Acute regulation of vascular tone by AMP-activated protein kinase in arteries of healthy, hypertensive and aged rats

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.

Rebecca Jill Ford.

Abstract

Background, Rationale and General Purpose: Several seminal observations suggest that AMPK mediates vascular tone: 1) in endothelial cell culture and in vitro isolated protein experiments, activation of AMPK stimulates nitric oxide (NO) production via phosphorylation of endothelial nitric oxide synthase (eNOS), 2) stimuli associated with AMPK activation relax isolated vascular smooth muscle preparations from healthy animals, and 3) acute activation of AMPK *in vivo* induces hypotension in normotensive animals, an effect that could be indicative of reduced vascular tone. Together these findings prompt the logical hypothesis that acute activation of AMPK induces relaxation that is both endothelium-, NO-dependent and also vascular smooth muscle dependent; however the direct effects of AMPK activation on the regulation of vascular tone in the context of intact healthy arteries *in vitro* or *in situ* have not been tested. AMPK activation is dysregulated in essential hypertension and aging, conditions both characterized by vasomotor dysfunction. The integrity of AMPK-mediated vasomotor effects has not been evaluated in any model of vascular dysfunction or in the presence of AMPK dysregulation, and so it is unknown if or to what extent, activation of AMPK alters vascular tone in vessels with these impairments. The *mechanisms* of AMPK-mediated vasomotor effects have also not been delineating in healthy or dysfunctional arteries. Studying basic vascular signalling mechanisms in both healthy and dysfunctional models is important for understanding physiological function and regulation of vascular tissue, as well as to understand vascular pathology and aid in the development of therapeutic interventions. Collectively these considerations present compelling reasons to investigate the role of AMPK in vasomotor function in health and disease. The unifying purpose of this thesis was therefore to investigate the role of AMP-activated protein kinase in regulating vascular tone in arteries of healthy, hypertensive and aged rats.

Experimental Approach and Main Findings: The global objective of the thesis is satisfied by four main studies that utilize a combination of *in vitro* isolated artery preparations to assess vasomotor

function, biochemical analyses and *in vivo* hemodynamic assessments. In **Study 1**, we characterize the basic nature of the vasomotor response generated acutely by the pharmacological AMPK activator AICAR *in vitro* in isolated aorta of normotensive (Wistar-Kyoto rats; WKY) and hypertensive rats (Spontaneously Hypertensive rats; SHR), and the mechanisms mediating these responses. In these experiments, acute activation of AMPK using AICAR induced dose-dependent relaxation of isolated, precontracted arteries from WKY and SHR that was dependent in part on both the endothelium and vascular smooth muscle, and vasorelaxation to AICAR was *enhanced* in aortic rings of SHR versus those of WKY. In WKY, the endothelium-dependent component of relaxation to AICAR was solely NO-mediated, while in SHR it was dependent on both elevated NO-bioactivity and blunted COX-dependent contraction. In **Study 2**, we investigate the mechanisms responsible for AMPK-mediated inhibition of endothelium- and cyclooxygenase-dependent vasocontraction in aorta from WKY and SHR (a response enhanced in arteries of hypertensive rats that contributes to vasomotor dysfunction). Pre-activation of AMPK blunted endothelium-dependent contractions to acetylcholine in isolated, non-precontracted WKY and SHR aortic rings. The mechanisms accounting for this effect of AICAR were endothelium-specific, occurring via inhibition of the ACh-stimulated production/release of 6-keto-prostaglandin $F_{1\alpha}$, the major product of prostacyclin, which is the key prostanoid responsible for endothelium-dependent contractions in aorta of WKY and SHR. AMPK activation had no effect on vascular smooth muscle responsiveness to TP-receptor agonists, ruling out a contribution of vascular smooth muscle mechanisms. In **Study 3**, we examine responses and mechanisms associated with acute pharmacological AMPK activation on vascular tone of isolated mesenteric resistance arteries *in vitro*, and on *in vivo* hemodynamics in WKY and SHR. These experiments revealed that administration of AICAR acutely *in vivo* acutely reduced blood pressure by ~70mmHg in SHR and this effect was partly NO-dependent. In contrast, AICAR had no effect on blood pressure in WKY. Activation of AMPK also produced vasodilation of isolated, precontracted WKY and SHR resistance mesenteric arteries *in vitro*, and this was dependent on NO to a greater extent in SHR than in WKY. Together, the parallel reductions in blood pressure *in vivo* and relaxation of isolated arteries *in vitro* support reduced vascular resistance as a potential explanation for the *in vivo* blood pressure

effects. Finally, **Study 4** characterizes the basic vasodilatory responses to acute AMPK activation and mechanisms associated with these responses in aorta from aged animals and their young counterparts (male Sprague Dawley rats) to glean insight using an additional model of vasomotor dysfunction. In this study, acute activation of AMPK using AICAR generates relaxation in a dose-dependent manner that is partly endothelium-, NO-dependent and partly reliant on vascular smooth muscle in precontracted aorta of both young and aged rats. Similar to the findings of Study 1 in SHR versus WKY, vasodilatory response to AICAR were also enhanced in dysfunctional aorta of aged rats versus healthy aorta of young animals. Other agents shown to activate AMPK in other tissues and models, the anti-diabetic drug metformin and the polyphenol resveratrol, generated varying amounts of relaxation in vascular smooth muscle of young and aged aortic rings. These effects were only associated with AMPK activation in rings treated with metformin but not resveratrol.

Conclusions and Perspectives: These findings are the first to characterize the vasomotor responses generated by acutely activating AMPK in intact arteries of any hypertensive or aging model, and to delineate mechanisms mediating these responses in healthy and dysfunctional vessels. Despite vasomotor dysfunction and dysregulated AMPK activity in arteries of hypertensive and aged rats, acute AMPK activation still generates robust relaxation responses via endothelium- and NO-dependent relaxation, inhibition of enhanced endothelium-dependent contractions in SHR, and direct relaxation of the vascular smooth muscle; effects that would aid in reversing the dysfunctional characteristics of arteries from these animals, and may recommend AMPK as a useful therapeutic target for interventions aimed at improving vasomotor function. Future studies will be necessary to reveal whether AMPK plays a role in generating acute changes in vessel tone induced by AMPK-activating physiological stimuli *in situ* (i.e. such as shear stress during exercise). Together these data continue to support AMPK as a novel regulator of vascular tone, yield valuable, novel, mechanistic insight into AMPK-mediated regulation of vasomotor function in arteries during health, disease and aging, and highlight the need for continued investigation into a vasoregulatory function for AMPK in health and disease.

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List of Abbreviations

AA; arachadonic acid

ACC; acetyl-CoA carboxylase

ACh; acetylcholine

AICAR; 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AMPK activator)

AMPK; adenosine monophosphate-activated protein kinase

BH_4; tetrahydrobiopterin

BP; blood pressure

cAMP; cyclic adenosine monophosphate

CaM; calmodulin

CaMKII; Ca^{2+}/Calmodulin protein kinase II

CC; Compound C (AMPK inhibitor)

cGMP; cyclic guanosine monophosphate

CIF; calcium influx factor

CO; carbon monoxide

COX; cyclooxygenase (COX-1 or COX-2 denotes cycooxygenase isoforms 1 or 2)

$cPLA_2$; cytosolic (calcium-dependent) phospholipase A_2

DAG; diacylglycerol

CPI-17; PKC-potentiated myosin phosphatase inhibitor of 17 kDa

EDCF; endothelium-derived contracting factor

EDHF; endothelium-derived hyperpolarizing factor

EDRF; endothelium-derived relaxing factor

EETs; epoxyeicosatrienoic acids

eNOS; endothelial nitric oxide synthase

ER; endoplasmic reticulum

FAD; flavin adenine dinucleotide

FMN; flavin mononucleotide

G_x; G-proteins (x denotes subtype)

20-HETEs; 20-hydroxyeicosatetraenoic acids

HR; heart rate

H_2S; hydrogen sulphide

HSP90; heat shock protein 90

INDO; indomethacin (cyclooxygenase inhibitor)

