 diabetes mellitus: 1) increased flux through the aldose reductase/polyol pathway; 2) activation of PKC; 3) increased formation of advanced glycation end-products; and 4) increased flux through the hexosamine pathway (19; 36). Together these alterations contribute to various endothelial impairments including: further enhancement of oxidative stress; intra- and extracellular protein damage leading to altered protein and cellular function; increased vascular permeability; increased expression of pro-inflammatory transcription factors, cytokines, growth factors, and enzymes; reduced NO production; and decreased arterial elasticity. The collective result of this compendium of perturbations is endothelial dysfunction and enhanced susceptibility to common diabetes-related vascular complications such as atherosclerosis (11; 19; 36; 104). However, in addition to the overt hyperglycemia that characterizes type 1 diabetes, several other metabolic abnormalities are present in type 2 diabetes (e.g. insulin resistance, hyperinsulinemia, hypertriglyceridemia, obesity). Superimposing these supplementary metabolic derangements onto hyperglycemia in type 2 diabetes could potentiate the deleterious hyperglycemic consequences on endothelial function (11; 36; 104). All of these metabolic abnormalities are exhibited by female ZDF (34) and could therefore influence aortic vasomotor responses in these animals.

Macrovascular complications (e.g. atherosclerosis) greatly impact morbidity and mortality in type 2 diabetic patients (11; 104). Studies examining endothelial dysfunction in large conduit arteries could illuminate mechanisms responsible for the increased susceptibility to these conditions as well as potential therapeutic targets. Reduced endothelium-dependent relaxation is consistently reported in male (28; 57; 112; 162) and

sex-unspecified (18) ZDF, in other male (14; 71; 72; 95; 96; 122) and sex-unspecified (107; 123) animal models of type 2 diabetes, and in type 2 diabetic human patients (93; 145). Interestingly, inhibition of superoxide anion production (28) and the presence of a peroxynitrite scavenger (18) both improve aortic relaxation in ZDF, and acute intraarterial administration of vitamin C, which has anti-oxidant properties, improves forearm blood flow responses in type 2 diabetic humans (145). Additional indices of oxidative stress are present in ZDF aorta in the form of elevated superoxide anion production (28; 108; 109; 111), pro-oxidant enzyme expression (28) and activity (162), and lipid and protein damage (18). Therefore, similar to the vascular etiology of essential hypertension, elevated vascular oxidative stress occurs in male and sex-unspecified ZDF and appears to play a role in the aortic endothelial dysfunction observed in these animals. Further understanding of the role of oxidative stress in promoting type 2 diabetic macrovascular perturbations and how this role changes with current anti-diabetic therapies may point towards a more effective and comprehensive approach to treatment.

In addition to impaired endothelial relaxation, male ZDF (79; 149) and other male (72; 152; 160) and sex-unspecified (107) animal models of type 2 diabetes exhibit augmented α adrenergic vasocontraction responses. This elevation appears to involve enhancement of both PKC and rho kinase (ROCK) contraction pathways (72; 149; 152). Hypersensitivity to α adrenergic stimulation could counterbalance endothelium-dependent vasodilatory signals and thus contribute to vasomotor dysfunction in type 2 diabetes.

The influences of anti-diabetic therapies on macrovascular function are understudied

Various anti-diabetic pharmacological interventions and lifestyle modifications (e.g. physical activity) are commonly prescribed for the treatment of type 2 diabetes (11; 104) and prevent development of hyperglycemia in ZDF (116; 134). Improved *micro*vascular endothelial function in male ZDF results from chronic administration of drugs typically used for treatment of traditional cardiovascular diseases, such as statins (108; 111), angiotensin converting enzyme inhibitors (108; 111), and a dual inhibitor of angiotensin converting enzyme and neutral endopeptidase (109; 125). Additionally, the anti-diabetic drug, metformin (91), and exercise training (97) improve microvascular function in other male animal models of type 2 diabetes. In contrast, the effects of therapeutic interventions on *macro*vascular impairments in type 2 diabetes are less well characterized. While one group has reported that aortic superoxide anion levels are reduced in male ZDF following chronic cardiovascular drug treatment (e.g. statins, angiotensin converting enzyme inhibitors, and a dual inhibitor of angiotensin converting enzyme and neutral endopeptidase) (108; 109; 111), no studies have examined the influence of pharmacological treatment on macrovascular vasomotor dysfunction in any animal model of type 2 diabetes. Chronic exercise training, on the other hand, improves endothelium-dependent relaxations in aorta of various male rodent models of type 2 diabetes other than ZDF (71; 72; 95; 96; 122). Type 2 diabetic patients also exhibit improved endothelial microvascular dilation following chronic metformin treatment (90) and improved endothelial micro- and macrovascular dilation following a chronic exercise training regimen (87). Therefore several aspects of pharmacological treatment, exercise training, and the combination of these therapies remain unstudied in the realm of

macrovascular function in type 2 diabetes. Understanding the ability for these interventions to improve macrovascular insufficiencies in this disease is important for their efficacious application.

Vascular function of ageing females with hypertension or type 2 diabetes is understudied

It is important to emphasize that the vast majority of the referenced studies examining mechanisms of endothelial vasomotor function in hypertension or diabetes have been performed in males. The one study that was specifically designed to examine sex differences in endothelial function in SHR reported that males exhibit greater blunting of endothelial responses compared to females and that these sex differences are largely due to enhanced COX-derived EDCF in males (70). This observation supports the idea that COX-dependent sex differences in endothelial function exist in hypertension. However, this study examined relatively young animals (16 wk old) and thus does not provide any insight into the influence of advancing age in female SHR. Studies of endothelial responses in human hypertensive patients report declining function in ageing males beginning in early adulthood but preservation of function in ageing females until the onset of menopause, at which point dysfunction begins to develop (139). These data indicate that ageing-related deterioration of endothelial function occurs in hypertensive females, however little is known about the mechanisms responsible for this phenomenon. Understanding if and how endothelial function is differentially modified in ageing female vs. ageing male SHR will shed light on sex-specific vascular behaviour and mechanisms responsible. Additionally, these

observations could allow for generation of therapeutic interventions that are tailored to the specific needs of ageing *female* hypertensive patients.

Similar to the limited numbers of studies examining endothelial function in hypertensive females, there are few reports of this phenomenon in type 2 diabetic females. A series of three studies from one group examined sex differences in the endothelium-dependent systemic hemodynamic responses of anesthetized ZDF to intraarterial ACh infusion. Greater and longer lasting ACh-induced reductions in mean arterial pressure (MAP) occurred in female compared to male ZDF (1). Additionally, the hypotensive ACh response became slightly reduced and greatly heightened following gonadectomy of female and male ZDF, respectively (2). Finally, hypotensive responses were enhanced in both sexes following chronic androgen receptor blockade in gonad-intact ZDF (3). Collectively these data indicate that in vivo resistance artery endothelial function is impaired in male compared to female animals in the presence of type 2 diabetes-like metabolic derangements. Moreover, protective and deleterious roles may exist for female and male sex hormones, respectively, in this animal model. One strength of a systemic outcome measurement such as BP fluctuation is that it accounts for all of the varied responses of the body (e.g. neural, humoral, endogenous production of vasoactive substances). However, it is important to keep in mind that this is also a major caveat of examining in vivo responses. It is extremely difficult to record compensatory activation or inhibition of the plethora of multiple and redundant pathways that govern vasomotor responses in vivo. Isolating mechanisms responsible for a particular phenomenological observation thus becomes nearly impossible in this experimental setting.

Therefore, we have very limited knowledge of alterations in endothelial function, from either a phenomenological or mechanistic standpoint, in both ageing hypertensive females and in type 2 diabetic females. The lack of studies examining female vasomotor function is a major issue when translating from basic mechanistic observations to a patient population. Assessment of the sex differences or similarities in these diseases would shed light on sex-specific vascular behaviour and mechanisms responsible. These findings may also point to important factors when designing and implementing therapeutic strategies in female hypertensive and type 2 diabetic patients.

1.7 Specific purposes, hypotheses, and the anticipated contributions of the findings

As stated above, the global purpose of this thesis is to describe and to further our understanding of mechanisms of vasomotor dysfunction in the female sex in hypertension and type 2 diabetes mellitus. The specific purposes of the thesis are numbered below, the methods used to address each purpose are described, and reference to the corresponding experimental chapters is included. Each specific purpose is accompanied by several explicit hypotheses and by a statement of the anticipated value and contributions of the main findings to the literature.

1. To characterize potential sex differences in vasomotor responses of ageing hypertensive rats. For this specific purpose, vasomotor responses mediated by NO

and by COX-derived prostanoids were assessed in aortic segments isolated from male and female WKY and SHR at 16 wk and 30 wk of age. Endothelium-dependent, ACh-mediated relaxations and contractions are presented in **Chapter 2** and **Chapter 3**, respectively. It was hypothesized that:

i. endothelium-dependent vasorelaxation would be blunted in 30wk female SHR vs. 16wk;

ii. a similar rate of deterioration in endothelial function would occur in male and female SHR between 16 and 30 wk old (i.e. 16wk values will be blunted less in female than in male SHR and the final extent of decline will be less in 30wk female vs. male SHR); and

iii. endothelium-dependent contraction of quiescent vessels would be enhanced in 30 wk old female SHR compared to 16 wk old female SHR, and to a similar level as 30 wk old male SHR.

These experiments will provide the first description of the influence of ageing on vasomotor function of female SHR, and will be the first sex comparison in SHR older than 19 wk. These findings could direct research towards defining sex-specific treatments and issues/considerations concerning endothelial health in human hypertension.

2. To provide understanding of mechanisms governing vasomotor responses of ageing hypertensive female rats. For this specific purpose, the potential roles for COX-1, COX-2, and TP receptor in mediating development of endothelial dysfunction in

aorta of female SHR between 16 wk and 30 wk of age. **Chapter 2** first determines a role for the COX-TP receptor pathway in the impaired endothelium-dependent relaxation. **Chapter 3** examines in further detail the involvement of COX-1 and -2 in both the endothelium-dependent relaxation and contraction responses of ageing female SHR. **Chapter 3** also assesses TP receptor sensitivity to exogenous PGH_2. It was hypothesized that:

i. the reduced vasorelaxation of 30wk female SHR would be COX- and TP receptor-mediated, and would be accompanied by increased aortic expression of COX and aortic release of PGI_2;
ii. robust endothelium-dependent relaxation would be restored and endothelium-dependent contraction would be abolished in 30 wk old female SHR with preferential inhibition of COX-1 or antagonism of the TP receptor;
iii. preferential COX-2 inhibition would impart only modest improvements in endothelium-dependent vasomotor responses of 30 wk old female SHR; and
iv. enhanced PGH_2-mediated contraction would be exhibited by 30 wk old female SHR compared to WKY.

These data will shed light on potential mechanisms underlying the vasomotor responses noted in ageing female SHR in experiments described in Specific Purpose #1. This information could help refine current strategies for treatment of hypertension in ageing female patients.

3. To assess the ability of chronic dietary DHA (22:6 n-3) supplementation to improve the endothelial vasomotor dysfunction established in a male rat model of essential hypertension. For this specific purpose, the endothelium-dependent and -independent vasomotor responses of aorta isolated from adult male SHR were examined after 8–12 wk of consuming a diet supplemented with 0.5 % wt/wt DHA. These experiments are described in **Chapter 4**. It was hypothesized that:
    i. DHA would abrogate the endothelium-dependent re-contraction to higher ACh doses (i.e. endothelium-dependent vasomotor dysfunction) that occurs in adult male SHR and that involves the COX-TP receptor axis (see Chapters 2 and 3), and
    ii. DHA would not reduce hypertension in adult male SHR.

    These data will provide the first characterization of endothelium-dependent responses of adult SHR with established vasomotor impairments and hypertension to dietary n-3 PUFA supplementation. The information gained may help determine the importance of exploring the therapeutic potential of n-3 PUFAs, and specifically DHA, on endothelial health in hypertension.

4. To characterize vasomotor responses of female rats exhibiting type 2 diabetes-like symptoms. For this specific purpose, α_1 adrenergic vasocontraction and endothelium-dependent vasorelaxation were examined in aorta isolated from female type 2 diabetic ZDF rats and Zucker lean counterparts. These experiments are described in **Chapter 5**. It was hypothesized that:

i. elevated α_1 adrenergic vasocontraction would be observed in type 2 diabetic ZDF compared to Zucker lean;

ii. reduced endothelium-dependent vasorelaxation would be observed in type 2 diabetic ZDF compared to Zucker lean; and

iii. the presence of the SOD mimetic, Tempol, would improve endothelium-dependent vasorelaxation of type 2 diabetic ZDF towards the level of Zucker lean.

These observations will be a novel characterization of macrovascular endothelial function in *female* type 2 diabetic animals and will therefore shape our nascent understanding of the state of their vasomotor function.

5. To examine the influence of physical activity and anti-diabetic pharmacotherapy, alone and in combination, on vasomotor responses of a female rat model of type 2 diabetes. For this specific purpose, the α_1 adrenergic contraction and endothelium-dependent relaxation responses of aorta isolated from type 2 diabetic female ZDF were assessed following 8 wk of aerobic exercise training and anti-diabetic metformin therapy, alone and in combination. These experiments are described in **Chapter 5**. It was hypothesized that:

   i. none of the therapeutic interventions would alter the enhanced α_1 adrenergic vasocontraction observed in type 2 diabetic ZDF;

   ii. all three therapeutic interventions would restore endothelium-dependent vasorelaxation of type 2 diabetic ZDF towards the level of Zucker lean; and

iii. the presence of Tempol would not further improve endothelium-dependent vasorelaxation in type 2 diabetic ZDF that had received one of the therapeutic interventions.

These data will explore potential synergistic benefits of adding physical activity to ongoing pharmacotherapy strategies on macrovascular endothelial function of female type 2 diabetic patients.

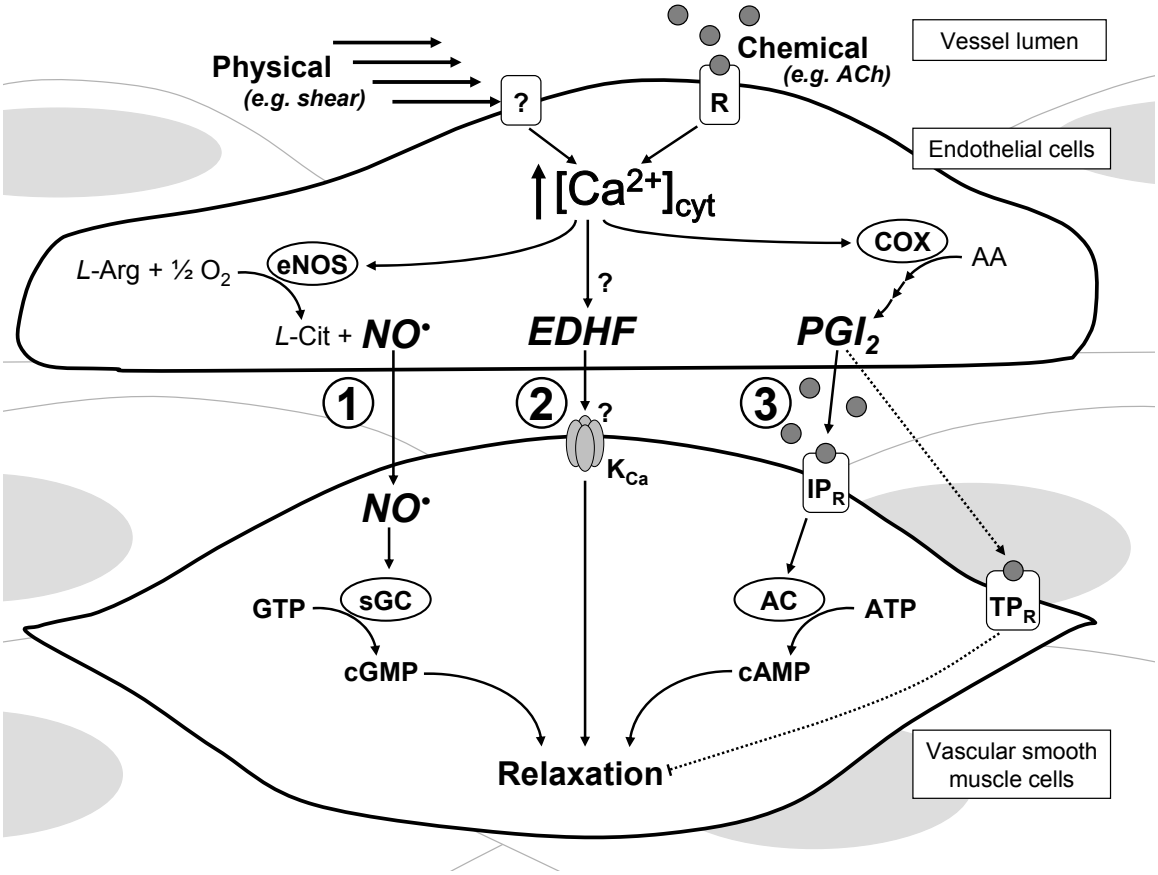

Figure 1-1 The three major endothelium-dependent vasorelaxation pathways in healthy arterial walls. See *Endothelium-dependent, NO-mediated vasorelaxation as a marker of overall endothelial function* sub-section for details. Solid arrows, activation. Hatched arrow, less pronounced activation. Hatched line with perpendicular terminal, inhibition. ?, unknown mechanism. R, receptor. $[Ca^{2+}]_{cyt}$, cytosolic calcium concentration. K_{Ca}, calcium-activated potassium channel. IP_R, IP receptor. TP_R, TP receptor. AC, adenylate cyclase.

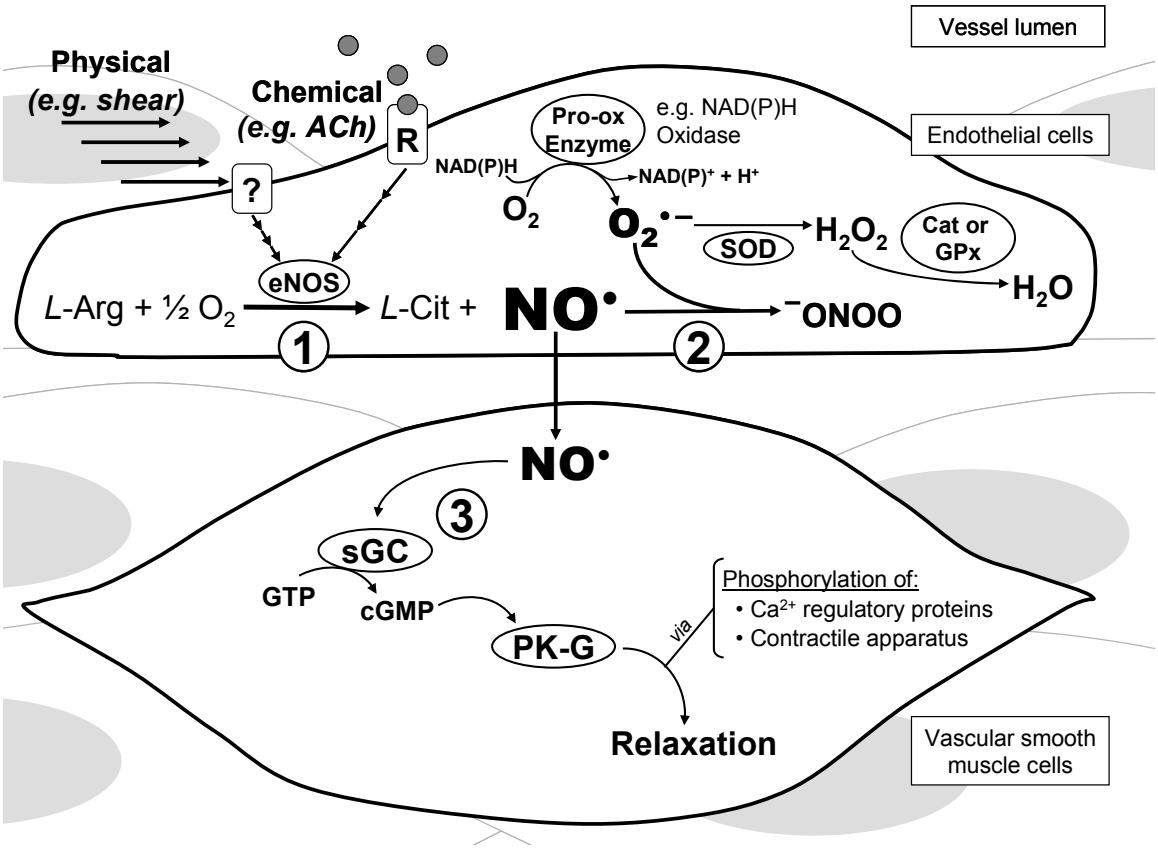

Figure 1-2 The three main determinants of NO bioavailability. See *Determinants of NO bioavailability* sub-section for details. Solid arrows, activation. $O_2^{\bullet -}$, superoxide anion. $^-$ONOO, peroxynitrite. H_2O_2, hydrogen peroxide. Cat, catalase. GPx, glutathione peroxidase.

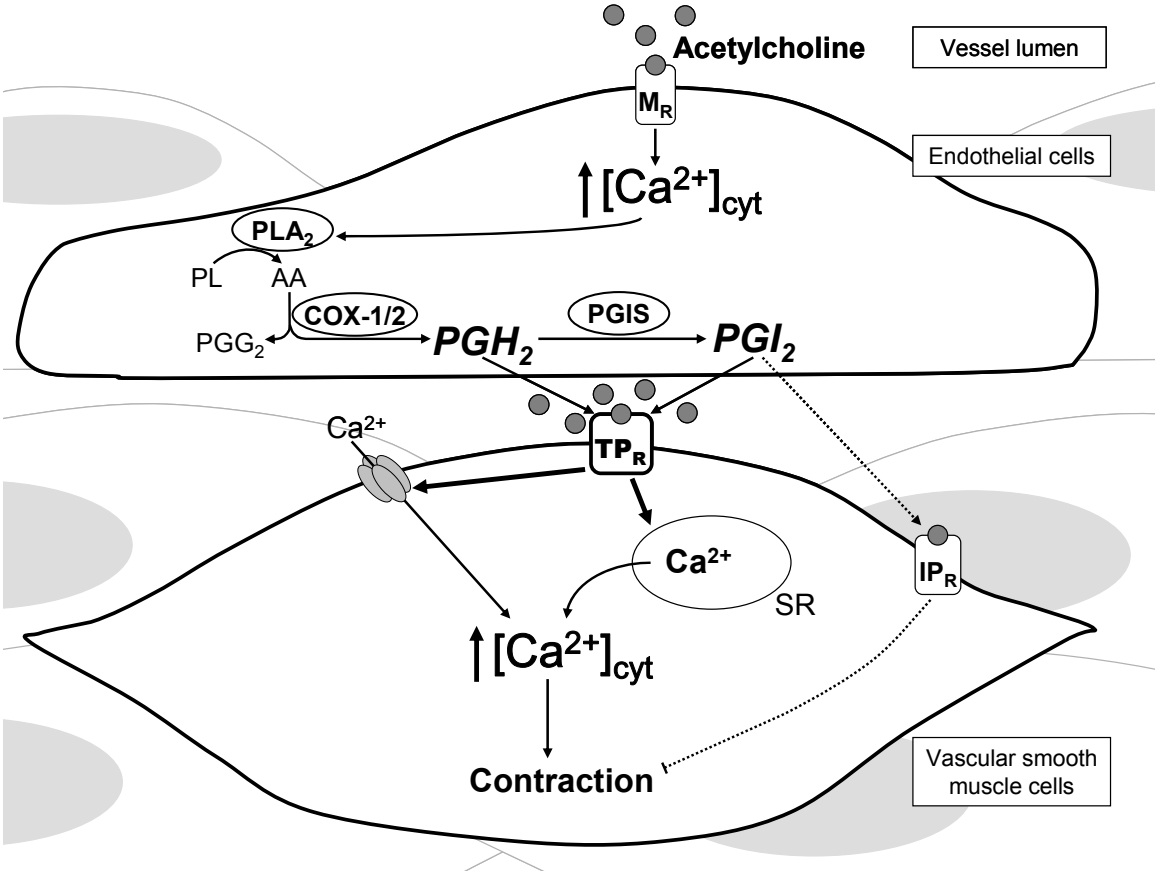

Figure 1-3 The COX-mediated production of EDCF. See *Prostanoid-mediated vasoconstriction* sub-section for details. Solid arrows, activation. Hatched arrow, less pronounced activation. Hatched line with perpendicular terminal, inhibition. M_R, muscarinic receptor. PLA_2, phospholipase A_2. PL, phospholipid. PGIS, PGI_2 synthase. SR, sarcoplasmic reticulum.

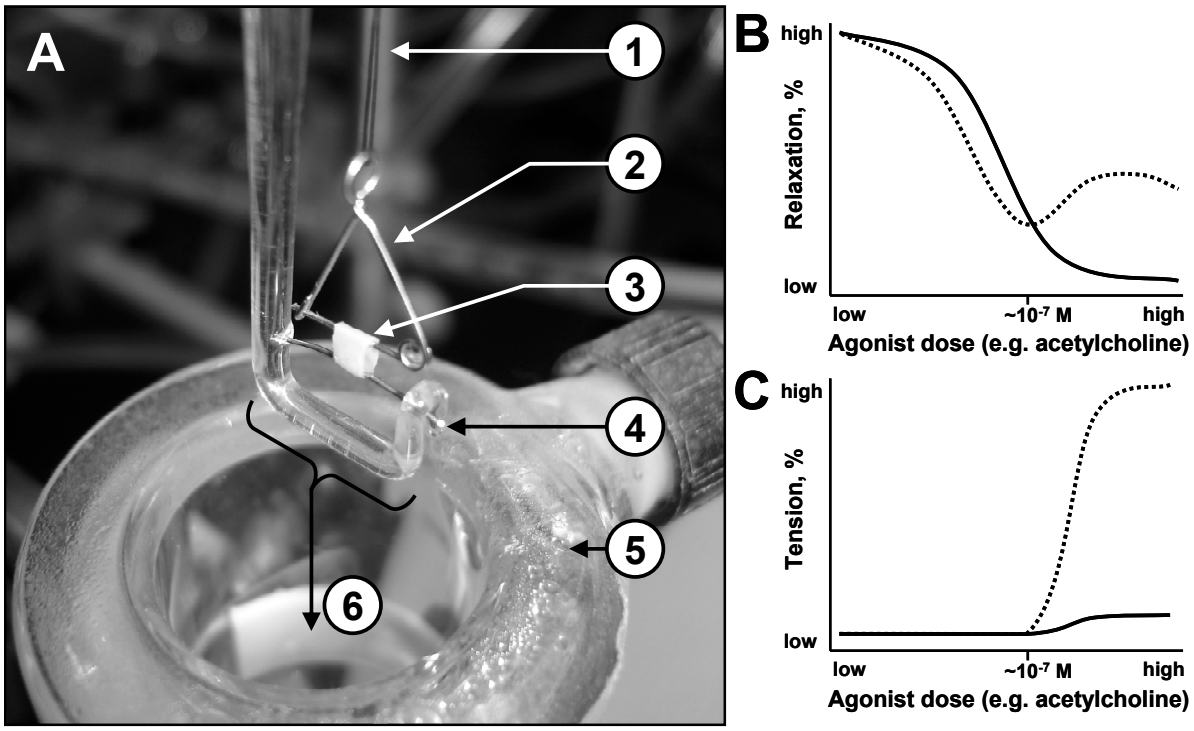

Figure 1-4 The vascular myography apparatus and schematics of endothelium-dependent dose-responses. A close-up photograph of the apparatus (panel A) showing an isolated ring segment (#3) suspended between an upper triangular tissue support (#2) and a lower fixed wire (#4). A length of suture (#1) connects the triangular tissue support to an isometric force transducer (not shown). Throughout the protocol, a pump-perfused water-jacketed tissue bath (#5) maintains the physiologic conditions (37 ^{o}C, pH 7.4, continuously aerated with 95:5 % O_2:CO_2) of the buffer in which the ring segment is submersed (#6). Schematic representations of unimpaired (solid line) and impaired (dotted line, e.g. as occurs in hypertension and with ageing) endothelium-dependent (e.g. acetylcholine-stimulated) relaxation (panel B) and contraction (panel C) dose-responses.

Chapter 2

Cyclooxygenase and thromboxane/prostaglandin receptor contribute to reduced aortic endothelium-dependent relaxation in ageing female spontaneously hypertensive rats

2.1 Synopsis

Cyclooxygenase (COX)-derived vasoconstrictory prostanoids contribute to impaired endothelium-dependent vasorelaxation in aging male (m) spontaneously hypertensive rats (SHR), however vasomotor responses in aging female (f) SHR and sex differences in aging SHR are unknown. Examining mechanisms governing dysfunction in aging fSHR will contribute to understanding sex-dependent vascular complications in advanced hypertension. Aortic endothelium-dependent relaxation dose-responses (ACh) of 16wk- and 30wk-old mSHR and fSHR and normotensive Wistar-Kyoto (WKY) rats were examined in the absence (no drug control, ND) and presence of COX inhibition (indomethacin, Indo) and thromboxane/PG (TP) receptor inhibition (SQ29548). ND-treated 16wk mSHR exhibited considerable blunting of the peak relaxation response to ACh (e.g. 77±4 % relaxation to 10^{-5} mol/l) versus WKY controls (89±6 %) and greater dysfunction occurred in 30wk mSHR (63±2 %). Interestingly, ACh relaxations of fSHR were unimpaired at 16wk (101±2 % to 10^{-5} mol/l) but blunted in 30wk (76±4 %). Indo and SQ29548 restored robust ACh

vasorelaxation in all groups (e.g. 113±3 % and 112±3 %, respectively, in Indo- and SQ29548-treated 30wk fSHR). Aortic COX-1 protein expression was elevated by 75 % in 30wk vs. 16wk fSHR, while group-averaged ACh-stimulated aortic PGI_2 release (assessed as 6-keto-$PGF_{1\alpha}$) was 30 % greater in 30wk vs. 16wk fSHR (9926±890 vs. 7621±690 pg/ml/mg dry wt, respectively) although this did not reach significance (p=0.0758). Dramatic deterioration of endothelium-dependent vasomotor function in fSHR across this age range involves COX- and TP receptor, supporting a mechanism of impairment similar to that which occurs in aging mSHR.

Key Words: endothelium-derived contracting factor, prostanoid, nitric oxide, sex difference, endothelium-dependent relaxation, PGI_2, TP receptor, SQ29548

2.2 Introduction

Hypertension (59; 63; 68; 70; 76; 77; 81; 85; 114; 137; 139; 141; 144) and aging (59; 63; 74; 75; 77; 139; 141) are associated with diminished endothelium-dependent vasomotor function in humans and laboratory animals, and these reductions appear to be abated in females (69; 70; 139). Blunted endothelium-dependent vasorelaxation (59; 63; 68; 70; 74-77; 85; 144) and elevated endothelium-derived contracting factor (EDCF)-mediated contraction (49; 70; 77; 85; 119) occur in aorta of young (i.e. <19 wk old) male spontaneously hypertensive rats (SHR) compared to young normotensive Wistar-Kyoto rats

(WKY), and to female SHR. Similar dysfunction also occurs in aorta of aging male SHR and WKY compared to their young counterparts. Cyclooxygenase (COX)-derived vasoconstrictory prostanoids are EDCF that appear to be central to this aortic vasomotor dysfunction: inhibition of COX (59; 68; 70; 74; 75; 85; 119; 144) or of thromboxane/PG (TP) receptors (63; 68; 77; 128; 144) corrects the impaired endothelium-dependent vasomotor response in young SHR (vs. WKY), in young male SHR (vs. females), and in aging male SHR and WKY (vs. young). COX-generated PGH_2 and PGI_2 (prostacyclin) have been proposed as two primary candidate EDCFs that contribute to endothelial impairments of male SHR, and to a greater extent with aging (6; 43; 48; 52; 63; 106; 119). Specifically with aging in male SHR, the predominant functional result of PGI_2 appears to shift from PGI_2 receptor (IP receptor)-mediated vasorelaxation to TP receptor-mediated vasocontraction (43; 52; 119). Interestingly, another common EDCF, thromboxane A_2 (TxA_2), does not appear to contribute significantly to vasomotor impairments in aorta of SHR (6; 43; 52; 68; 75; 85; 128). Furthermore, expression and activity of several components of the COX-EDCF pathway are altered in aorta of male SHR (vs. WKY) and in aging males of both strains (vs. young) including: elevated COX (48; 52; 106; 154), elevated PGI_2 synthase (52; 63; 106; 119), and reduced IP receptor (106).

It is unknown whether aging-related alterations in vasorelaxation occur in female SHR and whether a role exists for COX-derived EDCF in this potential phenomenon. Furthermore, there are no reports comparing the effects of advancing age (i.e. >19 wk old) on vasomotor function between male and female SHR. Characterization of the pathways controlling endothelial vasomotor function in aging female SHR could elucidate sex-specific

aging effects in hypertensive vascular dysfunction that may be important from a basic science perspective and potentially from a clinical perspective regarding vascular mechanisms determining the efficacy of specific prevention and treatment modalities before the onset of frank disease.

The present study therefore examined endothelium-dependent vasomotor responses mediated by nitric oxide (NO) and COX-derived prostanoids, and the release of PGI_2 and TxA_2 (assessed as the respective stable metabolites 6-keto-$PGF_{1\alpha}$ and of TxB_2), in aorta segments isolated from male and female WKY and SHR at 16 wk and 30 wk of age. The rationale for using animals at these two ages was that by 16 wk old SHR had reached a stable elevation in blood pressure (BP) compared to WKY for several weeks. By 30 wk of age, animals would have been exposed to this elevated BP for an additional 14 wk without yet having the confounding influences of secondary conditions related to diseases common in older hypertensive animals (e.g. heart failure) or of cessation of reproductive cycling, which occurs between 10 and 12 months of age in female SHR (46; 120). These were important considerations to facilitate distinguishing the effects of aging and hypertension in the two sexes within the age range studied. Moreover, we had observed a consistent pattern in preliminary data from unrelated experiments in which, unlike young male SHR, 16wk female SHR exhibited unimpaired endothelial vasomotor responses that were similar to WKY, while 30wk female SHR had reductions in function. This provoked our interest in systematically studying age-dependent effects in this age range. It was hypothesized that: 1) endothelium-dependent vasorelaxation would be blunted in 30wk vs. 16wk female SHR; 2) a similar rate of deterioration in endothelial function would occur in male and female SHR between 16 and

30 wk old (i.e. 16wk values will be blunted less in female than in male SHR and the final extent of decline will be less in 30wk female vs. male SHR); and 3) the reduced vasorelaxation of 30wk female SHR would be COX- and TP receptor-mediated, and would be accompanied by increased aortic expression of COX and aortic release of PGI_2.

2.3 Materials and Methods

Animals. Male (m) and female (f) WKY and SHR (Harlan, Madison, WI) were raised to 16wk or 30wk of age. Eight experimental groups resulted: 16wk mWKY (n=20), 16wk mSHR (n=20), 16wk fWKY (n=8), 16wk fSHR (n=8), 30wk mWKY (n=15), 30wk mSHR (n=16), 30wk fWKY (n=16), and 30wk fSHR (n=29). Endpoint age ranges were 16.2–17.7 wk (for 16wk) and 29.7–30.6 wk (for 30wk). All rats were group-housed in sex-specific cages (4–5/cage) in the same room, which was temperature- and humidity-controlled (12 h:12 h light:dark cycle), and were fed standard chow (Teklad 22/5 Rodent Diet, Harlan) and tap water *ad libitum*. The University of Waterloo Animal Care Committee approved all animal procedures. All chemicals and drugs were purchased from Sigma (St. Louis, MO) or BioShop (Burlington, ON).

Blood pressure measurements. Body mass (BM) was recorded and anesthesia was induced by sodium pentobarbital (65 mg/kg BM, i.p.). Left common carotid artery was exposed and cannulated with a Mikro-Tip™ Pressure Transducer catheter (Millar

Instruments, Houston, TX), and a stable intra-arterial BP tracing was recorded for several minutes using a PowerLab data acquisition unit (ADInstruments, Colorado Springs, CO). Mean arterial BP (MAP) was determined from the raw BP tracing using the Cyclic Measurements function in the Chart 5.5 software (ADInstruments). To maintain body temperature during anesthesia and BP measurement, rats were placed on a pump-perfused, water-jacketed heating pad (Gaymar, Orchard Park, NY) set to 37.5 ^{o}C.

Tissue harvesting. Anesthetized animals were euthanized by cardiac excision and exsanguination. Descending thoracic aorta was placed in cooled Krebs-bicarbonate buffer (4 ^{o}C, pH 7.4: 131.5 mmol/l NaCl, 5.0 mmol/l KCl, 2.5 mmol/l $CaCl_2$-$2H_2O$, 1.2 mmol/l NaH_2PO_4-H_2O, 1.2 mmol/l $MgCl_2$, 11.2 mmol/l D-glucose, 13.5 mmol/l $NaHCO_3$, 2.5 μmol/l EDTA-$2H_2O$) and adhering connective tissue was gently removed. Rings (~2 mm) were cut for vasomotor function experiments and the remainder was gently blotted dry and stored at – 80 ^{o}C for Western blotting. Atria and aortic stump were removed from the heart and discarded. Outer right ventricular wall (RV) was separated from outer left ventricular wall plus septum (LV) and their masses recorded. LV-to-BM ratio (LV/BM) was calculated by dividing the organ mass (in mg) by BM (in g). Adhering fat was removed from left kidney (K) and its mass was recorded.

Vasomotor function experiments. Thoracic aorta was utilized in this study for several reasons: 1) it is a common vascular tissue studied in literature examining effects of aging, sex, and hypertension on vasomotor function and thus examining the novel aspect of aging in

female SHR using this artery allowed direct comparison of the current study with many previous reports (48; 49; 52; 59; 63; 68-70; 74-77; 85; 106; 119; 128; 144; 154); 2) impaired endothelium-dependent vasomotor responses of large conduit arteries are associated with conduit-specific diseases including atherosclerosis and stiffening (20); 3) the greater amount of tissue available per animal from the thoracic aorta allows for replicates of multiple drug treatments for vasomotor function experiments and other analyses; and 4) the use of large arteries avoids some technical difficulties of small vessel preparations.

Aortic rings were suspended on an isolated tissue myograph system (Radnoti Glass Technology, Monrovia, CA), immersed in continuously aerated (95 %:5 % O_2:CO_2) and warmed Krebs-bicarbonate buffer (37 ^{o}C, pH 7.4), and isometric tension development was recorded using a PowerLab data acquisition unit. Resting tension was gradually increased (0.5 g every 5 min) to the group-specific level of optimal final resting tension (Table 2-2), as determined in pilot testing by repeated exposure of rings to 60 mmol/l potassium chloride (KCl, depolarizing agent) at increasing levels of resting tension until KCl contractile response reached a plateau.