IP_3; inositol triphosphate

$iPLA_2$; calcium-independent phospholipase A_2

IP-receptor; prostaglandin I_2/prostacyclin receptor

$[Ca^{2+}]_i$; intracellular calcium concentration

KCl; potassium chloride

L-NAME; N_ω-nitro-L-arginine methyl ester (NOS inhibitor)

lysoPL; lysophospholipids

MAP; mean arterial pressure

MLC_{20}; myosin regulatory light chain 20

MLCK; myosin light chain kinase

MLCP; myosin light chain phosphatase

MYPT1; myosin phosphatase target subunit 1

NADPH; nicotinamide adenine dinucleotide phosphate

NEPI; norepinephrine

NO; nitric oxide

$O_2^{\bullet-}$; superoxide anion

$OONO^{\bullet-}$; peroxynitrite

p-ACC, P(Ser79)-ACC; acetyl-CoA carboxylase phosphorylated at serine 79

p-AMPK, P(Thr172)-AMPK; AMPK phosphorylated at threonine 172

p-eNOS, P(Ser1177)-eNOS; eNOS phosphorylated at serine 1177

PE; phenylephrine

PG; prostaglandin

PGD_2; prostaglandin D_2

PGE_2; prostaglandin E_2

$PGF_{1\alpha}$; prostaglandin $F_{1\alpha}$

PGH_2; prostaglandin H_2

PGI_2; prostacyclin

PI3K; phosphoinositide 3-kinase

PK's; protein kinases

PKA; protein kinase A

PKB; protein kinase B

PKC; protein kinase C

PKG; protein kinase G

PLs; phospholipids

PLC; phospholipase C

RhoA; Ras homolog gene family member A

Rho-GEFs; Rho family of guanine exchange factors

ROCK, Rho-kinse; Rho-associated protein kinse

ROS; reactive oxygen species

S-1-P; sphingosine-1-phosphate

SERCA; sarco/endoplasmic reticulum Ca^{2+}-ATPase

sGC; soluble guanylate cyclase

SHR; Spontaneously hypertensive rats

SOC; store-operated calcium channel

SR; sarcoplasmic reticulum

TP-receptor; thromboxane-prostanoid receptor

TxA_2; thromboxane A_2

VEGF; vascular endothelial growth factor

VGCC; voltage-gated calcium channels

VSM; vascular smooth muscle

WKY; Wistar-Kyoto rats

Chapter 1

General Introduction

1.1 Orientation to the thesis

1.1.1 Global Purpose and Approach

The unifying purpose of this thesis is to investigate the role of AMPK in regulating arterial vascular tone in models of health and dysfunction (essential hypertension and aging). Our primary experimental approach employs *in vitro* isolated artery preparations to evaluate vasomotor function while pharmacologically manipulating AMPK activation and vasomotor pathways of interest. These measures are complimented with biochemical analyses and *in vivo* hemodynamic assessments.

1.1.2 Thesis Organization

The thesis begins with background information, rationale for the thesis topic, an overview of the thesis studies (the specific objectives and hypotheses for each study), and descriptions of the major models and methods chosen for the experiments. The thesis is comprised of four main studies that are each presented in manuscript form. In **Study 1**, entitled *Endothelium-dependent vasorelaxation to the AMPK activator AICAR is enhanced in aorta from hypertensive rats and is NO- and EDCF-dependent* (published in the American Journal of Physiology Heart and Circulatory Physiology), we investigate the vasomotor responses generated by acutely activating AMPK in isolated aorta of normotensive and hypertensive rats *in vitro,* and the mechanisms associated with these responses. In **Study 2,** entitled *Activation of AMP-activated protein kinase blunts contractions to acetylcholine by endothelium-dependent mechanisms in aorta of normotensive and hypertensive rats*, we explore mechanisms mediating the effects of AMPK activation on endothelium-dependent contractions (a hallmark feature of vasomotor dyfunction in hypertension), using isolated aorta of normotensive and hypertensive rats. **Study 3** is entitled *AMPK activator AICAR acutely lowers blood pressure and relaxes resistance arteries of hypertensive rats* (accepted for publication at the Journal of Hypertension) and investigates alterations in

blood pressure in response to acute AMPK activation *in vivo* in normotensive and hypertensive animals, and the acute vasomotor effects of AMPK activation on isolated mesenteric resistance arteries of these animals *in vitro*. The final study (**Study 4),** entitled *Vasomotor responses generated by acute exposure to AMP-activated protein kinase activators in isolated aorta of young and aged rats***,** mirrors Study 1 in that we evaluate the vasodilatory effects of acute AMPK activation, but in this study we use isolated aorta from young and aged rats. Each experimental chapter is also succeeded by an addendum containing related data that is not part of the manuscript. The thesis concludes with a general summary of the main findings, conclusions, overall interpretation, potential implications, future directions, limitations, and perspectives derived from the collective body of work.

1.2 Preface: The physiological and pathological big picture -- the importance of studying vascular signalling systems in health and disease

The arterial wall is a dynamic tissue that is capable of sensing, integrating and responding to complex patterns of environmental signals to serve many important physiological functions. It consists of three distinct layers that include (moving from the lumen to vessel wall exterior): 1) the tunica intima, a single layer of endothelial cells anchored to a connective tissue matrix, 2) the tunica media, composed of vascular smooth muscle (VSM) cell layers aligned circumferentially around the vessel lumen, and 3) the tunica adventia, a membrane of collagen and elastin fibers overlayed by perivascular fat, nerves and fibroblasts[158]. Arteries play an essential role in maintaining homeostatic control of hemostasis, thrombogenesis and fibrinolysis, vascular permeability, tissue perfusion, and vascular resistance[62,65,89]. The endothelium is the central orchestrator of many of these functions, and accordingly, dysregulation of the endothelial function is particularly debilitating. Endothelial dysfunction is a hallmark feature of major cardiovascular disease risk factors such as aging[17,211], smoking[17,108], menopause[17,210], diabetes mellitus[17], hypercholesterolemia[17], and hypertension[17,172,209], and contributes to the progression and exacerbation of a host of overt cardiovascular diseases including atherosclerosis[231], coronary artery

disease[187,231], heart failure[109], and peripheral vascular disease[84] (Figure 1-1). Cardiovascular disease is a major health concern, accounting for ~ 30% of the human mortality rate in Canada[105] and globally[255], and represents a substantial societal financial burden that costs the Canadian economy more that 22 billion dollars annually[106].

Since the signalling systems within and between vascular cells are the underlying mechanisms that dictate the capacity of the vasculature to perform its physiological functions, it is not surprising that investigations aimed at understanding the basic mechanisms that govern vascular function in health and disease have become an important research priority. Substantial progess has been made towards understanding vascular signalling processes in physiological and pathophysiological circumstances, and in the translation of this knowledge into treatment strategies; however even with these advancements, existing therapeutic interventions still do not completely restore proper endothelial function, or only address some of the contributing factors [62]. Therefore, continuing to study novel and existing vasomotor signalling mechanisms is important for moving forward, to both expand our comprehension of the normal physiological function of the vasculature, and to uncover how vascular signalling processes are compromised in disease to facilitate the development of more efficacious therapeutic strategies. The aim of this thesis is to understand how a novel signalling pathway contributes to the regulation of vascular tone in healthy and in dysfunctional arteries, with the greater goal of ultimately enhancing our comprehension of vascular function in physiological and pathophysiological conditions.

1.3 Thesis focus: the regulation of vascular tone

Although vascular tissue has many important functions, the focus of this thesis is on regulation of vascular tone. The ability of arteries to properly regulate vessel tone is also indicative of the health of vascular signalling systems, and thus provides some insight regarding their ability to carry out other functions (such as anti-thrombotic, anti-inflammatory, anti-proliferative roles). Vascular tone is the net level of contraction present in the vascular wall, and influences the diameter of the vessel lumen. The

capacity to tightly regulate vascular tone is important, because it confers upon the arterial system the ability to divert and direct blood flow to meet metabolic demand, and to contribute to the homeostatic regulation of blood pressure by maintaining appropriate levels of vascular resistance[158]. The net level of vascular tone is ultimately determined by the balance between the multiple vasodilatory and vasoconstrictory signals that converge on the contractile machinery of the VSM [89] (Figure 1-1). *In situ,* signal input to the VSM originates from the vessel lumen via the endothelium (i.e. the endothelium senses physical, hormonal, and chemical signals in the blood stream and generates signal output to the underlying VSM) and from other sources that act on the VSM directly (i.e. neural input, pressure changes; Figure 1-1). *In vitro*, vascular tone of isolated arteries can be manipulated using endothelium-dependent and endothelium-independent (VSM dependent) agonists to study endothelial and VSM vasomotor signalling pathways in isolation. The following section describes the vascular signalling systems of the endothelium and VSM that are involved in regulating vascular tone in healthy arteries, and then describes how these become dysfunctional in essential hypertension and aging.