Rings were equilibrated at final resting tension (30 min). Following the equilibration, final resting tension was recorded (Table 2-2) and rings were then incubated (30 min) in one of four drug conditions: no drug control (ND), indomethacin (Indo, inhibitor of COX-1 and -2, $5x10^{-6}$ mol/l), N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME, inhibitor of NO synthase (NOS), 10^{-4} mol/l), or co-incubation with Indo and L-NAME (I+L-N). Rings were contracted twice with KCl (60 mmol/l), and each exposure was separated by two washes (5 min) and return to baseline tension. Stable contraction to phenylephrine (PE, α_1-

adrenoceptor agonist, 10^{-7} mol/l) was achieved and the endothelium-dependent response to cumulative doses of ACh (muscarinic agonist, 10^{-10}–10^{-4} mol/l) was performed, followed by three washes. Rings were contracted again (10^{-7} mol/l PE) and the endothelium-independent vasorelaxation to cumulative doses of sodium nitroprusside (SNP, NO donor, 10^{-12}–10^{-4} mol/l) was assessed.

Rings from a subset of 16wk and 30wk male and female SHR were prepared as described above and used for experiments examining the endothelium-dependent responses to ACh in the absence (ND) or presence of TP receptor inhibition with [1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenyl amino)carbonyl]hydrazine]methyl]-7-oxabycyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ29548, 10^{-6} mol/l, Cayman Chemical, Ann Arbor, MI).

Vasomotor function data were collected from duplicate rings for ND, L-NAME, and SQ29548, and from singlet rings for Indo and I+L-N drug conditions. All drugs were dissolved in distilled water except Indo and SQ29548, which were dissolved in DMSO and 70 % ethanol, respectively. Final in-bath concentrations of DMSO and ethanol were 0.05 % and 0.07 % vol/vol, respectively. Pilot studies in our laboratory and others (48; 63; 68) indicated that these concentrations of DMSO or ethanol alone had no detectable effects on vasomotor responses to a variety of vasoactive agents as used in this study compared to the absence of DMSO or ethanol. Absolute vasomotor response (tension in g) to a given dose of agonist was recorded from the steady-state plateau response to that concentration. This absolute value was then expressed as a percentage of the prior contraction to PE (in g) on a ring-by-ring basis, and used for group mean and SEM calculations. EC_{50} and maximum

relaxation values were obtained by non-linear regression curve fitting using Prism 4.03 software (GraphPad, San Diego, CA).

Western blotting. Frozen sections of thoracic aorta were pulverized under liquid nitrogen with a mortar and pestle and homogenized on ice in ~200 µl of ice-cold lysis buffer (pH 7.4: 20.0 mmol/l HEPES, 10.0 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, 1.0 mmol/l DTT, 20.0 % vol/vol glycerol, 0.1 % vol/vol TRITON® X-100) using a ground glass homogenizer. Homogenates were supplemented with Complete protease inhibitor cocktail (40 µl/ml, Roche Diagnostics, Mannheim, Germany) prior to centrifugation (2,000 *g*, 10 min, 4 ^{o}C) and recovery of supernatant. Total protein content of the supernatant was determined in triplicate by bicinchoninic acid assay as described previously (131). Samples were diluted to a total protein concentration of 1.0 mg/ml using 25 % of total volume sample buffer (1.46 mol/l sucrose, 7.5 % wt/vol SDS, 62.5 mmol/l Tris-HCl, 2.0 mmol/l EDTA-$2H_2O$, 200.0 mmol/l DTT, 0.01 % vol/vol Bromophenol Blue), appropriate volume of concentrated sample, and the remaining volume made up with lysis buffer. Dilute samples and Rainbow^{TM} (GE Healthcare/Amersham, Little Chalfont, UK) and biotinylated (Cell Signaling Technology, Beverly, MA) protein ladders were warmed (5 min, 95 ^{o}C) prior to SDS-PAGE of 30 µg of sample protein and semi-dry transfer to a poly-vinylidene-difluoride membrane (Roche Diagnostics). Membranes were blocked in 5 % wt/vol BSA dissolved in Tris-buffered saline with Tween®-20 (TBS-T; pH 7.5, room temperature: 20.0 mmol/l Tris base, 137.0 mmol/l NaCl, 0.1 % vol/vol Tween®-20), transferred to primary antibody (rabbit anti-COX-1: 1:200, Cayman Chemical; rabbit anti-COX-2: 1:200, Cayman Chemical; mouse anti-eNOS: 1:500,

BD Biosciences, Franklin Lakes, NJ), rinsed (3 x 5 min) in TBS-T, and transferred to secondary antibody (goat anti-rabbit: 1:5000 (COX-1) and 1:2000 (COX-2), Santa Cruz Biotechnology, Santa Cruz, CA; goat anti-mouse: 1:2000, Santa Cruz Biotechnology), and rinsed (3 x 5 min) in TBS-T. Enhanced chemiluminescence (GE Healthcare/Amersham) and gel documentation (Syngene, Cambridge, UK) were used to detect protein signal. Inter-gel chemiluminescent Western blot signals were standardized to a common thoracic aortic homogenate run in all gels and values were then expressed as fold changes relative to 16wk mWKY. α-actin was originally used as an internal control to ensure equal protein loading and transfer, but we observed a systematic treatment group effect on α-actin expression. Thus, as an alternative we performed ponceau red staining of the membrane and used integration of the densitometry signal from a consistent five band pattern as a means to rule out lane-to-lane differences in total protein loading and transfer.

Prostanoid release. Prostanoid release was measured in buffer following cumulative ACh exposure, as previously cited in male SHR (68). Isolated aortic rings from a subset (n=4 per group) of 16 and 30wk mSHR and fSHR were suspended on a myography system as described and exposed to cumulative ACh doses (10^{-10}–10^{-4} mol/l) in the presence of L-NAME (10^{-4} mol/l) and without pre-contraction. Krebs-bicarbonate buffer (5 ml) was collected and snap frozen in liquid nitrogen after a steady-state tension level was achieved following the last ACh dose. Rings were collected, blotted dry, and snap frozen in liquid nitrogen. Buffer and rings were stored at –80 ^{o}C until analysis. Levels of TxB_2 (stable metabolite of TxA_2) and 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) were determined by EIA

kits according to the manufacturer's instructions (Cayman Chemical). Buffer samples for measurement of TxB_2 were concentrated 6.37-fold using Clean-Up® C18 sorbent-type extraction columns (UCT, Bristol, PA) before performing the assay. Rings were freeze-dried under vacuum pressure for 3 h at –30 ^{o}C followed by 23 h at room temperature. Ring dry weights were then measured on an ultrasensitive balance and used for normalization of prostanoid release to dry tissue mass on a ring-by-ring basis.

Statistics. Values reported are mean±SEM. Data were analysed using ANOVA and least squares means *post-hoc* was conducted where an interactive term was significant. $p<0.05$ was considered significant. SAS 9.1.3 software (SAS Institute, Cary, NC) was used to perform all statistical analyses.

2.4 Results

Physical characteristics and mean arterial pressure. BM was elevated in males vs. females, and in 30wk vs. 16wk with the exception of fSHR (Table 2-1). LV/BM was elevated in: SHR vs. WKY, females vs. males, and 30wk vs. 16wk SHR. MAP was higher in SHR vs. WKY. 16wk fWKY had higher MAP vs. 16wk mWKY, whereas the opposite was true within 30wk WKY. A lower MAP was observed in 30wk females vs. 16wk counterparts. Supplementary BP, heart rate, and organ mass measurements are presented in the Online Data Supplement (Table A-1).

Contractile vasomotor responses. Exposure of ND rings to 60 mmol/l KCl resulted in similar contractions across all 16wk groups (Table 2-2). KCl-induced contractions were elevated in 30wk vs. 16wk males of both strains, and to a greater extent in SHR. Contractile responses to 10^{-7} mol/l PE were higher in males vs. female counterparts, except within 16wk SHR where the contractions were similar. PE contractions were also greater in 30wk vs. 16wk males. KCl and PE contractile responses in the presence of Indo and SQ29548 are presented in the Online Data Supplement (Table A-2).

Endothelium-dependent vasomotor responses. High ACh concentrations caused modest re-contraction (i.e. reversal of ACh-mediated relaxation) in both 16wk and 30wk mWKY that was slightly accentuated in 30wk (Figure 2-1A). Both 16wk and 30wk mSHR exhibited considerable re-contraction to high ACh doses, and this response was exaggerated in 30wk (Figure 2-1B). Interestingly, mSHR exhibited both greater relaxation to some low ACh doses ($10^{-8.5}$–$10^{-7.5}$ mol/l) and greater re-contraction to high ACh doses (peak re-contraction occurred at $10^{-5.0}$ mol/l ACh in all groups) vs. age-matched mWKY.

ACh-mediated relaxations to low doses were slightly greater in 16wk vs. 30wk fWKY and these two groups had similar responses to high doses of ACh with no apparent re-contraction (Figure 2-1C). ACh responses of 16wk and 30wk fWKY (Figure 2-1C) were greater than those of age-matched mWKY counterparts (Figure 2-1A) at all ACh concentrations that elicited a detectable response. 16wk fSHR exhibited robust relaxation (Figure 2-1D) that was greater than 16wk fWKY to low ACh doses ($10^{-8.5}$–10^{-7} mol/l) and

similar to this group at high ACh doses (Figure 2-1C). As well, the ACh relaxation response of 16wk fSHR (Figure 2-1D) at all ACh doses was greater than that of 16wk mSHR (Figure 2-1B). In contrast 30wk fSHR exhibited: a severely impaired ACh response compared to 16wk fSHR (Figure 2-1D); a blunted relaxation to high ACh compared to the 30wk fWKY group (Figure 2-1C); and only slightly greater relaxation to high ACh doses compared to 30wk mSHR (Figure 2-1B).

Inhibition of COX with Indo eliminated the re-contraction to high concentrations of ACh observed in 16wk mSHR, 30wk mSHR, and 30wk fSHR, but enhanced the relaxation to low ACh only in the 30 wk fSHR (Figures 2-2A, B, and D). In the presence of Indo, the robust relaxation exhibited by 30wk fSHR (Figure 2-2D) was similar to that seen in 16wk fSHR (Figure 2-2C) and greater than that of 30wk mSHR (Figure 2-2B). NOS inhibition with L-NAME completely abolished ACh-induced relaxation in all groups regardless of the presence of Indo (data omitted from figures for clarity).

TP receptor inhibition with SQ29548 also abolished the re-contractions induced by high doses of ACh in 16wk mSHR, 30wk mSHR, and 30wk fSHR (Figures 2-3A, B, and D). SQ29548-treated rings from 16wk mSHR (Figure 2-3A) and 30wk fSHR (Figure 2-3D) both had slightly greater maximal relaxation to ACh compared to 30wk mSHR (Figure 2-3B). SQ29548 did not affect relaxation at low ACh except for a small enhancement in 30wk mSHR.

Endothelium-independent vasorelaxation. Similar maximal relaxation to SNP was observed in curve-fit dose-responses of all groups (average 109±1 %, Figure 2-4). Likewise,

EC_{50} values averaged 1.14±0.12 nmol/l across all groups, and there were no age or hypertension-related group differences. The only significant group difference in EC_{50} values was between 16wk fSHR (0.54±0.21 nmol/l) and 16wk mSHR (1.50±0.38 nmol/l, p=0.0512). Dose-by-dose comparisons revealed some small group differences at certain low doses of SNP.

Protein expression. Relative aortic expression of COX-1 protein was lower (–33 %) in 16wk fSHR vs. 16wk mSHR and was elevated in 30wk fSHR to levels higher (+75 %) than in 16wk fSHR and similar to those in 30wk mSHR (Figure 2-5A). In fWKY, COX-1 protein expression was also higher (+24 %) in 30wk vs. 16wk groups. Relative aortic COX-2 protein expression was higher in 16wk mSHR (+54 %) and 30wk mSHR (+85 %) vs. WKY counterparts (Figure 2-5B). In 30wk animals, COX-2 protein levels were higher (+41 %) in mSHR vs. f SHR, and in fWKY vs. mWKY (+44 %). Relative aortic eNOS protein levels were similar across all groups (n=4–6, data omitted from figure for clarity).

Prostanoid release. ACh-stimulated release of PGI_2 (assessed as 6-keto-$PGF_{1\alpha}$) was similar in 16wk mSHR and fSHR (Figure 2-6). 6-keto-$PGF_{1\alpha}$ was elevated in 30wk mSHR vs. 16wk mSHR and 30wk fSHR. Within fSHR, the ~30 % higher group mean for the 30wk vs. 16wk group did not reach significance (p=0.0758). However, 6-keto-$PGF_{1\alpha}$ values were positively correlated to the degree of peak endothelial dysfunction, assessed by the contractile response to 10^{-5} mol/l ACh measured during the prostanoid release experiments,

in individual data pairs across groups (r^2=0.4288, p=0.0081). ACh-induced TxB_2 release was much lower than that of 6-keto-$PGF_{1\alpha}$ and was similar in all groups (Figure 2-6).

2.5 Discussion

The present study examined the endothelium-dependent aortic vasomotor responses of male and female WKY and SHR at 16 and 30 wk of age and mechanisms contributing to these responses. The major novel findings of this study are that: 1) female SHR exhibited reductions in endothelium-dependent vasomotor responses between 16 and 30 wk of age; 2) the decline of endothelium-dependent vasomotor function in SHR across the age range studied occurred at a greater rate in females than in males; and 3) the COX-TP receptor axis contributed to the vasomotor impairments of 30wk female SHR based on functional responses using pharmacological blockers and assessments of COX-1 expression and PGI_2 release.

A robust vasorelaxation to ACh occurred in arteries from 16wk female animals and was similar between 16wk fSHR and 16wk fWKY at high ACh doses. By 30wk of age, however, female SHR had a greatly blunted ACh response compared to both 16wk and WKY counterparts, maintaining only a slightly greater response to high ACh doses compared to 30wk mSHR, which exhibited deteriorating vasomotor function compared to the younger males in the present study and others (63; 74; 75). These results demonstrate a decline in function in arteries of female SHR between 16wk and 30wk of age, confirming our first

hypothesis. The data further suggest that the rate of impairment of endothelial vasomotor function was accelerated in fSHR compared with mSHR between the ages of 16wk and 30wk, in contrast to our second hypothesis. This finding is a simple function of the facts that: 1) at 16 wk old the response was already blunted in males but not females; and 2) that by 30 wk old the male and female responses were reduced to similar levels.

The differences between impairments of endothelial vasomotor function of male and female *SHR* as they age from 16 wk to 30 wk of age observed in the present study are contrasted by very modest functional reductions across this age range in *WKY* that are similar in males and females. Previous studies have also reported a greater decline of endothelium-dependent vasomotor function in aging male SHR compared to WKY (63; 77; 127) which has been presented as a premature aging ("endothelial senescence") in SHR (77; 127; 147). To our knowledge, though, the current study is the first report of: sex differences in vasomotor function in SHR older than 19 wk; and alterations in vasomotor function of female SHR across any age range.

The decline of endothelium-dependent vasomotor function as fSHR age from 16 wk to 30 wk old in the present study was abolished by blockade of the COX-TP receptor pathway in vitro at two levels: COX inhibition with Indo, and TP receptor inhibition with SQ29548. Additionally, elevation of COX-1 expression occurred in aorta of 30wk vs. 16wk fSHR and the aging-related increases in 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) release from aorta and endothelial dysfunction were significantly positively correlated. Collectively these data confirm the first two parts of our third hypothesis and support a COX- and TP

receptor-mediated decline in endothelium-dependent vasomotor function as fSHR age from 16 wk to 30 wk old.

A role for COX-1-derived EDCF, most likely in the form of PGH_2 and/or PGI_2, has been established in the aortic endothelial vasomotor dysfunction of aging *male* SHR (6; 43; 48; 52; 63; 106; 119). Inhibition of COX in the present study and others (59; 74; 75; 119) and of the TP receptor in the present study and others (63; 77) enhanced endothelium-dependent relaxation and reduced endothelium-dependent contraction in aging male SHR. The elevated 6-keto-$PGF_{1\alpha}$ release in the present 30wk vs. 16wk mSHR corroborates previous observations (106) and adds to the growing support for a role for PGI_2 in the reduced endothelial function in aging male SHR (6; 43; 48; 52; 63; 106; 119). The low levels of TxB_2 (stable metabolite of TxA_2) that were released equally across male and female SHR of both ages, on the other hand, suggest a limited role for this prostanoid in the present sex and aging effects on vasomotor dysfunction observations and align with previous conclusions in male SHR (6; 43; 52; 68; 75; 85; 128). Overall, the present results suggest that a common COX-TP receptor pathway largely contributes to the aging-related impairment of aortic endothelium-dependent vasorelaxation in both female and male SHR across the age range studied. While PGI_2 appears to play a prominent role in the aging effect in both male and female SHR, it is possible that the proportional contribution of COX-derived EDCF, such as PGI_2 and PGH_2, to aging-related endothelial impairments differs between the sexes. This issue was not specifically studied in the current experiments, but the difference in the degree to which PGI_2 release is affected by age in male vs. female SHR is consistent with this idea. Additionally, it is unclear at present what stimuli ultimately

determine the sex difference in COX- and TP receptor-mediated vasomotor responses in 16 wk old SHR, and how this changes with aging over the 16-to-30 wk period.

Although there is good general agreement between the data presented in the current study and previous work in this area, there has been one previous report (157), which disagrees with the finding of impaired ACh responses in 16 wk old male vs. female SHR reported in the current study and others (70). Additionally, one previous study (77) disagrees with the with the finding in the present study and others that inhibition of the TP receptor (63; 68) or of COX (68; 74; 75; 85) restored a robust ACh response in male SHR between the ages of 30 wk and 80 wk old. Possible reasons for the sex and aging discrepancies between these previous studies (77; 157) and the present data are not readily apparent from the methods described in the papers in question. It is noteworthy, however, that the endothelium-dependent relaxation responses in these two dissenting studies (77; 157) did not exhibit the characteristic re-contraction to high ACh doses that has been commonly observed: in SHR in the present study and others (63; 68; 70; 74-76; 85; 144); with aging in SHR in the present study and others (63; 74; 75); and to a greater extent in male compared to female SHR in the present study and others (70).

Limitations. The unexpected finding of reductions in MAP recorded in the 30wk fWKY and fSHR compared to 16wk counterparts in this study is inconsistent with previous observations that BP did not decrease between 4 and 8 months of age in female SHR (46), and indeed with other preliminary data from our own laboratory. Endpoint carotid arterial BP measurements, recorded over a relatively small time period under anesthetized conditions, were obtained for the purpose of simply documenting that SHR were

hypertensive compared to WKY counterparts, which was confirmed. We did not design our BP assessment approach to take ambulatory BP measurements or to compare the BP changes with vascular function changes among groups. This is an experimental design limitation of this study, as it is possible that the age-related BP reductions in female animals reported herein could be particularly compromised by limitations in the procedures employed (e.g. timing of collection, anesthesia, etc).

PE-mediated pre-contractions were not similar across treatment groups. This is explicitly illustrated in Table 2-2. In keeping with convention, we expressed dose-response data to dilatory agonists as a percentage of the preceding PE-mediated contraction. This is the most suitable manner in which to express the relaxation data from a common pre-contractile stimulus level (dose of PE) as it normalizes the response for differences that might result from potentially different mass of the rings across treatment groups (69; 157), which we did not directly assess in the current study. Group differences in optimum final resting tension and in tension development to 60 mmol/l KCl followed a similar pattern to that observed in the pre-contraction response to PE, suggesting a common, rather than a specific, mechanism (e.g. altered aortic smooth muscle content).

A central role has been attributed to sex hormones in governing the sex differences in vasomotor function in young (i.e. 16–17 wk old) SHR (35; 70; 148). Precise characterization of the mechanism(s) through which the COX-TP receptor pathway is differentially controlled in male vs. female SHR and changes with aging in SHR would contribute to the field. These experiments, however, would require very specific manipulation of sex hormones (e.g. gonadectomy with and without sex hormone replacement) and are beyond the scope of the

present study which was designed to functionally characterize sex- and aging-dependent effects and to establish whether the COX-TP receptor axis was involved specifically in the female aging response in hypertension. The vasomotor function changes observed with aging between 16 and 30 wk old in female SHR observed in this study, however, likely did not depend on changes in estradiol per se, as it has been previously reported that SHR do not stop cycling until 10–12 months of age (46; 120). This does not discount the possibility that some aspect of changes in sex hormone production or sensitivity may contribute, as might be revealed in studies designed to specifically elucidate this involvement.

Conclusions. This is the first report characterizing endothelium-dependent vasomotor responses of aging female SHR across any age range, sex differences between male and female SHR of any age greater than 19 wk old, and mechanisms involved in the decrements of function in the latter. Deterioration of endothelium-dependent aortic relaxation occurred in female SHR between the ages of 16 wk old, when relaxations were normal and robust, and 30 wk old, when the blunted responses of females were nearly as impaired as those of age-matched male SHR. Across this age range, female SHR exhibit a greater rate of impairment than male SHR. In the presence of the COX inhibitor, indomethacin, or the TP receptor inhibitor, SQ29548, the endothelium-dependent vasomotor response of 30 wk old female SHR was restored to the level of 16 wk counterparts. Increased aortic protein levels of COX-1 in 30 vs. 16 wk-old female SHR does not prove, but is consistent with the suggestion that this isoform could be involved in coordinating the aging-related blunting of endothelium-dependent relaxation observed in these animals. While the potential role of TxA_2 as an EDCF in the age-related endothelial dysfunction in fSHR appears to be limited, PGI_2 likely

does contribute to this dysfunction, although differential roles for PGI_2 and PGH_2 between male and female aging effects could exist. Furthermore, it must be noted that differences in sensitivity of the vascular smooth muscle to prostanoid products from the endothelium could contribute to age-, sex-, and hypertension-related functional differences. This issue has not been addressed in the current study. The results of this study, implicating the COX-EDCF-TP receptor axis as a contributing factor to the vasomotor impairment that develops as female SHR age from 16 to 30 wk old, parallel previously published observations in aging *male* SHR (48; 52; 59; 63; 74; 75; 77; 106; 119), and our own observations of male SHR across the age range reported herein. Therefore, the endothelium-dependent vasomotor dysfunction that develops in female SHR between 16 and 30 wk of age appears, at least to a significant extent, to be COX (likely COX-1)-dependent and to be mediated through the TP receptor, thus sharing commonalities with the mechanisms implicated in the vasomotor dysfunction occurring in aging male SHR.

2.6 Acknowledgements

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Table 2-1 Physical characteristics and MAP.

		mWKY	mSHR	fWKY	fSHR
BM, g	16wk	310±4	340±3[a]	187±3[a]	187±2[b]
	30wk	349±5[a]	364±4[be]	214±1[ce]	198±4[fg]
LV/BM, mg/g	16wk	2.25±0.02	2.56±0.02[a]	2.50±0.04[a]	2.73±0.05[bc]
	30wk	2.20±0.03	2.67±0.04[be]	2.55±0.07[e]	2.96±0.03[dfg]
MAP, mm Hg	16wk	92±3	176±10[a]	109±4[a]	173±2[c]
	30wk	90±3	178±9[e]	73±5[ce]	163±4[dg]

Values are mean±SEM. n=8–29 (BM and LV/BM). n=6–8 (MAP). $p<0.05$ vs.: [a] 16wk mWKY, [b] 16wk mSHR, [c] 16wk fWKY, [d] 16wk fSHR, [e] 30wk mWKY, [f] 30wk mSHR, [g] 30wk fWKY.

Table 2-2 Final resting tension and contractile responses to PE and KCl stimuli in the ND condition.

	mWKY	mSHR	fWKY	fSHR
		Final resting tension, g		
16wk	6.96±0.01	6.95±0.01	5.95±0.01	6.45±0.01
30wk	7.93±0.01	7.91±0.01	5.47±0.01	6.44±0.01
		KCl contraction (ND condition), g		
16wk	1.45±0.05	1.58±0.12	1.45±0.07	1.61±0.09
30wk	1.74±0.06[a]	1.92±0.07[be]	1.61±0.06	1.76±0.06
		PE contraction (ND condition), g		
16wk	1.77±0.06	1.68±0.06	1.33±0.10[a]	1.54±0.13
30wk	2.04±0.05[a]	2.01±0.06[b]	1.58±0.10[e]	1.74±0.07[f]

Values are mean±SEM. n=8–16 in duplicate rings. Final resting tension, tension recording prior to 30 min drug incubation. KCl contraction, contractile response to 60 mmol/l KCl. PE contraction, contractile response to 10^{-7} mol/l PE prior to ACh relaxation dose-response. $p<0.05$ vs.: [a] 16wk mWKY, [b] 16wk mSHR, [e] 30wk mWKY, [f] 30wk mSHR.

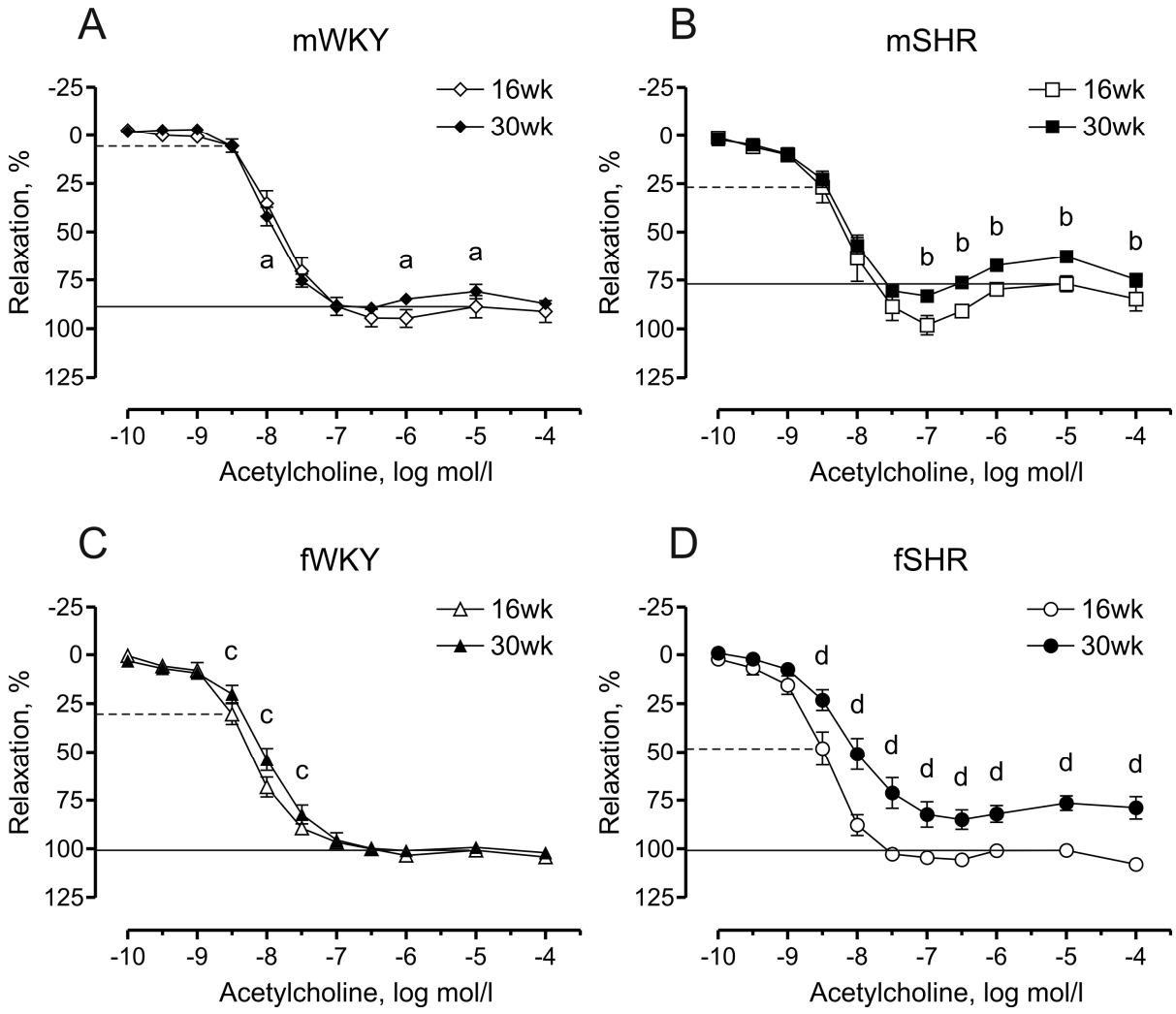

Figure 2-1 Endothelium-dependent relaxation to ACh in the ND condition of thoracic aortic rings isolated from 16wk and 30wk male and female WKY and SHR. For inter-panel comparison, horizontal lines have been drawn corresponding to the percent relaxation observed in 16wk to $10^{-8.5}$ mol/l (dashed line) and 10^{-5} mol/l (solid line) ACh. Values are mean±SEM, expressed as a percentage of PE (10^{-7} mol/l) contraction. n=8–16 in duplicate rings. L-NAME drug condition has been omitted for clarity. $p<0.05$ vs.: [a] 16wk mWKY, [b] 16wk mSHR, [c] 16wk fWKY, [d] 16wk fSHR.

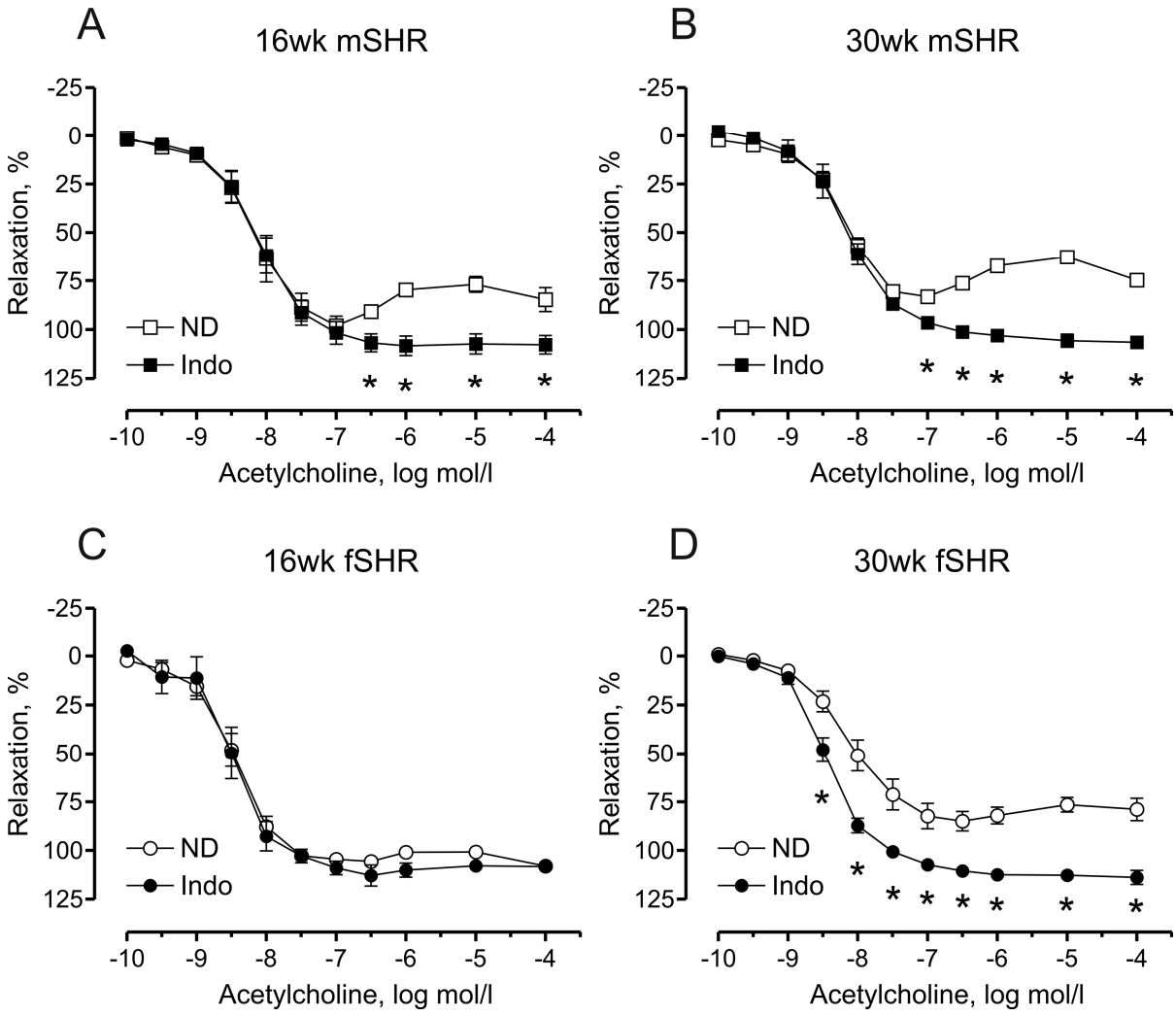

Figure 2-2 Endothelium-dependent relaxation to ACh in the absence (ND, transposed from Figure 2-1 for reference) or presence (Indo) of COX inhibition of thoracic aortic rings isolated from 16wk and 30wk male and female SHR. Values are mean±SEM, expressed as a percentage of PE (10^{-7} mol/l) contraction. n=8–16 in duplicate rings (ND). n=4–8 in singlet to duplicate rings (Indo). I+L-N drug condition has been omitted for clarity. $p<0.05$ vs.: * ND.

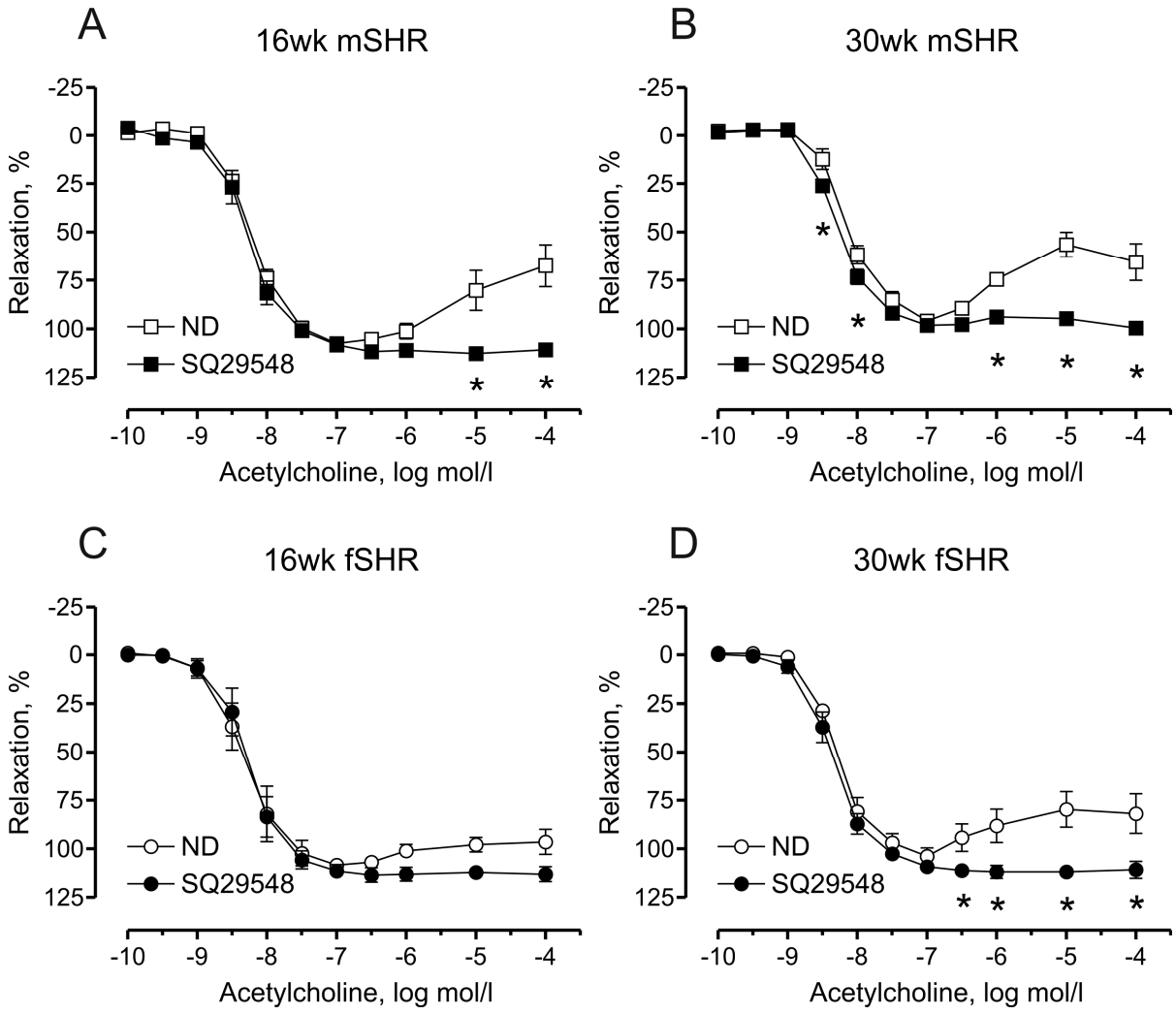

Figure 2-3 Endothelium-dependent relaxation to ACh in the absence (ND) or presence (SQ29548) of TP receptor inhibition of thoracic aortic rings isolated from a subset of 16wk and 30wk male and female SHR. Values are mean±SEM, expressed as a percentage of PE (10^{-7} mol/l) contraction. n=4 in duplicate rings. $p<0.05$ vs.: * ND.

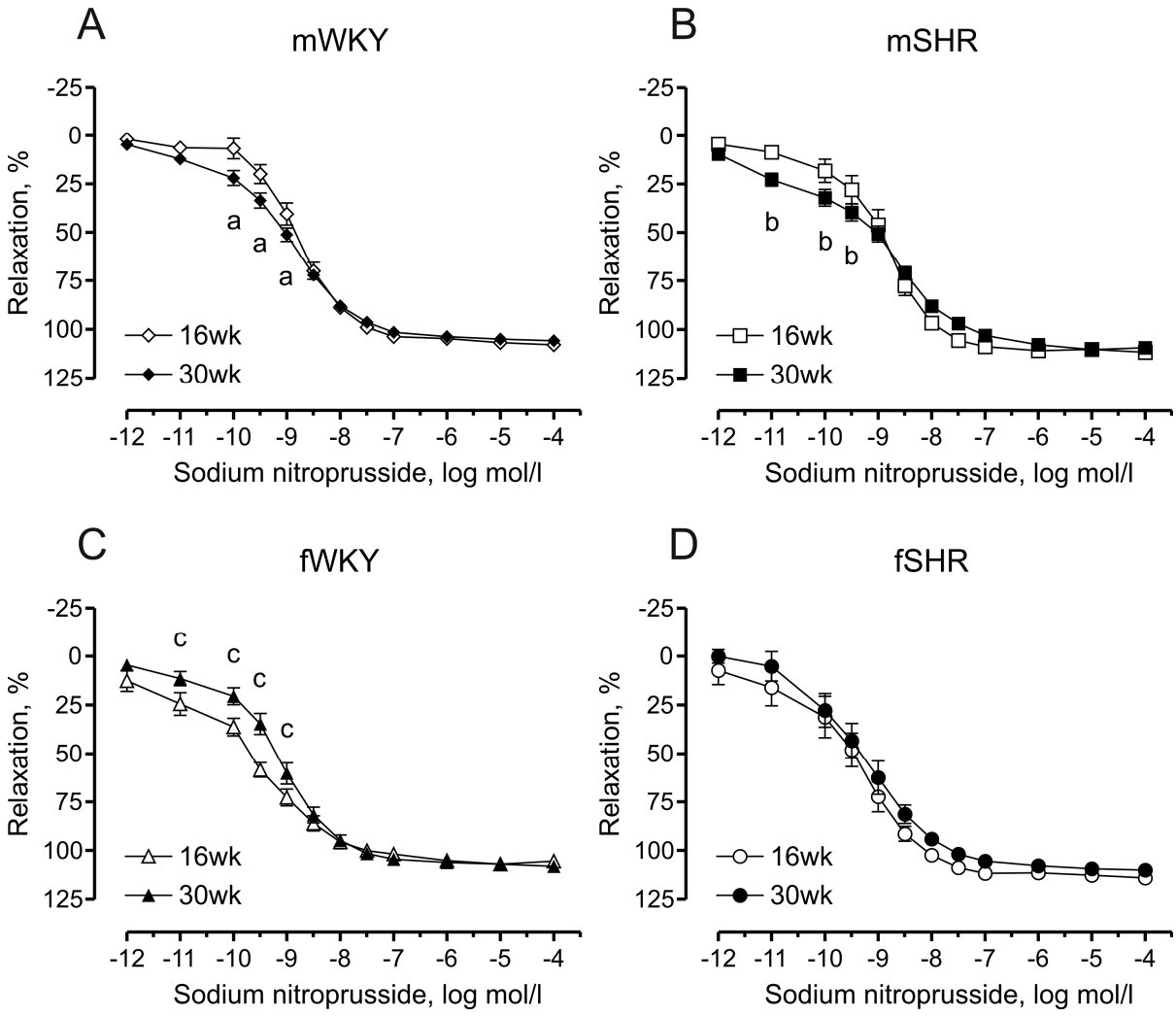

Figure 2-4 Endothelium-independent relaxation to SNP in the ND condition of thoracic aortic rings isolated from 16wk and 30wk male and female WKY and SHR. Values are mean±SEM, expressed as a percentage of PE (10^{-7} mol/l) contraction. n=8–15 in duplicate rings. L-NAME, Indo, and I+L-N drug conditions have been omitted for clarity. $p<0.05$ vs.: [a] 16wk mWKY, [b] 16wk mSHR, [c] 16wk fWKY.

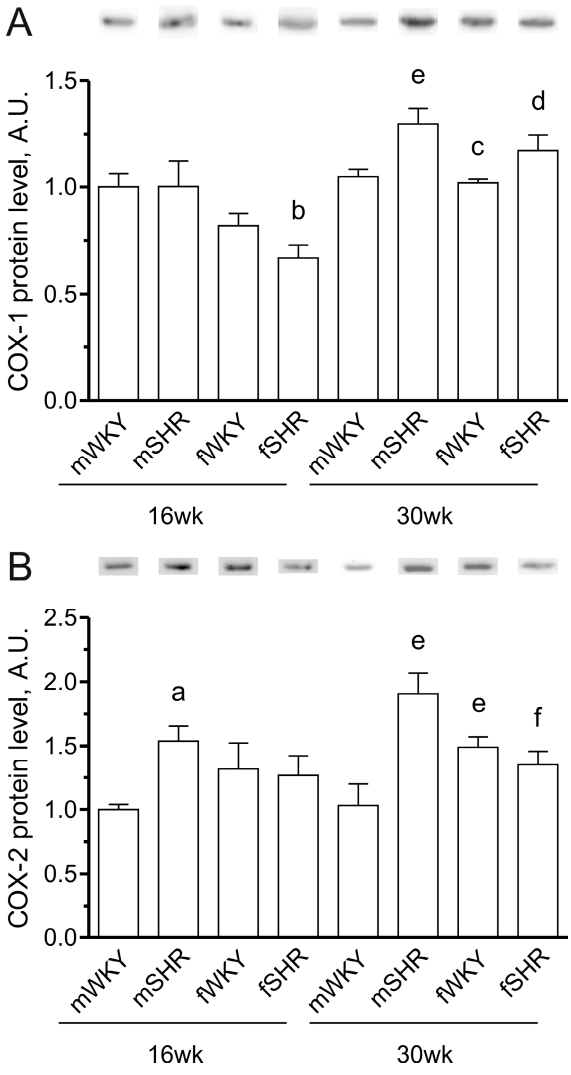

Figure 2-5 Relative protein levels of COX-1 (panel A) and COX-2 (panel B) as measured by Western blot in thoracic aortic homogenates from 16wk and 30wk male and female WKY and SHR. Upper panels show representative signals. Values are mean±SEM, expressed as a fold difference from the 16wk mWKY group. n=4–6 in singlet to triplicate. $p<0.05$ vs.: [a] 16wk mWKY, [b] 16wk mSHR, [c] 16wk fWKY, [d] 16wk fSHR, [e] 30wk mWKY, [f] 30wk mSHR.

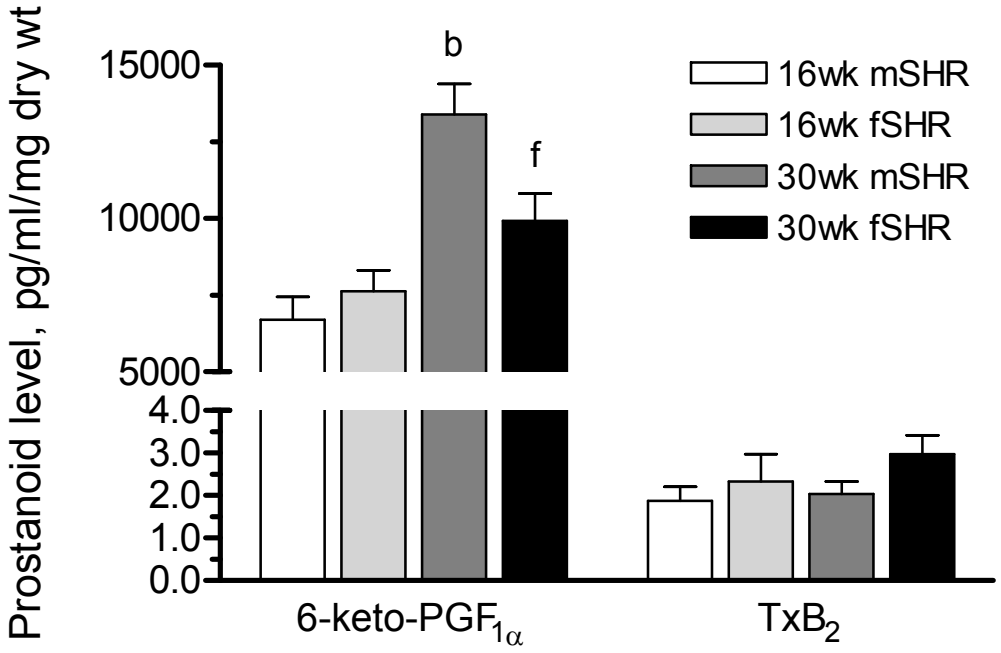

Figure 2-6 ACh-induced release of 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2) and TxB_2 (stable metabolite of TxA_2) from aortic rings isolated from 16wk and 30wk male and female SHR in the presence of L-NAME (10^{-4} mol/l). Values are mean±SEM of data normalized to tissue dry weight on a ring-by-ring basis. n=4 in duplicate. $p<0.05$ vs.: [b] 16wk mSHR, [f] 30wk mSHR.

Chapter 3

Mechanisms of impaired aortic endothelial vasomotor function in ageing female and male SHR are similar and involve both cyclooxygenase-1 and -2

3.1 Synopsis

Endothelium-dependent contraction and relaxation responses to acetylcholine (ACh) and prostaglandin (PG) H_2-mediated contraction were assessed in aortic segments isolated from 16- (16wk) and 30-wk-old (30wk) male (m) and female (f) normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR). Within fSHR, contraction to 10^{-5} M ACh (quiescent ring preparation) was moderate and similar in 30wk (59±7 %) and 16wk (50±4 %), whereas relaxation to 10^{-5} M ACh (phenylephrine (PE)-contracted ring preparation) was robust in 16wk (102±3 %) and blunted in 30wk (84±5 %). 16wk and 30wk mSHR had elevated ACh contraction (76±7 and 91±3 %, respectively) and reduced ACh relaxation (82±8 and 58±5 %, respectively) compared to fSHR counterparts, and these impairments within mSHR were amplified in 30wk. Endothelium-dependent function of all SHR to higher ACh doses (>10^{-7} M) was restored with preferential inhibition of cyclooxygenase (COX)-1 (valeroyl salicylate, VAS) or thromboxane A_2/prostaglandin (TP) receptor (SQ 29548), but only partially improved with COX-2 inhibition (NS-398). Interestingly, at lower ACh doses (<10^{-7} M) NS-398 improved relaxation of all SHR and

VAS blunted relaxation of all SHR except for 30wk mSHR. PGH_2 contraction was not influenced by sex and was reduced with ageing within SHR (range: 105±5 to 124±7 %, 10^{-6} M). Therefore, higher doses of ACh initiate endothelial vasomotor impairments of female SHR across the age range studied primarily by COX-1- and TP receptor-dependent mechanisms, while COX-1 and -2 appear to enhance and blunt relaxation to lower ACh doses, respectively. Similar mechanisms govern endothelial dysfunction in age-matched male SHR. Also, increased TP receptor responsiveness to PGH_2 does not influence the sex- or ageing-related dysfunction observed in SHR.

Key words: nitric oxide, acetylcholine, prostanoid, TP receptor, hypertension, sex

3.2 Introduction

COX-derived prostanoid-mediated aortic vasomotor dysfunction has been well characterized in male hypertensive SHR as compared to male normotensive WKY rat counterparts and with ageing in male SHR. Elevated endothelium-dependent contraction (49; 63; 119; 154) and greater re-contraction of endothelium-dependent relaxation (59; 63; 68; 70; 76; 85; 127; 144) occur in aorta of male SHR vs. male WKY and this dysfunction is restored with preferential inhibition of COX-1 or TP receptor (48; 52; 59; 62; 63; 68; 70; 85; 144; 154). Ageing further increases endothelium-dependent contraction (63; 119) and exacerbates re-contraction of endothelium-dependent relaxation (63; 74; 75; 127) in male SHR aorta in a COX- and TP receptor-dependent manner (63; 74; 75; 119). Additionally,

elevated aortic protein expression of COX-1 (48; 106) and PGI_2 synthase (106), as well as greater release of vasoconstrictory prostanoids (e.g. PGH_2 and PGI_2) (6; 48; 52; 63; 106; 119), occur in male SHR vs. male WKY and with ageing in male SHR. Therefore, the major mechanism contributing to the impaired vasomotor function in male SHR vs. WKY and in ageing male SHR is stimulation of TP receptors by PGH_2 and/or PGI_2 derived from COX (most likely COX-1).

Few studies, however, have examined the contribution of the COX-TP receptor pathway to vasomotor responses in female SHR (vs. male) or with ageing in female SHR (vs. young). One study (70) reported that the elevated endothelium-dependent contraction and the blunted endothelium-dependent relaxation (i.e. greater re-contraction) observed in male vs. female SHR were both COX-mediated, but the findings were limited to 16 wk-old animals. Experiments in Chapter 2 extended these observations by demonstrating that female SHR experienced a loss of endothelium-dependent relaxation between the ages of 16 wk old, when the response was robust compared to the impaired response of age-matched male SHR, and 30 wk old, when the response was blunted nearly to the level of 30 wk-old male SHR. Data from Chapter 2 further showed that inhibition of either COX or TP receptors fully restored endothelium-dependent relaxation in 30 wk old female SHR, suggesting that the impairment occurred through a COX-TP receptor mechanism, similar to that which has been well characterized in ageing male SHR. However, a more detailed understanding of the mechanisms of endothelial impairment in hypertensive females during ageing is essential for the development of efficacious treatment for this population.

The present study examines the potential roles for COX-1, COX-2, and TP receptor in mediating development of endothelial dysfunction in female SHR across an age range where endothelium-dependent relaxation of female SHR deteriorates from robust and unimpaired (at 16 wk old) to blunted (at 30 wk old) in a COX- and TP receptor-mediated manner. Endothelium-dependent contraction (quiescent ring preparation), endothelium-dependent relaxation (PE-contracted ring preparation), and exogenous PGH_2 contraction dose-responses were performed in aortic segments isolated from male and female WKY and SHR at 16 wk and 30 wk of age. It was hypothesized that: 1) endothelium-dependent contraction of quiescent vessels would be enhanced in 30 wk old female SHR compared to 16 wk old female SHR, and to a similar level as 30 wk old male SHR; 2) endothelium-dependent contraction would be abolished and robust endothelium-dependent relaxation would be restored in 30 wk old female SHR with preferential inhibition of COX-1 or antagonism of the TP receptor; 3) preferential COX-2 inhibition would impart only modest improvements in endothelium-dependent vasomotor responses of 30 wk old female SHR; and 4) enhanced PGH_2-mediated contraction would be exhibited by 30 wk old female SHR compared to WKY.

3.3 Materials and Methods

Animals. Male (m) and female (f) WKY and SHR were obtained from Harlan (Madison, WI) and raised to either 16 wk (16wk) or 30 wk (30wk) of age. Eight experimental groups resulted: 16wk mWKY (n=13), 16wk mSHR (n=19), 16wk fWKY

(n=8), 16wk fSHR (n=16), 30wk mWKY (n=9), 30wk mSHR (n=17), 30wk fWKY (n=8), and 30wk fSHR (n=15). Endpoint age ranges were 16.1–17.6 wk (16wk) and 29.1–30.6 wk (30wk). All animals were group-housed in sex-specific cages (4–5/cage) in the same room, under controlled temperature and humidity (reverse 12 h:12 h light:dark cycle) and with free access to standard rat chow (Teklad 22/5, Harlan) and tap water. All animal protocols were approved by the University of Waterloo Animal Care Committee. All chemicals were obtained from Sigma (St. Louis, MO) or BioShop (Burlington, ON) unless otherwise stated.

Endpoint arterial BP. Body mass (BM) was recorded and the rats were anesthetized with sodium pentobarbital (0.65 mg/kg BM, i.p.). Carotid intra-arterial blood pressure (BP) was recorded in anesthetized rats as described in Chapter 2. Mean arterial pressure (MAP) was calculated from the raw BP tracing using the Cyclic Measurements function in Chart software (v. 5.5.6, ADInstruments, Colorado Springs, CO).

Tissue harvesting. Anesthetized rats were euthanized by cardiac excision and exsanguination. Descending thoracic aorta was harvested and immediately placed in cold Krebs-bicarbonate buffer (4 ^{o}C, pH 7.4, in mM: 131.5 NaCl, 13.5 $NaHCO_3$, 11.2 D-Glucose, 5.0 KCl, 2.5 $CaCl_2$-$2H_2O$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 0.025 EDTA). Thoracic aorta was gently dissected and 2-mm rings were cut (6–10 rings/rat) as described in Chapter 2. Remaining thoracic aortic tissue was frozen in liquid nitrogen and stored at –80 ^{o}C. The left kidney and cardiac ventricles were harvested and their masses recorded as described in

Chapter 2. Left ventricle (LV)-to-BM ratio (LV/BM) was calculated as LV mass (in mg) divided by BM (in g).

Vasomotor function. Rings were loaded into isolated tissue myography baths containing warm Krebs-bicarbonate buffer (37 ^{o}C, pH 7.4) and resting tension was gradually raised to group-specific optimal levels determined in pilot testing for Chapter 2. After 15 min equilibration at optimal resting tension, rings were exposed twice to 60 mM KCl (depolarizing agent). Buffer was replaced at 5-min intervals after each exposure and resting tension returned to baseline. Steady-state tension development to the second exposure to 60 mM KCl was used as the reference contraction for normalization of contraction dose-responses described below. Rings were assigned to one of the following dose-response curves: 1) endothelium-dependent relaxation to ACh (10^{-10}–10^{-4} M, muscarinic agonist) from a prior sub-maximal steady-state contraction to PE (10^{-7} M, α_1 adrenergic agonist); 2) endothelium-dependent contraction to ACh (10^{-10}–10^{-4} M) with no prior contraction to PE (i.e. quiescent ring preparation); or 3) contraction to PGH_2 (10^{-9}–10^{-6} M, TP receptor agonist, Cayman Chemical, Ann Arbor, MI). SHR rings used for ACh relaxation or contraction dose-responses were incubated (30 min) immediately prior to the curve with one of four drug conditions: No Drug control (ND); VAS ($3x10^{-3}$ M, preferential COX-1 inhibitor, Cayman Chemical); N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398, 10^{-6} M, preferential COX-2 inhibitor, Cayman Chemical); or [1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenyl amino)carbonyl]hydrazine]methyl]-7-oxabycyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ 29548, 10^{-6} M, TP receptor antagonist, Cayman Chemical). ACh relaxation and contraction

responses of WKY rings were examined in the ND condition only. All four drug conditions for ACh contraction dose-responses were supplemented (30 min) with Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 10^{-4} M, nitric oxide (NO) synthase (NOS) inhibitor). All rings used for PGH_2 contraction dose-responses were incubated (30 min) immediately prior to the curve with L-NAME (10^{-4} M) and indomethacin (Indo, $5x10^{-6}$ M, COX-1 and -2 co-inhibitor).

Vasomotor responses were performed in duplicate rings. Doses are reported as final in-bath concentrations. For relaxation data, steady-state loss of tension (in g) to each ACh dose was expressed as a percentage of the preceding contraction to 10^{-7} M PE on a ring-by-ring basis. For contraction data (to ACh and PGH_2), steady-state tension development (in g) to each dose of agonist was expressed as a percentage of the second reference contraction to 60 mM KCl on a ring-by-ring basis. Mean values calculated from duplicate rings within a given rat and drug condition were used for statistical analyses and for generating group mean and SEM data. ACh, PE, and L-NAME were dissolved in distilled water. PGH_2 (supplied in acetone) was divided into single-use aliquots and stored under nitrogen gas at –80 ^{o}C. Immediately prior to use, one aliquot was placed on ice, the acetone was evaporated under a steady stream of nitrogen gas and PGH_2 was immediately reconstituted in ice-cold, nitrogen gas-purged ethanol (maximum final in-bath ethanol concentration: 0.49 % vol/vol). VAS, NS-398, and SQ 29548 were dissolved in ethanol (final in-bath ethanol concentration: 0.07 % vol/vol). Indo was dissolved in DMSO (final in-bath DMSO concentration: 0.05 % vol/vol). Pilot testing in our laboratory indicated that these concentrations of ethanol and DMSO alone did not detectably alter vasomotor responses to the agents used in this study.

Statistics. Data are presented as mean±SEM. Statistical analyses were performed by 1-way or repeated-measures ANOVA, with least squares means post-hoc test where appropriate, using SAS software (v. 9.1, SAS Institute, Cary, NC).

3.4 Results

Physical characteristics and MAP. BM was greater in males vs. females, in 30wk vs. 16wk, and in mSHR vs. mWKY (Table 3-1). Additionally, 30wk fWKY had a greater BM vs. 30wk fSHR. LV/BM was greater in SHR vs. WKY and in fSHR vs. mSHR. MAP was greater in SHR vs. WKY, in mSHR vs. fSHR, and in 30wk fWKY vs. 16wk. Additional BP, heart rate, and physical data are presented in Appendix B (Table B-1).

Contraction to KCl and PE. Contractile response to the second (reference) exposure to 60 mM KCl was greater in males vs. females, except within 16wk WKY in which the response was similar (Table 3-2). KCl also elicited greater contraction in 30wk vs. 16wk, except within fWKY in which the response was similar. A similar response was observed in WKY vs. SHR, except for an elevated response in 30wk fSHR vs. WKY. Contraction to 10^{-7} M PE in the ND condition was greater in male vs. female counterparts, except within 16wk SHR in which the response was similar. Compared to 16wk counterparts, PE contraction was greater in 30wk mSHR and lower in 30wk fWKY. PE response was greater in WKY vs.

SHR (with the exception of 30wk males which exhibited a similar contraction) and within 30wk females (where contraction of SHR were greater than WKY). Response to PE in the presence of VAS, NS-398, and SQ29548 are presented in Appendix B (Table B-2).

Endothelium-dependent vasocontraction of quiescent rings. 16wk mWKY rats #5–8 exhibited detectable endothelium-dependent vasocontraction to ACh doses of 10^{-9} M and greater, whereas contraction was only detectable at doses of 10^{-7}–$10^{-6.5}$ M and greater in 16wk mWKY #1–4 (Figure B-1, Appendix B) and in all other animals in the present study (Figure 3-1). Experiments using 16wk mWKY #5–8 were performed between the hours of 9:30 p.m. and 4:00 a.m. (i.e. during the 'lights on', asleep time for the rats), whereas all other experiments in the study were performed between 8:30 a.m. and 9:00 p.m. (i.e. during the 'lights off', awake time for the rats). It is unclear whether timing of experiments influenced ACh contraction responses of 16wk mWKY #5–8, however, due to the systematic effect observed, ACh contraction data from these rats were excluded from the study.

ACh contraction in the ND condition was greater in 16wk mWKY vs. 30wk (Figure 3-1A) and reduced in 16wk mSHR vs. 30wk (Figure 3-1B). Greater ACh contraction was observed in 30wk and 16wk mSHR (Figure 3-1B) vs. WKY (Figure 3-1A) to all ACh doses that elicited a detectable response, and this elevation was exacerbated within 30wk mSHR (vs. 16wk). fWKY exhibited very little contraction regardless of age, however the response was slightly but significantly greater in 30wk vs. 16wk (Figure 3-1C). 16wk and 30wk fWKY (Figure 3-1C) exhibited reduced responses compared to male counterparts (Figure 3-1A). ACh contraction was similar between 16wk and 30wk fSHR (Figure 3-1D).

Additionally, 16wk and 30wk fSHR exhibited responses that were greater than fWKY counterparts (Figure 3-1C) and that were lower than mSHR counterparts (Figure 3-1B).

ACh contraction was abolished in the presence of preferential COX-1 inhibition with VAS such that responses of all SHR were similar (Figure 3-2). NS-398-induced preferential COX-2 inhibition partially blunted the responses of all SHR, however the contraction remained greater in 30wk mSHR (Figure 3-2B) vs. fSHR (Figure 3-2D). Additionally, both 30wk mSHR (Figure 3-2B) and 30wk fSHR (Figure 3-2D) exhibited greater ACh contractions compared to respective 16wk counterparts (Figures 3-2A and C) in the presence of NS-398.

SQ 29548-induced TP receptor antagonism greatly reduced ACh contractions such that responses were similar in all SHR (Figure 3-3), with the exception that SQ 29548-treated rings of 30wk mSHR (Figure 3-3B) and fSHR (Figure 3-3D) exhibited slightly greater contraction vs. 16wk counterparts (Figure 3-3A and B). Contraction in the presence of SQ 29548 was also slightly greater in 30wk mSHR (Figure 3-3B) vs. fSHR counterparts (Figure 3-3D).

Endothelium-dependent vasorelaxation. Preferential inhibition of COX-1 with VAS restored robust relaxation responses to higher doses of ACh and abolished all re-contractions that occurred in the ND condition, resulting in similar relaxation responses across all SHR in this dose range (Figure 3-4). VAS had no effect on relaxation responses to lower ACh doses in 30wk mSHR (Figure 3-4B) and it caused slight reductions in relaxation in this range of ACh in all other SHR (Figures 3-4A, C, D). Preferential COX-2 inhibition with NS-398

resulted in greater relaxation to lower ACh doses within all SHR (vs. ND) such that similar responses were observed across all SHR in this range (Figure 3-4). However, NS-398 only partially tempered the re-contraction to higher ACh doses in all SHR with the exception of 16wk fSHR (Figure 3-4C), in which little re-contraction occurred in the ND condition. 30wk mSHR (Figure 3-4B), in which the re-contraction was most prominent in the ND condition, maintained greater re-contraction to higher ACh doses in the presence of NS-398 vs. both 16wk mSHR (Figure 3-4A) and 30wk fSHR (Figure 3-4D) counterparts.

ACh-mediated relaxation responses in the ND condition (Figure B-2, Appendix B) confirmed the main observations from Chapter 2. Briefly, 16wk fSHR exhibited unimpaired relaxations that were similar to WKY, whereas moderate re-contractions were detected in 30wk fSHR. Re-contraction was also observed to higher ACh doses in 16wk and 30wk mSHR and was exacerbated in the latter. However, in addition to these findings that corroborate data from Chapter 2, two unexpected group differences were observed. Compared to 30wk counterparts, reduced ACh relaxation was exhibited by both 16wk mWKY (to all ACh doses that elicited a detectable response) and fWKY (to lower ACh doses only). The most likely explanation for these discrepancies is the elevated pre-contractions to 10^{-7} M PE observed in 16wk mWKY and fWKY in this Chapter compared to Chapter 2. However, unlike the ACh contraction dose-response, the reduced ACh relaxation and elevated PE pre-contraction of the present 16wk mWKY (vs. Chapter 2 16wk mWKY) appear to be independent of diurnal vs. nocturnal timing of experiments. All 8 rats in the present 16wk mWKY group were examined for ACh relaxation (and thus PE pre-

contraction) and no systematic differences were observed in the ACh relaxation or PE pre-contraction responses of animals tested during the 'awake' vs. the 'asleep' period.

ACh relaxation in the presence of SQ 29548 (Figure B-3, Appendix B) also confirmed the finding from Chapter 2 that TP receptor antagonism restored relaxation to higher ACh doses in all SHR groups.

PGH_2-mediated vasocontraction. PGH_2 elicited slightly greater contraction to higher doses in 16wk vs. 30wk counterparts, with the exception of mWKY in which responses were similar across the age range studied (Figure 3-5). SHR exhibited greater contraction vs. WKY counterparts. Greater PGH_2 contraction was observed in 30wk mWKY (Figure 3-5A) vs. fWKY (Figure 3-5C), however no other sex differences occurred.

3.5 Discussion

The major novel findings of the present study are that: 1) the previously-impaired endothelium-dependent vasomotor function (i.e. re-contraction of relaxation and enhanced contraction) to higher ACh doses observed in 30wk fSHR was restored with preferential inhibition of COX-1 or antagonism of TP receptor and only partially restored with preferential inhibition of COX-2; 2) endothelium-dependent relaxation to lower ACh doses was improved with preferential COX-2 inhibition (all SHR) and was blunted with preferential COX-1 inhibition (all SHR except 30wk males); and 3) PGH_2-mediated contraction was elevated in SHR vs. WKY and in 16wk vs. 30wk, but was similar between

male and female SHR. These data indicate that the COX-1-TP receptor pathway is a major contributor to the endothelial dysfunction observed in ageing female SHR in response to higher ACh doses. This mechanism of impairment is similar to that observed in ageing male SHR. In contrast, suppression of endothelium-dependent vasorelaxation in ageing male and female SHR appears to involve COX-2 at lower doses of ACh. Furthermore, TP receptor responsiveness to PGH_2 could contribute to endothelial impairments in SHR (vs. WKY) but not sex differences or exacerbations with ageing within SHR.

30wk fSHR had an ACh contraction response that was similar to 16wk fSHR and that was 65 % of the maximal response of 30wk mSHR. These data do not support our first hypothesis, since it was expected that 30wk fSHR would have exhibited an ACh contraction response that was greater than 16wk and similar to male counterparts. Interestingly, the deterioration of endothelium-dependent relaxation exhibited by fSHR between 16 wk (robust and unimpaired response) and 30 wk of age (re-contraction to higher ACh doses) in the ND condition in the present study mimics age-related reductions in relaxation occurring in male SHR (63; 119; 127) and corroborates previous observations of female SHR in Chapter 2. It is possible that 16wk, but not 30wk, fSHR are able to effectively balance the release of vasoconstrictory molecules with increased bioavailability of vasodilatory substances like NO and that this moderate level of ACh contraction in 16wk fSHR is only realized when NO production is inhibited with the presence of L-NAME during the ACh contraction dose-response. This interpretation is supported by the finding that quiescent rings isolated from aorta of 16 wk old female SHR exhibited moderate ACh contraction in the presence, but not the absence, of NO synthase inhibition (70). Therefore, it could be that ACh stimulation

results in the release moderate levels of vasoconstrictory prostanoids in both 16wk and 30wk fSHR, but that the latter are unable to counteract this vasoconstrictory signal with a compensatory elevation in bioavailability of vasodilatory molecules thus resulting in impaired vasorelaxation. Male SHR, on the other hand, have been shown to exhibit depressed relaxation and robust contraction mediated by higher ACh doses by 16 wk of age compared to normotensive counterparts (59; 63; 70; 76; 127; 144) and these responses are magnified with ageing (63; 119; 127). These findings in male SHR are supported by the present results and those in Chapter 2 that robust contraction and reduced relaxation (re-contraction) responses to higher ACh doses are exhibited by 16wk mSHR and, to a greater extent, 30wk mSHR.

The present results demonstrate that both the ACh contraction response and the re-contraction of relaxation to higher ACh doses of 30wk fSHR are largely mediated through COX-1 and TP receptors, since both of these responses were VAS- and SQ 29548-inhibitable. These data support our second hypothesis and further the Chapter 2 findings that endothelium-dependent relaxation to higher ACh doses is blunted in 30wk fSHR and is restored by COX-1 and -2 co-inhibition with Indo or inhibition of the TP receptor with SQ 29548. Moreover, these thesis data collectively build on previous literature reporting that a COX- and TP receptor-dependent mechanism of impairment is predominantly responsible for the loss of endothelium-dependent function occurring in aorta of male SHR vs. male WKY (59; 63; 68; 70; 76; 85; 144) and vs. female SHR (70), as well as with ageing in male SHR (63; 74; 75; 127). The present thesis results support the conclusion that COX-1-mediated TP receptor stimulation plays a major role in the endothelium-dependent vasomotor dysfunction

to higher ACh doses observed in ageing female SHR, and that this mechanism is similar to that occurring in ageing male SHR.

Preferential COX-2 inhibition with NS-398 imparted only partial abrogation of ACh contraction and partial restoration of re-contraction to high ACh doses in SHR in the present study, supporting our third hypothesis. A similar partial tempering of aortic ACh contractions by NS-398 has been reported previously in 30–52 wk old male SHR (48; 52; 154). Two potential mechanisms have been proposed: 1) partial inhibition of *COX-1* by NS-398 (which is possible at high concentrations of NS-398) (48; 52; 154); or 2) a secondary contribution of COX-2-derived prostanoids to the contraction response (52). The former possibility is unlikely in the present study that used 1 μM NS-398, since the IC_{50} of NS-398 on COX-1 has been reported to be 220 μM in enzyme extracted from sheep cotyledons (64) and since 100 μM NS-398 had no inhibitory effect on COX-1 in rat skin inflammation model (89). Additionally, the present observation that NS-398 enhanced ACh relaxation in the lower range of ACh doses (10^{-9}–$10^{-7.5}$ M) in all SHR suggests that COX-2-derived vasoconstrictory prostanoids may suppress ACh-mediated relaxation in this dose range. This interpretation agrees with a report in aorta of 104 wk-old male Wistar rats that showed improved relaxation to lower, but not higher, ACh doses in the presence of NS-398, while preferential COX-1 inhibition with VAS restored relaxation to higher, but not lower, ACh doses (55). The authors of this study concluded that both COX-1 and -2 likely contribute to blunting of different parts of the ACh relaxation response in aorta of ageing male Wistar rats. In contrast, the present observation of slightly reduced relaxation to lower ACh doses in the presence of VAS suggests a role for COX-1 in the enhancement of endothelial responses in

this dosage range. It is interesting that this VAS-inhibitable effect does not occur in 30wk mSHR and thus may be lost specifically in ageing *male* SHR or may be a deterioration that occurs only in more advanced stages of endothelial dysfunction. Therefore, while a secondary role may exist for COX-2-derived vasoconstrictory prostanoids in the impairment of endothelial vasomotor responses to *higher* ACh doses, this COX isoform appears to play a more definite role in suppressing vasorelaxation to *lower* ACh doses in male and female SHR across the age range studied. Moreover, a product of COX-1 may oppose this effect of COX-2 at lower ACh doses, illuminating the subtle complexities of prostanoid influences on endothelial vasomotor responses of ageing hypertensive rats.

Direct stimulation of the TP receptor with PGH_2 (in the presence of L-NAME and Indo) resulted in robust contraction in all groups that was greater in all SHR compared to WKY counterparts, confirming our fourth hypothesis as well as previous reports of elevated PGH_2 contraction in male SHR vs. WKY of a similar age range (48; 49). The present data also extends these strain comparisons in male WKY and SHR to the effects of age and sex on PGH_2 contraction. It is noteworthy that PGH_2 responses were not influenced by sex and were reduced with ageing within SHR. It is therefore unlikely that elevated sensitivity of TP receptors to a given level of PGH_2 contributed to the male sex- or ageing-related impairments in endothelial vasomotor function that occur in SHR in the present study and in Chapter 2. However, several other possible reasons for these impairments remain: 1) enhanced endothelium-derived contracting factor (EDCF) production (likely PGH_2 and/or PGI_2) by COX-1 to a given ACh dose; 2) elevated sensitivity of TP receptors to an EDCF other than PGH_2 (e.g. PGI_2) that is released in response to ACh; and/or 3) reduced PGI_2-IP receptor-

mediated vasorelaxation due to lower expression of IP receptors and/or reduced sensitivity of these receptors to PGI_2.

Conclusions. This study examined mechanisms controlling vasomotor impairments of female SHR between the ages of 16 and 30 wk old, as compared to male SHR and to normotensive WKY counterparts. 16wk and 30wk fSHR both displayed moderate endothelium-dependent contraction (in the presence of L-NAME-induced NOS inhibition), whereas 30wk fSHR, but not 16wk, exhibited impaired endothelium-dependent relaxation (i.e. re-contraction) to higher ACh doses. These findings suggest that perhaps a counteractive enhancement of NO bioavailability occurs in 16wk fSHR, but not 30wk fSHR, in the face of elevated COX-EDCF pathway and contributes to the maintenance of robust endothelial relaxations in the former. All endothelial vasomotor dysfunction to higher ACh doses in fSHR was abolished with either preferential COX-1 inhibition or TP receptor antagonism but was only partially tempered with preferential COX-2 inhibition. These data indicate that COX-1-derived prostanoid(s) acting through the TP receptor is a primary mechanism for impaired vasomotor responses in this dosage range in ageing female SHR. COX-1- and TP receptor-mediated endothelial dysfunction was also observed in mSHR in the present study, corroborating previous literature in male SHR. Interestingly, relaxation of SHR aorta to lower ACh doses was blunted and improved with preferential inhibition of COX-1 and COX-2, respectively, suggesting that prostanoids derived from these two isoforms in this dosage range may exert opposing influences on endothelium-dependent relaxation of male and female SHR across the age range studied. Furthermore, PGH_2 dose-response contractions were similar between male and female SHR and were reduced with

ageing in SHR, indicating that altered sensitivity of TP receptors to PGH_2 did not greatly contribute to the sex- and ageing-related impairments in endothelial function observed in this strain. Collectively, these data support the conclusion that similar pathways contribute to endothelial vasomotor dysfunction in 30wk female SHR and in 16wk and 30wk male SHR. Specifically, stimulation of the TP receptor by COX-1-derived vasoconstrictory prostanoids mediates enhanced endothelium-dependent contraction and reduced endothelium-dependent relaxation induced by higher ACh doses in these groups. Additionally, endothelium-dependent relaxation stimulated by lower ACh doses appear to be enhanced and suppressed by prostanoids derived from COX-1 and COX-2, respectively.

3.6 Acknowledgements

We thank Kourtney Dupak for her excellent technical assistance. This study was funded by the Heart and Stroke Foundation of Canada (T6009) and the Natural Sciences and Engineering Research Council of Canada (RGPIN238342). James Rush holds the Canada Research Chair in Integrative Vascular Biology sponsored by the Canadian Institutes of Health Research. Drew Graham was supported by a Heart and Stroke Foundation of Canada Doctoral Research Award.

Table 3-1 Endpoint physical characteristics and MAP.

		mWKY	mSHR	fWKY	fSHR
BM, g	16wk	293±5	319±5[a]	185±2[a]	189±3[b]
	30wk	352±7[a]	377±3[be]	217±3[ce]	206±3[dfg]
LV/BM, mg/g	16wk	2.20±0.08	2.45±0.03[a]	2.28±0.06	2.78±0.06[bc]
	30wk	2.01±0.02[a]	2.50±0.06[e]	2.35±0.03[e]	2.87±0.02[fg]
MAP, mm Hg	16wk	94±6	190±6[a]	72±5[a]	169±2[bc]
	30wk	98±3	194±4[e]	92±4[c]	175±3[fg]

Values are mean±SEM. $p<0.05$ vs.: [a] 16wk mWKY, [b] 16wk mSHR, [c] 16wk fWKY, [d] 16wk fSHR, [e] 30wk mWKY, [f] 30wk mSHR, [g] 30wk fWKY.

Table 3-2 Final resting tension and reference contractions to KCl and to PE.

	mWKY	mSHR	fWKY	fSHR
		Final resting tension, g		
16wk	6.98±0.01	7.00±0.01	5.98±0.01	6.49±0.01
30wk	7.98±0.01	7.97±0.01	5.47±0.01	6.45±0.01
		KCl contraction, g		
16wk	1.70±0.06	1.81±0.04	1.62±0.05	1.65±0.04[b]
30wk	2.00±0.07[a]	1.98±0.03[b]	1.45±0.12[e]	1.83±0.03[dfg]
		PE contraction (ND condition), g		
16wk	2.10±0.03	1.36±0.11[a]	1.95±0.06[a]	1.46±0.08[c]
30wk	2.02±0.13	1.95±0.11[b]	1.11±0.13[c]	1.55±0.08[fg]

Values are mean±SEM. Final resting tension, tension recording prior to 30 min drug incubation. KCl contraction, contractile response to the second reference contraction to 60 mM KCl. PE contraction, contractile response to 10^{-7} M PE prior to initiation of ACh relaxation dose-response. $p<0.05$ vs.: [a] 16wk mWKY, [b] 16wk mSHR, [c] 16wk fWKY, [d] 16wk fSHR, [e] 30wk mWKY, [f] 30wk mSHR, [g] 30wk fWKY.