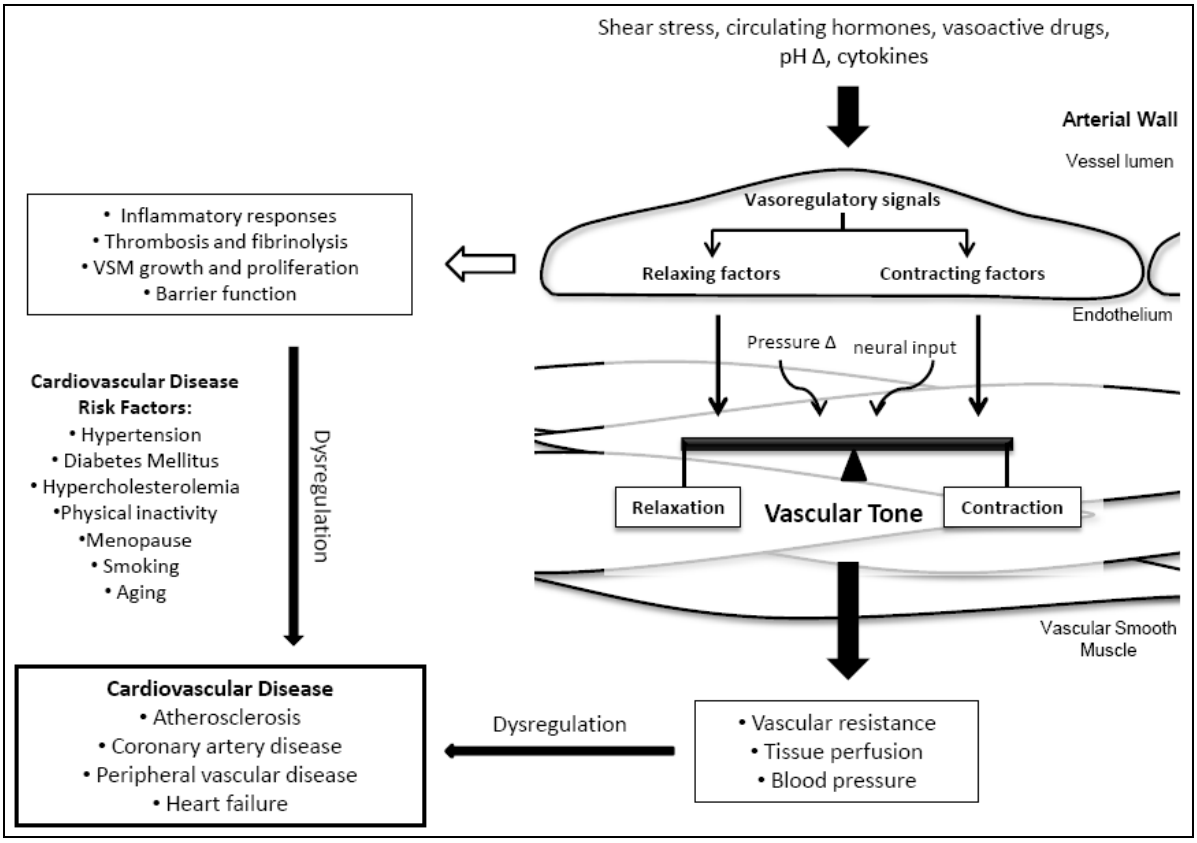


Figure 1-1. Regulation and function of the vascular wall, and its dysregulation in cardiovascular disease. The arterial wall is comprised of endothelial and vascular smooth muscle cell layers. External signals act via the endothelium or directly on the VSM to influence vessel tone, which is important for regulating vascular resistance, tissue perfusion and contributes to the maintenance of blood pressure. The endothelium is also a central regulator of other important functions of the vascular wall, such as thrombosis and fibrinolysis, vascular inflammatory responses, VSM growth and proliferation and barrier function. Dysregulation of vascular function is a hallmark feature that co-exists in the presence of cardiovascular disease risk factors, and ultimately contributes to the development of cardiovascular disease. See text for further details.

1.3.1 Endothelium-derived vasomotor signals

In 1980, Furchgott and Zawadski performed a seminal experiment that demonstrated for the first time the importance of endothelium in directing VSM tone [76]. Extensive research in endothelial biology over the 30 years since then has revealed that endothelial cells sense and respond to signals including physical factors (shear stress, changes in pressure), substances released from nerves (autonomic, sensory) and platelets, pH changes, circulating hormones, vasoactive drugs, and cytokines[62]. Exposure to these factors *in vivo* or *in vitro,* stimulate the endothelium to produce and release a plethora of vasodilatory

(endothelium derived relaxing factors; EDRFs)[55] and vasoconstrictory (endothelium-derived contracting factors; EDCFs)[62] signalling intermediates that act on the VSM. EDRFs produced and released by the endothelium include nitric oxide (NO), COX-derived prostacyclin (PGI_2), adenosine, hydrogen peroxide, epoxyeicosatrienoic acids (EETs), C-natriuretic peptide (CNP) and endothelium-derived hyperpolarizing factors (EDHFs; a broad term for substances including H_2S, CO etc)[62,216]. EDCFs generated by the endothelium can include substances such as angiotensin II, endothelin-1, uridine adenosine tetraphosphate, superoxide anion, 20-hydroxyeicosatetraenoic acid (20-HETEs) and other COX-derived endoperoxides and prostanoids (prostaglandin H_2 (PGH_2), prostacyclin (PGI_2), thromboxane A_2 (TxA_2), prostaglandin $F_{1\alpha}$, prostaglandin E_2 and others)[62]. EDRFs and EDCFs diffuse in a paracrine fashion to the underlying VSM to stimulate relaxation or contraction respectively, and maintaining a proper balance between EDRF and EDCF signalling is important for healthy vasomotor function and regulation of vascular tone. The particular compliment of vasoactive factors generated by the endothelium varies and is dependent upon the agonist/stimulus applied, on the arterial bed involved (i.e. heterogeneity exists in vasoactive factors generated to the same stimulus across vascular beds) and on the health status of the vessel (diseased endothelium generate a different compliment of vasoactive factors compared to their health counterparts) [229]. For example: of the EDRFs, NO is the major contributor to vasorelaxation in large conduit arteries [64,144,231], where as this predominance is offset by the contribution of EDHFs in resistance vasculature [216]; although the production and release of vasoconstrictor COX-derived prostanoids are observed in healthy blood vessels and likely play a role in the physiological regulation of vascular tone, over-production of these products occurs in arteries with aging or disease and contributes to vasomotor dysfunction[62].

Of the EDRFs and EDCFs that contribute to vascular tone, the signalling systems regulating NO and COX-derived prostanoids will be the focus of this thesis. These two systems are of particular interest because reduced NO bioavailability and enhanced endothelium-COX-EDCF-TP-receptor mediated contractions are two main factors that individually or jointly contribute to endothelial dysfunction in

hypertension and aging [62,65], and these are the main pathways of interest in terms of their potential interaction with our enzyme of interest (AMPK). Thus for clarity and brevity, only the vasomotor signalling systems pertaining to NO and COX-derived prostanoids will be described in detail in the following section. Although mechanical forces like fluid shear stress and hormonal stimuli play a major role in the physiological regulation of vascular tone *in vivo* via activation of endothelium-dependent signalling mechanisms, G-protein coupled receptor agonists also contribute to the regulation of vascular tone, and are useful for studying common endothelial signalling systems *in vitro* (which was the approach used to evaluate vasomotor pathways in the thesis experiments). Therefore the following discussion also focuses on how these systems are modulated by G-protein coupled receptor agonists.

1.3.2 Endothelium-derived vasomotor signals: a focus on nitric oxide and cyclooxygenase-derived prostanoids

The production of NO and COX-derived prostanoids by arterial endothelium can be initiated by agonists such as acetylcholine, bradykinin, adenine nucleotides (ATP and ADP) and others that stimulate specific G-coupled protein receptors (i.e. M_3-muscarinic, B_2- bradykinin, P_2Y-purigenic receptors respectively) on the endothelial cell surface [55,190,230]. G-proteins exist as several different subtypes, and the receptor/G-proteins combination determines the downstream signalling processes initiated. These downstream effects typically include activation of protein kinase signalling cascades and/or the release of calcium from intracellular stores. G_i (pertussis toxin-insenstive, coupled to serotonin, thrombin, sphingosine-1-phosphate receptors) initiate PI3K-Akt/PKB kinase signalling cascades[55,190,230]; G_s (responsive to shear stress) activate PI3K-Akt/PKB cascade as well as adenylate cyclase-cAMP mediated activation of protein kinase A (PKA)[190]; and G_q (pertussis toxin-sensitive, coupled to bradykinin, purigenic, muscarinic receptors) activate phospholipase Cβ-mediated production of IP_3 and DAG from membrane phospholipids. In the case of G_q stimulation, IP_3 acts on IP_3-sensitive receptors on the endoplasmic reticulum (ER) to trigger Ca^{2+} release from intracellular stores [55,190,230]. Elevation of endothelial $[Ca^{2+}]_i$ is from the ER is a central mechanism responsible for the production of both NO and

COX-derived prostanoids, where as the protein kinase signalling cascades contribute to production of NO.

Nitric oxide

NO is produced by endothelial nitric oxide synthase (eNOS) from L-arginine, oxygen, and NADPH in the presence of heat shock protein 90, and FMN, FAD and BH_4 co-factors [55,190]. eNOS is activated by increases in $[Ca^{2+}]_i$, which facilitate the formation of Ca^{2+}-calmodulin complexes. Ca^{2+}-calmodulin binds to eNOS and stimulates NO production by increasing the efficiency of electron flow through the eNOS active complex[55]. eNOS can also be activated covalently upon phosphorylation at its serine 1177 and serine 633 residues by upstream kinases such as PI3K-Akt/PKB, CaMKII, PKA, or AMPK [55,190]. NO presumably diffuses across the intracellular space or through gap junctions to the VSM where it stimulates relaxation via activation of soluble guanylate cyclase (sGC) in the VSM[190,230,231]. NO can also contribute to relaxation via other mechanisms independently of sGC, such as by direct activity on potassium channels (BK_{Ca}, K_v, K_{ATP}; promoting K^+ efflux, hyperpolarization and relaxation)[55,64], Na^+-K^+-ATPase[64] and SERCA (to promote re-uptake of intracellular Ca^{2+} and relaxation) via protein nitrosylation [34] in the VSM (mechanisms of VSM relaxation will be discussed in more detail below). The signalling pathways involved in NO-mediated vasomotor function are depicted in Figure 1.2.

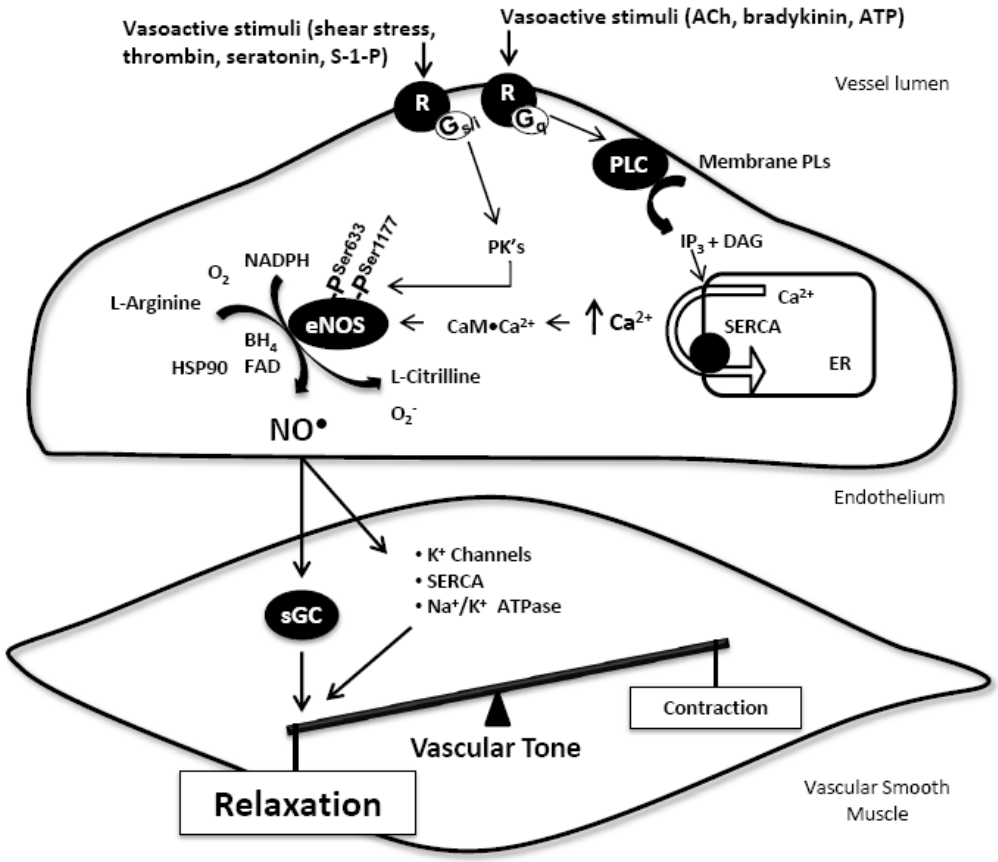


Figure 1-2. Endothelium- and nitric oxide-dependent regulation of vascular tone. See text for details.

Cyclooxygenase-derived endoperoxides and prostaglandins

Elevation of endothelial $[Ca^{2+}]_i$ by agonist-mediated mechanisms, or via compounds that bypass agonist-dependent machinery to facilitate the entrance of extracellular Ca^{2+} (i.e. calcium ionophores like A23187), stimulate the production and release of COX-derived prostanoids. The current working hypothesis for the signalling mechanisms mediating agonist-stimulated prostanoid production by the endothelium is depicted in Figure 1-3. Briefly, stimulation of G_q-coupled receptors (by ACh, bradykinin, adenine nucleotides) results in IP_3-mediated release of Ca^{2+} from the ER (described above). Although not yet demonstrated in endothelial cells, results from experiments in other cell types suggest that depletion of ER Ca^{2+} content triggers the release of Ca^{2+} influx factor (CIF) from the ER [177], and that CIF displaces calmodulin from its inhibitory association with Ca^{2+}-independent phospholipase A_2 β ($iPLA_2$) [12,169,194].

Activated endothelial $iPLA_2$ then releases lysophospholipids from membrane lipids that interact with store-operated Ca^{2+} channels on the endothelial cell membrane to permit the entrance of extracellular Ca^{2+} into the cytoplasm [12,195,250]. This influx of Ca^{2+} actives cytosolic phospholipase A_2 α ($cPLA_2$; a Ca^{2+}-dependent isoform), which liberates arachondonic acid from lipid membranes to act as a substrate for COX [63,250]. COX-1 and COX-2 isoforms both generate endoperoxides such as PGH_2 from arachadonic acid. PGH_2 is the central substrate for the family of prostaglandin (PG) synthases (i.e. prostacyclin, thromboxane, PGD, PGE and PGF sythnase) that convert this compound into a number of specific prostanoid by-products (PGI_2, TxA_2, PGD_2, PGE_2 and $PGF_{2\alpha}$ respectively). These substances (PGH_2 and prostanoids) subsequently exert vasomotor effects by their interaction with VSM receptors [63].

At the VSM, endoperoxides and prostaglandins interact with prostanoid receptors, which are part of the seven transmembrane G-protein coupled receptor family [63]. Although there are five subtypes of these receptors that function to preferentially interact with specific prostanoids mentioned above, prostaglandin I_2/prostacyclin (IP)- and thromboxane prostanoid (TP)- are the two main types important for understanding the current work. IP-receptors have a preferential affinity for PGI_2 and initiate vasorelaxation, where as TP-receptors may be activated by PGH_2, PGI_2, TxA_2 and others (i.e. not only by TxA_2; order of affinity for prostanoids is TxA_2 > U46619 >> 8-isoprostane = $PGF_{2\alpha}$ = PGH_2 > PGE_2 = PGD_2 > PGI_2) and initiate VSM contraction[65]. In healthy arteries, PGI_2 acts predominantly on IP-receptors to function as a vasodilator; however, under circumstances in which PGI_2 concentrations are supra-normal, in the absence of functional IP-receptors, or hyper-sensitivity of TP-receptors (as can occur in dysfunctional arteries, discussed below in section *1.4 Dysregulation of vascular tone in essential hypertension and aging*), PGI_2 paradoxically stimulates TP-receptors and acts as a vasoconstrictor[63,65].