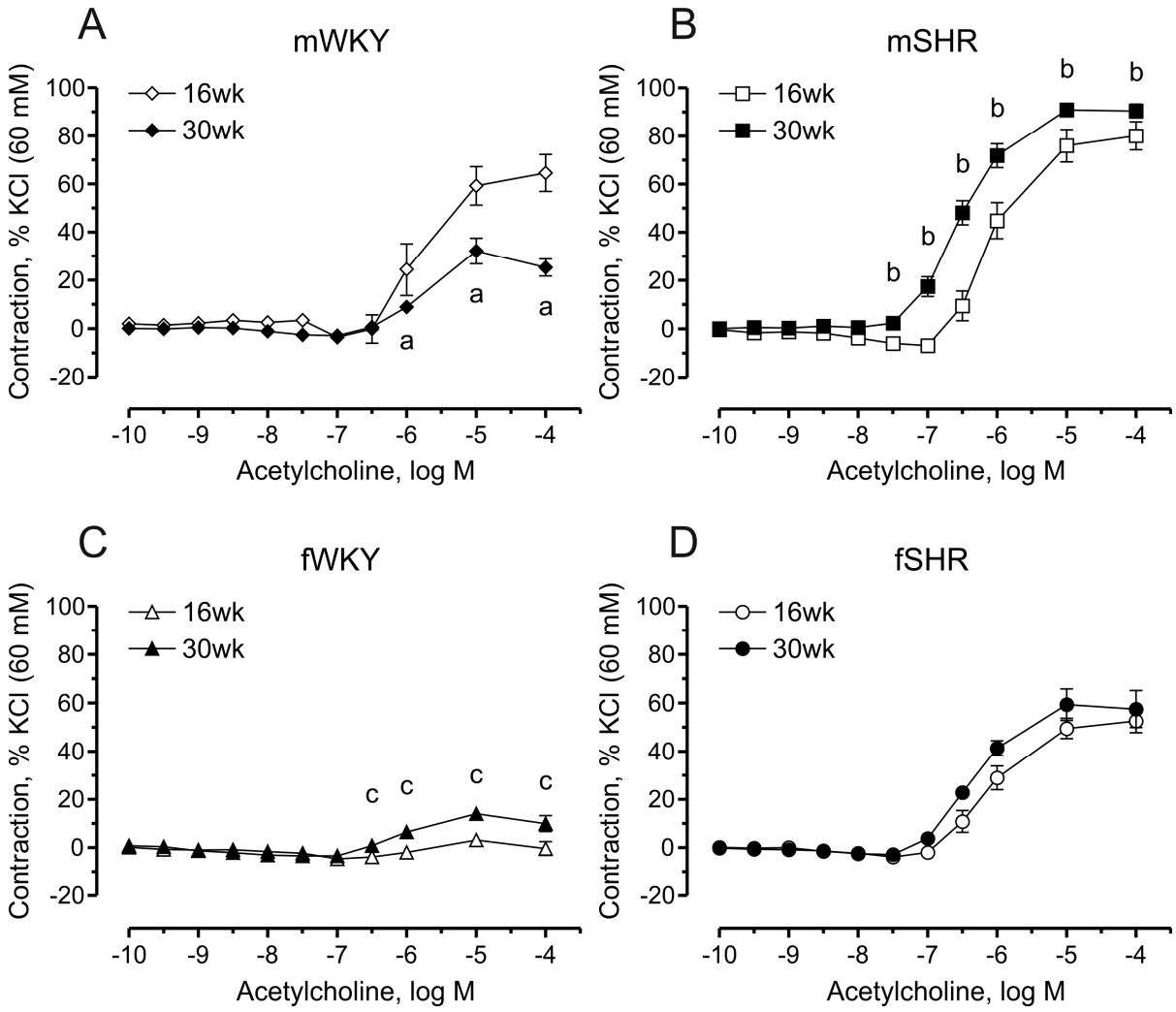

Figure 3-1 ACh-induced endothelium-dependent contraction of quiescent thoracic aortic rings isolated from 16wk and 30wk male and female WKY and SHR in the ND control condition. All rings exposed to L-NAME (10^{-4} M, NOS inhibitor). Values are mean±SEM, expressed as a percentage of the tension development to the 60 mM KCl reference contraction. n=4–9 in singlet to duplicate rings. $p<0.05$ vs.: [a] 16wk mWKY, [b] 16wk mSHR, [c] 16wk fWKY.

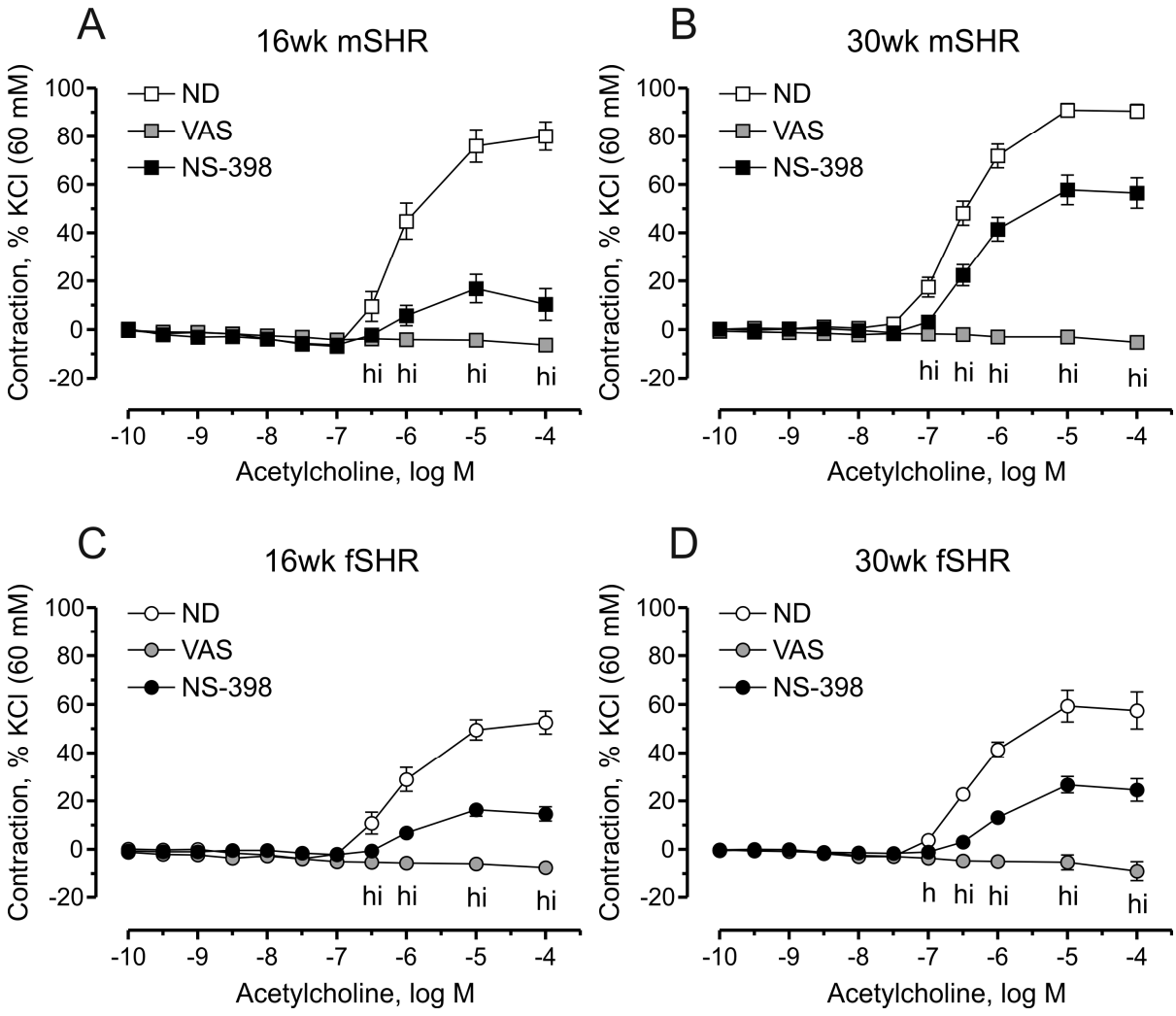

Figure 3-2 ACh-induced endothelium-dependent contraction of quiescent thoracic aortic rings isolated from 16wk and 30wk male and female SHR exposed to VAS ($3x10^{-3}$ M, preferential COX-1 inhibitor) or NS-398 (10^{-6} M, preferential COX-2 inhibitor). ND control values transcribed from Figure 3-1 for reference. All rings exposed to L-NAME (10^{-4} M, NOS inhibitor). Values are mean±SEM, expressed as a percentage of the tension development to the 60 mM KCl reference contraction. n=4–9 in singlet to duplicate rings. $p<0.05$ ND vs.: ^{h} VAS, ^{i} NS-398.

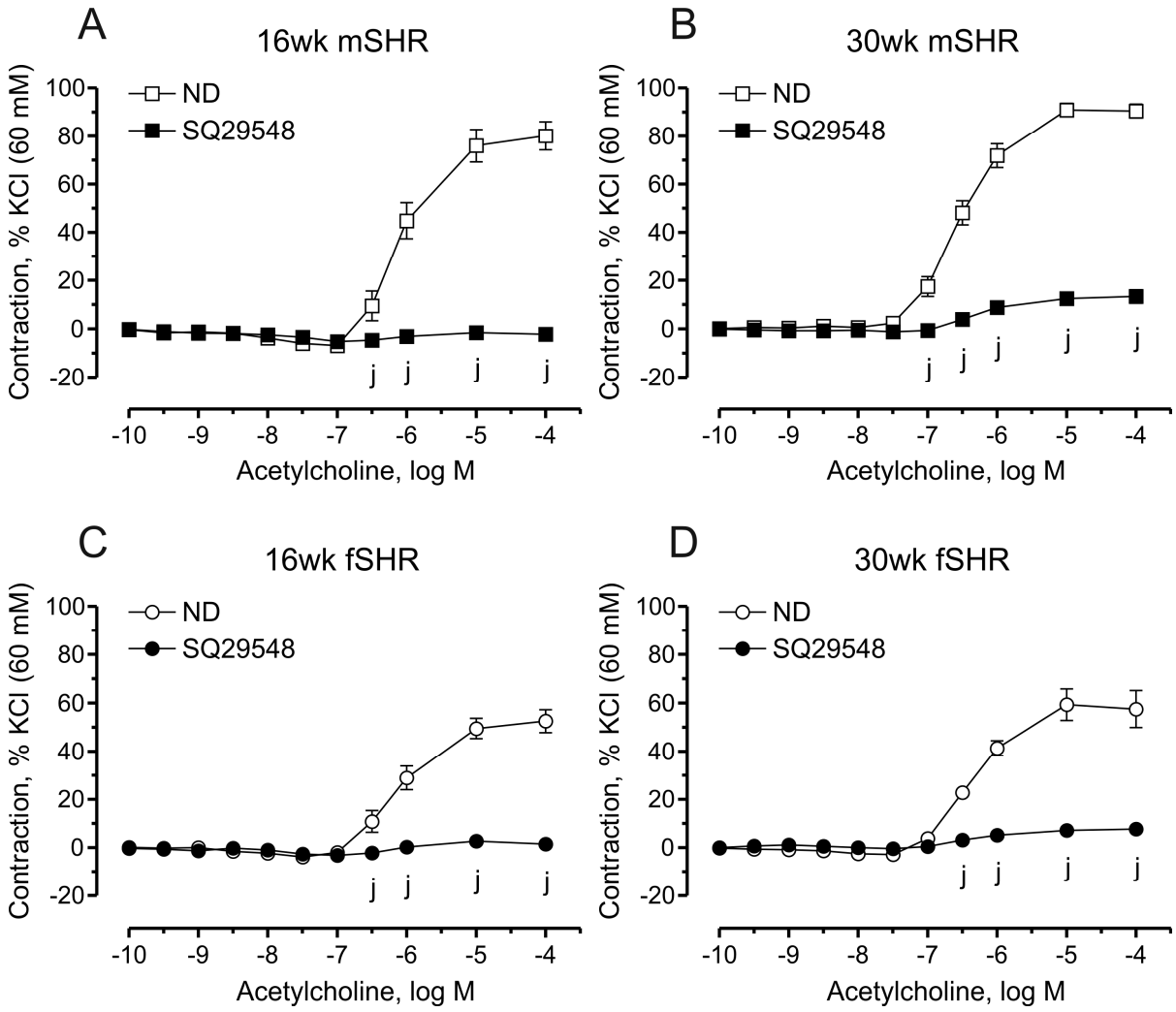

Figure 3-3 ACh-induced endothelium-dependent contraction of quiescent thoracic aortic rings isolated from 16wk and 30wk male and female SHR exposed to SQ 29548 (10^{-6} M, TP receptor antagonist). ND control values transcribed from Figure 3-1 for reference. All rings exposed to L-NAME (10^{-4} M, NOS inhibitor). Values are mean±SEM, expressed as a percentage of the tension development to the 60 mM KCl reference contraction. n=4–9 in singlet to duplicate rings. $p<0.05$ vs.: ^{j} ND.

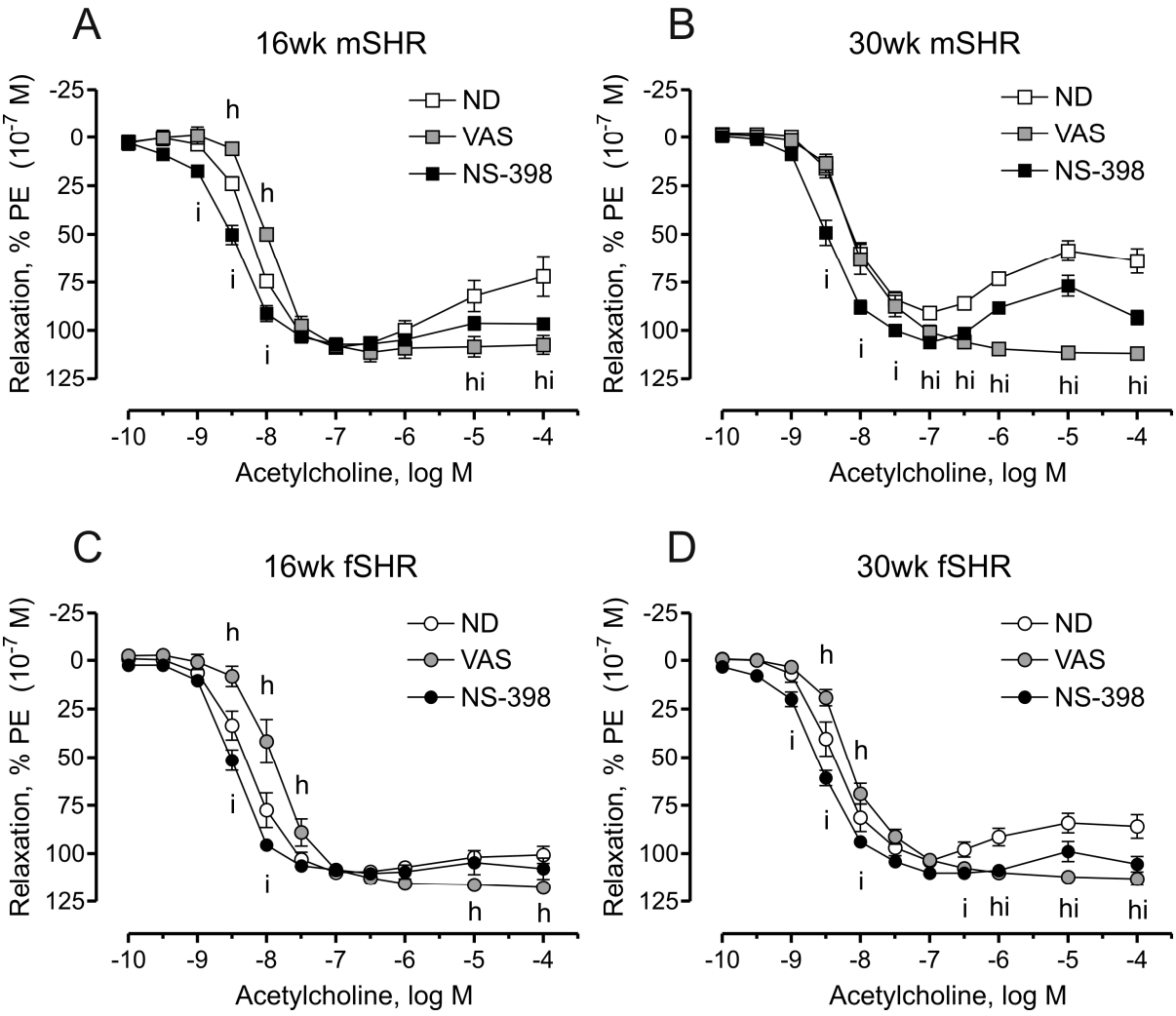

Figure 3-4 ACh-induced endothelium-dependent relaxation of thoracic aortic rings isolated from 16wk and 30wk male and female SHR exposed to the ND control condition, VAS ($3x10^{-3}$ M, preferential COX-1 inhibitor), or NS-398 (10^{-6} M, preferential COX-2 inhibitor). Values are mean±SEM, expressed as a percentage of the prior tension development to 10^{-7} M PE. n=6–9 in singlet to duplicate rings. $p<0.05$ ND vs.: ^{h} VAS, ^{i} NS-398.

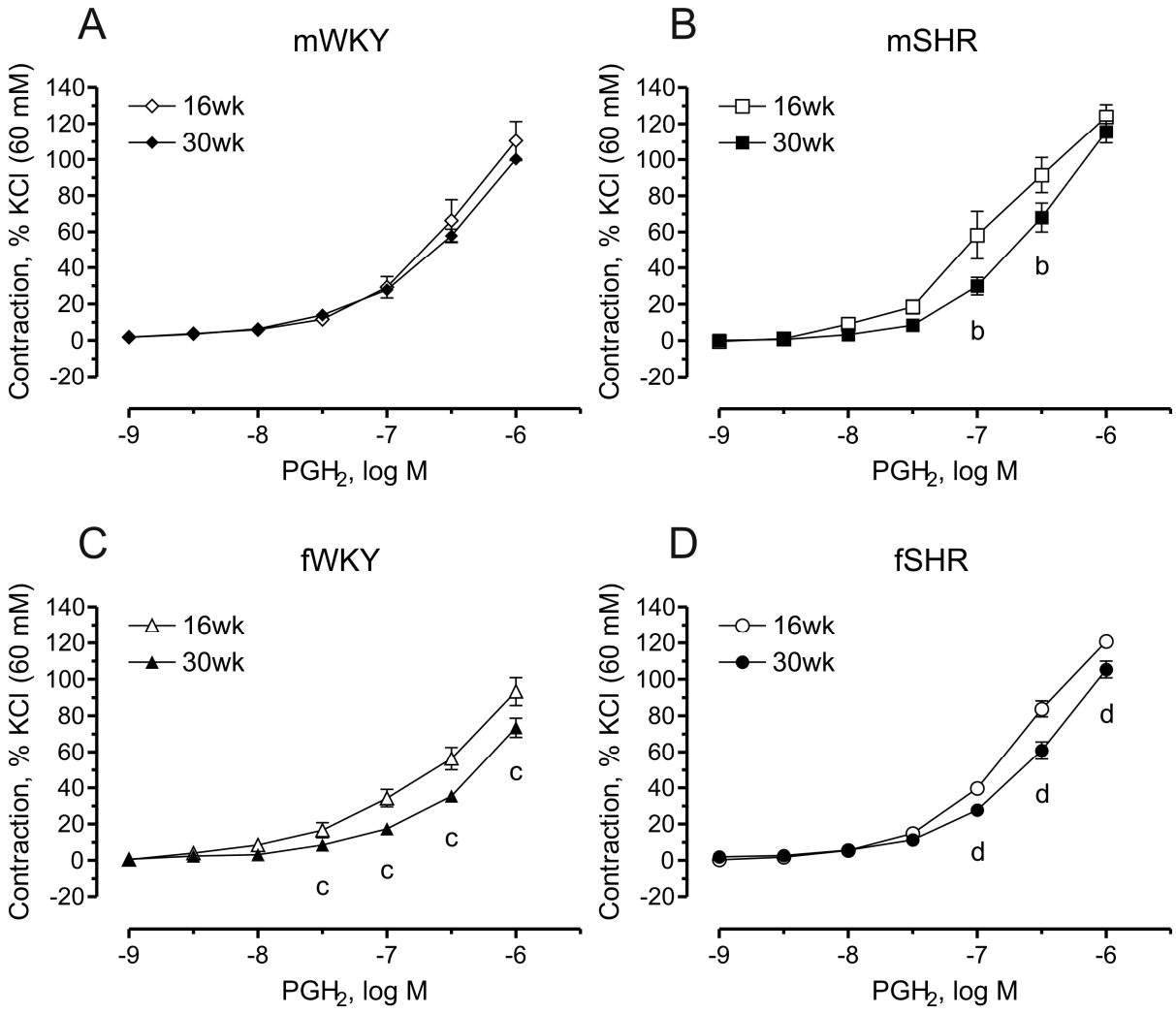

Figure 3-5 PGH_2-induced contraction of thoracic aortic rings isolated from 16wk and 30wk male and female WKY and SHR. All rings exposed to L-NAME (10^{-4} M, NOS inhibitor) and Indo ($5x10^{-6}$ M, COX-1 and -2 co-inhibitor). Values are mean±SEM, expressed as a percentage of the tension development to the 60 mM KCl reference contraction. n=4–9 in singlet to duplicate rings. $p<0.05$ vs.: [b] 16wk mSHR, [c] 16wk fWKY, [d] 16wk fSHR.

Chapter 4

Dietary docosahexaenoic acid does not reduce blood pressure or improve aortic endothelium-dependent vasomotor function in adult male spontaneously hypertensive rats

4.1 Synopsis

Dietary consumption of n-3 polyunsaturated fatty acids (PUFAs) appears to have wide ranging cardiovascular benefits. Specifically, blood pressure (BP) reduction and improvement of endothelium-dependent vasomotor function could target two major pathological concerns in essential hypertension. To date, most animal studies examining BP and endothelial function outcomes began dietary supplementation at an early age before full establishment of hypertension. Thus the present study supplemented 20–24 wk old "adult" male (m) spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats with docosahexaenoic acid (DHA, 22:6 n-3, 0.5 % wt/wt diet) for 8–12 wk and examined intraarterial systolic BP (SBP) and in vitro vasomotor dose-responses of isolated aortic rings. Carotid arterial SBP (n=4–5/group) was elevated in control diet (CON)-fed mSHR (197±5 mm Hg) vs. CON mWKY (136±8 mm Hg) and was unaltered by DHA in either strain (201±3 vs. 133±2 mm Hg, respectively). Endothelium-dependent vasomotor response (n=10–12/group) to lower acetylcholine (ACh) doses ($10^{-9.5}$–$10^{-7.5}$ M) was slightly enhanced in CON mSHR vs. CON mWKY. Higher ACh doses (10^{-7}–10^{-4} M) elicited re-contraction in

CON mSHR that resulted in a reduced response vs. CON mWKY. DHA did not alter the responses of either strain to ACh. Endothelium-independent relaxation (n=9–13/group) was enhanced in CON mSHR vs. CON mWKY to lower (10^{-12}–10^{-9} M) but was equal to higher sodium nitroprusside (SNP) doses. Relaxation to SNP (10^{-12}–10^{-7} M) was blunted in DHA mWKY vs. DHA mSHR and CON mWKY. DHA did not alter SNP relaxation in mSHR. Collectively these data indicate that dietary DHA does not diminish hypertension or endothelial vasomotor dysfunction already established in adult male SHR. Additionally, DHA appears to reduce endothelium-independent vasorelaxation in mWKY.

Key words: n-3 polyunsaturated fatty acid, fish oil, acetylcholine, blood pressure, nitric oxide

4.2 Statement of Authorship

It should be noted that a fellow graduate student, Steven G. Denniss, contributed considerably to the collection of the data herein. Steve is responsible for approximately 50 % of endpoint BP data and tissue harvesting, and for approximately 15 % of vasomotor function experiments. It is anticipated that Steven Denniss will be a co-author on the manuscript resulting from this chapter.

4.3 Introduction

High dietary intake of n-3 PUFAs, largely from marine sources such as fatty fish, is associated with reduced risk of cardiovascular disease, including coronary artery disease, myocardial infarction, stroke, and sudden cardiac death (117). Furthermore, n-3 PUFA consumption appears to: improve serum lipid profile and endothelium-dependent relaxation; reduce vascular levels of chemoattractants, growth factors, and adhesion molecule expression; and provide anti-inflammatory, anti-thrombotic, anti-arrhythmic, and anti-hypertensive benefits to the cardiovascular system (23). Specifically, n-3 PUFA-mediated reduction in BP (12; 13; 25; 41; 47; 65; 66; 78; 88; 92; 124; 126; 129; 158) and enhancement of endothelial vasomotor function (13; 158; 159) could address two major pathological issues in essential hypertension.

BP in the SHR model of essential hypertension begins to rise at a young age (54) and remains greatly elevated compared to normotensive WKY counterparts throughout the lifespan of the animal (10; 40). Additionally, endothelium-dependent vasorelaxation of male SHR is blunted (vs. WKY). Male SHR exhibit "re-contraction" (reversal of prior relaxation) to higher doses of endothelium-dependent vasodilators (59; 63; 68; 70; 76; 85; 127; 144). This re-contraction has been attributed to the action of cyclooxygenase (COX)-generated endothelium-derived contracting factors (EDCF) on the thromboxane A_2/prostaglandin (TP) receptors of the underlying vascular smooth muscle (VSM) (48; 52; 59; 62; 63; 68; 70; 85; 144; 154). These characteristics (i.e. hypertension and endothelial vasomotor dysfunction)

make SHR an appropriate model for studying the potential therapeutic effects of dietary n-3 PUFA supplementation on the cardiovascular pathology of essential hypertension.

Various combinations of dietary n-3 PUFAs abrogate the *development* of hypertension (SBP: –9 to –40 mm Hg) in "younger" (i.e. <12 wk old) male SHR, when supplementation begins before full establishment of the disease (12; 13; 25; 41; 47; 65; 66; 78; 88; 92; 124; 126; 129; 158). In "adult" (i.e. >12 wk old) male SHR, however, 6–9 wk of dietary supplementation with either DHA (4.5 % wt/wt diet), eicosapentaenoic acid (EPA, 20:5 n-3, 4.5 % wt/wt diet) (92), or "fish oil", a combination of EPA plus DHA (EPA 0.3 % + DHA 0.2 % wt/wt diet) (103) does not reduce BP. In contrast, 2 wk administration of pure EPA by gavage (300 mg/kg/d) (78) or 11 wk of dietary supplementation with the n-6 PUFA γ-linolenic acid (GLA, 18:3 n-6, 0.6 % wt/wt diet) in addition to fish oil (EPA 0.5 % + DHA 0.3 % wt/wt diet) (12) abrogate SBP (–14 to –21 mm Hg) in adult SHR. Therefore a specific combination of dosing strategy, duration of treatment, and composition of PUFAs administered may determine the BP response of adult SHR with established hypertension.

Reports of the potential effects of n-3 PUFA supplementation on endothelial function in SHR are currently limited to younger male SHR, in which the characteristic endothelial dysfunction has not been fully established. Aortic endothelial relaxation of younger male SHR is improved by 10 d gavage administration of EPA (280 mg/kg/d) (159) or 4–13 wk of dietary fish oil (EPA 1.9–3.6 % + DHA 1.5–2.4 % wt/wt diet) (13; 158). Additionally, n-3 PUFA supplementation improves endothelium-dependent vasomotor function in human patients with type 2 diabetes mellitus (94), hypercholesterolemia (27), hyperlipidemia/obesity (98), and coronary artery disease (142). Interestingly, 6 wk of dietary

DHA (0.4 % wt/wt diet) beginning at 7 wk of age did not improve aortic endothelial responses of male SHR (41). It is possible that the lower concentration and/or shorter duration of treatment used in the Engler et al. study (41) may have played a role in the lack of effect of DHA on endothelium-dependent relaxation. Collectively, these data suggest that, when given in sufficiently high doses and for sufficiently long durations, n-3 PUFAs can blunt the *development* of endothelium-dependent dysfunction in male SHR and ameliorate vasomotor function in several other disease states in humans. The effect of n-3 PUFA supplementation on endothelium-dependent vasomotor function in humans with essential hypertension, in the absence of other diseases, has not been assessed. Additionally, the responsiveness of the established endothelial vasomotor dysfunction of adult male SHR to supplementation with any n-3 PUFA is unknown. While the potential benefits of n-3 PUFA supplementation are promising, more research is needed to elucidate these benefits on BP and endothelial function in situations where hypertension is already established.

The present study examines intraarterial BP and in vitro vasomotor responses of aortic segments isolated from 28–35 wk old adult male WKY and SHR that had been fed standard lab chow supplemented with either 2.0 % wt/wt diet soybean oil (as a control diet) or with 1.5 % soybean oil + 0.5 % wt/wt diet DHA for the preceding 8–12 wk. This age range was chosen since supplementation began at 20–24 wk of age, when SHR would have experienced a stable elevation in BP compared to WKY for approximately 8–12 wk. Additionally, this age range allowed for 8–12 wk of supplementation while maintaining an endpoint age in which SHR would likely not experience secondary conditions (e.g. heart failure, stroke) that arise in long-term exposure to severe hypertension. It was hypothesized

that: 1) DHA would abrogate the endothelium-dependent re-contraction to higher ACh doses (i.e. endothelium-dependent vasomotor dysfunction) that occurs in adult male SHR and that involves the COX-TP receptor axis (see Chapters 2 and 3); and 2) DHA would not reduce hypertension in adult male SHR.

4.4 Materials and Methods

Animals and diet. Male normotensive WKY (mWKY, n=19) and hypertensive SHR (mSHR, n=25; Harlan, Madison, WI) were raised to 20–24 wk of age in group-housed conditions (4–5/cage) in a temperature- and humidity-controlled room (12 h:12 h light:dark) with free access to food (Teklad 22/5 rodent diet, Harlan) and tap water. Rats of each strain were then randomly assigned to one of two diets for 8–12 wk: control diet (CON, 5 % wt/wt diet from fat supplemented with 2 % wt/wt diet soybean oil) or DHA-supplemented diet (DHA, 5 % wt/wt diet from fat supplemented with 1.5 % wt/wt diet soybean oil and 0.5 % wt/wt diet DHA, Harlan). Nutrient composition (Table 4-1) and fatty acid (Table 4-2) analyses were performed by the supplier (Harlan). All animal protocols were approved by the University of Waterloo Animal Care Committee. All chemicals were obtained from Sigma (St. Louis, MO) or BioShop (Burlington, ON) unless otherwise stated.

BP measurement. Body mass (BM) was recorded and anesthesia was induced with sodium pentobarbital (65 mg/kg, i.p.). Left common carotid artery was cannulated with a

saline-filled PE-50 catheter connected to a pressure transducer (Harvard Apparatus, Holliston, MA) and readings of SBP were obtained from the display every 10 s for 50 s consecutively, recorded by hand, and averaged within a rat.

Tissue harvesting. Rats were euthanized by cardiac excision and exsanguination. Thoracic aorta was carefully isolated, submersed in cold Krebs-bicarbonate buffer (4 ^{o}C, pH 7.4), and cleaned of adhering connective tissue as described in Chapter 2. Six rings (~2 mm) were cut for use in vasomotor function experiments and the remaining tissue was gently blotted dry and stored at –80 ^{o}C. Masses of the left (LV) and right cardiac ventricles and left kidney were obtained as described in Chapter 2. Organ-to-BM ratios were calculated as described in Chapter 2.

Vasomotor function. Thoracic aortic rings were loaded onto tissue supports and submersed into isolated tissue myography baths containing warm Krebs-bicarbonate buffer (37 ^{o}C, pH 7.4) that was continuously aerated with 95 %:5 % O_2:CO_2. Resting tension was initially set to 0.5 g and was gradually raised at a rate of 0.5 g every 5 min to a pre-determined group-specific level of resting tension that was optimal for tension development (determined in pilot testing for Chapter 2). Rings were equilibrated for 15 min at optimal resting tension and tension was adjusted as needed. Two rings per rat were incubated (30 min) in the absence (No Drug control, ND) or presence of N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME, nitric oxide (NO) synthase (NOS) inhibitor, 10^{-4} M). Contraction of rings was induced twice by exposure to KCl (membrane depolarizing agent, 60 mM) with

buffer replacement and full relaxation to baseline tension after each exposure. Rings were then assigned to either a Relaxation Protocol or a Contraction Protocol. For the Relaxation Protocol, steady-state contraction was induced by phenylephrine hydrochloride (PE, α_1 adrenergic agonist, 10^{-7} M) and endothelium-dependent relaxation to ACh (muscarinic agonist, 10^{-10}–10^{-4} M) was assessed. Rings were then rinsed 3 x 5 min with fresh buffer and allowed to return to baseline resting tension. Steady-state contraction was induced with PE (10^{-7} M) and then endothelium-independent relaxation to SNP (NO donor, 10^{-12}–10^{-4} M) was assessed. For the Contraction Protocol, contractile dose-response to KCl (10–100 mM) was assessed and rings were rinsed 3 x 5 min with fresh buffer and allowed to return to baseline resting tension. Contractile dose-response to PE (10^{-10}–10^{-4} M) was then assessed.

Vasomotor function responses were obtained in duplicate rings for Relaxation Protocol and in singlet rings for Contraction Protocol. Final in-bath doses are reported. All drugs were dissolved in distilled water. For the Relaxation Protocol, loss of tension (in g) to each dose of ACh or SNP was expressed relative to the preceding contraction (in g) to 10^{-7} M PE on a ring-by-ring basis. The mean response of duplicate rings within an animal and drug condition were calculated and used for group mean and SEM calculations. For the Contraction Protocol, steady-state tension development (in g) to each dose was expressed in absolute terms.

Statistics. Data are mean±SEM. Repeated measures ANOVA was used with least squares means post-hoc test in cases where the interaction term was significant. SAS (v.9.1, SAS Institute, Cary, NC) was used for all statistical procedures.

4.5 Results

Physical characteristics and SBP. BM was similar in all groups (Table 4-3). LV-to-BM ratio (LV/BM) and SBP were elevated in mSHR vs. mWKY regardless of diet and these parameters were unaffected by diet. Additional organ mass data are presented in Appendix C (Table C-1).

Contraction to KCl and to PE. Contraction to 60 mM KCl in the ND condition was similar in all groups with the exception of DHA mSHR, which exhibited a greater contractile response compared to CON mSHR and to DHA mWKY (Table 4-4). Contractile response to 10^{-7} M PE was reduced in mSHR vs. mWKY regardless of diet and this response was unaffected by diet. Contractions to single doses of KCl and PE in the presence of L-NAME are presented in Appendix C (Table C-2). Dose-response to higher KCl doses was slightly greater in CON mSHR vs. CON mWKY, and response to lower KCl doses was elevated in mSHR following DHA feeding (Figure C-1, Appendix C). DHA feeding did not affect the KCl dose-response of mWKY, and responses of DHA-fed mWKY and mSHR were similar. Dose-response to PE was reduced in CON mSHR vs. CON mWKY (Figure C-2, Appendix C). DHA feeding elevated the PE dose-response within mSHR but not mWKY (vs. strain-matched CON), however response of DHA mSHR remained lower than that of DHA mWKY.

Endothelium-dependent relaxation. Relaxation to lower doses of ACh in the ND condition was greater in mSHR vs. mWKY regardless of diet (Figures 4-1A and B). However, mSHR exhibited reduced relaxation (due to marked re-contraction) to higher ACh doses vs. mWKY (no re-contraction). Diet did not alter ACh responses within either strain (Figures 4-1C and D). Responses to ACh in the presence of L-NAME are presented in Appendix C (Figure C-3).

Endothelium-independent relaxation. Relaxation to lower doses of SNP in the ND condition was enhanced in CON mSHR vs. CON mWKY (Figure 4-2A). SNP relaxation was also greater in DHA mSHR vs. DHA mWKY across a wider range of doses (Figure 4-2B). DHA feeding reduced SNP relaxation within mWKY (Figure 4-2C) but did not alter the response of mSHR (Figure 4-2D). Relaxation responses to SNP in the presence of L-NAME are presented in Appendix C (Figure C-4).

4.6 Discussion

The major findings of this study are that chronic supplementation of adult male SHR with DHA: 1) did not improve endothelial vasomotor impairments to higher ACh doses that are known to be mediated by the COX-TP receptor axis; 2) did not affect endothelium-independent relaxation to SNP; 3) did not reduce arterial SBP; and 4) partially restored the blunted PE dose-response. These data suggest that some of the previously reported

cardiovascular protective effects of n-3 PUFAs (e.g. blunting of arterial hypertension, improvement of endothelium-dependent vasorelaxation) in younger male SHR do not occur with 8–12 wk of dietary supplementation with 0.5 % w/w diet DHA in adult male SHR with established hypertension.

DHA supplementation does not improve the impaired endothelium-dependent vasorelaxation in adult male SHR. The present data indicate that dietary DHA (0.5 % wt/wt) supplementation does not restore endothelial vasomotor dysfunction in adult male SHR, and thus rejects our first hypothesis. Both CON- and DHA-fed mSHR exhibited a similar degree of re-contraction of thoracic aortic rings to ACh doses above 10^{-7} M, while there was a lack of re-contraction in both mWKY groups. Re-contraction to higher ACh doses has been observed in adult mSHR in previous studies of this thesis (Figures 2-1 and B-2) and in the literature (59; 63; 68; 70; 76; 85; 144), and has been attributed to the action of COX-derived EDCF on VSM prostanoid receptors. These present data indicate that dietary DHA alters neither aortic re-contraction nor, by association, the COX-EDCF pathway in adult male SHR.

The lack of effect of dietary DHA on aortic endothelium-dependent relaxation in the present adult male SHR corroborates a similar finding in younger male SHR. DHA supplementation (0.4 % wt/wt diet) of male SHR for 6 wk beginning at 7 wk of age did not alter aortic responsiveness to ACh (41). It appears that the present study and that of Engler et al. (41) are the only two reports of the effects of dietary DHA supplementation alone on vascular reactivity of SHR. In addition to these findings in SHR, 8 wk of DHA supplementation (1.4 % wt/wt diet) did not restore impaired ACh-induced relaxation in aorta of male Wistar rats with chemically-induced type 1 diabetes mellitus (53). In contrast, 8–13

wk of DHA (7.0 % wt/wt diet) feeding beginning at 5 wk of age in male stroke-prone SHR (a sub-strain of SHR) prevented blunting of ACh relaxation in perfused mesenteric arterial bed (102). It is unclear if the divergence of the present data from that of Murakami et al. (102) is due to the much higher concentration of DHA administered in the latter (0.5 % vs. 7.0 % wt/wt diet) or to possible differences in responsiveness to DHA between strains (SHR vs. SHR-SP) or across vascular beds (aorta vs. mesenteric artery). Nevertheless, the present findings support the conclusion that dietary DHA supplementation alone does not improve aortic endothelium-dependent vasomotor dysfunction in adult male SHR.