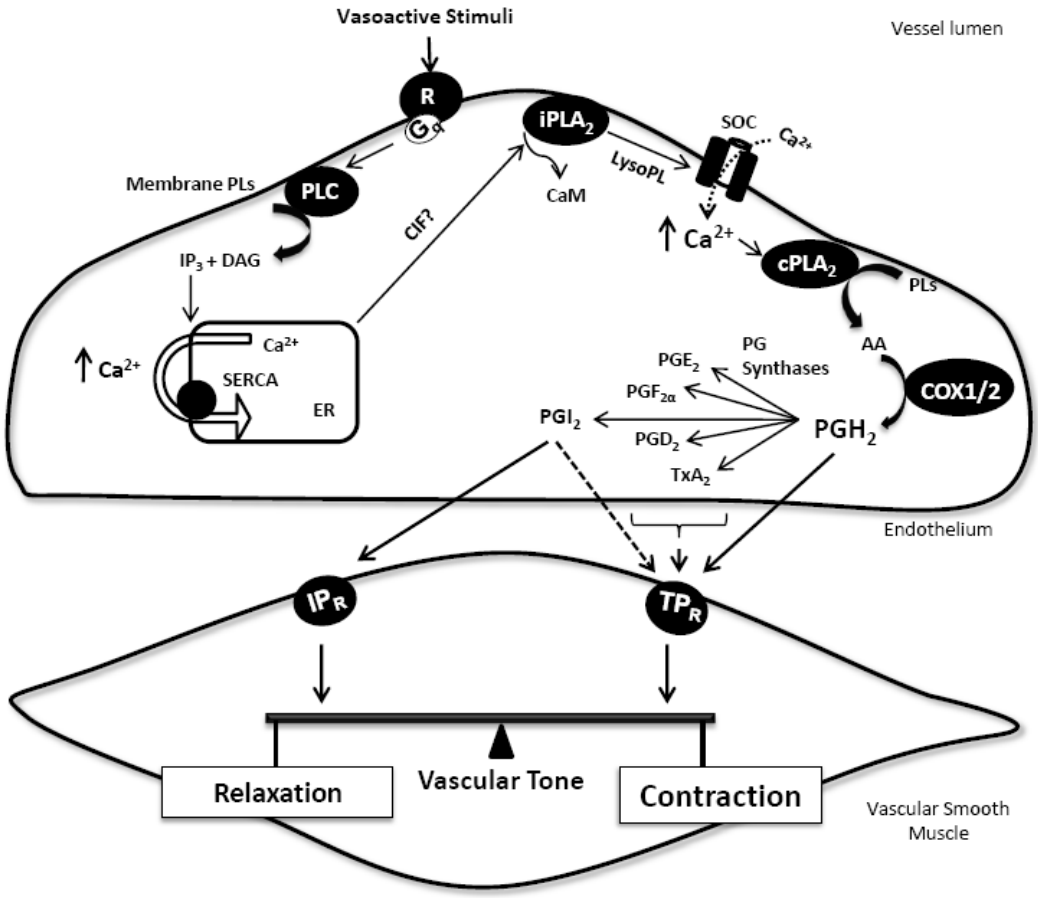


Figure 1-3. Cyclooxygenase-derived endoperoxide and prostanoid mediated regulation of vascular tone. See text for details.

1.3.3 Convergence of EDRFs and EDCFs on the contractile machinery: signalling systems of the vascular smooth muscle

Relaxation and contraction of the VSM can be initiated by either receptor-mediated signalling pathways (i.e. sGC by NO, TP and IP receptors by prostanoids, α-adrenergic receptors by phenylephrine) or by inducing changes in the VSM membrane potential (i.e. EDHFs, potassium chloride etc.). These mechanisms are coupled to one or both of the two main signalling axes within VSM cells that govern active tone: the Ca^{2+}-dependent and Ca^{2+}-independent (or Ca^{2+}-sensitization) signalling pathways [164,198]. The activation of these systems independently or synergistically converge to increase phosphorylation of

the 20 kDa myosin light chain regulatory subunit on smooth muscle myosin (MLC20), which is the final step required to promote cross bridge cycling and contraction [164,198] (Figure 1-4).

Ca^{2+}-dependent contraction is induced by agonists, stretch or depolarization of the VSM resting membrane potential. Depolarization (by agents like potassium chloride or action potentials) initiates elevated $[Ca^{2+}]_i$ by triggering an influx of extracellular Ca^{2+} through voltage dependent L-type Ca^{2+} channels (VGCC) that may be augmented by the release of Ca^{2+} from intracellular SR stores[164]. Agonists (like EDCFs or phenylephrine) stimulate receptors coupled to $G_{q/11}$ proteins activate phospholipase C to produce IP_3 and DAG; IP_3 acts on IP_3-sensitive Ca^{2+} release channels on the SR to initiate the release of Ca^{2+} into the cytosol[164]. Elevated $[Ca^{2+}]_i$ by either mechanism interacts with calmodulin to activate Ca^{2+}-sensitive myosin light chain kinase (MLCK), which is responsible for phosphorylating MLC20 of VSM myosin to initiate cross bridge cycling and generate contraction[164].

Ca^{2+}-sensitization pathways are activated by agonist stimulation of receptors coupled to the $G_{12/13}$ subtypes of the G-protein super-family (i.e. TP-receptors), which stimulate mobilization of Rho-GEFs to activate RhoA-Rho kinase[198]. Agonist receptors coupled to $G_{q/11}$ proteins can also activate Ca^{2+} sensitization in addition to stimulating Ca^{2+} release, as DAG generated by PLC activates PKC that in turn activates the kinase CP1-17[198]. These kinases (Rho kinase and CPI-17) phosphorylate the regulatory subunit (MYPT1) of myosin light chain phosphatase (MLCP), to *inhibit* its activity; since MLCP is responsible for *de*phosphorylation of MLC20, inhibition of MLCP ultimately leads to contraction by allowing phosphorylation of MLC20 by MLCK to persist/increase (in essence enhancing the sensitivity of the contractile apparatus to a given concentration of Ca^{2+})[164,198].

Vasorelaxation can be achieved by: 1) decreasing activity of MLCK via reduced VSM $[Ca^{2+}]_i$ and/or 2) inhibition of the Ca^{2+} sensitization pathway, reversing the suppression of MLCP activity to allow de-phosphorylation at MLC20. One of the main ways in which both of these effects are initiated is via central coordinate activity of VSM PKA and PKG[198]. Activation of soluble guanylate cyclase

(resulting in the production of cGMP from GTP) by NO or activation of IP-receptors by PGI_2 (coupled to G_s proteins and adenylate kinase that generates cAMP from ATP) both stimulate PKA and/or PKG (activated by cAMP and cGMP respectively)[198]. PKA and PKG exert multiple functions that result in lower $[Ca^{2+}]_i$ (by activating SERCA, extrusion by plasma membrane Ca^{2+}-ATPase, inhibiting SR Ca^{2+} release by IP_3-mediated receptor channels, and inducing cell hyperpolarization via activation of Ca^{2+}- or ATP-dependent K^+ efflux channels, inward rectifying K^+ channels or Na^+-K^+ ATPase)[133] and decreased Ca^{2+} sensitization (via phosphorylation of an inhibitory site on MLCK and MLCK regulatory protein telokin)[198]. A simplified representation of the signalling pathways modulating VSM tone is depicted in Figure 1-4.

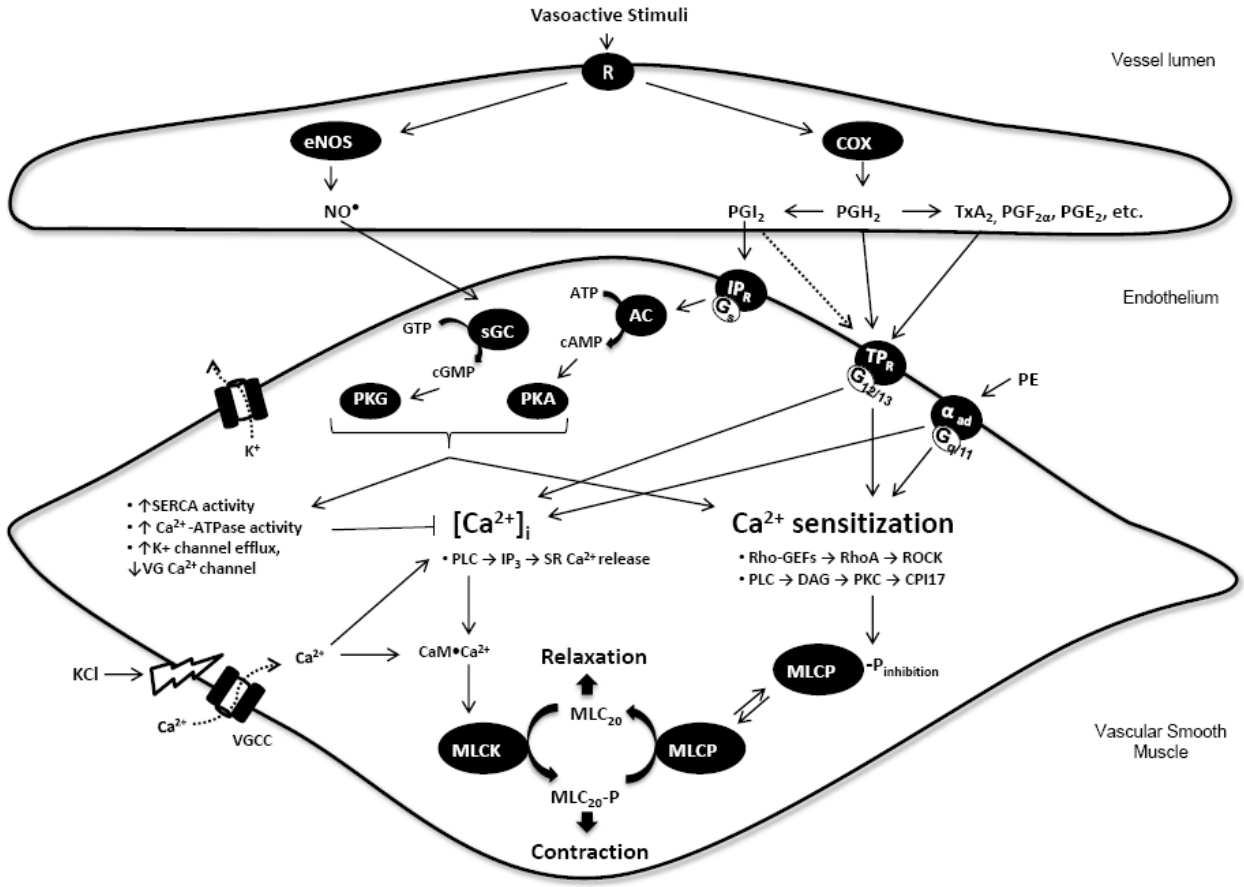


Figure 1-4. The signalling pathways of the vascular smooth muscle that dictate vascular tone. See text for details.

1.4 Dysregulation of vascular tone in essential hypertension and aging

In both hypertension and aging, homeostatic regulation of vascular tone is impaired, resulting in vascular dysfunction. Vasomotor dysfunction results from an imbalance between vasodilatory and vasoconstrictory signals that converge on the VSM contractile apparatus, and can be exacerbated by both dysregulation of the endothelium-dependent signals reaching the VSM (endothelial dysfunction is a major contributor to vasomotor dysfunction), and by altered VSM responsiveness. Endothelial dysfunction is characterized by impaired vasorelaxation and enhanced vasocontraction responses to vasoactive stimuli [64]. Reduced NO bioavailability, enhanced production of prostanoids (such as prostacyclin, thromboxane $A_{2,}$ etc.), impaired EDHF responses, and increased release of endothelin-1 are some of the common mechanisms that can individually or co-ordinately contribute to endothelial dysfunction; however the extent to which each of these contribute to the dysfunctional phenotype varies markedly depending on the pathological state, vascular bed and stimulus studied[62]. In arteries of both hypertensive and aging animals and humans, the major mechanisms contributing to the impaired ability of the endothelium to generate endothelium-dependent relaxation *in vitro* and *in vivo* include both reduced NO bioavailability [42,62,89,93,101,115,183,196,200,209,211,221,224,225,228] and enhanced COX-dependent contraction [48,49,63,86,112,191,230,237,240,251]. The vasoreactivity of the VSM is also altered with age [5,46,149,228] and hypertension [111,133,148,233], although the specific nature of the alterations varies depending on the stimulus and vascular bed examined. Endothelial dysfunction (rather than dysregulation of the VSM) is the major focus of this thesis, and since reduced NO bioavailability and enhanced COX-derived EDCFs are the main mechanism contributing to endothelial dysfunction in conduit arteries of Spontaneously hypertensive rats (SHR) and aged rats (the two models of dysfunction used in the experimental chapters), these mechanisms will be the focus of the more detailed description below.

Reduced NO bioavailability

The bioavailability of NO is determined by three main factors: 1) NO production, 2) the degradation of NO by reactions involving reactive oxygen species (ROS; i.e. superoxide anion) and 3) the sensitivity of the target VSM to NO[183]. Of the three factors, elevated levels of ROS primarily contribute to reduced NO bioavailability in the vascular wall [62,249,260]. ROS (superoxide anion in particular) originate from a number of sources, although in vascular tissue enhanced production is accounted for largely by the activity of NADPH oxidase[92,225], xanthine oxidase[94], mitochondria[225-227] and uncoupled eNOS (resulting from deficiency of necessary co-factors, oxidation or substrate deficiency) [94]. In the vasculature of SHR, the production of NO is generally not affected or enhanced; however elevated levels of superoxide anion interact with NO to generate peroxynitrite and thus quench NO bioavailability and vasodilatory potential[91,222]. In aging arteries, reduced NO bioavailability is generated by an increase in arginase activity (reducing L-arginine bioavailability and competing with eNOS for a common substrate)[7], reduced tetrahydrobiopterin bioavailability[193], augmented production of free radicals[54,224,225,228], and reduced expression[38,212,252] and activity[7,196] of eNOS. The sensitivity to NO is generally unaltered (in some examples it is slightly enhanced or depressed), in arteries of hypertensive[47,85,86] and aged animals[46,49,86,252,253].

Enhanced endothelium-dependent contractions

Alterations that contribute to enhanced endothelium- and COX-dependent contractions can include dysregulation of endothelial $[Ca^{2+}]_i$ in response to agonist stimulation (i.e. exaggerated elevations in intracellular calcium following exposure to agonists), elevated levels of ROS (which activate COX-mediated prostanoid production), increased expression of COX-1 and/or COX-2 (COX-1 and COX-2 can both contribute to endothelium-dependent contractions depending on the model and artery bed studied, although COX-1 is the mainly responsible for enhanced EDCF production/release observed in aorta of hypertensive rats[229]), greater production vasoconstrictory prostanoids (i.e. PGI_2, thromboxane A_2 etc.),

impaired IP-receptor function, and increased TP-receptor sensitivity in dysfunctional vessels from compared to those of healthy arteries[65,232,233]. These observations have been derived largely from arteries of hypertensive rats (SHR) compared to their normotensive control strain (Wistar-Kyoto rats; WKY). The mechanisms that account for enhanced endothelium- and COX-dependent contractions with age are less well defined, but increases in COX-1 and/or COX-2 protein expression have been documented in arteries of aged rats[112,191,251]. Since the studies that deal predominantly with the COX-prostanoid pathway in this thesis are performed using SHR and WKY, the examples given below will focus on how these alterations contribute to enhanced contractions in arteries from this model.

Dysregulated endothelial calcium content is a major contributor to enhanced endothelium-dependent contractions in aorta of SHR. The production of COX-derived EDCFs is triggered by abnormally high concentrations of intracellular calcium (i.e. under normal circumstances, regular levels of calcium do no initiate production of COX-derived EDCFs to the same extent) [213,216]. Stimulation of SHR aortic endothelial cells results in greater accumulation of intracellular Ca^{2+} (from intracellular stores) than that observed in endothelial cells of WKY [213,216], suggesting that dysfunctional Ca^{2+} handling is an important upstream impairment contributing to the elevated production of EDCFs [216]. All other downstream alterations in the COX-dependent EDCF signalling pathway therefore act to amplify this critical impairment [216].

The compliment of vasoconstrictor prostanoids released by the endothelium to generate contractions varies depending on the vascular bed, stimulus applied, age and disease condition. Endothelium-dependent contractions are usually evoked by a combination of these compounds rather than exclusively due to one particular substance[216]. However, there are instances where it is likely that one prostanoid in particular is a *predominant* contributor to the enhanced contractions observed in dysfunctional vessels. In conduit arteries of SHR and WKY rats, contractions to acetylcholine are accompanied by the release of PGI_2, PGE_2, $PGF_{2\alpha}$, and TxA_2 [65]. However, there are a number of

important observations that support PGI_2 as the prodominant EDCF responsible for enhanced contraction in SHR aorta (these have been reviewed in detail previously by others [65]): 1) prostacyclin synthase is by far the most abundant prostanoid synthase expressed in the endothelium [215], and expression is greater in aorta of SHR versus WKY [161,215], 2) the release of prostacyclin is 10 to 100 times greater than all other prostanoids, and is ~2 times greater in aorta of SHR than WKY [79-81] 3) in aorta of SHR and aging WKY, PGI_2 acts predominantly as a constrictor and not a vasodilator, 4) PGI_2 is a more potent constrictor in SHR than WKY[65] 5) at higher concentrations, PGI_2 stimulates activation of the TP-receptor (to signal contraction), rather than that of the IP receptor (mediating relaxation) [65], and 6) the release of prostacyclin and endothelium-dependent contractions are affected similarly by COX inhibitors[65]. Thus PGI_2 is likely the main factor accounting for enhanced endothelium- and COX-dependent contractions in aorta of SHR rats[65].

Altered components of VSM signalling mechanisms may also play a role in enhanced responsiveness to EDCFs. For example, the IP-receptor is not functional in aortic vascular smooth muscle of SHR as early as 12 weeks of age [65]. Some experiments have also shown that IP-receptor gene expression decreases with age and is lower in arteries of SHR versus those of normotensive WKY, although this remains controversial[65,161,215]. Both of these effects could be responsible for greater activation of the TP-receptor by PGI_2 (due to "spill-over") in arteries of SHR versus WKY, and in arteries of aged versus young animals. TP-receptor expression is not altered between SHR and WKY arteries[215], but TP-receptors are more sensitive to endoperoxides (generate greater contraction to the same stimulus) in SHR versus WKY aorta [78]. Elevated activation of the RhoA-Rho kinase signalling axis (greater Rho-kinase expression, RhoA activity) has also been observed in arteries of SHR versus normotensive WKY rats[48,133].