In contrast to DHA alone, supplementation with fish oil (EPA 2.0–3.6 % + DHA 1.5–2.4 % wt/wt diet) (13; 158) or EPA alone (280 mg/kg/d gavage) (159) improved ACh responses in aorta of male SHR. It is noteworthy, however, that supplementation of SHR in these studies (13; 158; 159) *appears* to have begun at a younger age and before large deficits in endothelial function would have been established. The Bexis et al. study began the fish oil diet at 4 wk of age (13). While neither Yin et al. study explicitly states the age of male SHR at the start of supplementation, the lower initial BM values (200–250 g) (158; 159) reported and a SBP that is still rising over the course of the study (158) indicate that PUFA supplementation began in animals that were <12 wk old. This earlier commencement of treatment may have been an important contributing factor to the beneficial effects of fish oil and EPA on vasomotor responses of male SHR in these studies. Collectively these functional data indicate that EPA, but not DHA, may be an active component in fish oil that can improve endothelium-dependent vasomotor dysfunction in hypertension, especially if administered early in the progression of the disease.

DHA supplementation does not alter endothelium-independent relaxation in adult male SHR. SNP-induced aortic relaxation of adult male SHR was not influenced with DHA feeding (0.5 % wt/wt diet) in the present study. This observation compliments previous reports that DHA (0.4 % wt/wt diet) (41), EPA (280 mg/kg/d gavage) (159), or fish oil (EPA 3.6 % + DHA 2.4 % wt/wt diet) (158) supplementation do not affect SNP relaxation in aorta of younger male SHR. The present lack of DHA effect on SNP relaxation indicates that diet-induced changes in aortic VSM sensitivity to NO did not occur and thus did not influence endothelium-dependent responses in mSHR. These data are contrasted by the reduction of SNP relaxation induced in mWKY by DHA feeding in the present study. If this truly represents a DHA-induced reduction in VSM responsiveness to NO in mWKY, it is possible that these arteries could have compensated for this lower VSM responsiveness by enhancing ACh-stimulated endothelial NO release and/or reducing NO destruction. This type of compensation could account for the similar ACh responses but lower SNP responses observed in DHA mWKY vs. CON mWKY. Interestingly, in contrast to the present SNP data in mWKY (blunted SNP response with DHA) and in mSHR (no effect of DHA), 4 wk of fish oil supplementation (EPA 1.8 % + DHA 1.2 % wt/wt diet) was found to *improve* SNP relaxation of perfused mesenteric bed of younger male SHR (158). Possible explanations for these discrepancies are that the effects of n-3 PUFAs on endothelium-independent vasomotor mechanisms may be highly dependent on the age of the animal, stage of hypertension development (or lack thereof in the case of the present WKY), and/or vascular bed/vessel size. Nonetheless, it can be collectively concluded from the present functional data that

DHA supplementation alters neither the endothelium-dependent NO-mediated vasomotor response nor the responsiveness of VSM to NO in aorta of adult male SHR.

DHA supplementation does not reduce arterial BP in adult male SHR. The potential anti-hypertensive effects of dietary n-3 PUFA supplementation have been studied extensively in both the SHR model and in human hypertensive patients. Many of the SHR studies, however, began supplementation at a young age, before hypertension was completely established (12; 13; 25; 29; 41; 47; 65; 66; 88; 92; 124; 126; 129; 143; 158; 159). This timing of supplementation before the establishment of chronic, steady-state hypertension allows conclusions to be made regarding the ability of dietary n-3 PUFAs to alter the *development* of hypertension. The reason for the lack of hypotensive effects of n-3 PUFA supplementation in young SHR reported in a few studies (29; 30; 143; 159), however, is unclear. It is possible that a shorter treatment time (2–5 wk) (29; 30; 159) or the use of flaxseed oil (143), which is mainly rich in α-linolenic acid (ALA, 18:3 n-3), could have prevented n-3 PUFAs from blunting development of hypertension in SHR in these studies.

The present study aimed to examine the potential of dietary DHA (0.5 % wt/wt for 10–12 wk) to reduce SBP in adult male SHR beginning at 24 wk old, in which a steady-state elevation in arterial BP would have been established for approximately 12 wk. The lack of hypotensive effect of DHA supplementation in the present study confirms the second hypothesis and corroborates other reports that either DHA or EPA, alone (each 4.5 % wt/wt diet) (92) or in combination (EPA 0.3 % + DHA 0.2 % wt/wt diet) (103), do not reduce BP in adult male SHR with steady-state hypertension already established at the start of supplementation. One consideration is that SHR may be more resistant to the hypotensive

effects of PUFAs in this adult age range. This is supported by the finding that a mixture of GLA (18:3 n-6, 0.6 % wt/wt diet) and fish oil (EPA 0.5 % + DHA 0.3 % wt/wt diet) began to elicit hypotensive effects 5 wk after beginning treatment in 5 wk-old and in 51 wk-old SHR, whereas this effect was not seen until after 8 wk of treatment in 20 wk-old SHR (12). Additionally, while some n-3 PUFAs, such as EPA and DHA, can abrogate the development of hypertension in younger SHR, they may not provide as potent a hypotensive stimulus to older SHR with established hypertension as other PUFAs (e.g. 18:3 n-6 GLA). This is supported by findings from one research group showing that supplementation with fish oil (EPA 0.3 % + DHA 0.2 % wt/wt diet) does not reduce BP in adult SHR (103), whereas BP was lowered (–14 mm Hg) in similar animals by adding GLA (0.6 % wt/wt diet) to the same fish oil mixture (12). Furthermore, GLA alone (2.4–14.4 mg/kg/d i.p.) reduces arterial BP (–35 mm Hg) in adult male SHR with as little as 5 d of treatment (135). These data suggest that the n-6 PUFA, GLA, may have a hypotensive effect of its own in adult SHR. Thus, timing of administration (i.e. age range of SHR), the duration of treatment, and the type of PUFA administered may influence the success of generating a hypotensive effect in adult male SHR.

The strategy taken in the present study was to begin administration after development of steady-state hypertension in adult SHR. This study design may provide a more realistic model for some clinical populations who present with established essential hypertension and who wish to explore dietary manipulation as a therapeutic strategy. Unfortunately, human studies examining the effects of n-3 PUFA supplementation (usually a combination of EPA and DHA) on BP in hypertensive patients are inconclusive. Fish oil (EPA + DHA)

supplementation either does not alter (38; 82; 99) or has mild hypotensive effects (8; 17; 73; 105; 118) in hypertensive human patients. A meta-analysis of randomized controlled trials concluded that fish oil supplementation (average intake of 4.1 g per day for 12 wk) offered modest reductions in BP to hypertensive patients, estimated to be in the order of –4.0/–2.5 mm Hg for SBP/diastolic BP (DBP) (51). More research is needed to identify and characterize the potential hypotensive benefits of dietary n-3 PUFA supplementation. Additionally, mechanisms responsible for these effects require further elucidation. The present data add to the literature the observation that chronic DHA feeding at the dose used does not offer hypotensive benefits to adult male SHR with established hypertension.

Limitations. It is possible that the present experimental conditions using animals under barbiturate anesthesia could have concealed a BP effect of DHA. Most other reports of n-3 PUFA supplementation in SHR measure BP by tail cuff sphygmomanometry in conscious animals (12; 13; 25; 29; 30; 41; 47; 65; 66; 78; 88; 92; 103; 124; 126; 129; 135; 158; 159). However, the tail cuff method necessitates restraining and heating the rat during BP measurement and any procedure-induced stress response generated could influence values despite efforts to acclimatize the animals. It is important to be mindful of these caveats when interpreting BP data.

Conclusions. The present data indicate that chronic dietary DHA supplementation does not reduce arterial SBP or correct aortic vasomotor dysfunction in adult male SHR with established hypertension. The present findings further suggest that DHA may not influence the aortic COX-TP receptor axis, which has been shown to be the primary mechanism responsible for the functional impairments in male SHR. Finally, DHA feeding appears to

partially restore α_1 adrenergic receptor-mediated contraction in male SHR. Although not discussed in detail, this finding may present an interesting avenue for future research. The present study adds to the relatively small body of literature that has assessed the influence of n-3 PUFA supplementation on hypertension-related pathologies in SHR. Furthermore this appears to be the first evaluation of vasomotor function in *adult* SHR following n-3 PUFA supplementation. Examining treatment efficacy in adult animals, in which hypertension and its related tissue impairments are already established, may be more applicable to clinical populations in whom symptoms often remain sub-clinical until more advanced stages of this disease.

4.7 Acknowledgements

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Table 4-1 Macronutrient, vitamin, and 'other' composition of the diets administered.

Component	CON	DHA
Total fat*	7.1	7.1
Saturated fat**	1.03	1.22
Monounsaturated fat**	1.47	1.40
Polyunsaturated fat**	3.82	3.66
Total carbohydrates	63.2	63.1
Total protein	14.7	14.8
Ash	4.7	4.9
Moisture	10.3	10.1
α-tocopherol, mg/kg	110	116
Vitamin C, mg/kg	< 10	–
Calories, kcal/100 g	376	376

Values are reported in % wt/wt diet unless otherwise indicated. *, calculated by hydrolysis. **, calculated as acids. –, value not reported.

Table 4-2 Fatty acid composition of diets administered.

Fatty acid* (common name)	CON	DHA
16:0 (palmitic)	0.83	0.87
17:0	< 0.01	< 0.01
18:0 (stearic)	0.20	0.15
18:1 n-9 (oleic)	1.49	1.40
18:2 n-6 (linoleic)	3.62	3.07
18:3 n-6 (γ-linolenic)	< 0.01	< 0.01
18:3 n-3 (α-linolenic)	0.36	0.28
18:4 n-3	< 0.01	< 0.01
20:0	0.02	0.02
20:1 n-9	0.03	0.03
20:2 n-6	< 0.01	< 0.01
20:4 n-6 (arachidonic)	< 0.01	< 0.01
20:3 n-3	< 0.01	< 0.01
20:5 n-3 (eicosapentaenoic)	< 0.01	< 0.01
22:0	0.03	0.02
22:1 n-9	< 0.01	< 0.01
22:5 n-3	< 0.01	< 0.01
22:6 n-3 (docosahexaenoic)	< 0.01	0.49
24:0	0.01	0.01

Values are reported in % wt/wt diet. *, calculated as triglycerides.

Table 4-3 Feeding duration and endpoint age, BM, LV/BM ratio, and SBP.

		mWKY	mSHR
Age at start of feeding, wk	CON	19.9±0.0	23.8±0.6
	DHA	19.9±0.0	23.6±0.5
Endpoint age, wk	CON	28.3±0.1	35.8±0.5
	DHA	27.5±0.0	33.9±0.5
Feeding duration, wk	CON	8.4±0.1	12.0±0.2
	DHA	7.7±0.0	10.3±0.0
BM, g	CON	357±6	359±7
	DHA	361±8	351±11
LV/BM, mg/g	CON	2.08±0.04	2.74±0.04[a]
	DHA	2.10±0.03	2.78±0.16[c]
SBP, mm Hg	CON	136±8	197±5[a]
	DHA	133±2	201±3[c]

Values are mean±SEM. n=9–13 (ages). n=14–18 (BM and LV/BM). n=4–5 (SBP). $p<0.05$ vs.: [a] CON mWKY, [c] DHA mWKY.

Table 4-4 Developed tension of thoracic aortic rings to KCl and PE in the ND condition.

	mWKY	mSHR
KCl contraction (60 mM), g		
CON	1.79±0.17	1.90±0.08
DHA	1.67±0.16	2.20±0.11[bc]
PE contraction (10^{-7} M), g		
CON	2.00±0.12	1.41±0.07[a]
DHA	1.92±0.13	1.38±0.13[c]

Values are mean±SEM. n=8–12. KCl contraction, contractile response to the second exposure to 60 mM KCl. PE contraction, contractile response to 10^{-7} M PE prior to ACh relaxation dose-response. $p<0.05$ vs.: [a] CON mWKY, [b] CON mSHR, [c] DHA mWKY.

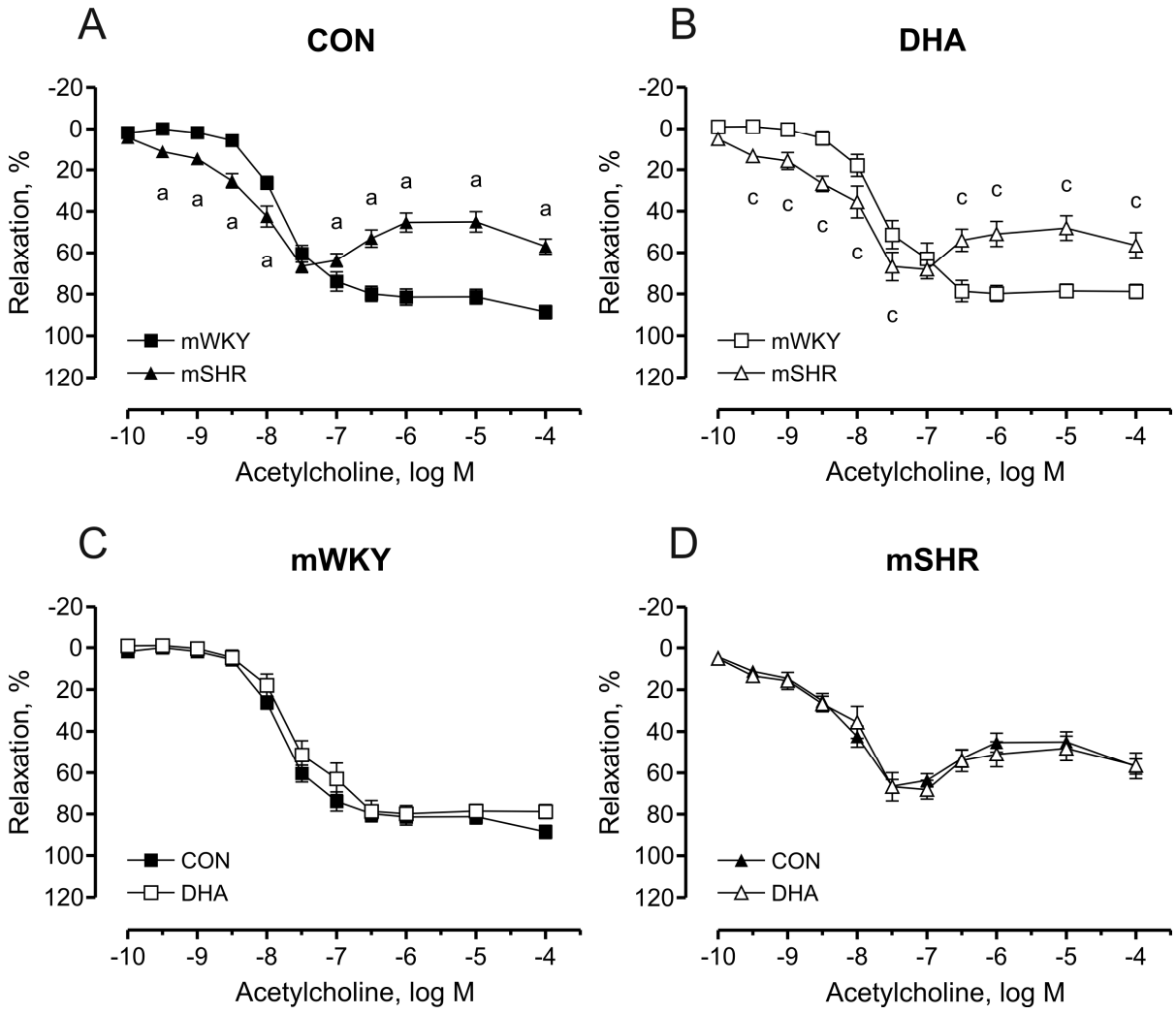

Figure 4-1 Endothelium-dependent relaxations to ACh in the absence of L-NAME (ND condition) of thoracic aortic rings isolated from male WKY and SHR following 8–12 wk of CON or DHA diet. Responses of CON (panel A) and DHA groups (panel B) are plotted for comparison of strain (within each diet). Responses of WKY (panel C) and SHR (panel D) are re-plotted separately for comparison of diet (within each strain). Values are mean±SEM, expressed as a percentage of the preceding contraction to PE (10^{-7} M). n=10–12 in duplicate rings. $p<0.05$ vs.: [a] CON mWKY, [c] DHA mWKY.

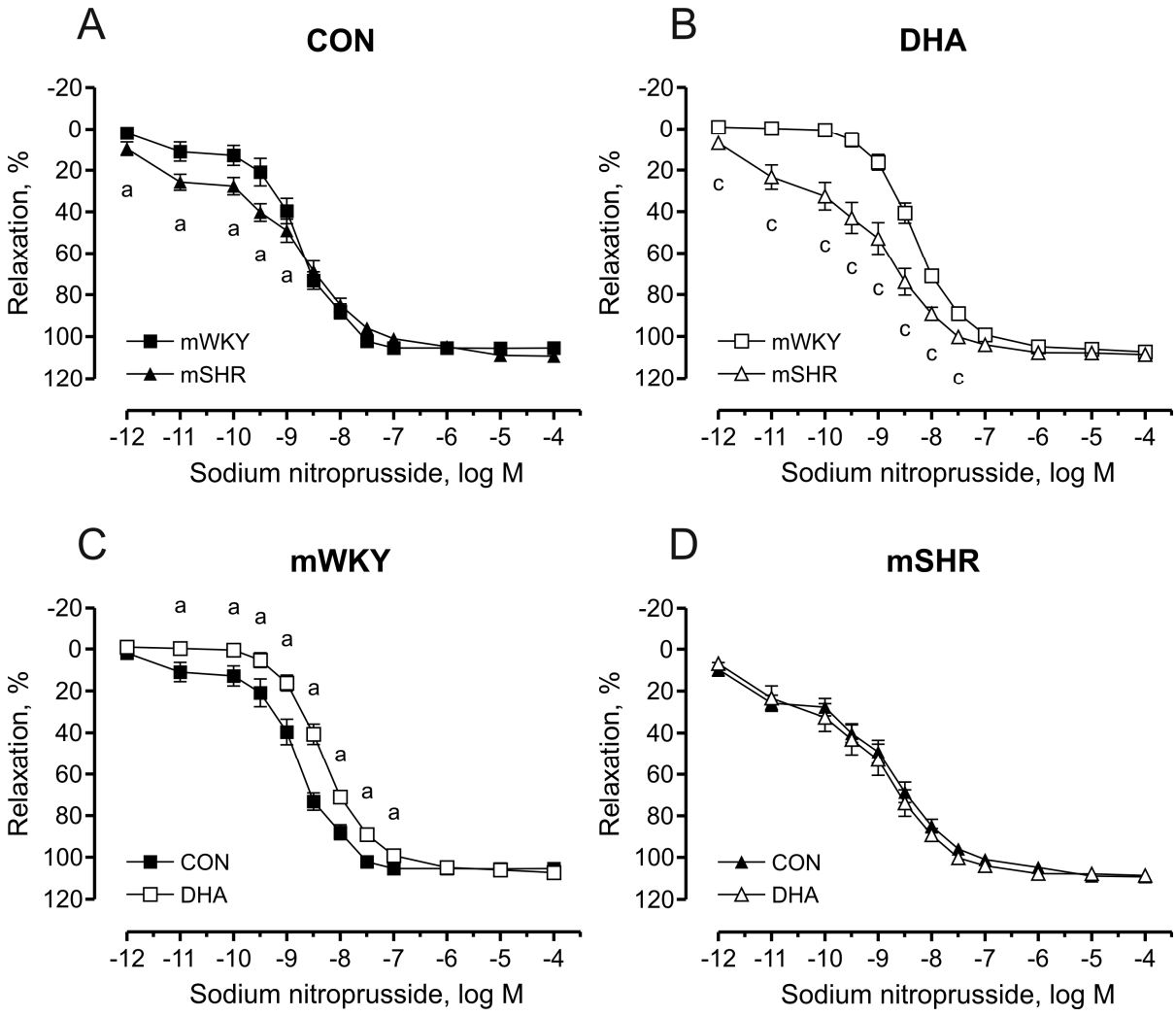

Figure 4-2 Endothelium-independent relaxations to SNP in the absence of L-NAME (ND condition) of thoracic aortic rings isolated from male WKY and SHR following 8–12 wk of CON or DHA diet. Responses of CON (panel A) and DHA groups (panel B) are plotted for comparison of strain (within each diet). Responses of WKY (panel C) and SHR (panel D) are re-plotted separately for comparison of diet (within each strain). Values are mean±SEM, expressed as a percentage of the preceding contraction to PE (10^{-7} M). n=9–13 in duplicate rings. $p<0.05$ vs.: [a] CON mWKY, [c] DHA mWKY.

Chapter 5

Aortic α_1 adrenergic contraction is reduced in exercise-trained but not metformin-treated female Zucker diabetic fatty rats

5.1 Synopsis

Vascular dysfunction is common in type 2 diabetes mellitus and negatively influences cardiovascular outcomes. Anti-diabetic drugs and lifestyle interventions (e.g. physical activity) are prescribed to type 2 diabetics, however, little is known of the potential benefits of these interventions on vasomotor function in this disease. In vitro aortic α_1 adrenergic contraction to phenylephrine (PE) and endothelium-dependent relaxation to acetylcholine (ACh) dose-responses were assessed in female Zucker diabetic fatty rats (ZDF), a model of high fat diet-induced type 2 diabetes with obesity, following an 8 wk administration of: 1) no therapeutic intervention (high fat diet alone, HF); 2) the anti-diabetic drug, metformin (HF-Met); 3) chronic exercise training (HF-Ex); or 4) metformin and exercise (HF-E+M). Zucker lean rats (Lean) and normal fat-fed ZDF (Con) served as lean and obese non-diabetic controls, respectively. Con (1.70±0.21 g) and HF (1.69±0.16 g) exhibited a maximal α_1 adrenergic contraction that was 2.4-fold the response of Lean (0.71±0.13 g) and this elevation was blunted in HF-Ex (1.02±0.17 g). The superoxide dismutase (SOD) mimetic, 4-hydroxy-TEMPO (Tempol), increased maximal PE contraction in Lean (1.29±0.14 g) and

HF-Ex (1.36±0.15 g) to the level of all other groups, which remained unaltered. Aortic protein expression of α_1 adrenergic receptor was slightly elevated in Con (+27 %) versus Lean, but was similar in all other groups. Endothelium-dependent relaxation was unimpaired and similar in all groups (10^{-4} M: 78.1±2.4 %). Tempol improved maximal relaxation in HF-Ex only (No Drug control (ND) vs. Tempol: 78.7±4.7 vs. 93.6±4.7 %, respectively). Aortic protein expression of endothelial nitric oxide (NO) synthase (eNOS), SOD-1, and SOD-2 were similar across groups. Together, these data suggest a hyperresponsive α_1 adrenergic contraction and apparently normal endothelium-dependent vasorelaxation in aorta of obese, hyperglycemic, and hyperinsulinemic female ZDF. Chronic exercise training, but not metformin pharmacotherapy, normalized the α_1 adrenergic contractions, supporting further examination of detailed mechanisms by which exercise training can improve vascular function, and possibly clinical cardiovascular outcomes, in type 2 diabetes.

Key words: endothelium, superoxide dismutase, endothelial nitric oxide synthase, Tempol

5.2 Statement of Authorship

It should be noted that this study was performed in conjunction with the laboratory of Dr. David Dyck in the Department of Human Health and Nutritional Sciences at the University of Guelph. Dr. Angela Smith, a doctoral student in Dr. Dyck's laboratory at the time, is primarily responsible for the study design and preparation of the animals to the point of sacrifice (including treatment administration). Additionally, all body mass and fasting

blood glucose and plasma insulin data were collected and reported by Smith et al. (130) and are merely transcribed in the thesis for reference. It is anticipated that Drs. Smith and Dyck will be co-authors on the manuscript resulting from this chapter.

5.3 Introduction

Impaired macrovascular function, including arterial vasomotor dysfunction, is a prominent characteristic of type 2 diabetes mellitus that appears to be related to many cardiovascular complications that are closely tied to morbidity and mortality in this disease (104). Specifically, enhanced α adrenergic vasocontraction has been reported in human type 2 diabetic patients (56) and in several rodent models of type 2 diabetes: male ZDF rats (37; 79; 149), male Otsuka-Long-Evans-Tokushima Fatty (OLETF) rats (160), male *db/db* mice (72; 152), and sex-unspecified *ob/ob* mice (107). Additionally, endothelium-dependent vasorelaxation is blunted in various arteries isolated from male (28; 32; 33; 57; 108-113; 125) and sex-unspecified (18; 50) ZDF, male OLETF (91; 95), male (71; 72; 96; 97) and sex-unspecified (123) *db/db*, and sex-unspecified *ob/ob* (107), as well as in human type 2 diabetic patients (93). The impaired relaxation response in rodent models of type 2 diabetes is associated with elevated oxidant stress (18; 28; 32; 91; 96; 97; 108-112; 123) and is partially recovered with anti-oxidant treatment (18; 28; 50; 96; 97; 108; 112; 123). Therefore, both enhanced α adrenergic vasocontraction and reduced endothelium-dependent vasorelaxation appear to contribute to arterial vasomotor dysfunction in type 2 diabetes.

Lifestyle interventions (e.g. physical activity) and various anti-diabetic pharmacological therapies (e.g. the biguanide-class drug, metformin) are often prescribed to type 2 diabetic humans and have resulted in improved glycemic control in male ZDF (116; 134) and in type 2 diabetic patients (83; 87; 90), however studies examining the potential benefits of these interventions on diabetic vasomotor dysfunction are limited. Chronic exercise training improved endothelium-dependent relaxation in male OLETF rats (95), in male *db/db* mice (71; 96; 97), and in human type 2 diabetic patients (87), however it failed to reduce the augmented α_1 adrenergic vasocontraction in male *db/db* mice compared to wildtype controls (72). Endothelium-dependent relaxation was also improved in male ZDF treated with rosiglitazone, a thiazolidinedione-class anti-diabetic drug (108), and in metformin-treated male OLETF rats (91) and type 2 diabetic humans (90).

No reports of vasomotor function exist, however, in a *female* animal model of type 2 diabetes. Likewise, the effects of therapeutic interventions such as chronic exercise training or metformin treatment have not been examined in female type 2 diabetic animals. Understanding the potential therapeutic role for these interventions in alleviating the type 2 diabetic vasomotor dysfunction in females is important for the efficacious application of these therapies.

α_1 adrenergic vasocontraction and endothelium-dependent vasorelaxation were examined in thoracic aorta isolated from female Zucker lean fed a normal fat content, non-diabetogenic diet and from ZDF fed either a normal fat content diet or a high fat content, diabetogenic diet. Three additional groups of high fat-fed ZDF underwent 8 wk of: chronic exercise training, anti-diabetic metformin treatment, or both in combination. Furthermore, in

vitro supplementation with the SOD mimetic compound, Tempol, was studied to address the role of elevated oxidant stress on vasomotor dysfunction in this model. We hypothesized that: 1) elevated α_1 adrenergic vasocontraction would be observed in high fat-fed ZDF compared to Zucker lean, and would not be altered by exercise training, metformin treatment, or a combination of the two; 2) reduced endothelium-dependent vasorelaxation would be observed in high fat-fed ZDF compared to Zucker lean, and would be restored with exercise training, metformin treatment, and a combination of the two; and 3) the presence of Tempol would improve endothelium-dependent vasorelaxation in high fat-fed ZDF towards the level of Zucker lean and would not further improve vasorelaxation in high fat-fed ZDF that were exercise-trained, metformin-treated, or both.

5.4 Materials and Methods

Animals. Female Zucker lean (+/?) or ZDF (*fa/fa*) rats (n=72) were purchased from Charles River Laboratories (Senneville, QC) at 5 wk of age and were individually housed in a light- (12 h reverse cycle) and environment-controlled room with ad libitum access to standard Purina Formulab 5008 lab chow (normal fat content: 16.7 % kcal fat, PMI Nutrition International, St. Louis, MO) and tap water. Animal-related procedures in this study were approved by the University of Guelph Animal Care Committee. All animals in this chapter were also examined for metabolic studies in skeletal muscle (130). Chemical supplies were purchased from Sigma (St. Louis, MO) or BioShop (Burlington, ON) unless otherwise stated.

Diet. Rats were acclimatized for 1 wk in the animal facility and then assigned one of two ad libitum diet protocols for the 8 wk duration of the study. Zucker lean animals were fed Purina Formulab 5008 and remained lean and non-diabetic (Lean, n=12). ZDF littermates were fed either Purina Formulab 5008 and became obese, serving as a non-diabetic obesity control (Con, n=12), or a high fat diabetogenic diet (48.0 % kcal fat, Research Diets C13004, New Brunswick, NJ) and became obese and exhibited diabetic-like symptoms (n=48). High fat-fed ZDF were sub-divided into four groups: high fat diet only (HF, n=12), HF supplemented with the anti-diabetic agent metformin (HF-Met, n=12, details below), HF that were chronically exercise trained (HF-Ex, n=12, details below), and HF that received both Met and Ex interventions (HF-E+M, n=12).

Anti-diabetic treatment. HF-Met and HF-E+M received metformin that was dissolved in tap water (50 mg/ml) and mixed into the high fat diet (which was in a meal form) to produce a net dose of 250 mg/kg body mass (BM) for the first week and then increased to 500 mg/kg BM for the remaining 7 wk.

Chronic exercise training. HF-Ex and HF-E+M were acclimatized to forced treadmill running with two exercise bouts for no more than 5 min at 13 m/min and 0 % grade before commencement of the 8 wk training protocol. Exercise work rate and duration increased progressively from 15 min/d at 15 m/min and 0 % grade at the beginning of the training protocol, to a final target work rate of 120 min/d at 18–19 m/min and 10 % grade by

the end of the 4th week. This final work rate and duration were maintained for the remaining 4 wk of training. Endpoint testing occurred 48 h following the last exercise training session.

Endpoint tissue harvesting. All rats were approximately 14 wk of age at endpoint. Following an overnight fast, BM was recorded and rats were anesthetized with sodium pentobarbital (15 mg/kg BM, i.p.) and euthanized by cardiac excision and exsanguination. Descending thoracic aorta was excised and immersed in cold Krebs-bicarbonate buffer (4 ^{o}C, pH 7.4: 131.5 mM NaCl, 5.0 mM KCl, 2.5 mM $CaCl_2$-$2H_2O$, 1.2 mM NaH_2PO_4-H_2O, 1.2 mM $MgCl_2$, 11.2 mM D-glucose, 13.5 mM $NaHCO_3$, 2.5 μM EDTA-$2H_2O$). Adhering connective tissues were gently dissected away and the intact aorta was stored in cold (4 ^{o}C) Krebs-bicarbonate buffer for the duration of tissue collection (1 rat/group/testing day, harvested serially and in random order). Approximately 100–130 min elapsed from the beginning of tissue collection to the start of the myography protocol.

Vasomotor function. Adjacent rings (~2 mm axial length) were cut from each aorta with a razor blade. Remaining aortic tissue was gently blotted dry and stored at –80 ^{o}C for Western blotting. Rings were then loaded into the isolated myography baths and submersed in warmed (37 ^{o}C, pH 7.4) and continuously aerated (95 %:5 % O_2:CO_2) Krebs-bicarbonate buffer for isometric force recording as described in Chapter 2.

Initial resting tension was set at 1.0 g and increased by 0.5 g step-wise increments every 5 min until a level of 5.0 g was reached. Rings were incubated for 15 min in the absence (ND control) or presence of 4-hydroxy-TEMPO (Tempol, SOD mimetic, 10^{-4} M)

during which resting tension was adjusted to 5.0 g as needed. Steady-state contraction was elicited with two sequential exposures to KCl (membrane depolarizing agent, 60 mM) and the buffer changed to allow relaxation back to baseline after each exposure. Steady-state contraction was attained with PE (α_1 adrenergic receptor agonist, 10^{-7} M) and endothelium-dependent dose-response to ACh (muscarinic agonist, 10^{-10}–10^{-4} M) was examined, followed by wash out (3 x 5 min). In preliminary experiments, pre-incubation with Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, NOS inhibitor, 10^{-4} M) abolished ACh relaxation regardless of the presence of Tempol. Rings were contracted with PE (same manner as above) and endothelium-independent dose-response to sodium nitroprusside dihydrate (SNP, NO donor, 10^{-12}–10^{-4} M) was performed, followed by wash out (3 x 5 min). Dose-responses to PE (10^{-10}–10^{-4} M) were then examined.

Drugs and chemicals for vasomotor function experiments were dissolved in distilled water and final in-bath concentrations are reported. Absolute tension recordings (in g) for relaxation responses were expressed as a percentage of the preceding developed tension to PE (in g) on a ring-by-ring basis. Dose-responses were fit to a sigmoidal curve using non-linear regression analysis (Prism 4.03; GraphPad Software, La Jolla, CA) to obtain EC_{50} and maximal responses of each agonist.

Fasting blood glucose and plasma insulin. These data were reported by Smith *et al.* (130) and are transcribed in the present study for reference (Figure 5-1).

Protein expression. Frozen sections (2–4 mm) of thoracic aorta were prepared for Western blotting as described in Chapter 2 with the following modifications. In the present study, samples were hand-homogenized in ~100 µl ice-cold urea buffer and centrifuged (8000 *g*, 2 min, 4 ^{o}C). Uniformity of protein loading and transfer was confirmed by ponceau red stain. Membranes were blocked in 5 % wt/vol BSA (eNOS and α_1 adrenergic receptor) or 10 % wt/vol skim milk (SOD-1 and -2) dissolved in tris-buffered saline with Tween®-20 (TBS-T). Primary antibodies: mouse anti-eNOS (1:500; BD Biosciences, Franklin Lakes, NJ), rabbit anti-SOD-1 and -SOD-2 (both 1:1000; Assay Designs/Stressgen, Ann Arbor, MI), rabbit anti-α_1 adrenergic receptor (recognizes all subtypes of α_1 adrenergic receptor; 1:400; Sigma). Secondary antibodies: goat anti-mouse (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-rabbit (1:2000 for SOD-1, SOD-2, and α_1 adrenergic receptor; Santa Cruz Biotechnology).

Statistics. Values are mean±SEM. Data were analyzed by 1-way or repeated measures ANOVA (vasomotor function) and unpaired two-tailed t-test (protein expression). Significance was accepted at $p<0.05$. SAS 9.1 (SAS Institute, Cary, NC) and Excel software (Microsoft, Redmond, WA) were used for statistical analyses.

5.5 Results

Model verification. These physical and metabolic data are reported by Smith et al. (130) and are transcribed in the thesis for reference. Endpoint BM was similarly elevated in

all ZDF groups vs. Lean (Figure 5-1). Fasting blood glucose was elevated in HF vs. Lean, and this hyperglycemia was partially blunted in HF-Met, HF-Ex, and HF-E+M such that it was lower in these groups vs. HF. The greatest treatment-induced reductions in hyperglycemia occurred in HF-Ex and HF-E+M, which had similar glucose levels to Con. Fasting plasma insulin was higher in all high fat-fed ZDF vs. Lean.

Contractile responses to a single dose of KCl or PE. Contraction induced by 60 mM KCl in the ND condition was elevated in HF, HF-Ex, and HF-E+M vs. Lean (Table 5-1). Additionally, HF-E+M exhibited greater KCl contraction vs. both Con and HF-Met. The KCl response was lowered in HF-E+M in the presence of Tempol, and responses were similar across all Tempol-treated groups. Contraction to 10^{-7} M PE prior to ACh dose-response in the ND condition was greater in all ZDF vs. Lean (Table 5-1). HF-E+M responses were additionally elevated vs. Con and HF. Tempol reduced the PE response in HF-E+M and similar contractions were observed in all groups.

α_1 adrenergic vasocontraction. PE dose-responses were elevated to a similar extent in Con (10^{-7}–10^{-4} M) and HF ($10^{-6.5}$–10^{-4} M) vs. Lean (Figure 5-2A). Compared to HF, PE contractions were blunted in HF-Ex ($10^{-6.5}$–10^{-4} M) and in HF-E+M ($10^{-6.5}$–10^{-4} M), and to a greater extent in the former. PE contractions were similar between HF and HF-Met. Sensitivity to PE was reduced (greater EC_{50}) in HF-E+M vs. Lean, Con, HF, and HF-Ex, whereas it was similar across all other groups (Figure 5-2C). Maximal response to PE in the ND condition was greater in all groups vs. Lean, with the exception of HF-Ex which

exhibited a maximal PE contraction that was lower than Con and HF, and that was similar to Lean.

PE dose-responses (Figures 5-2B, 5-3A and 5-3E) and maximal contractions (Figure 5-2D) were elevated in the presence of Tempol (vs. ND) in Lean and HF-Ex but were not altered within any other group (Figures 5-2D, 5-3B–D and 5-3F), such that maximal PE contractions were similar across all Tempol-treated groups (Figures 5-2B and 5-2D). Sensitivity to PE (EC_{50}) was not altered by Tempol (vs. ND) within any group (Figure 5-2C). PE sensitivity was greater in Tempol-treated Lean, HF, and HF-Met vs. both HF-Ex and HF-E+M. Sensitivity was also greater in Tempol-treated Con vs. HF-Ex.

α adrenergic contractile dose-responses are most commonly presented as absolute tension development (in units of force) in the literature reviewed in the thesis (72; 101; 107; 112; 133; 152). However, to account for intergroup variation in smooth muscle content, adrenergic contractions are sometimes normalized to tissue mass (69; 157) or to a reference contraction elicited by a maximal dose of KCl (4; 21; 107). The present PE contractile dose-responses were normalized to the second reference contraction to 60 mM KCl to confirm that the chosen method of data presentation did not affect the conclusions drawn. Similar to absolute data presented in Figures 5-2 and 5-3, normalized PE contractions in the ND condition were approximately 2-fold higher in Con and HF vs. Lean, and these elevations were abolished in HF-Ex, but not in HF-Met (Figure D-1, Appendix D). However, in contrast to the absolute PE response, HF-E+M exhibited a clear reduction in normalized PE contraction that was similar to Lean and HF-Ex. Tempol enhanced normalized PE

contractions in Lean and HF-Ex only (Figure D-2, Appendix D), similar to its effect on absolute responses.

Endothelium-dependent vasomotor function. Endothelium-dependent vasorelaxation elicited by ACh was similar across all groups within ND or Tempol drug conditions (Figures 5-4A and B). Compared to ND, Tempol-treated HF-Ex exhibited greater relaxation to ACh ($10^{-7.5}$–10^{-4} M, Figure 5-4C) and an elevated maximum relaxation to ACh (ND vs. Tempol: 78.7±4.7 vs. 93.6±4.7 %, respectively, $p<0.05$). Tempol did not alter the ACh dose-response or maximum relaxation (ND vs. Tempol: 78.7±2.7 vs. 83.0±2.6 %, respectively) within any other group, or the EC_{50} (ND vs. Tempol: 55.0±5.3 vs. 52.8±9.2 nM, respectively) within any group.

Endothelium-independent vasomotor function. Endothelium-independent vasorelaxation to SNP was similar across all groups in the ND condition (Figure 5-5A). ANOVA of the SNP dose-response revealed a significant group-by-dose interaction term in the Tempol drug condition (Figure 5-5B) however between-group differences were very small and difficult to interpret. Non-linear regression revealed a similar EC_{50} (4.7±0.6 nM) and maximal relaxation (105.3±0.6 %) to SNP across all groups (within drug condition) and drug conditions (within group).