Reactive oxygen species generated in the endothelium may amplify endothelium-dependent contractions by both directly activation COX to enhance the production of EDCFs, and by reducing the

bioavailability and thus the functional, balancing, vasodilatory influence of NO [232]. ROS are generated by COX in response to stimulation with ACh (ROS production to ACh is prevented by the COX-inhibitor indomethacin) and the burst of ROS is greater in aorta of SHR than WKY[213]. These ROS facilitate production of vasoconstrictor prostanoids by VSM COX[259], and likely reach the VSM by travelling through myoendothelial gap junctions that exist between endothelial and VSM cells[214]. Thus although the production of ROS by COX seems to be a secondary event to that of PGH_2 production, ROS do contribute to amplification of the vasoconstrictory response [232]. The identity of the ROS that contribute to enhanced contractions could be superoxide anion, hydrogen peroxide and/or hydroxyl radicals (tiron and diethyldithiocarbamic acid (DETCA), intracellular scavengers of superoxide anion and hydrogen peroxide respectively, both reduce endothelium-dependent contractions to ACh in aorta of SHR)[259].

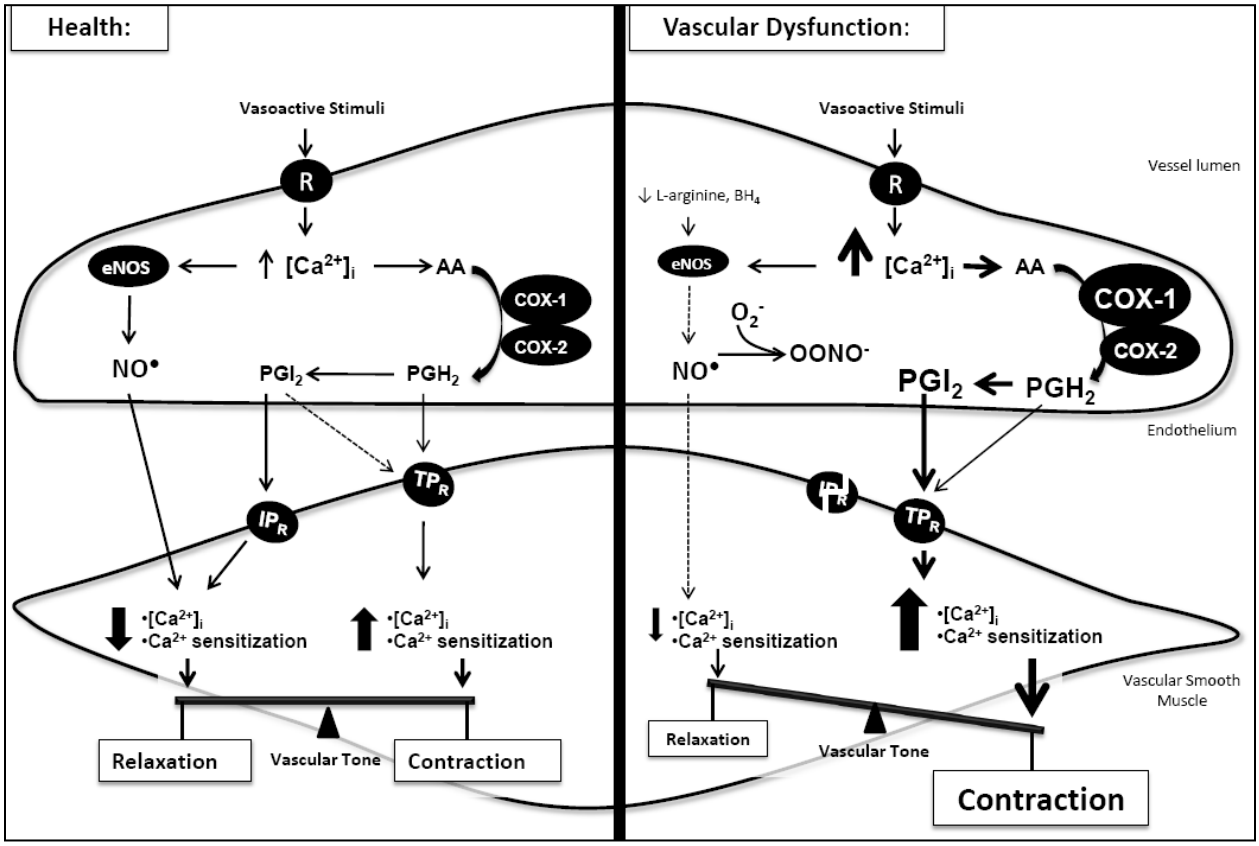


Figure 1-5. Pathological alterations in vascular signalling mechanisms that lead to vasomotor dysfunction. The *left panel* of the figure depicts the intact signalling processes of healthy arteries, leading to an appropriate balance between relaxation and contraction, and proper responses to vasoactive stimuli. The *right panel* of the figure highlights changes in the vascular signalling pathways that may contribute to

vasomotor dysfunction depending on the model and artery bed observed. See text for a detailed description of the alterations depicted above.

The preceding discussion provided a basic introduction to vasomotor signalling systems of interest in this thesis, their involvement in regulating vascular tone, and the dysregulation of these processes in vascular dysfunction. The focus of the following section will now shift to address the enzyme of interest, AMP-activated protein kinase, and its potential contribution to the regulation of vascular tone.

1.5 AMP-activated protein kinase as a novel regulator of vascular tone in health and disease

AMPK has only very recently been recognized as a potential mediator of arterial tone, and is therefore a new topic of study in the field of vascular biology. Although it has been studied extensively in other capacities for the past 30-40 years, AMPK was not identified in endothelial cells until 1999 by Dagher et al[40], and was reported in vascular smooth muscle in 2005 by Rubin and colleagues[181]. The first experiment associating AMPK activation and changes in vascular tone was published by Rubin et al in 2005 [181]. Therefore, at the onset of this thesis, the relationship between AMPK and vasomotor function was virtually unexplored. The sections that follow describe AMPK and the rationale for studying it as a novel regulator of vascular tone and function.

1.6 A basic introduction to AMP-activated protein kinase

1.6.1 The identification of AMPK and its multiplicity of functions

The enzyme we now call AMPK was first described in the early 1970's as both acetyl-CoA carboxylase kinase-3[23] and 3-hydroxy-3-methylglutaryl kinase[6] and was part of the second protein kinase signalling cascade ever described [98,121] . In 1988, Munday, Campbell, Carling and Hardie described it as "AMP-activated protein kinase" [152], a name assigned based on the ability of the enzyme to

be activated by AMP and inhibited by ATP. The sensitivity of AMPK to these substrates enabled it to sense changes in the [ATP]/[ADP][AMP] ratio, endowing upon it the capacity to sense and respond to changes in intracellular energy status [22,98,152,163]. This property, coupled with its ubiquitous expression in eukaryotic cells, quickly made AMPK a promising research target as a central regulator and integrator of fundamental metabolic processes. As such, AMPK is most well-studied for its ability to sense changes in intracellular energy status (and has been famously labelled the "energy sensing" serine-threonine protein kinase), and for its ability to respond by activating energy producing pathways (fatty acid and glucose catabolism) and inhibiting energy consuming processes (glucose, glycogen and lipid biosynthesis) [98,99,205]. However since this description, knowledge regarding the role of AMPK has expanded to include government of numberous cellular processes (such as cell growth and senescence, cell death, mitochondrial biogenesis, protein and nucleotide synthesis), and the regulation of whole body energy status through the neural control of appetite [56,57,98,205]. In addition to responding to changes in intracellular energy status, we now know that AMPK activity can also be regulated by a variety stimuli beyond changes in intracellular adenine nucleotide ratios, including a variety of hormones[56], cytokines[56,207] and intracellular calcium[70,98]. It is also appreciated that AMPK exerts regulatory control not only acutely via phosphorylation signalling cascades, but also chronically through regulation of transcription factors and gene expression [99,205]. Thus, our knowledge of AMPK is expanding beyond mere regulation of cellular energy metabolism, to encompass important roles in other vital cell and whole body functions, including the recently identified regulation of vascular tissue.

1.6.2 The structure and expression of AMPK

AMPK is comprised of three subunits: a catalytic alpha subunit, containing the serine-threonine protein kinase domain and a Thr^{172} regulatory residue; a beta subunit, which maintains the structural integrity of the enzyme via important contacts with the other subunits and also hosts a glycogen binding domain; and a regulatory gamma subunit, the site of two nucleotide binding sites (Bateman domains)[98,99,205]. Each subunit exists as multiple isoforms (α1, α2, β1, β2, λ1, λ2, λ3) [143,204], creating 12

possible isoform combinations for the expression of AMPK. The ability to express distinct subunit combinations affords variation in subcellular localization[185,248], sensitivity to stimuli[68,130,185,248], substrate specificity [143,254] and general functional diversity of the enzyme.