Protein expression. Aortic protein expression of α_1 adrenergic receptor was elevated in Con (+27 %) vs. Lean and was similar across all other groups (Figure 5-6A). eNOS

protein expression was similar across all groups, with the exception of a reduction in HF-Ex (–41 %) vs. HF-Met (Figure 5-6B). All groups exhibited similar aortic expression of SOD-1 and SOD-2 proteins (Figures 5-6C and D).

5.6 Discussion

This study examined type 2 diabetes- and obesity-related impairments of aortic vasomotor responses of female Zucker lean and ZDF rats, and the potentially therapeutic effects of anti-diabetic metformin administration and chronic exercise training, alone and in combination. The major novel findings include: 1) α_1 adrenergic contractions to PE were greatly elevated in Con and HF compared to Lean and were blunted by chronic exercise training alone such that responses in HF-Ex were similar to Lean; 2) endothelium-dependent, NO-mediated vasorelaxation to ACh was similar across all groups, despite the severe obesity and hyperinsulinemia in all ZDF, and the additional extreme hyperglycemia in high fat-fed ZDF; and 3) Tempol did not alter the ACh response in any group, with the exception of HF-Ex in which relaxation was improved.

α_1 adrenergic-mediated contraction is elevated in ZDF and is attenuated by chronic exercise training. Contractile dose-responses to PE were greatly elevated in HF compared to Lean in the present study, confirming the first part of our first hypothesis and indicating that high fat diet-induced hyperglycemia and hyperinsulinemia would be associated with α_1 adrenergic hyperresponsiveness in female ZDF aorta. This finding corroborates previous reports of elevated α adrenergic contractile responses in various arteries isolated from male

ZDF (79; 149), male OLETF (160), male *db/db* (72; 152), and sex-unspecified *ob/ob* (107). Interestingly, normal fat-fed Con animals, which displayed a blunted endpoint hyperglycemia and hyperinsulinemia compared to HF (Figure 5-1), exhibited similar levels of obesity (Figure 5-1) and elevations in α_1 adrenergic vasocontraction (Figure 5-2) compared to HF. These data suggest that either: 1) there is an obesity-dependent component of the elevation in α_1 adrenergic contraction; or 2) a threshold of hyperglycemia exists, above which enhanced α_1 adrenergic contraction occurs, and that blood glucose levels of both Con and HF surpassed this threshold. The increased protein expression of α_1 adrenergic receptors in Con vs. Lean in the present study could be one contributor to the observed adrenergic hyperresponsiveness in the former, however there are likely other mechanisms responsible since the elevation of α_1 adrenergic receptor expression was small and did not parallel functional findings across all groups. Previous reports have also suggested roles for protein kinase C (PKC) and rho kinase (ROCK) pathways in governing the adrenergic hyperresponsiveness seen in male ZDF (149) and *db/db* (72; 152).

Exercise training alone imparted the greatest suppression of absolute α_1 adrenergic contraction in high fat-fed ZDF. This finding disagrees with the second part of our first hypothesis as well as with a study that reported no chronic exercise training-induced reduction of the elevated PE contractions of male *db/db* mice compared to wildtype (72). The blunted PE response in HF-Ex does, however, corroborate previous reports of reduced aortic α adrenergic contraction in exercise-trained male spontaneously hypertensive (26) and normotensive Sprague-Dawley rats (133). It is plausible that exercise training in the present study blunted PE contractions by inhibition of the PKC or ROCK signaling pathways, which

link α_1 adrenoceptors to myosin activity in vascular smooth muscle. Regardless of the exact mechanisms through which exercise training acts, it seems as though α_1 adrenergic responsiveness differentially sensitive to exercise training in female ZDF rats and in male *db/db* mice (72). The finding that metformin alone had no statistical effect on PE contraction partially confirms the second part of our first hypothesis. This observation, plus the fact that combination treatment of exercise plus metformin imparted a smaller reduction of absolute PE contraction than exercise alone, suggests that metformin counteracts the chronic exercise training-induced blunting of α_1 adrenergic contractions. If exercise training does in fact blunt PE vasocontraction by inhibiting either the PKC or ROCK pathways, it could be that co-treatment with metformin might counteract this exercise effect by directly activating these pathways. To our knowledge, the present data from female ZDF and another report examining aortic responses of male *db/db* mice (72) are the only reports that have systematically examined the effects of chronic exercise training on vascular α adrenergic contractile responses in a model of obesity and type 2 diabetes. Additionally, no previous studies have examined the effects of an anti-diabetic drug (e.g. metformin) on vascular adrenergic responses in a model of type 2 diabetes.

Interestingly, the presence of Tempol elevated PE contractions in Lean and HF-Ex but did not alter responses in other groups, resulting in similar contractions in all Tempol-treated groups. These findings were unexpected and may suggest a role for superoxide anion in *suppressing* the PE contractile response in Zucker lean and in ZDF following exercise training. Alternatively, it is possible that Tempol may produce these effects in another

manner altogether, for example by shifting redox balance within the vessel and altering expression of redox-sensitive genes.

Endothelium-dependent vasomotor function is unimpaired in female ZDF and is unaltered by metformin, exercise training, or a combination of both. Aortic endothelium-dependent vasorelaxation to ACh of HF was robust and similar to that of Lean and Con groups in the present study. This observation was in contrast to the first part of our second hypothesis that the extreme obesity and type 2 diabetic-like symptoms exhibited by HF would be associated with endothelial vasomotor dysfunction. Additionally, endothelium-independent vasorelaxation to SNP was robust and similar across all groups, indicating that there were no differences in vascular smooth muscle responsiveness to NO. Impaired aortic endothelium-dependent vasorelaxation has been observed in male ZDF (57), and ZDF of unspecified sex (18; 112) over the age of 22 wk, in which blood glucose and BM would have been elevated for several weeks (34; 115) and in which plasma insulin levels would have begun to decline due to exhaustion of the pancreatic β cells (115). Aortic endothelial vasomotor dysfunction has also been described in male ZDF as young as 9–11 wk (28). These animals would have exhibited extreme diabetic-like symptoms for a shorter duration than the present 14 wk female ZDF (34; 42; 115), which exhibited severe hyperglycemia and hyperinsulinemia vs. Lean and Con groups for 4–8 wk prior to testing (130). Two studies, however, showed that 8–24 wk old male and sex-unspecified ZDF exhibited robust aortic ACh relaxations similar to Zucker lean counterparts (18; 112). Based on the observed aortic vasomotor impairments in male ZDF (18; 28; 57; 112), it was reasonable to hypothesize that 14 wk old female ZDF that had been exposed to the high fat diabetogenic stimulus for 8 wk

would have exhibited signs of deteriorating endothelial function. It is possible that the female sex and/or the young adult age of the present ZDF contributed to preservation of endothelium-dependent vasomotor responses, however the present study design did not allow for sex or age comparisons to be made.

Chronic administration of the anti-diabetic drug metformin and exercise training, alone or in combination, abrogated the extreme hyperglycemia in high fat-fed ZDF, however the second part of our second hypothesis was rejected with the observation that ACh responses were not augmented following these interventions. Both metformin (67; 90; 91) and chronic exercise training (71; 87; 95-97) have separately been shown to enhance endothelium-dependent vasorelaxation in various arteries isolated from male animal models of insulin resistance (67) and type 2 diabetes (91; 95-97) as well as in the forearm vasculature of type 2 diabetic humans (87; 90). The fact that endothelial vasorelaxation was robust and unimpaired in all groups (including Con and HF) in the present study could have precluded any potential enhancements of vasomotor function via metformin and/or exercise training despite significant attenuation of high fat diet-induced hyperglycemia with these interventions. Additionally, differences between the experimental models referenced cannot be excluded as a potential influence since to our knowledge the present study is the first to examine the effects of anti-diabetic metformin drug treatment or a lifestyle intervention such as exercise training on vascular function in the ZDF rat.

Tempol improves endothelium-dependent relaxation in chronically exercise-trained ZDF, but not in sedentary ZDF. Previous studies have shown that the reduction in endothelial vasomotor function in ZDF compared to lean counterparts is associated with

elevated vascular reactive oxygen species (ROS) and oxidant stress markers (18; 28; 32; 108; 110; 111). Manipulation of vascular ROS with supplemental anti-oxidants or inhibition of cellular ROS-generating mechanisms has been shown to improve endothelial vasorelaxation (18; 28; 50; 108; 112) and to reduce vascular ROS levels (108) and markers of oxidant stress (18; 108) in ZDF. In the present study the SOD mimetic Tempol caused a small elevation in relaxation to $10^{-7.5}$–10^{-4} M ACh in HF-Ex but did not alter the response in any other group. This finding was in contrast to our third hypothesis; however, in light of the fact that no impairment of relaxation was observed in any group, it is not surprising that Tempol was unable to improve this response in most groups. Furthermore, these data suggest that small chronic exercise-induced enhancements in NO-mediated vasorelaxation may have been suppressed by concomitant elevations in vascular oxidant stress and that this benefit of exercise training was unmasked in the presence of Tempol. The aortic protein expression of eNOS was lower in HF-Ex vs. HF-Met, but this specific reduction is of questionable physiological relevance since ACh relaxations were similar between these two groups. Protein content of the anti-oxidant enzymes SOD-1 and SOD-2 were also similar across all groups, indicating that any perturbation of cellular redox status that may have occurred in HF-Ex was not enough to elicit a compensatory upregulation of aortic SOD. While a single bout of moderate- to high-intensity exercise can acutely elevate vascular oxidant stress, the long-term benefits of a chronic exercise training regimen include an overall lowering of vascular oxidant stress (reviewed in (121)). It is unlikely that residual elevations of oxidant stress from the last exercise bout could have accounted for the Tempol-induced improvement in the ACh relaxation in HF-Ex since the last training session occurred 48 h before

experiments were performed. Additionally, if the last exercise bout had been responsible for the Tempol-induced improvement of vasomotor function in HF-Ex, then a similar effect might also have been expected in HF-E+M, which it was not.

Conclusions. The present study examines α_1 adrenergic receptor-mediated contraction and endothelium-dependent relaxation responses in aortic segments isolated from a female diet-induced rat model of obesity and type 2 diabetes mellitus. α_1 adrenergic contractions to PE were greatly elevated in obese and obese-diabetic ZDF compared to Zucker lean counterparts. This hyperreactivity was largely reversed following 8 wk of exercise training alone, but was only partially abrogated with 8 wk of anti-diabetic metformin treatment in combination with exercise and remained unaltered with metformin treatment alone. Based on previous reports in male ZDF, it was expected that impaired endothelial function would accompany the severe hyperglycemia and hyperinsulinemia in obese-diabetic animals. However, in contrast to our hypothesis, endothelium-dependent, ACh-mediated vasorelaxation was robust and similar in all groups regardless of obesity, diabetic status, or treatment intervention. The precise mechanisms providing vascular protection in the face of severe obesity and substantial disruptions in systemic glucose and insulin homeostasis remains unclear, however candidates include those related to the animals' female sex, young adult age, or duration of exposure to the high fat diabetogenic stimulus. Together, these data support alterations in aortic α_1 adrenergic contractility, but not endothelium-dependent relaxation, in the high fat-fed female ZDF. Additionally, chronic exercise training, but not anti-diabetic metformin treatment, is able to abrogate the diabetes-induced hyperresponsiveness to α_1 adrenergic stimulation.

5.7 Acknowledgements

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Table 5-1 Contractile responses to single doses of KCl and PE.

		ZDF				
	Lean	Con	HF	HF-Met	HF-Ex	HF-E+M
		KCl contraction (60 mM), g				
ND	0.74±0.10	0.87±0.15	1.10±0.13[a]	1.07±0.15	1.21±0.12[a]	1.45±0.09[abd]
Tempol	0.80±0.08	0.90±0.16	1.09±0.10	0.67±0.13	1.13±0.15	0.95±0.13[f]
		PE contraction (10^{-7} M, prior to ACh dose-response), g				
ND	1.26±0.12	1.62±0.17[a]	1.72±0.13[a]	1.82±0.15[a]	1.90±0.14[a]	2.15±0.08[abc]
Tempol	1.37±0.08	1.61±0.17	1.65±0.09	1.45±0.13	1.68±0.18	1.57±0.18[f]

Values are mean±SEM. n=8–11 in singlet to duplicate rings. $p<0.05$ vs.: [a] Lean, [b] Con, [c] HF, [d] HF-Met, [e] HF-Ex. $p<0.05$ vs.: [f] ND.

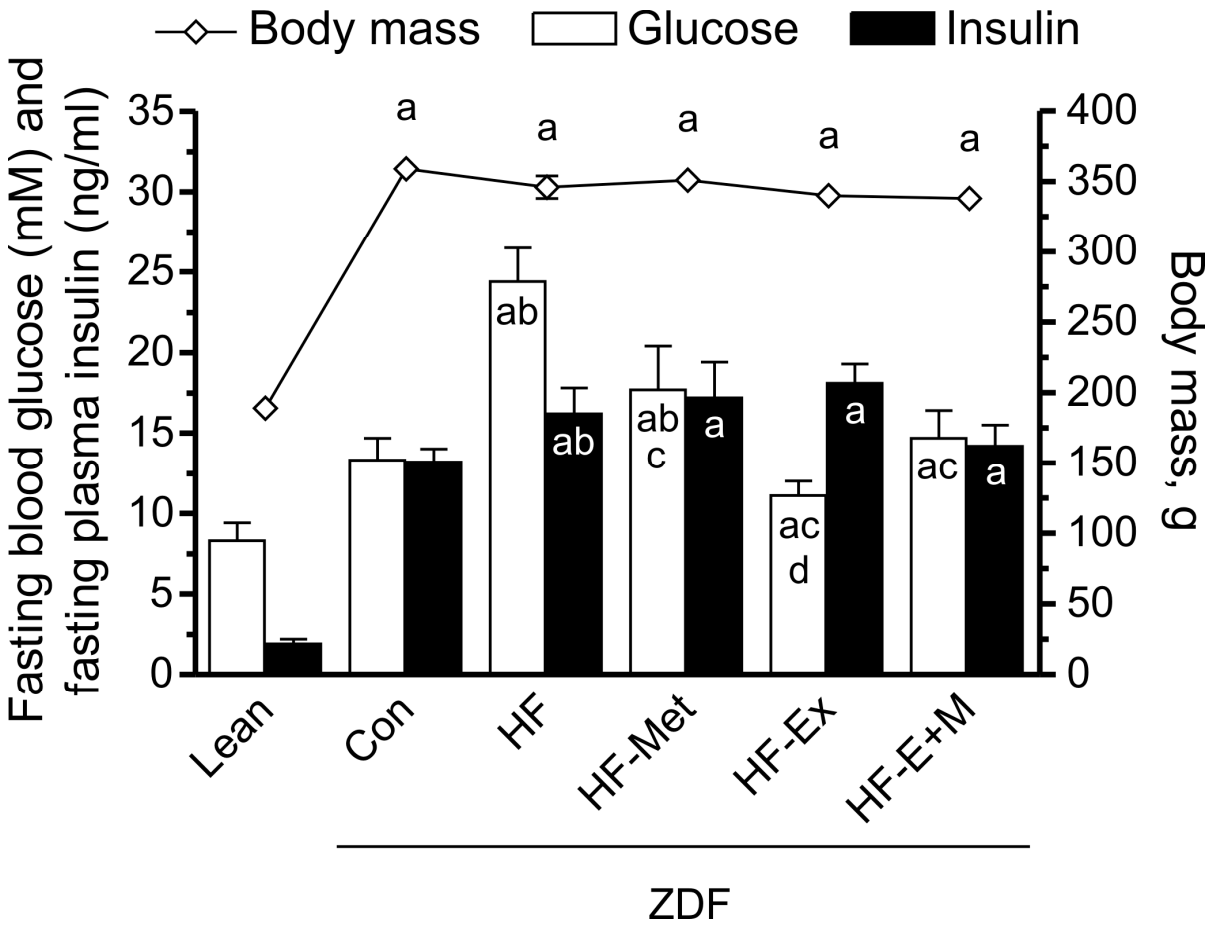

Figure 5-1 Fasting blood glucose (open bars, left y-axis, in mM), fasting plasma insulin (closed bars, left y-axis, in ng/ml), and BM (line graph, right y-axis, in g) of 14 wk-old female Zucker lean and ZDF rats. Values are mean±SEM. n=12. $p<0.05$ (within a measurement) vs.: [a] Lean, [b] Con, [c] HF, [d] HF-Met. This data has been published previously (130) and is transcribed in the thesis for reference purposes.

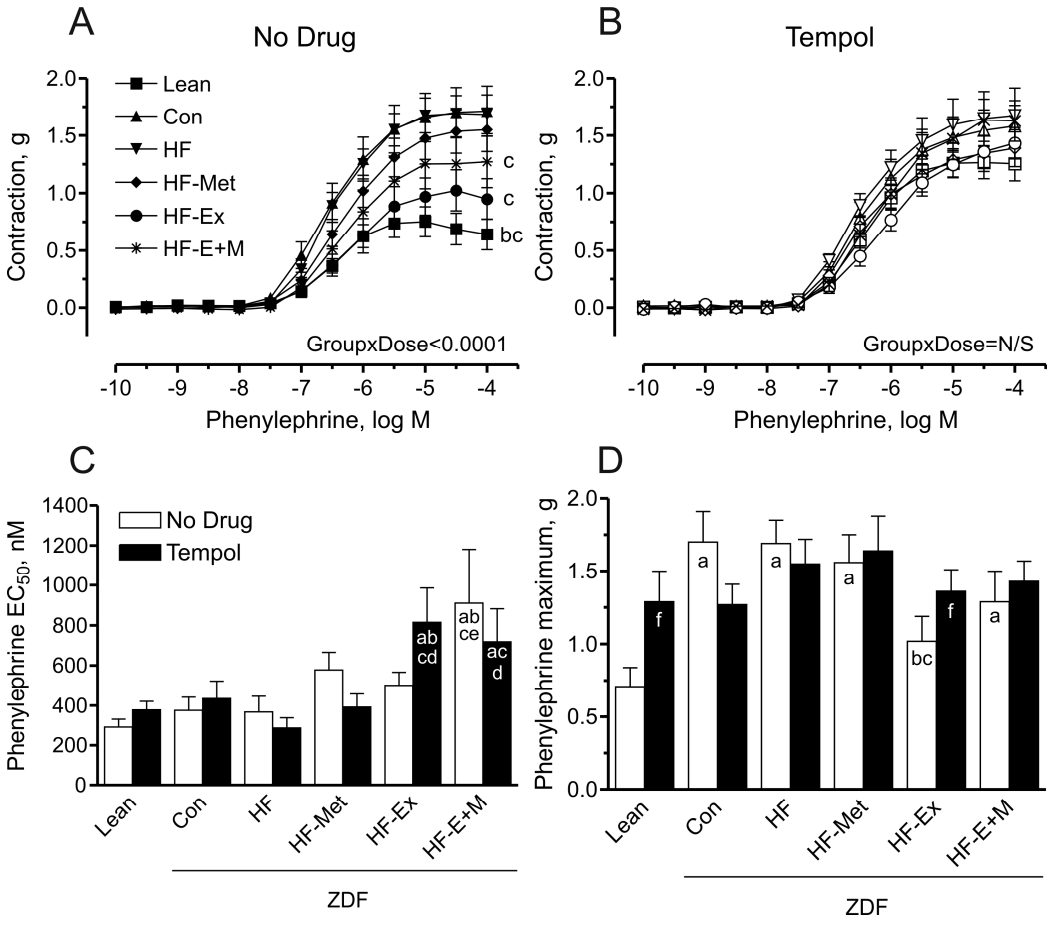

Figure 5-2 Aortic α_1 adrenergic receptor-mediated vasocontractile dose-response to PE of 14 wk-old female Zucker lean and ZDF rats in the absence (ND control, panel A) and in the presence of the SOD mimetic, Tempol (panel B). EC_{50} (panel C) and maximum contraction (panel D) characteristics of the PE dose-response were generated by non-linear regression curve fitting. Values are mean±SEM, expressed as grams of developed tension (panels A, B, and D) and as concentration (in nM) of PE required to elicit 50 % of the maximal contraction (panel C). n=7–11 in singlet rings. Panel A: $p<0.05$ vs.: [b] Con (10^{-7}–10^{-4} M), [c] HF ($10^{-6.5}$–10^{-4} M), respectively. Panels C and D: $p<0.05$ vs.: [a] Lean, [b] Con, [c] HF, [d] HF-Met, [e] HF-Ex. $p<0.05$ vs.: [f] ND.

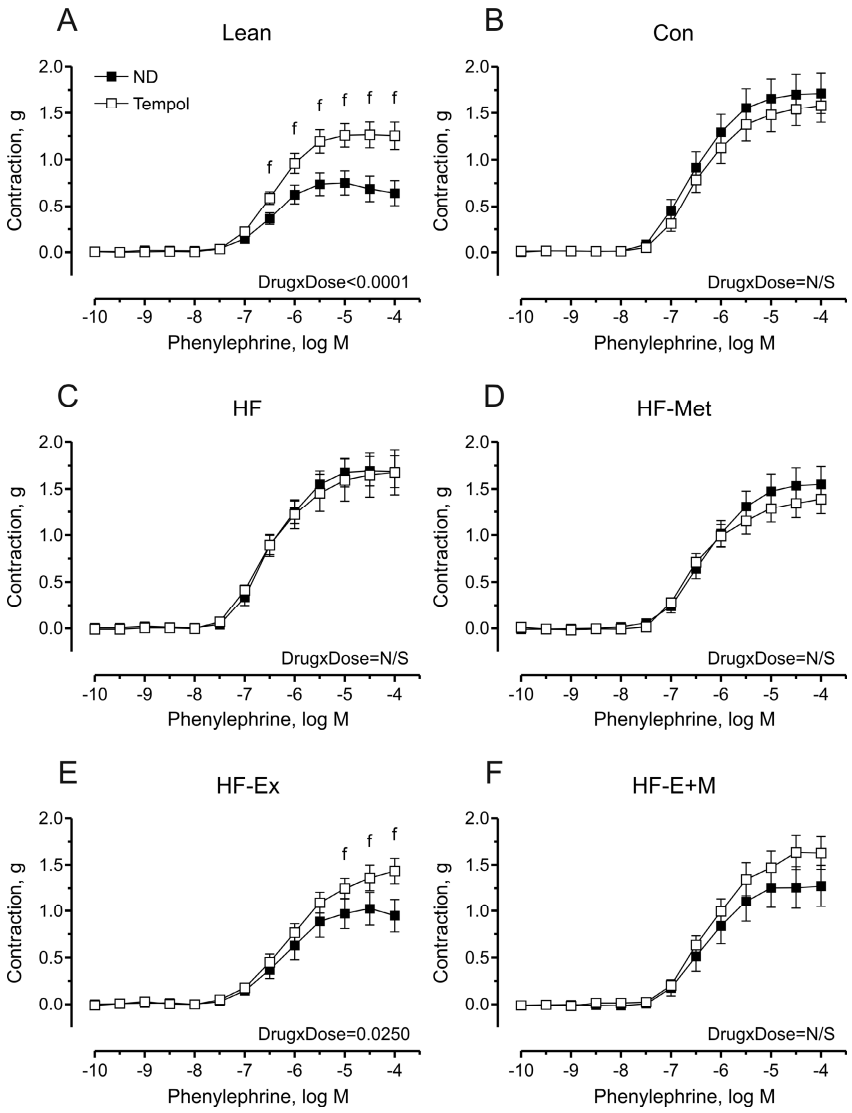

Figure 5-3 Aortic α_1 adrenergic receptor-mediated vasocontractile dose-response to PE of 14 wk-old female Zucker lean (panel A) and ZDF rats (panels B–F) in the absence (ND control, closed symbols) and in the presence of the SOD mimetic, Tempol (open symbols). These data are transcribed from Figures 5-2A and B and re-plotted by group for easier comparison of intra-group drug conditions. Values are mean±SEM, expressed as grams of developed tension. n=8–11 in singlet rings. $p<0.05$ vs.: ^{f} ND.

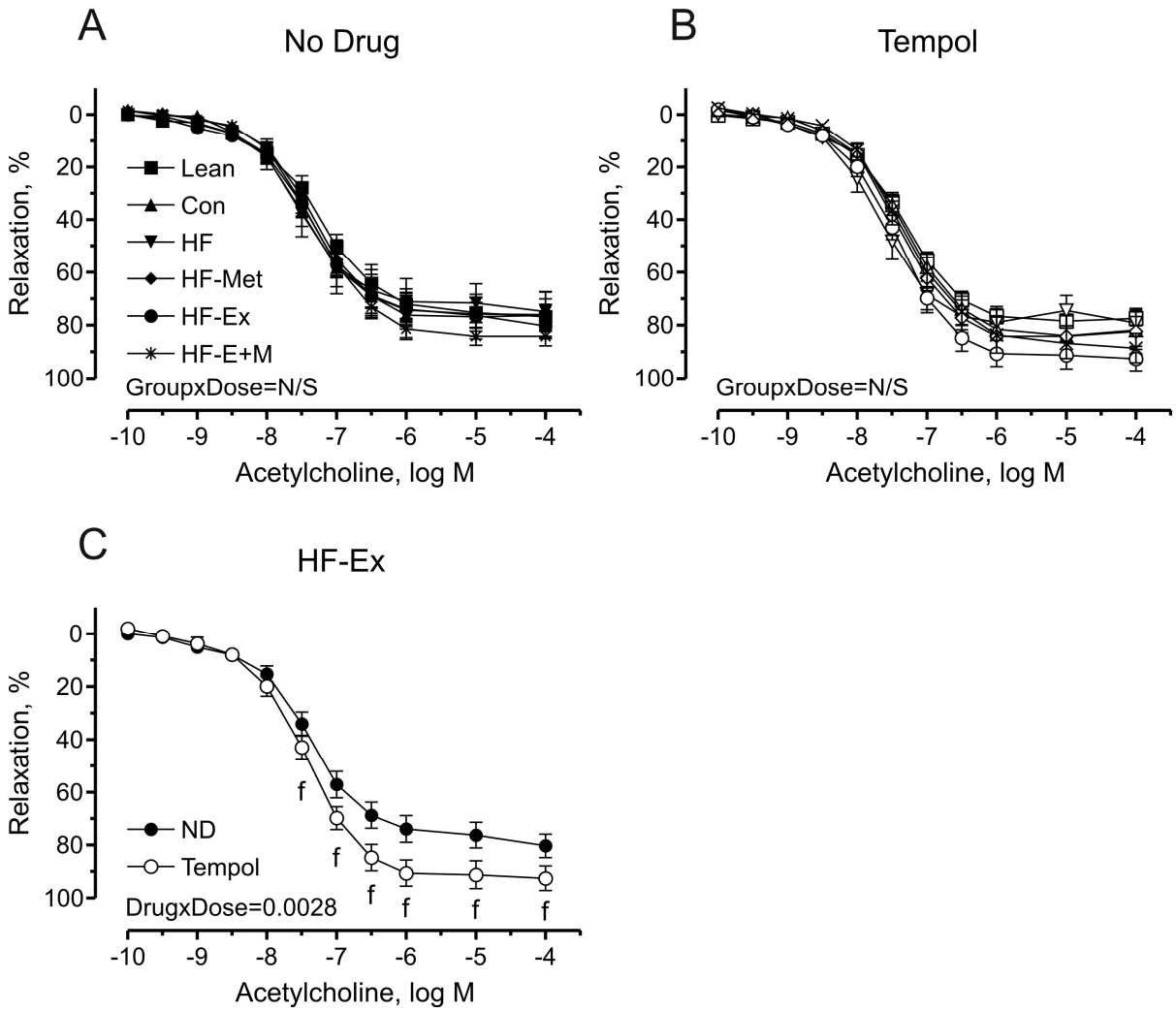

Figure 5-4 Aortic endothelium-dependent, NO-mediated relaxation to ACh of 14 wk-old female Zucker lean and ZDF rats in the absence (ND control, panel A) and presence of the SOD mimetic, Tempol (panel B). Responses of HF-Ex in the ND (closed symbols) and Tempol (open symbols) drug conditions are re-plotted in panel C. Values are mean±SEM, expressed as a percentage of the prior contraction to 10^{-7} M PE. n=8–11 in singlet to duplicate rings. $p<0.05$ vs.: ^{f} ND.

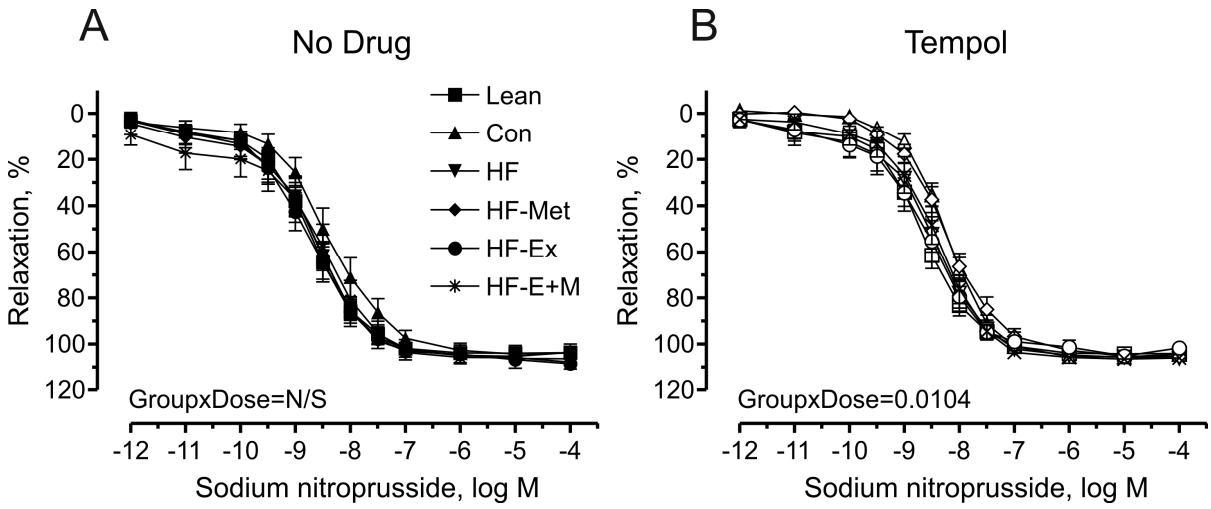

Figure 5-5 Aortic endothelium-independent, NO-mediated relaxation to SNP of 14 wk-old female Zucker lean and ZDF rats in the absence (ND control, panel A) and presence of the SOD mimetic, Tempol (panel B). Values are mean±SEM, expressed as a percentage of the prior contraction to 10^{-7} M PE. n=8–11 in singlet rings.

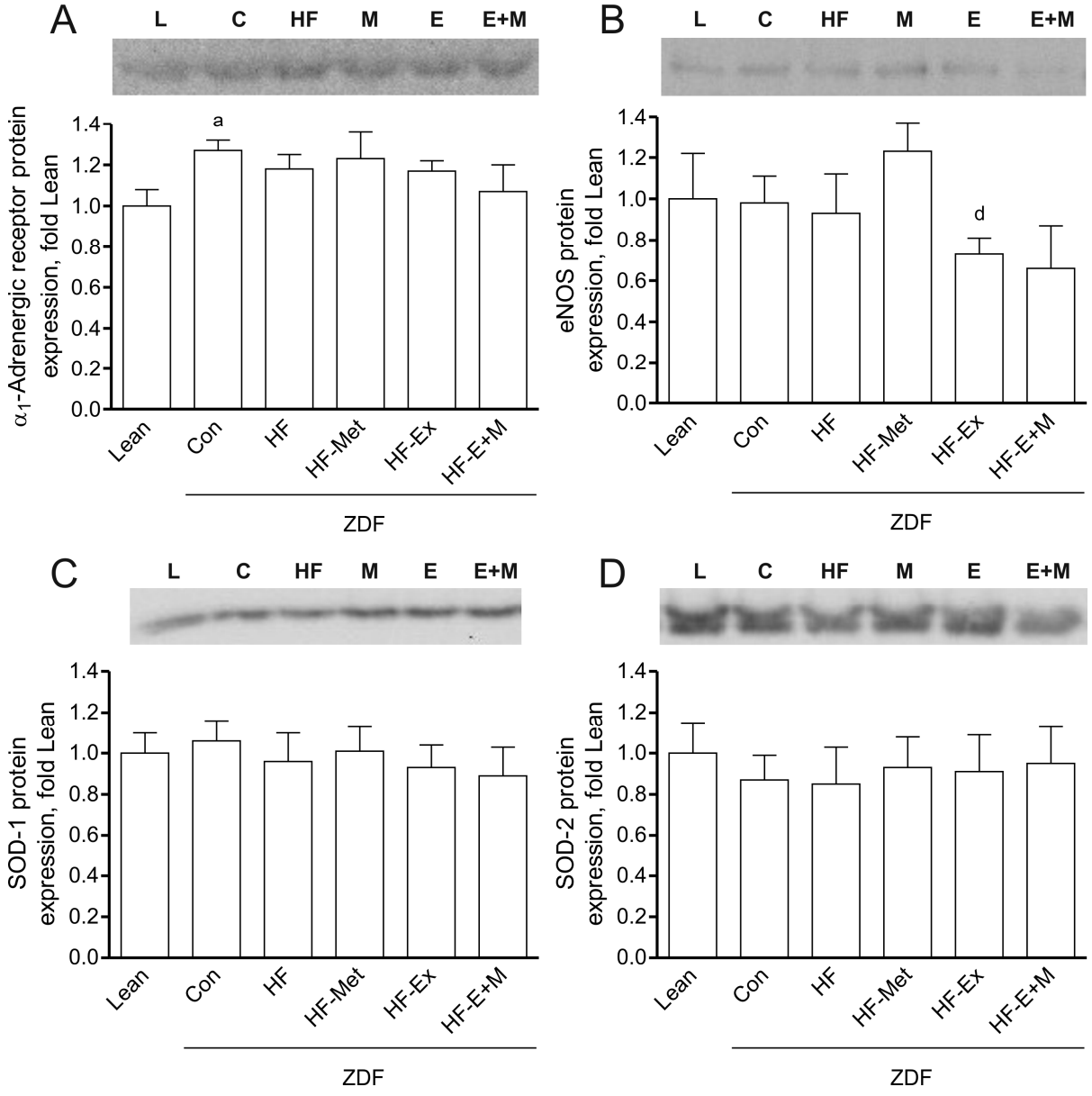

Figure 5-6 Relative aortic protein expression of α_1 adrenergic receptor (panel A), eNOS (panel B), SOD-1 (panel C), and SOD-2 (panel D). Representative Western blots are shown above each panel. Values are mean±SEM, expressed as fold differences compared to the Lean group. n=6 in duplicate (panel A). n=3–4 in singlet to duplicate (panel B). n=7–10 in singlet to quadruplicate (panels C and D). $p<0.05$ vs.: [a] Lean, [d] HF-Met.

Chapter 6

Discussion

The global purpose of this thesis is to identify and characterize the pathways controlling impaired vasomotor function in female animal models of two chronic disease states: hypertension and type 2 diabetes mellitus. Based on studies in male animals and in humans, it is apparent that impaired endothelial function is central to macrovascular complications that arise in these disease states. However, there are few studies that have examined these parameters in hypertensive or type 2 diabetic *female* animals. Comparing endothelial responses of females to those of males will illuminate sex-specific phenomena controlling vascular function. It is especially important to understand female sex-specific differences in hypertension and type 2 diabetes since impaired endothelial function governs many additional cardiovascular conditions associated with these diseases. In this thesis, endothelium-dependent vasomotor function was assessed in ageing female spontaneously hypertensive rats (SHR) and compared to male counterparts. Furthermore, mechanisms governing endothelial impairments in ageing female SHR were examined. The therapeutic potential of chronic dietary docosahexaenoic acid (DHA, 22:6 n-3 polyunsaturated fatty acid (PUFA)) supplementation was assessed in restoring the endothelium-dependent relaxation in adult male SHR. The endothelium-dependent relaxation and α_1 adrenergic contraction responses of female type 2 diabetic Zucker diabetic fatty rats (ZDF) were also characterized. Finally, the effects of established anti-diabetic therapeutic interventions, metformin

pharmacotherapy and chronic exercise training, alone and in combination, on vascular function of female ZDF were tested. The overriding goal of the thesis is that these data will contribute to the currently limited knowledge of vasomotor function in hypertensive and type 2 diabetic females, and that this new information may eventually lead to clinical interventions that more appropriately address sex-specific needs.

The thesis Discussion is organized into one sub-section per specific purpose listed in the thesis Introduction. The sub-sections are organized in a common manner to improve readability. The first paragraph of each sub-section describes the experimental approach, the novelty and importance of the specific purpose, and generalized major findings. The main paragraphs of each sub-section describe the more specific main findings, how the data fits with the literature, and speculate on mechanisms responsible where appropriate. The final paragraph of each sub-section discusses any overall observations and themes relating to the specific purpose and the thesis, directions for future research, and potential clinical relevance of the main findings. The thesis Discussion is concluded with a brief section describing overall conclusions and relevance of the thesis work as a whole.

6.1 Specific Purpose #1: To characterize potential sex differences in vasomotor responses of ageing hypertensive rats

This first specific purpose was approached by comparing endothelial vasomotor responses in 16 and 30 wk old male and female SHR. The importance of this specific purpose is that it is the first characterization of ageing-related alterations in vasomotor

responses in *female* SHR. The major finding is that sex differences occur in 16 wk old SHR and are abrogated in 30 wk old SHR.

30wk fSHR exhibited a significant reduction in endothelium-dependent acetylcholine (ACh) relaxation compared to 16wk counterparts, such that responses in the former were nearly as impaired as those of 30wk mSHR (Figure 2-1). Interestingly, endothelium-dependent contractions were not altered with ageing in fSHR (Figure 3-1). These relaxation data compliment previous reports of ageing-induced impairments of endothelium-dependent relaxation and enhanced endothelium-dependent contraction in male SHR (48; 52; 59; 62; 63; 68; 70; 85; 144; 154) and extend these ageing observations to the female sex. The sex differences in endothelial relaxation observed at 16 wk of age in SHR in this thesis and previously (70) appear to be nearly abolished by 30 wk of age in this strain. One factor contributing to the lack of an ageing effect in endothelial contraction of fSHR is that *16wk* fSHR exhibited a moderate contraction response compared to males that was still much more pronounced compared to 16wk fWKY counterparts. This finding in 16wk fSHR seems to contradict the essentially normal ACh relaxations in this group that were similar to age-matched fWKY. One plausible explanation is that higher ACh doses induce the aortic endothelium of 16wk fSHR to produce moderate levels of vasoconstrictory signaling molecules (e.g. cyclooxygenase (COX)-generated endothelium-derived contracting factors (EDCF)) and a compensatory elevation in nitric oxide (NO) production. It may be only in the presence of NO synthase (NOS) inhibition (Nω-nitro-L-arginine methyl ester (L-NAME) was used in all ACh contraction experiments) that this moderate level of vasoconstrictory molecule production is unmasked. Despite the observation that 16wk fSHR exhibit similar

aortic endothelial NOS (eNOS) and COX-2 protein expression and lower aortic COX-1 protein expression compared to 30wk counterparts (Figure 2-5 and data not shown), the potential contribution of altered activity of these enzymes and/or altered destruction/target tissue sensitivity (i.e. altered bioavailability) of their products cannot be discounted.

Therefore, the present data indicate that ageing induces impaired endothelial vasomotor function in female SHR and, in turn, blunts sex differences in this response. This raises the obvious question regarding the mechanism responsible for the sex difference that occurs in 16wk SHR and how it is influenced by ageing. It is possible that ageing alters sex hormone signaling in female SHR in a manner that suppresses endothelial vasomotor function. The studies comprising the thesis were not designed to examine potential roles for sex hormones in the sex differences observed. Examining this factor would involve very deliberate and controlled manipulation of sex hormone levels in SHR of both sexes. Nevertheless, the sex differences that exist in young SHR very clearly deteriorate with ageing. This finding will alert researchers to the importance of age when designing studies examining sex differences in vascular function. Additionally, it would be worthwhile exploring sex-specific treatment strategies for human hypertension, particularly in younger patients.

6.2 Specific Purpose #2: To provide understanding of mechanisms governing vasomotor responses of ageing hypertensive female rats

This second specific purpose was addressed by examining whether the COX-EDCF-thromboxane A_2/prostaglandin (TP) receptor pathway contributes to the reduced endothelial relaxation that develops in female SHR between 16 and 30 wk of age and to the moderate endothelial contractions of 16wk and 30wk fSHR, similar to observations in male ageing SHR. Understanding mechanisms controlling this deterioration of endothelial function in ageing female SHR may introduce new targets for therapy in hypertensive women. It was determined that COX-1, COX-2, and TP receptor all contribute to specific aspects of dysfunction in 30wk fSHR in a manner similar to that observed in male counterparts.

Detailed discussion of this mechanism will first focus on the COX-1- and TP receptor-dependent control of endothelium-dependent relaxation and contraction responses to *higher* ACh doses. It was initially observed that non-isoform-specific inhibition of COX-1 and -2 with indomethacin (Indo) restored the previously impaired ACh relaxation of 30wk fSHR (Figure 2-2). The use of more specific, isoform-preferential COX inhibitors subsequently revealed a primary role for a product of COX-1 in the reduced relaxation and moderate contraction to higher doses of ACh (Figures 3-2 and 3-4). At most, a secondary role may exist for COX-2 in mediating the endothelial impairments of ageing female SHR in this range of ACh doses. Furthermore, the relative contribution of COX-2 to the ACh contraction response does not appear to change across the age range of fSHR studied, since preferential COX-2 inhibition with NS-398 resulted in a similar reduction in contraction in

both 16wk and 30wk fSHR (Figure 3-2). This point is supported by the finding of unaltered aortic levels of COX-2 with ageing in fSHR (Figure 2-5). It was also determined that endothelium-dependent dysfunction of ageing female SHR depends on stimulation of the TP receptor (Figures 2-3 and 3-3). The few reports that do describe vasomotor responses of female SHR are limited to animals that are 14–19 wk of age (70; 128; 157). Since no study to date has examined endothelial function in female SHR of different ages, the literature cannot shed light on the effects of ageing in this sex and strain. The present novel observations establish a COX-1-TP receptor mechanism of endothelial impairment that develops in female SHR between 16 and 30 wk of age.

A second original and noteworthy finding of the present data is the establishment of a *similar* COX-1-TP receptor mechanism of ageing-induced endothelial impairment to higher ACh doses in both *female* and *male* SHR. By and large, the present observations in ageing male SHR are confirmatory of previous reports in the literature describing vasomotor impairments of male SHR to higher ACh doses (48; 52; 59; 62; 63; 68; 70; 85; 144; 154). However the male SHR in the present study provide a necessary control against which to compare the findings in ageing female SHR. 30wk male and female SHR exhibit blunted relaxation to higher ACh compared to 16wk counterparts (Figure 2-1). Relaxation was restored and contraction to higher ACh was abolished by inhibition of COX-1 (Figures 3-2 and 3-4) and of TP receptor (Figures 2-3 and 3-3) in both sexes. It was therefore concluded that ageing female SHR share a common COX-1-TP receptor mechanism of endothelial impairment with ageing male SHR.

Interestingly, while the contribution of COX-*2* to vasomotor responses is apparently unaffected by ageing within female SHR (see previous paragraph), this does not appear to be true within male SHR. A greater blunting of ACh contraction in the presence of preferential COX-2 inhibition was observed in 16wk mSHR compared to that of 30wk mSHR (Figure 3-2), suggesting that perhaps this isoform plays a more prominent role in the endothelial impairments of the former and that this contribution is reduced with ageing in 30wk mSHR. The relative NS-398-induced blunting of ACh contraction in 30wk mSHR in the present study (to ~65 % of the ND response to 10^{-5} M ACh) aligns well with previous findings in 30–36 wk-old male SHR (48; 154). However, the apparently greater contribution of COX-2 to ACh contraction in the present 16wk mSHR (vs. 30wk mSHR) cannot be corroborated by the literature since COX isoform-specific inhibition of ACh contraction has not previously been performed in male SHR younger than 30wk. Additionally, aortic COX-2 protein expression tended to *increase* in mSHR across the age range studied, however this effect did not reach statistical significance (Figure 2-5). It is possible that an ageing-related reduction in specific activity of COX-2 could have accounted for the discrepancies observed between protein expression levels and the noted NS-398 effects on ACh contraction responses, however this was not specifically examined in the thesis.

Finally, opposite roles appear to exist for COX-1 vs. -2 in the control of endothelial relaxation of female and male SHR to *lower* ACh doses. Preferential COX-1 inhibition *reduced* relaxation while preferential COX-2 inhibition *enhanced* relaxation of SHR to lower ACh (Figure 3-4). This observation of a COX-1-dependent enhancement of endothelium-dependent relaxation in this lower dosage range is curiously opposite to the well-established

COX-1-dependent blunting of relaxation to higher ACh doses. The present data revealing COX-2-dependent suppression of relaxation to lower ACh doses in all SHR corroborates a report of a similar occurrence in aorta of ageing male normotensive Wistar rats (55). However, the roles of COX-derived prostanoids in determining endothelial vasomotor function has focused primarily on *COX-1*-dependent responses to *higher* ACh doses in *male* SHR (48; 154; 155). The present observations are interesting and novel since roles appear to exist for *both* COX isoforms in determining relaxation to *lower* ACh doses in *male and female* SHR. The endothelium is capable of generating a plethora of vasodilatory (e.g. prostaglandin (PG) E_2, PGJ_2, PGI_2) and vasoconstrictory (e.g. PGH_2, PGI_2, thromboxane (Tx) A_2) prostanoids derived from catalysis of arachidonic acid (AA) by COX (15). One possible explanation for opposite influences of the two COX isoforms on relaxations is that lower ACh doses stimulate production of both COX-1-derived dilatory *and* COX-2-derived constrictory prostanoids. Regardless of the exact prostanoids responsible, the opposing actions of the two COX isoforms appear to counterbalance each other. Firstly, the relaxation to ACh within a given group is altered to similar magnitudes by preferential inhibition of either COX-1 or COX-2. Secondly, relaxations to lower ACh doses were not greatly altered in SHR by the presence of Indo (non-isoform-selective COX inhibitor) compared to the absence of inhibitors (i.e. No Drug (ND) condition, Figure 2-2). Nonetheless, while the physiological relevance of these two opposing COX-mediated actions may be insignificant when occurring simultaneously, the absence of one action and not the other could meaningfully influence vasomotor function. This is especially true since vasomotor tone is most sensitive to small changes in agonist concentration in this steep part of the curve. This

appears to be the case in 30wk mSHR. Preferential COX-2 inhibition improved relaxation to lower ACh doses in this group (compared to the ND condition) while preferential COX-1 inhibition had no effect (Figure 3-4). Interestingly, *16wk* mSHR exhibit the dual and opposing actions of both COX isoforms at lower ACh doses, suggesting that an ageing-related loss of COX-1-dependent enhancement of relaxation in this dosage range occurs in male SHR. Collectively, these data supplement a growing understanding of the involvement of COX-2 in several aspects of vasomotor function in hypertensive and normotensive male and female rats. Additionally, dual and opposing roles exist for COX-1 and -2 in regulating relaxation induced by lower ACh doses in SHR aorta. Finally, a loss of the COX-1-dependent enhancement of relaxation to lower ACh doses occurs in males between 16 and 30 wk of age.

These novel observations of the mechanisms controlling endothelium-dependent vasomotor dysfunction in ageing female SHR contribute to the understanding of how known sex differences in hypertension are altered with advancing age. Mechanistic observations of this nature have not been performed in human hypertensive patients. This knowledge is essential for developing anti-hypertensive therapeutic strategies that are appropriate for the patient's age and sex. Specifically, the established ageing-induced blunting of sex differences suggests that, while sex-specific considerations for treatment strategies may be prudent for younger hypertensive patients, the importance of this factor may diminish for the management of an ageing hypertensive population.

6.3 Specific Purpose #3: To assess the ability of chronic dietary DHA (22:6 n-3) supplementation to improve the endothelial vasomotor dysfunction established in a male rat model of essential hypertension

To approach this third specific purpose, endothelium-dependent and -independent vasomotor responses were determined in aortas isolated from 34–36 wk male SHR following 10–12 wk of dietary supplementation with DHA. These experiments provide the first assessment of the ability of n-3 PUFAs to restore endothelium-dependent vasomotor function to a model in which this impairment is established and is dependent on the COX-TP receptor axis. The data indicate that chronic DHA feeding does not improve aortic endothelial impairments or reduce systolic blood pressure (SBP) in adult male SHR.

The principle finding from this study is that aortic endothelium-dependent vasomotor dysfunction is not improved by DHA feeding in adult male SHR (Figure 4-1). This observation rejects the first hypothesis that endothelium-dependent vasorelaxation to higher ACh doses would be restored in these animals, an impairment that is primarily COX- and TP receptor-mediated. There are currently no reports examining the influence of n-3 PUFAs on functional endothelial responses of adult SHR with established hypertension (and accompanying vasomotor dysfunction) or in humans with hypertension (but in the absence of other cardiovascular diseases). The first hypothesis was therefore based on two observations from the literature: 1) improvement of aortic endothelium-dependent vasorelaxation in male SHR supplemented from a younger age, before the full establishment of functional impairments; and 2) the fact that dietary DHA supplementation abrogates platelet- and aortic-

generated production of vasoconstrictory prostanoids in male SHR as well as reduces aortic vasocontraction in adult male SHR in response to NOS inhibition. The rationale for deriving the present hypothesis from these observations is discussed in the two paragraphs that follow.

Firstly, reduced impairment of aortic relaxation of younger male SHR to ACh occurs with 10 d administration of eicosapentaenoic acid (EPA, 20:5 n-3, 280 mg/kg/d gavage) (159) or 4–13 wk supplementation with fish oil (EPA 1.8–3.6 % + DHA 1.2–2.4 % wt/wt diet) (13; 158). It is notable, however, that the SHR in only one (158) of these studies exhibit the well-established re-contraction of prior relaxation to higher ACh doses that are likely due to the release of COX-generated EDCF (48; 52; 59; 62; 63; 68; 70; 85; 144; 154). Therefore, while these functional data suggest that EPA, alone or in combination with DHA, can *abrogate the development* of aortic endothelial dysfunction in male SHR at an age before full establishment of vasomotor impairment, no conclusions can be made about: 1) the effect of DHA alone on endothelial function; or 2) the aortic response of adult male SHR with well-established COX- and TP receptor-mediated re-contraction. In contrast to the above-mentioned reports, 6 wk of DHA supplementation (0.4 % wt/wt diet) beginning at 7 wk of age does not alter the aortic ACh response of male SHR (41). When generating the first hypothesis, this finding (41) was weighed against the previously listed studies (13; 158; 159) that were able to improve vasomotor function by supplementation with EPA and DHA. It was felt that, compared to the methods of Engler et al. (41), the longer duration of DHA treatment (10–12 wk) at a slightly higher dose (0.5 % wt/wt diet) used in the present study would yield some restoration of function.

Secondly, there is speculation that n-3 PUFAs (i.e. DHA and EPA) mediate cardiovascular benefits through several mechanisms. These include: increasing cell membrane fluidity (53; 117); reducing circulating triglyceride levels (23; 117); direct anti-inflammatory effects (23; 47; 117); and slowing the progression of atherosclerosis by inhibition of chemoattractant/growth factor production in the plaque and of adhesion molecule expression on the surfaces of endothelial cells and circulating monocytes (23; 53). The proposed mechanism of action that may have the most impact on vasomotor function, however, is the potential for n-3 PUFAs to shift vascular prostanoid metabolism towards a more favourable profile. EPA and DHA compete with the n-6 PUFA, AA, for common enzymes required for their metabolism, including COX and lipoxygenase (117). The COX and lipoxygenase products resulting from metabolism of AA are 2-series prostanoids and 4-series leukotrienes, respectively, both of which promote a pro-inflammatory and pro-thrombotic environment (23; 117). The elevated vascular levels of EPA and DHA that occur with dietary supplementation result in these n-3 PUFAs out-competing AA for sites on COX and lipoxygenase, thus shifting eicosanoid production by these enzymes to 3-series prostanoids and 5-series leukotrienes, respectively. In contrast to the products of AA metabolism, these 3- and 5-series eicosanoids are anti-inflammatory in nature (13; 23; 41; 117), thus promoting a healthier vascular milieu. Indeed, 4–22 wk of dietary supplementation with fish oil (EPA 1.8–3.7 % + DHA 1.2–2.7 % w/w diet) reduced serum levels of TxB_2 (a stable metabolite of TxA_2) (13; 129; 158) and lowered aortic production of TxB_2 and 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) (158) in male SHR. Additionally, the contraction response induced by NOS inhibition in quiescent aortic rings (an index of

vasocontraction mediated by basal release of prostanoids) was reduced in 24 wk-old adult male SHR following 6 wk of dietary supplementation with DHA, but not EPA (both 4.5 % w/w diet) (92). Collectively these findings suggest that dietary DHA, alone or in combination with EPA, is able to blunt aortic production of vasoconstrictory prostanoids (e.g. PGI_2, PGH_2) in male SHR, in which a role for these COX products has been established in the observed endothelial vasomotor impairments.

Therefore, based on the ability of EPA + DHA to abrogate developing functional impairments in younger male SHR and on the DHA-induced reduction of vasoconstrictory prostanoid release (which are known to contribute to endothelial dysfunction in adult male SHR), it was reasonable to extrapolate the present hypothesis that 10–12 wk of dietary DHA supplementation (0.5 % w/w diet) would provide some amelioration of endothelium-dependent vasorelaxation to adult male SHR. The reason for the lack of effect of DHA in the present study is unclear. It is possible that DHA is able to favourably shift the prostanoid profile, but not to a degree sufficient for improving endothelium-dependent vasorelaxation in adult male SHR. Alternatively, 0.5 % wt/wt diet may simply be too low a concentration of DHA to exert an effect on prostanoid production or endothelium-dependent relaxation. Reports in the literature suggest that the duration of administration used in the present study (10–12 wk in SHR) is sufficient for detecting vasomotor benefits of other n-3 PUFAs, since improvements occur with as little as 10 d of EPA by gavage (159) and 4 wk of dietary fish oil (158). Regardless of the reason, the present data agrees with that of Engler et al. (41) and indicates that dietary administration of DHA alone (at the concentration used) does not improve endothelium-dependent vasomotor responses of male SHR.

The second major finding of the present study is that 10–12 wk of dietary DHA (0.5 % wt/wt diet) does not reduce SBP in adult male SHR (Table 4-1), and confirms the second hypothesis. This hypothesis was based on the findings that 6–9 wk of dietary DHA (4.5 % wt/wt diet) (92), EPA (4.5 % wt/wt diet) (92), or fish oil (EPA 0.3 % + DHA 0.2 % wt/wt diet) (103) did not reduce blood pressure (BP) in adult male SHR when supplementation began at 14–18 wk old, after establishment of a steady-state elevation in BP. Thus the present BP data specifically corroborate the lack of a BP-reducing effect reported by the McLennan et al. study (92) that supplemented with DHA alone, albeit at a much higher dose (4.5 % vs. 0.5 % wt/wt diet) and for approximately half of the duration (6 wk vs. 10–12 wk) of the present study.

Interestingly, these data from adult male SHR with established hypertension, both in the present study and others (92; 103), contrast BP effects of beginning n-3 PUFA supplementation in younger male SHR, in which hypertension is still developing. The rise in SBP is abrogated (–9 to –40 mm Hg) in these animals by: EPA fed in the diet (4.5 % wt/wt diet) (92); EPA administered by gavage (30–300 mg/kg/d) (66; 78; 159); EPA administered intraperitoneally (10–50 mg/d) (124); dietary DHA (0.4–4.5 % wt/wt diet) (41; 92); and dietary fish oil (EPA 1.7–3.9 % + DHA 1.2–2.7 % wt/wt diet) (13; 25; 65; 88; 92; 129; 158). Moreover, evidence from one study suggests that this hypotensive effect may be greatest with DHA alone, when compared alongside EPA alone or fish oil (92). Together with the lack of hypotensive effect of DHA alone in adult SHR in the present study and others (92; 103), it appears as though the mechanism through which DHA blunts the rise in BP in younger male SHR may exhibit changing sensitivity with age/progression of hypertension, or

that it is a completely different mechanism of control altogether between these two age groups. It is also notable that despite the aforementioned lack of BP-lowering effect of dietary EPA (92) or fish oil (103) in adult male SHR, just 2 wk of EPA administration by gavage (300 mg/kg/d) significantly reduces SBP (–21 mm Hg) in a similar cohort (78). It is unclear if route of administration and/or differences in dosage may be responsible for these disparate results. Furthermore, the n-6 PUFA γ-linolenic acid (GLA, 18:3 n-6), alone (2.4–14.4 mg/kd/d i.p.) (135) or in addition to fish oil (GLA 0.6 % + EPA 0.5 % + DHA 0.3 % wt/wt diet) (12), has a hypotensive effect (SBP: –14 to –35 mm Hg) in adult male SHR. This latter GLA+fish oil supplementation study (12) follows up a previous report by the same research group describing a lack of BP reduction in adult male SHR with fish oil alone (EPA 0.3 % + DHA 0.2 % wt/wt diet) (103). These data suggest that GLA may act independently of DHA and EPA to reduce BP in these animals. Nonetheless, collectively with the literature cited, the present findings confirm a lack of BP-lowering effect of dietary DHA when supplementation of adult male SHR occurs after a steady-state elevation in BP has been achieved.

The present study provides the first report of endothelial function following n-3 PUFA supplementation of adult male SHR with established vasomotor impairments and hypertension. It was observed that dietary DHA did not improve the impaired ACh response of segments isolated from aorta of adult male SHR. It may be inferred that DHA does not influence the COX-TP receptor axis since this pathway plays a major role in endothelial dysfunction in these animals (see Chapters 2 and 3). The present lack of hypotensive effect of DHA corroborates previous findings in adult male SHR with established hypertension.

Although dietary DHA does not appear to provide much benefit to male hypertensive rats, investigations of its therapeutic potential on endothelial function in human hypertensive patients have not been performed and thus cannot be discounted. Additionally, in cases where hypertension and endothelial dysfunction are in the developmental stages, the literature indicates that DHA may afford some abrogation of these symptoms. Moreover, there may be supplementary health benefits of increased n-3 PUFA consumption (either in purified form or from whole foods) in hypertensive individuals that are not discussed herein.

6.4 Specific Purpose #4: To characterize aortic vasomotor responses of female rats exhibiting type 2 diabetes-like symptoms

This fourth specific purpose was addressed by assessing α_1 adrenergic contraction and endothelium-dependent relaxation responses of isolated aortic rings from obese diabetic female ZDF compared to non-diabetic female Zucker Lean rats. This purpose recognizes and begins to address the very limited number of studies that have examined vasomotor responses in female type 2 diabetic animals. The present data describes a greatly enhanced α_1 adrenergic contraction, but unimpaired endothelium-dependent relaxation, in female ZDF compared to Zucker Lean.

The first important observation is that a doubling of maximal aortic α_1 adrenergic vasocontraction occurs in female ZDF compared to Zucker Lean counterparts (Figure 5-2). This result is consistent with the enhanced protein kinase C (PKC)- and rho kinase (ROCK)-dependent (72; 149; 152) α adrenergic contractions reported in various arteries of male (72;

79; 149; 152; 160) and sex-unspecified (107) rodent models of type 2 diabetes. Activation of PKC has been proposed as one of the 4 major mechanisms through which hyperglycemia induces endothelial dysfunction in type 2 diabetes (19; 36). Additionally, α_1 adrenoceptor stimulation results in a PKC-dependent phosphorylation and activation of myosin regulatory light chain in VSM, leading to enhanced contraction (132). It is thus plausible that hyperglycemia-induced activation of PKC contributes to enhanced α_1 adrenergic vasocontraction in aorta high fat-fed female ZDF (i.e. HF). There may also be an obesity-dependent component to this observation, since normal fat-fed ZDF (i.e. Con) had a similar elevation in body mass (Figure 5-1 and (130)) and in maximal vasocontraction to phenylephrine hydrochloride (PE; Figure 5-2), but a blunted rise in blood glucose and insulin levels compared to HF (Figure 5-1 and (130)). While these metabolic abnormalities were not as severe in Con as they were in HF, there may be a component of this impairment that is dependent on the *duration* of exposure to obesity, hyperglycemia, and hyperinsulinemia, as opposed to the endpoint level achieved. The present data expand the scope of the literature with a novel characterization of adrenergic contractile responses in obese and type 2 diabetic *female* animals. The physiological relevance of enhanced *aortic* α adrenergic contraction in type 2 diabetes, however, is unclear. α adrenergic alterations in the *resistance* arterial vasculature could contribute to systemic perturbations (e.g. altered distribution of organ blood flow and hypertension) in these animals. Unfortunately, BP recordings reported in ZDF are inconsistent. Some researchers observe hypertension in male (1; 16; 79; 112; 149) and female (1) ZDF vs. Zucker Lean while others observe that BP is similar in male ZDF (57; 108; 109) and ZDF of unspecified sex (18; 50; 136) compared to Zucker Lean.

Additionally, systemic infusion of PE elevates mean arterial pressure (MAP) in male ZDF (1; 3) and either increases (1) or does not alter (3) MAP in female ZDF. Therefore, enhanced α adrenergic contraction is consistently demonstrated in isolated arteries from male and, now, female type 2 diabetic animals. However, the noted discrepancies of BP measurements in the literature preclude confident conclusions about the meaning of these data until further research can garner information regarding the physiological relevance of the augmented α adrenergic contraction observed in type 2 diabetes.

The second major finding is that obese and type 2 diabetic female ZDF display robust and unimpaired endothelium-dependent aortic vasorelaxations compared to Zucker Lean. Considering the emerging picture of endothelial macrovascular dysfunction in male ZDF, it was an unexpected observation that high fat-fed (diabetic) female ZDF exhibited an ACh relaxation response similar to that of non-diabetic Zucker Lean control animals (Figure 5-4). This is especially surprising given the severe hyperglycemia (which can lead to elevated mitochondrial superoxide anion generation and many downstream consequences (19)), hyperinsulinemia, and obesity experienced by this group for 4–8 wk before tissue harvest (endpoint values: Figure 5-1; progression values: see (130)). However, if vascular overproduction of superoxide anion had been occurring in the present animals, it would likely have been detectable by a reduction of NO-mediated endothelial relaxation and a recuperation of function in the presence of the superoxide dismutase (SOD) mimetic Tempol. In retrospect, the essentially normal endothelial function of diabetic female ZDF supports the observation that Tempol did not augment this response in this group (Figure 5-4). Therefore,

it can be concluded that endothelium-dependent NO-mediated vasorelaxation remains intact in severely hyperglycemic, hyperinsulinemic, and obese female ZDF rats.

The endpoint age of the animals used in the present study was 14 wk. A clear parallel can be drawn between these "young-adult" female ZDF and the 16 wk old female SHR studied in the first two experimental chapters of the thesis. Quite possibly in both cases, the younger age and/or the female sex of the animals offered some means of vasculoprotection, even in the face of the severely disrupted BP (in SHR, Tables 2-1 and 3-1) and metabolic (in ZDF, Figure 5-1) profiles of these animals. The maintenance of endothelium-dependent, NO-mediated vasorelaxation has been proposed to be synonymous with maintenance of NO bioavailability (121). It follows that the apparently normal aortic endothelium-dependent NO-mediated relaxation responses of younger female ZDF indicates a robust aortic NO bioavailability in these animals. If this is the case, then it may offer younger female ZDF additional protection against the loss of other beneficial macrovascular roles for NO, including inhibition of: coagulation, thrombosis, platelet activation, vascular smooth muscle (VSM) proliferation, leukocyte adherence and transmigration. As mentioned above in the context of SHR (see *Specific Purpose #1*), addressing the *cause(s)* of preservation of endothelial function in younger female ZDF (vs. males) would be an important, albeit ambitious, scientific venture. Due to the onerous requirement of very calculated removal and supplementation of several specific sex hormones in both male and female rats, this task was beyond the scope and purpose of the thesis.

The present data in female ZDF (and SHR) nevertheless provide a foundation of phenomenological data that would support further studies detailing specific roles for sex

hormones in these responses. Given the accelerated development and increased prevalence of cardiovascular diseases when superimposed onto type 2 diabetes mellitus (11; 104), furthering our understanding of the tissue and cellular level disturbances associated with these conditions is therefore of considerable clinical value.

6.5 Specific Purpose #5: To examine the influence of physical activity and anti-diabetic pharmacotherapy, alone and in combination, on vasomotor responses of a female rat model of type 2 diabetes

To address this fifth specific purpose, α_1 adrenergic contraction and endothelium-dependent relaxation responses were assessed in aortic segments isolated from female obese and type 2 diabetic ZDF rats following an 8 wk administration of anti-diabetic metformin drug therapy, aerobic exercise training, or a combination of the two. Defining the ability of these interventions to positively alter vasomotor responses in type 2 diabetes has significant clinical value. It was observed that of all the interventions, chronic exercise training monotherapy led to the greatest tempering of α_1 adrenergic contraction in female ZDF. In contrast, none of the interventions elicited improvement of endothelium-dependent relaxation in these animals.

The adrenergic contractile responses will be discussed first. It was hypothesized that the elevated α_1 adrenergic contractions to PE observed in female ZDF would not be affected by any of the therapeutic interventions. The part of this hypothesis referring to the exercise training intervention was based on the only study examining α adrenergic contraction in an

animal model of type 2 diabetes (72). This group reported 8 wk of forced aerobic exercise does not affect aortic PE contraction of male *db/db* mice (72). As mentioned above, various animal models of type 2 diabetes consistently exhibit elevated adrenergic contractions in several vascular beds compared to non-diabetic controls (72; 79; 107; 149; 152; 160). Therefore, the *elevation* of adrenergic responsiveness in type 2 diabetes appears to be *independent* of factors such as animal model used and vascular bed studied. However, the exercise training-induced blunting of α_1 adrenergic contraction observed in the present female ZDF (Figures 5-2 and 5-3) but not in male *db/db* mice (72) indicates that the *sensitivity* of this response to an exercise training stimulus per se may be *dependent* on these factors.

The part of the hypothesis stating that metformin would also not alter α_1 adrenergic contraction was formed from the following reasoning. In addition to their indirect vascular benefits, such as improvement of blood glucose and insulin profiles (116; 134), metformin and exercise training interventions also exert direct vascular benefits in type 2 diabetic animals and humans by improvement of endothelium-dependent relaxation (67; 87; 90; 91; 95-97; 122). It stands to reason that if these two therapies achieve these direct benefits through similar mechanisms in the vascular wall, then they could also exert similar effects on α_1 adrenergic vasomotor pathways. In contrast to the exercise intervention, however, metformin treatment alone offered no reduction in PE contractions. Moreover, metformin tempered the exercise training-induced reductions in the absolute PE response when the two therapies were administered in combination (Figure 5-2). This suggests that metformin counteracts the exercise training-induced reduction in α_1 adrenergic vasocontraction, possibly

through a direct and opposite stimulation of the target(s) on which exercise training acts (e.g. possibly on PKC- and ROCK-mediated pathways downstream of the α_1 adrenoceptor). Since these data are the first report of the influence of any anti-diabetic drug treatment on α adrenergic vasomotor function in type 2 diabetes, it is unfortunately not possible to draw much interpretation from the literature. One conclusion that can be made from these data is that α_1 adrenergic responsiveness of female ZDF aorta is differentially sensitive to metformin and exercise training interventions.

One mechanism through which exercise training could have tempered α_1 adrenergic contraction responses of the present female ZDF is by suppression of hyperglycemia. Stimulation of α_1 adrenergic receptors on the VSM cell membrane initiates PKC activation via a phospholipase C-diacylglycerol (DAG) cascade. Activated PKC inhibits myosin phosphatase, leading to maintained phosphorylation of myosin regulatory light chain and thus VSM contraction (132). As mentioned in the Introduction of the thesis (see *Hyperglycemia-induced oxidative stress mediates endothelial dysfunction in diabetes*), many downstream vascular consequences result from hyperglycemia, including enhanced PKC activation (19; 36). The 8 wk high fat diabetogenic diet and therapeutic interventions began at 6 wk of age in the present female ZDF, at which point blood glucose was similar in all ZDF and lean groups (130). Chronic exercise training resulted in both delayed onset (130) and reduced endpoint level (Figure 5-1 and (130)) of hyperglycemia compared to untreated diabetic ZDF. Observations in type 1 diabetic rats indicate that prolonged hyperglycemia leads to enhanced aortic DAG content and PKC activity that persist well into a subsequent period of euglycemia (60). In contrast, a relatively brief episode of hyperglycemia does not

initiate a long-term elevation in DAG content or PKC activity (61). Together, the present blood glucose data and the observations of Inoguchi and colleagues (60; 61) collectively suggest that chronic exercise training could contribute to suppression of aortic α_1 adrenergic contraction in female ZDF through an anti-hyperglycemic inhibition of PKC activation. The fact that metformin therapy caused a similar delaying of onset of hyperglycemia, albeit resulting in endpoint blood glucose levels that remained higher than those of exercise-trained ZDF, suggests that metformin may be tempering the exercise training-induced reduction in PE vasocontraction through a pathway(s) other than a reduction of blood glucose levels. Metformin does activate 5'-AMP-activated protein kinase via a PKC-ζ-dependent mechanism in endothelial cells (151), resulting in many cardiovascular benefits, including improvement of hyperglycemia and insulin action as well as correction of endothelial dysfunction (58; 150). In contrast, protein expression of PKC-α (101), -β2 (60; 61; 101), and -ε (101), are increased in the vasculature of experimental animal models of diabetes and enhanced activation of PKC-α and -ε occur with adrenergic stimulation of arteries from these animals, suggesting roles for these isoforms in the progression of diabetic vascular complications (58; 101). These previous reports, together with the present findings, collectively suggest that metformin may act on multiple pathways in the vasculature and that PKC-isoform-specific effects could account for the apparently conflicting beneficial (reduction of hyperglycemia) and detrimental (abrogation of the exercise training-induced blunting of α_1 adrenergic contraction) effects induced by metformin. Substantiation of this speculation would require a detailed assessment of PKC pathway activation (e.g. DAG production, isoform-specific PKC expression and activity) alongside specific/independent

manipulation of blood glucose levels and exercise training stimulus. A study of this nature would help elucidate the exact mechanism(s) through which chronic exercise training preserves aortic α_1 adrenergic contractility in type 2 diabetes mellitus.

The second hypothesis relating to Specific Purpose #5 is that all three interventions would restore endothelium-dependent relaxation of female ZDF to the level of non-diabetic Zucker Lean counterparts. This hypothesis was rejected since none of the interventions altered the ACh relaxation response. This is primarily due to the fact that diabetic female ZDF that received no intervention exhibited robust and unimpaired endothelium-dependent vasomotor function (Figure 5-4). The restorative abilities of exercise training and anti-diabetic pharmacotherapy on endothelial responses of male type 2 diabetic animals (71; 91; 95-97; 108) and in human type 2 diabetic patients (87; 90) suggest that these therapies could have offered similar benefits to female ZDF, had these animals exhibited initial dysfunction. If the apparent vasculoprotection exhibited by the present 14 wk old female ZDF is dependent on the younger age of the animals, as discussed above, then it is possible that ageing female ZDF, like ageing female SHR, could begin to show signs of deteriorating endothelial function. It would be interesting and worthwhile to examine the ageing effects on vasomotor responses in female ZDF as well as the influence of anti-diabetic drug and lifestyle interventions on these ageing effects.

This is the first report of any treatment intervention on macrovascular function of ZDF of either sex. The present data indicate that the enhanced α_1 adrenergic contraction of 14 wk old female ZDF is blunted by chronic exercise training. In contrast, these animals display unimpaired endothelium-dependent aortic vasomotor function that is not further

improved by chronic administration of an anti-diabetic drug and/or exercise training. If the exercise training-induced reduction in α_1 adrenergic responsiveness of female ZDF aorta translates to human type 2 diabetic vasculature, then this introduces a therapeutic avenue for addressing the augmented α adrenergic responsiveness of various vascular beds in these patients (56). Further research is clearly needed to isolate the important factors determining the sensitivity of the type 2 diabetes-related elevation of adrenergic responsiveness to anti-diabetic pharmacotherapy and/or exercise training.

6.6 Conclusions and relevance of the main findings of the thesis

The main findings of the thesis are that ageing induces a loss of endothelium-dependent vasomotor function in aorta of female hypertensive rats, and blunts sex differences in this response in these animals. Furthermore, ageing female and male hypertensive rats share a common pathway of aortic endothelial dysfunction that is mediated primarily by a COX-TP receptor axis. Chronic dietary supplementation with DHA does not improve the impaired aortic endothelium-dependent vasomotor function observed in adult male hypertensive rats. Interestingly, younger female hypertensive rats and younger female obese and type 2 diabetic rats both exhibit maintenance of robust aortic endothelium-dependent vasomotor function in the face of significantly disrupted BP in hypertensive rats and metabolism in type 2 diabetic rats. In contrast, considerable augmentation of α_1 adrenergic contraction was observed in aorta of younger female obese and type 2 diabetic rats. Finally,

chronic administration of exercise training, but not anti-diabetic metformin drug, prevented elevated aortic α_1 adrenergic contraction in female obese and type 2 diabetic animals.

The principal encompassing novelty of the present data is that it addresses the limited number of reports examining vasomotor responses in *female* animal models of essential hypertension and of type 2 diabetes mellitus. The relevance of studying endothelial *macro*vascular responses in these diseases is that the endothelium plays a fundamental role in maintaining homeostatic balance in the vascular wall. Deterioration of endothelial function is a primary initiating event of many macrovascular complications that arise in hypertension and type 2 diabetes, such as coronary artery disease, aneurysms, and stroke. Basic science must first elucidate female sex-specific endothelial responses in hypertension and type 2 diabetes before more comprehensive and directed treatment strategies can be developed for the female half of the patient population.

Appendix A

Supplementary data for Chapter 2

Table A-1 Supplementary BP, heart rate, and organ mass measurements.

		mWKY	mSHR	fWKY	fSHR
SBP, mm Hg	16wk	108±2	211±13[a]	123±5[a]	205±4[c]
	30wk	102±4	207±11[e]	85±5[ce]	190±5[dg]
DBP, mm Hg	16wk	78±3	149±7[a]	97±4[a]	148±2[c]
	30wk	79±2	155±8[e]	62±4[ce]	143±4[g]
PP, mm Hg	16wk	30±1	62±6[a]	25±4	57±2[c]
	30wk	23±4	52±7[e]	22±3	48±3[dg]
HR, beats/min	16wk	329±11	388±9[a]	327±17	357±13
	30wk	242±7[a]	360±17[e]	222±11[c]	344±7[g]
RV/BM, mg/g	16wk	0.61±0.02	0.60±0.02	0.66±0.04	0.71±0.02[b]
	30wk	0.49±0.02[a]	0.52±0.01[b]	0.65±0.04[e]	0.63±0.02[f]
H/BM, mg/g	16wk	2.86±0.03	3.16±0.03[a]	3.16±0.05[a]	3.44±0.04[bc]
	30wk	2.69±0.04[a]	3.19±0.05[e]	3.21±0.09[e]	3.59±0.04[dfg]
K/BM, mg/g	16wk	3.29±0.05	3.09±0.04[a]	3.20±0.06	3.00±0.03[c]
	30wk	3.00±0.06[a]	3.12±0.04	3.04±0.08	3.14±0.05

Values are mean±SEM. n=6–8 (BP, HR). n=8–29 (organ masses). DBP, diastolic BP. PP, pulse pressure. HR, heart rate. RV, right ventricular mass. H, heart (RV+LV) mass. K, kidney mass. $p<0.05$ vs.: [a] 16wk mWKY, [b] 16wk mSHR, [c] 16wk fWKY, [d] 16wk fSHR, [e] 30wk mWKY, [f] 30wk mSHR, [g] 30wk fWKY.

Table A-2 Contractile responses to PE and KCl stimuli in the Indo and SQ29548 drug conditions.

	mWKY	mSHR	fWKY	fSHR
KCl contraction (Indo condition), g				
16wk	1.37±0.07	1.52±0.14	1.39±0.09	1.42±0.05
30wk	1.65±0.10	1.55±0.08*	1.29±0.01[e]*	1.52±0.04[g]*
KCl contraction (SQ29548 condition), g				
16wk	N/A	2.01±0.13	N/A	1.47±0.17[b]
30wk	N/A	1.86±0.11	N/A	1.72±0.05
PE contraction (Indo condition), g				
16wk	1.68±0.03	1.41±0.14	1.15±0.19	1.22±0.10
30wk	1.66±0.16*	1.51±0.07*	1.17±0.11[e]*	1.20±0.09[f]*
PE contraction (SQ29548 condition), g				
16wk	N/A	1.33±0.14	N/A	1.33±0.09
30wk	N/A	1.79±0.05[b]	N/A	1.41±0.16

Values are mean±SEM. n=4–8 in singlet to duplicate rings (Indo). n=4 in duplicate rings (SQ29548). KCl contraction, contractile response to 60 mmol/l KCl. PE contraction, contractile response to 10^{-7} mol/l PE prior to ACh dose-response. N/A, not applicable since response in the presence of SQ29548 were not measured in WKY rats. $p<0.05$ vs.: [b] 16wk mSHR, [e] 30wk mWKY, [f] 30wk mSHR, [g] 30wk fWKY. $p<0.05$ vs.: * ND.

Appendix B

Supplementary data for Chapter 3

Table B-1 Endpoint BP, HR, and organ-to-BM ratios.

		mWKY	mSHR	fWKY	fSHR
SBP, mm Hg	16wk	109±7	228±8[a]	86±4[a]	199±2[bc]
	30wk	112±4	230±7[e]	105±4[c]	206±4[fg]
DBP, mm Hg	16wk	81±6	163±5[a]	60±6	145±2[bc]
	30wk	84±3	167±3[e]	80±4[c]	152±2[dfg]
PP, mm Hg	16wk	28±3	65±5[a]	26±3	54±1[bc]
	30wk	29±1	63±6[e]	25±1[e]	55±2[g]
HR, beats/min	16wk	290±13	393±6[a]	231±18[a]	355±9[bc]
	30wk	303±11	395±13[e]	276±12	356±7[fg]
RV/BM, mg/g	16wk	0.58±0.02	0.56±0.02	0.57±0.02	0.62±0.02[b]
	30wk	0.53±0.02[a]	0.55±0.01	0.59±0.02[e]	0.61±0.01[f]
H/BM, mg/g	16wk	2.79±0.09	3.02±0.04[a]	2.85±0.08	3.40±0.06[bc]
	30wk	2.54±0.03[a]	3.05±0.06[e]	3.03±0.09[e]	3.47±0.03[fg]
K/BM, mg/g	16wk	3.16±0.05	3.06±0.06	3.09±0.12	3.02±0.02
	30wk	2.85±0.04[a]	3.10±0.03[e]	3.05±0.05[e]	3.01±0.05

Values are mean±SEM. $p<0.05$ vs.: [a] 16wk mWKY, [b] 16wk mSHR, [c] 16wk fWKY, [d] 16wk fSHR, [e] 30wk mWKY, [f] 30wk mSHR, [g] 30wk fWKY.

Table B-2 Developed tension of thoracic aortic rings to PE in the presence of inhibition of COX-1 (VAS, $3x10^{-3}$ M), COX-2 (NS-398, 10^{-6} M) or TP receptor (SQ 29548, 10^{-6} M).

	16wk mSHR	16wk fSHR	30wk mSHR	30wk fSHR
	PE contraction (10^{-7} M), g			
VAS	0.81±0.15[h]	1.18±0.06[b]	1.00±0.11[h]	0.91±0.07[dh]
NS-398	1.05±0.13	1.23±0.15	1.46±0.12[bi]	0.97±0.13[fi]
SQ 29548	1.28±0.13	1.23±0.10	1.75±0.05[b]	1.40±0.10[f]

Values are mean±SEM. PE contraction, contractile response to 10^{-7} M PE prior to ACh relaxation dose-response. $p<0.05$ vs.: [b] 16wk mSHR, [d] 16wk fSHR, [f] 30wk mSHR. $p<0.05$ ND vs.: [h] VAS, [i] NS-398.

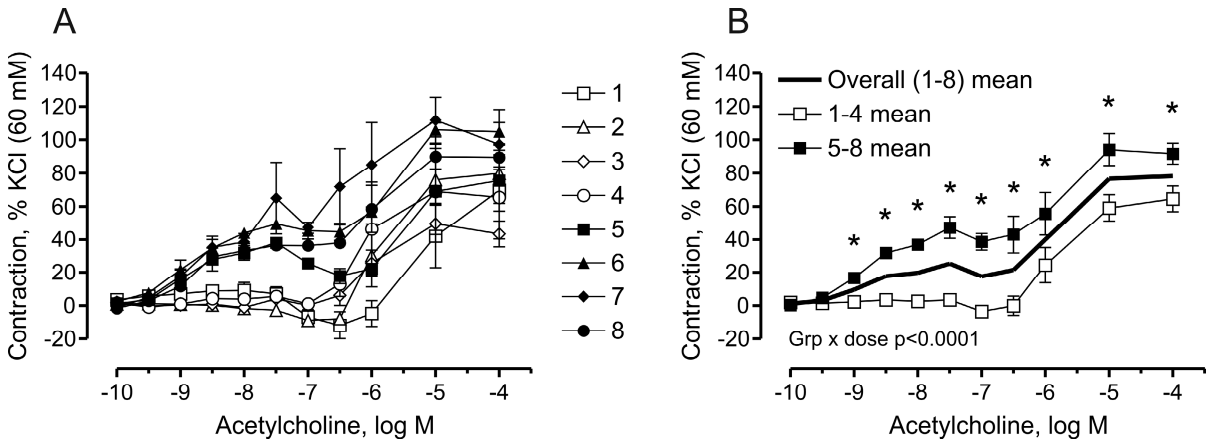

Figure B-1 ACh-induced endothelium-dependent contraction of thoracic aortic rings isolated from 16wk mWKY in the ND control condition. The lowest ACh dose that elicited a detectable contraction was systematically lower in 16wk mWKY #5–8 (i.e. 10^{-9} M) compared to 16wk mWKY #1–4 (i.e. 10^{-6}–$10^{-6.5}$ M, panel A) and to all other groups in the present study (i.e. 10^{-6}–10^{-7} M, Figure 3-1). The mean response of 16wk mWKY #5–8 also remained elevated compared to that of 16wk mWKY #1–4 to all ACh doses that elicited a detectable response in the former (10^{-9}–10^{-4} M, panel B). Due to the systematic nature of the elevated contraction exhibited by 16wk mWKY #5–8 to lower ACh doses, these data were excluded from comparison of mean responses between experimental groups. Data are mean±SEM, expressed as grams of tension development from baseline resting tension. n=2 rings/rat (panel A). n=4 rats/group, duplicate rings/rat (panel B). $p<0.05$ vs.: * 16wk mWKY #1–4.

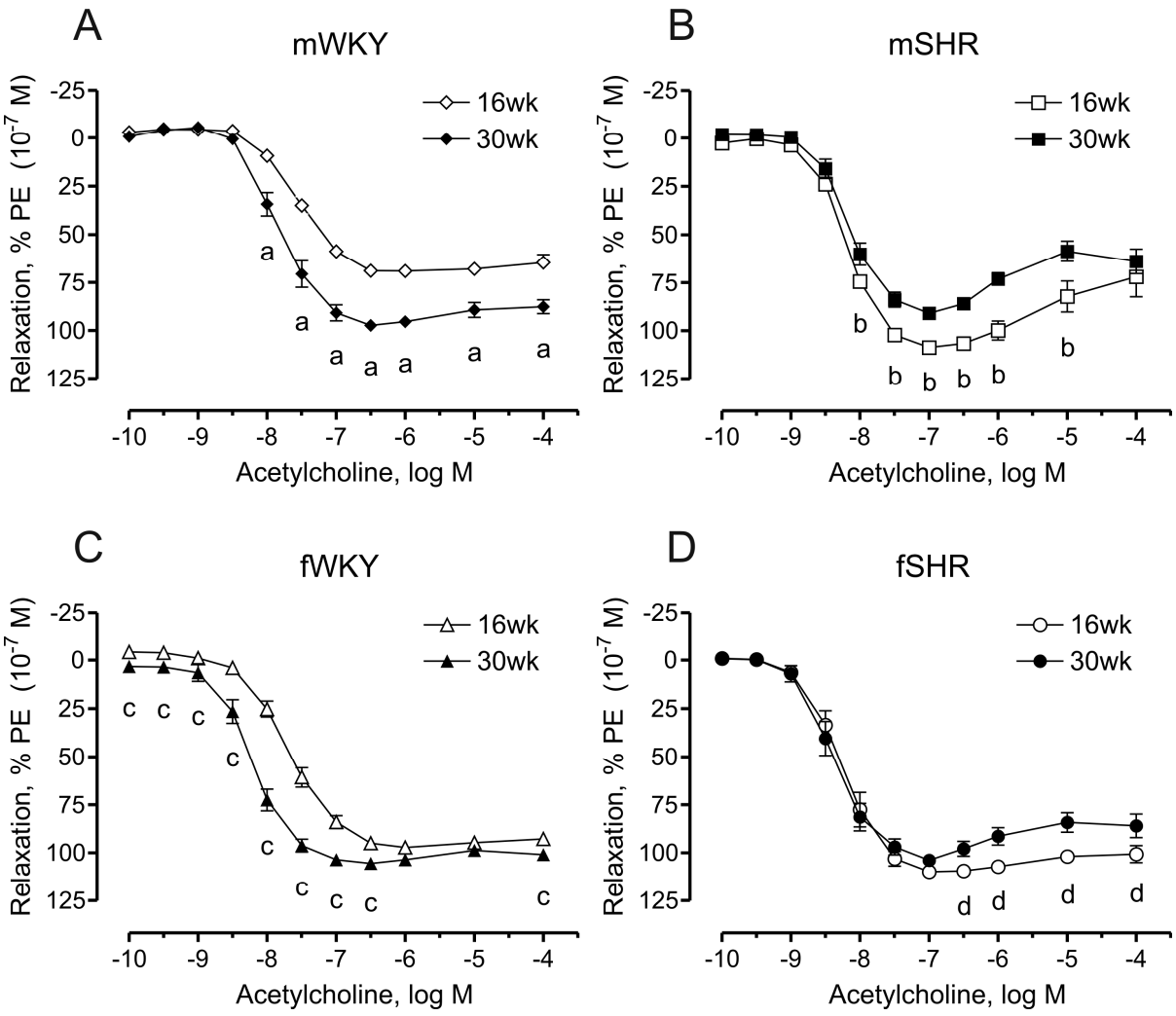

Figure B-2 ACh-induced endothelium-dependent relaxation of thoracic aortic rings isolated from 16wk and 30wk male and female WKY and SHR in the ND control condition. Values are mean±SEM, expressed as a percentage of the prior tension development to 10^{-7} M PE. n=8–9 in singlet to duplicate rings. $p<0.05$ vs.: [a] 16wk mWKY, [b] 16wk mSHR, [c] 16wk fWKY, [d] 16wk fSHR.

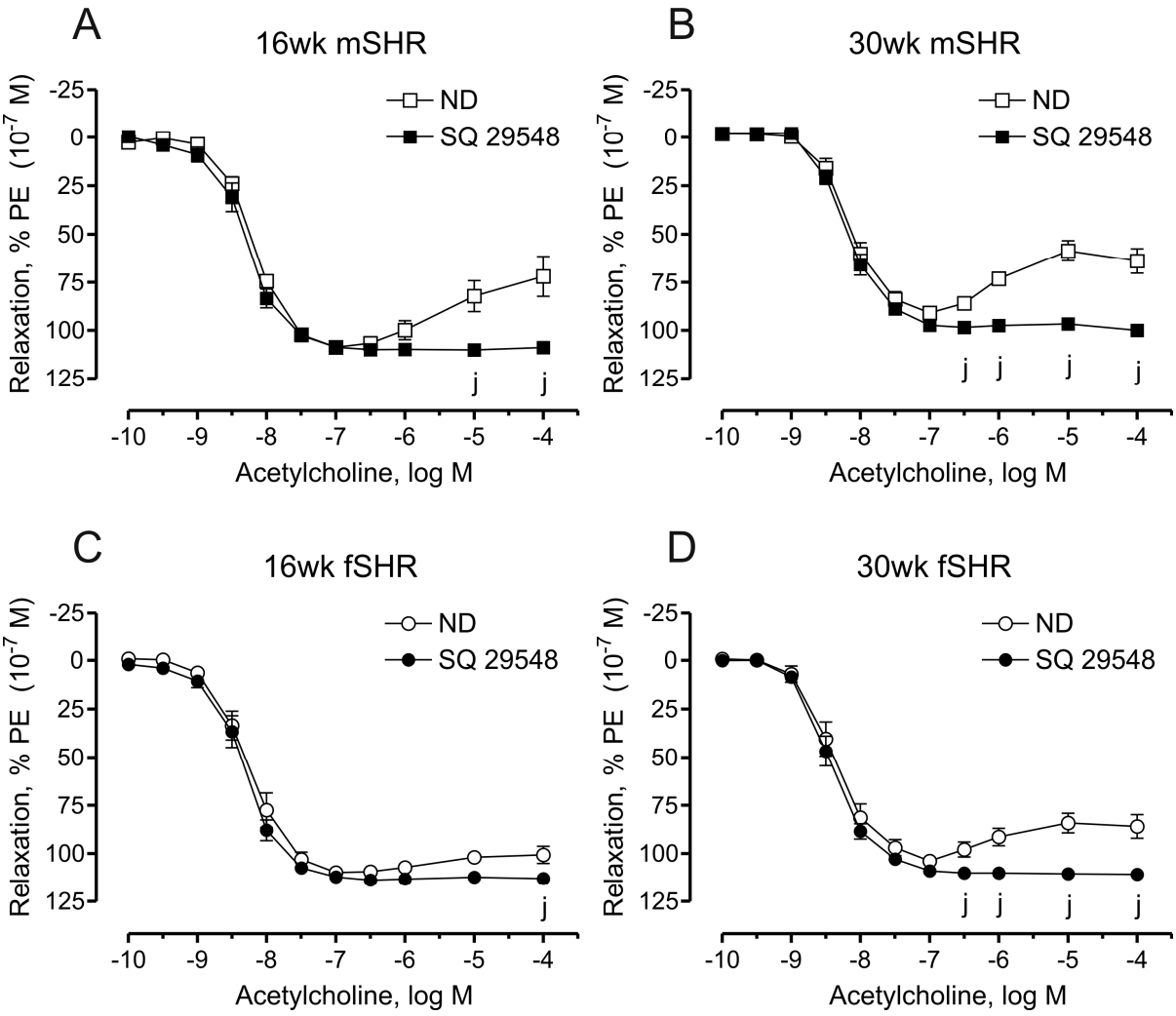

Figure B-3 ACh-induced endothelium-dependent relaxation of thoracic aortic rings isolated from 16wk and 30wk male and female SHR exposed to SQ 29548 (10^{-6} M, TP receptor antagonist). ND control values transcribed from Figure B-2 for reference. Values are mean±SEM, expressed as a percentage of the prior tension development to 10^{-7} M PE. n=8–9 in singlet to duplicate rings. $p<0.05$ vs.: ^{j} ND.

Appendix C

Supplementary data for Chapter 4

Table C-1 Endpoint organ-to-BM ratios.

		mWKY	mSHR
RV/BM, mg/g	CON	0.51±0.03	0.55±0.04
	DHA	0.48±0.03	0.56±0.04
H/BM, mg/g	CON	2.59±0.04	3.29±0.07[a]
	DHA	2.58±0.04	3.34±0.20[c]
K/BM, mg/g	CON	2.66±0.06	2.98±0.08[a]
	DHA	2.62±0.04	3.00±0.12[c]

Values are mean±SEM. n=14–18. $p<0.05$ vs.: [a] CON mWKY, [c] DHA mWKY.

Table C-2 Developed tension of thoracic aortic rings to KCl and PE in the presence of inhibition of NOS (L-NAME, 10^{-4} M).

	mWKY	mSHR
KCl contraction (60 mM), g		
CON	1.48±0.13	1.97±0.05[a]
DHA	1.68±0.13	1.92±0.15
PE contraction (10^{-7} M), g		
CON	1.85±0.16	1.68±0.06*
DHA	2.12±0.13	1.59±0.16[c]

Values are mean±SEM. n=8–12. KCl contraction, contractile response to the second exposure to 60 mM KCl. PE contraction, contractile response to 10^{-7} M PE prior to ACh relaxation dose-response. $p<0.05$ vs.: [a] CON mWKY, [c] DHA mWKY. $p<0.05$ vs.: * ND.

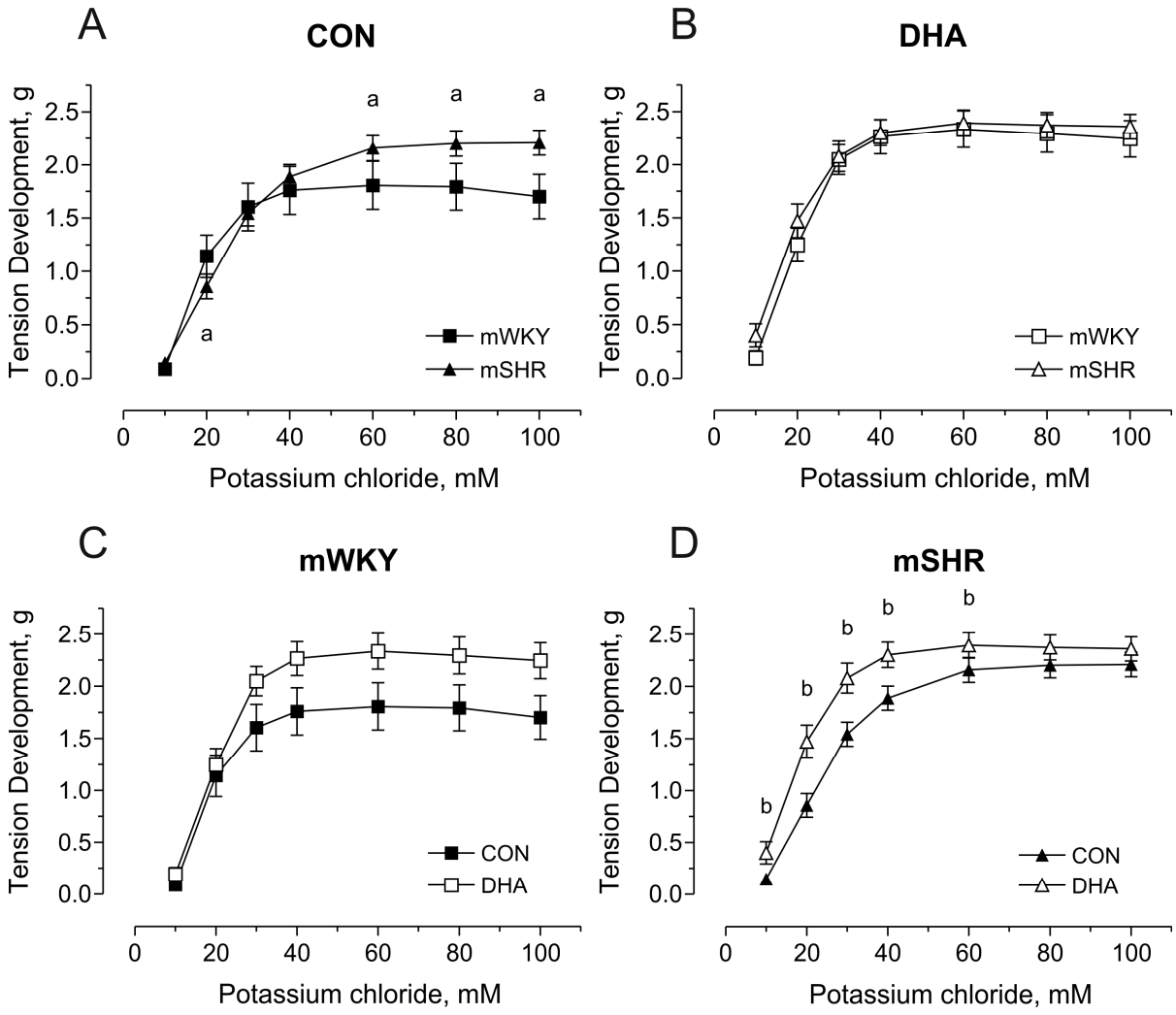

Figure C-1 Contractile response to KCl in the absence of L-NAME (ND condition) of thoracic aortic rings isolated from male WKY and SHR following 8–12 wk of CON or DHA diet. Responses of CON (panel A) and DHA groups (panel B) are plotted for comparison of strain (within each diet). Responses of WKY (panel C) and SHR (panel D) are re-plotted separately for comparison of diet (within each strain). Values are mean±SEM, expressed as tension development (in g). n=7–11 in duplicate rings. $p<0.05$ vs.: [a] CON mWKY, [b] CON mSHR.

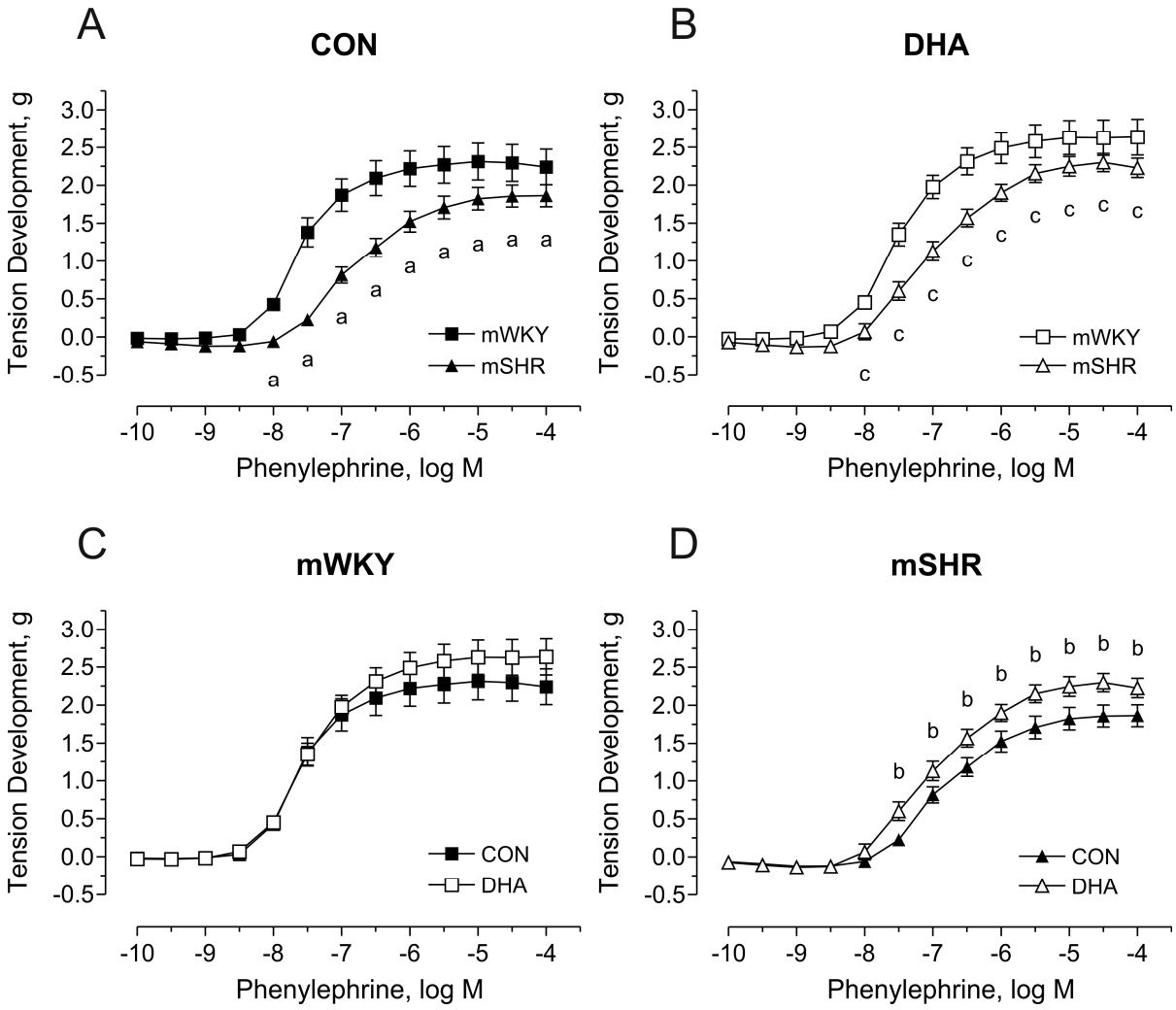

Figure C-2 α_1 adrenergic receptor-mediated contractile response to PE in the absence of L-NAME (ND condition) of thoracic aortic rings isolated from male WKY and SHR following 8–12 wk of CON or DHA diet. Responses of CON (panel A) and DHA groups (panel B) are plotted for comparison of strain (within each diet). Responses of WKY (panel C) and SHR (panel D) are re-plotted separately for comparison of diet (within each strain). Values are mean±SEM, expressed as tension development (in g). n=8–11 in duplicate rings. $p<0.05$ vs.: [a] CON mWKY, [b] CON mSHR, [c] DHA mWKY.

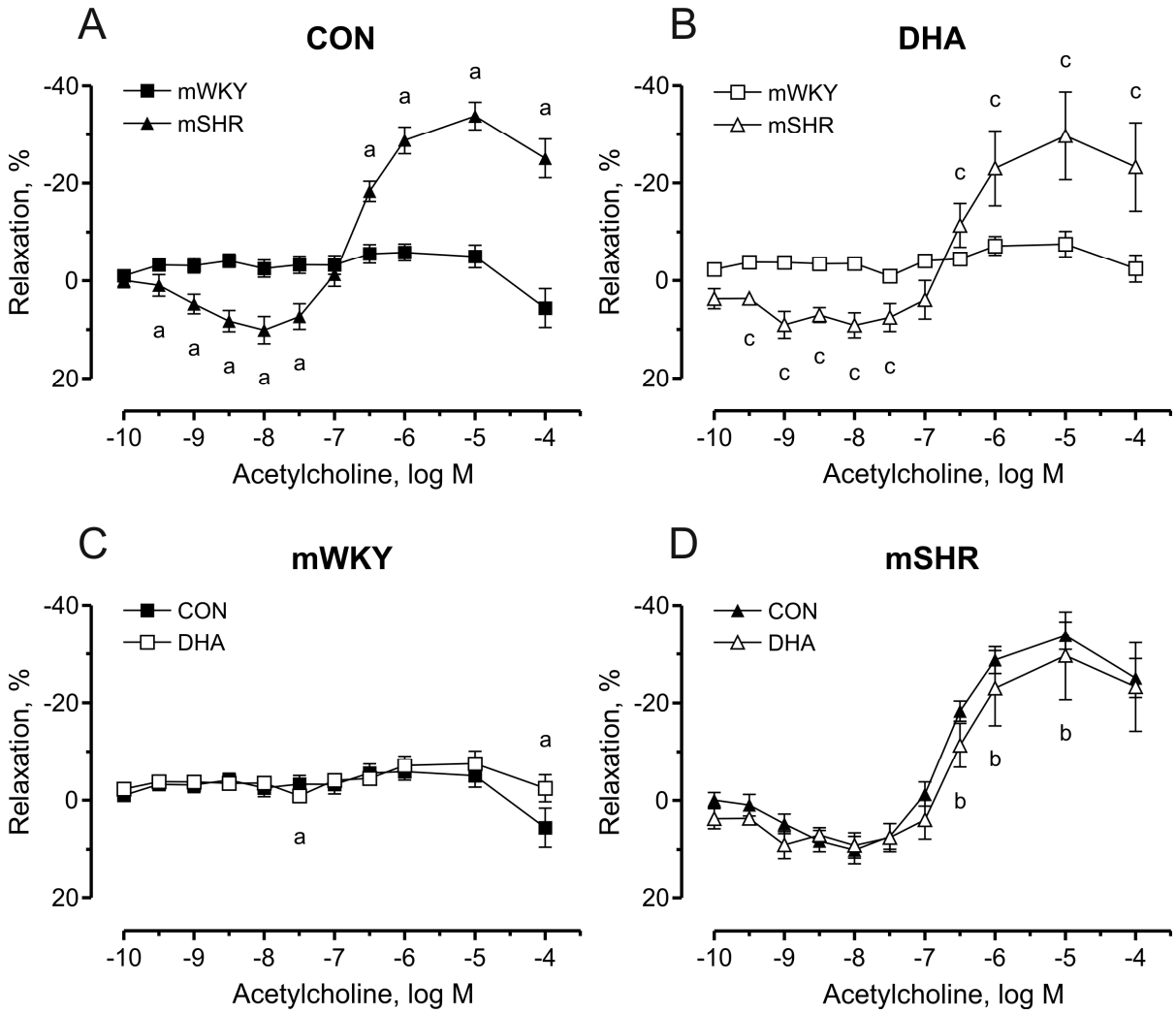

Figure C-3 Endothelium-dependent relaxation to ACh in the presence of L-NAME (10^{-4} M) of thoracic aortic rings isolated from male WKY and SHR following 8–12 wk of CON or DHA diet. Responses of CON (panel A) and DHA groups (panel B) are plotted for comparison of strain (within each diet). Responses of WKY (panel C) and SHR (panel D) are re-plotted separately for comparison of diet (within each strain). Values are mean±SEM, expressed as a percentage of the preceding contraction to PE (10^{-7} M). n=10–12 in duplicate rings. $p<0.05$ vs.: [a] CON mWKY, [b] CON mSHR, [c] DHA mWKY.

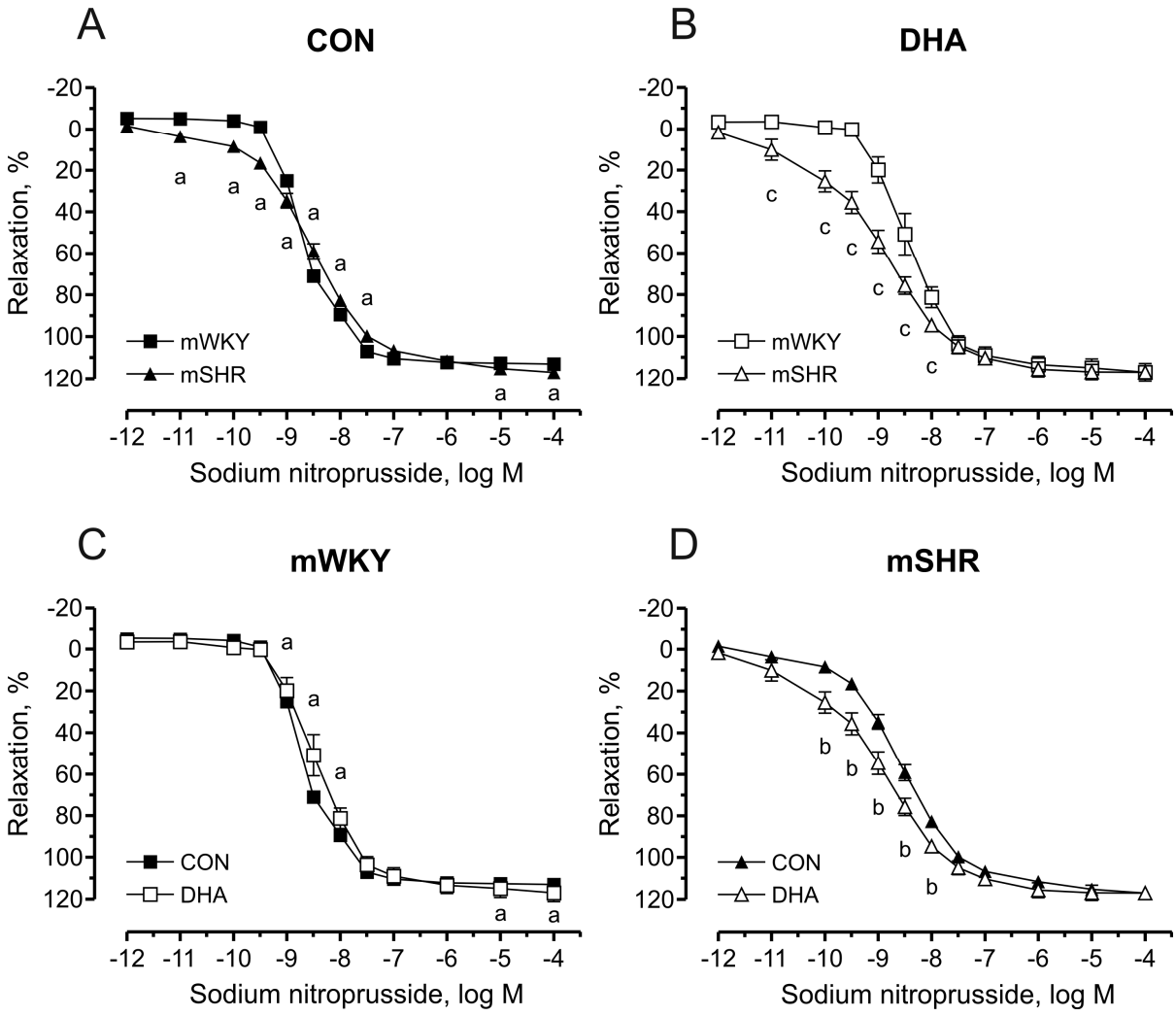

Figure C-4 Endothelium-independent relaxation to SNP in the presence of L-NAME (10^{-4} M) of thoracic aortic rings isolated from male WKY and SHR following 8–12 wk of CON or DHA diet. Responses of CON (panel A) and DHA groups (panel B) are plotted for comparison of strain (within each diet). Responses of WKY (panel C) and SHR (panel D) are re-plotted separately for comparison of diet (within each strain). Values are mean±SEM, expressed as a percentage of the preceding contraction to PE (10^{-7} M). n=9–13 in duplicate rings. $p<0.05$ vs.: [a] CON mWKY, [b] CON mSHR, [c] DHA mWKY.

Appendix D

Supplementary data for Chapter 5

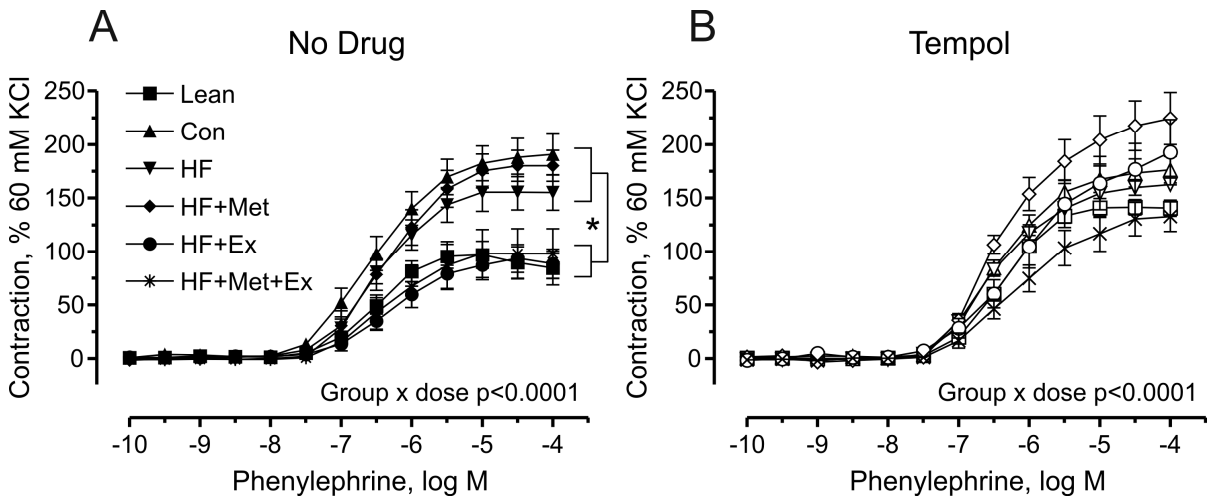

Figure D-1 Aortic α_1 adrenergic receptor-mediated vasocontractile dose-response to PE of 14 wk-old female Zucker lean and ZDF rats in the absence (ND control, panel A) and in the presence of the SOD mimetic, Tempol (panel B). Values are mean±SEM, expressed as a percentage of the developed tension to the second exposure to 60 mM KCl. n=6–10 in singlet rings. * $p<0.05$, relative PE contractions elicited by higher doses ($10^{-6.5}$–10^{-4} M) were elevated in Con, HF, and HF-Met compared to contractions of Lean, HF-Ex, and HF-E+M.

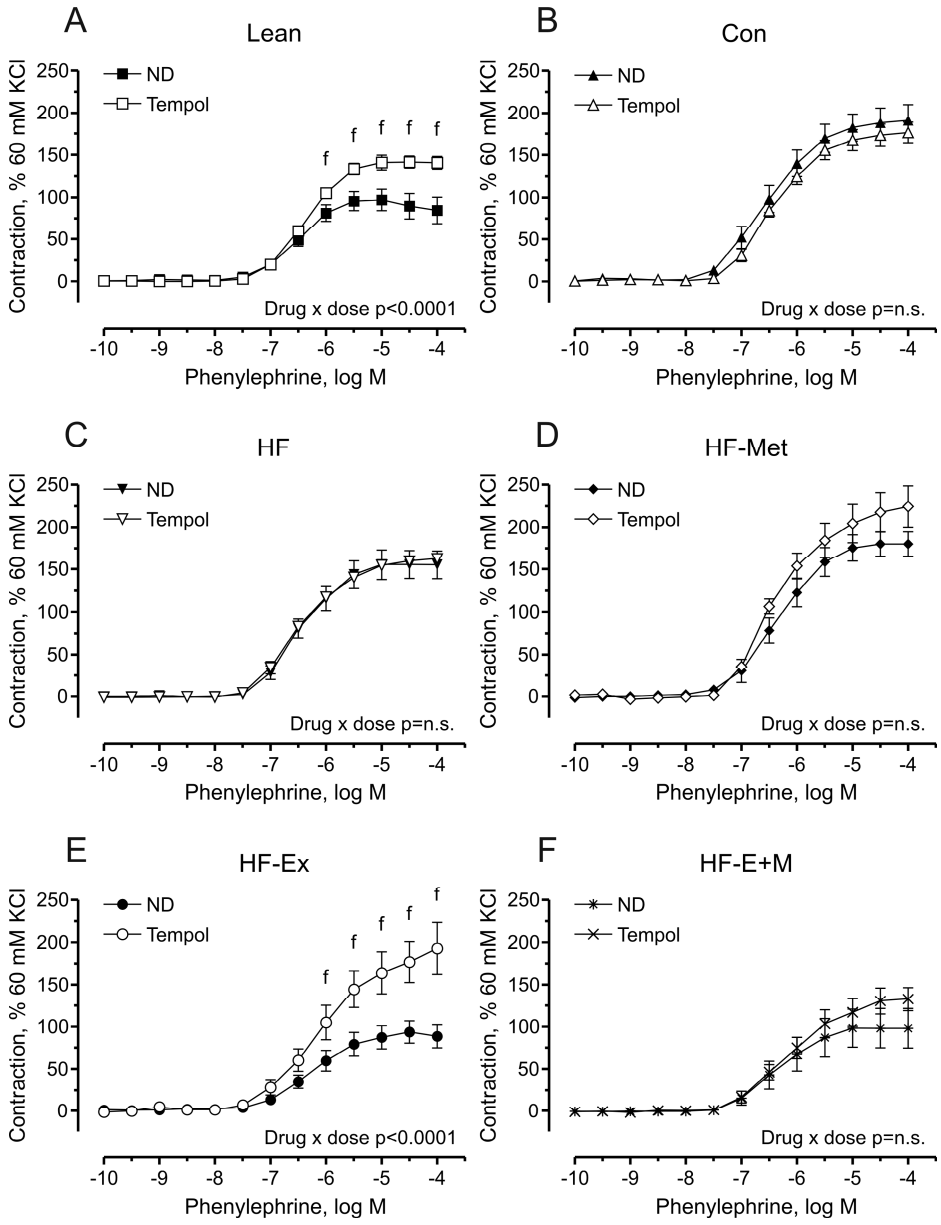

Figure D-2 Aortic α_1 adrenergic receptor-mediated vasocontractile dose-response to PE of 14 wk-old female Zucker lean (panel A) and ZDF rats (panels B–F) in the absence (ND control, closed symbols) and in the presence of the SOD mimetic, Tempol (open symbols). Values are mean±SEM, expressed as a percentage of the developed tension to the second exposure to 60 mM KCl. n=6–10 in singlet rings. $p<0.05$ vs.: ^{f} ND.

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