Endothelial and VSM cells both express AMPK[20,40]. The characterization of AMPK subunit isoform expression in vascular tissue is very limited, but the available data suggests that heterogeneity exists in AMPK subunit isoform expression patterns between endothelial and vascular smooth muscle cells, and within these cell types throughout the vascular tree. For example, cultured endothelial cells express predominantly AMPK α1 and much lower quantities of α2 [35,145,267]. Although vascular smooth muscle of porcine carotid[181], and rat 3rd order pulmonary arteries[60] contain α1 almost exclusively, rat mesenteric and main pulmonary arteries expresses similar levels of both α1 and α2 [60]. In endothelial cells express predominantly β1[35], whereas vascular smooth muscle contains both β1 and β2 (but β1 > β2, in porcine carotid arteries) [181] or solely β2 (rat pulmonary arteries) [60].

1.6.3 Regulation of AMPK enzymatic activity

The regulation of AMPK activity is complicated and occurs by both allosteric and covalent mechanisms. AMPK is activated covalently by phosphorylation at its threonine 172 (Thr^{172}) residue on the alpha subunit, a modification that is both necessary and sufficient to facilitate enzyme activity [99,104]. Thr^{172} is phosphorylated mainly by upstream kinases LKB1[29,256,257] and CaMKKβ [70,103,123,146,202] in vascular cells and is dephosphorylated by protein phosphatase 2C [186,208], such that the net phosphorylation at Thr^{172} is determined by the balance between upstream kinase and phosphatase activities. AMPK activity is also regulated allosterically by the competitive binding of AMP or ATP to the nucleotide binding sites (AMP stimulates activity where as ATP is inhibitory) [70,104,208]. The role of AMP in enzyme regulation becomes complex, as in addition to functioning as an allosteric regulator when bound to the Bateman domains, AMP also promotes phosphorylation of Thr^{172} by facilitating conformational changes in the enzyme structure to make it a more favourable substrate for upstream kinases and a poorer substrate

for upstream phosphatases (recent evidence is in favour of facilitated phosphatase rather than restricted kinase activity on Thr^{172})[70,180,186,208]. This dual role of AMP is important to the overall regulation of AMPK by AMP, because allosteric activation of AMPK upon binding of AMP produces only modest activation of < 10 fold, where as covalent phosphorylation at Thr^{172} produces a much more potent effect, increasing enzymatic activity by > 1000-fold [208].

1.6.4 Assessment of AMPK activation

Phosphorylation of AMPK at activation site Thr^{172}, and of its well known downstream target, serine 79 on acetyl-CoA carboxylase (ACC)[40,43,99], are commonly evaluated as reliable surrogate markers of AMPK activation (phosphorylation of the Ser 79 residue on ACC has been regarded as the "gold standard" target site for assessing AMPK activation status)[99]. Phosphorylation status at these sites can be easily evaluated by immunoblotting, providing a simple assay for AMPK activation using limited amounts of tissue. AMPK activity can also be measured using a SAMS peptide assay, whereby phosphorylation of the SAMS peptide substrate (engineered to mimic the sequence surrounding the serine 79 sequence on ACC) is an indicator of AMPK activity level [99,127]. However, this assay is inefficient in crude tissue extracts where there is a high level of background noise generated by other kinases and their substrates [127], and is not practical in experiments where sample matter is limited.

1.7 Specific rationale for the thesis topic: evidence of a role for AMPK in the regulation of vascular tone

Our hypothesis of a role for AMPK in regulating vascular tone developed from several seminal observations. These findings initiated our interested in investigating a potential acute vasomotor influence of AMPK, and provide the backbone on which the global and specific hypotheses of this thesis are based. A summary of these observations are as follows: 1) AMPK activator AICAR has hypotensive effects *in situ* that could be due to reduced vascular resistance, 2) AMPK stimulates the production of NO by eNOS

in biochemical experiments and cultured endothelial cells, 3) factors that activate AMPK generate relaxation of vascular smooth muscle preparations of healthy animals *in vitro*, and 4) AMPK is activated by a multitude of factors that exert vasomotor effects. Our premise for extending the investigation of AMPK-mediated vasomotor effects to include dysfunctional arteries of hypertensive and aged animals is partly based on observations showing that 5) AMPK activation is dysregulated in dysfunctional arteries of cardiovascular disease models and with age, a characteristic which may result in an impairment in any AMPK-mediated vasomotor effects.

1) AMPK activator AICAR has hypotensive effects in situ

The first vague indication for a potential role of AMPK in regulating vascular tone was derived from metabolic studies that incidentally observed hypotensive effects following acute or chronic administration of the pharmacological AMPK activator AICAR to healthy and diabetic animals respectively [19,71]. Similar observations were made in healthy, diabetic, and hypertensive models following chronic or acute administration of anti-diabetic drugs such as metformin[8,139,153,173,235] and thiazolidinediones[18,171,175] (TZDs), compounds later identified as possessing the ability to activate AMPK (at the time of the experiments, AMPK was not considered as a possible mediator of the effects observed in these studies). Although the hypotensive influence of these compounds could have resulted from sympathoinhibition of the central nervous system[153,173], suppression of cardiac function[113] or other factors regulating blood pressure, it is not possible to rule out decreased vascular resistance resulting from direct vasodilation of the vasculature as a potential contributor to these results. Furthermore, metformin and TZDs were able to stimulate vasodilation when administered directly to isolated rings *in vitro*[18,175] (although these studies did not assess AMPK activation), providing further support that the hypotensive effects of these compounds could be partially mediated by direct effects on vascular tone. Thus, although these data only provide loose, associated evidence for a link between AMPK and vasomotor effects, these observations initiated an interest in developing further studies to investigate a potential role for AMPK in mediating arterial vasodilation.

2) AMPK stimulates the production of nitric oxide by eNOS in biochemical experiments and cultured endothelial cells

In the late 1990's and early 2000's, *in vitro* biochemical and cell culture experiments revealed that AMPK is capable of stimulating NO production[27,29,31,145] by phosphorylating endothelial nitric oxide synthase (eNOS) at activation site Ser ^{1177} [27,145]. Activation of AMPK by AICAR[44,145,220], metformin[44], statins[30,31], shear stress[30,31,265], adiponectin [27,30,31], thrombin[220] and other stimuli has been associated with increased eNOS phosphorylation, activity and NO production in cell culture experiments. Since endothelium-derived NO is a major signalling intermediate responsible for vasodilatory responses generated in the vasculature, these data support the obvious hypothesis that AMPK may also be involved in mediating endothelium-dependent vasorelaxation though NO-dependent mechanisms. However, no data had been generated to test this hypothesis in the context of intact isolated vessel environment *in vitro* or *in vivo,* and the experiments of this thesis were designed to evaluate this question.

3) Acute activation of AMPK in vitro relaxes isolated arteries of healthy animals

The first study to directly investigate the association between AMPK activation and vascular tone was not presented until 2005 by Rubin and colleagues, who demonstrated that activation of AMPK by hypoxia/2-deoxyglucose is associated with vasodilation of isolated porcine carotid artery vascular smooth muscle *in vitro* (endothelium-*in*dependent relaxation, as their preparations were devoid of functional endothelium)[181]. Later, in 2007, Goirand et al. showed that AICAR dose-dependently produces vasodilation in isolated aortic rings of healthy mice, but that this response is largely blunted in aorta from AMPK$\alpha 1^{-/-}$ animals, suggesting dependency of AICAR-mediated relaxation on the α1 subunit of AMPK[82]. These observations provided the first strong support that activation of AMPK within the VSM can generate vasorelaxation and may be involved in regulating vascular tone.

4) *AMPK is activated by a multitude of vasoactive factors*

On-going investigations have revealed that AMPK can be activated by a wide array of vasoactive mediators including shear stress[29,265], bradykinin[100,145], adiponectin[29], histamine[220], thrombin[201,220], VEFG[136,146], sphingosine-1-phosphate[136,146], hypoxia[33,130], hydrogen peroxide[33,118,123], resveratrol[24,50], metformin[44] and AICAR[44,145]. The ability of AMPK to be stimulated by an overwhelming number of vasoactive stimuli also imply a role for this enzyme in the regulation of vascular tone.

5) AMPK activation is dysregulated in dysfunctional arteries of cardiovascular disease models and with age

Dysregulated AMPK activation (altered basal AMPK activation or responsiveness of the AMPK signalling axis to stimulation relative to that observed in healthy conditions) has been documented in tissues from a number of cardiovascular and metabolic pathologies as well as with age. AMPK activation is reduced in arteries of aged [132,179], streptozotocin-induced diabetic [243], Zucker diabetic fatty [10] and Otsuka Long-Evans Tokushima Fatty (OLETF)[135] rodents. These are all models where vasomotor dysfunction also exists. Pilot work in our lab demonstrated that AMPK activation is reduced in thoracic aorta of 20-24 week old spontaneously hypertensive rats (SHR), a finding that is consistent with the lower AMPK activity observed in left ventricle of 15 week old SHR [50]. However, it was unknown if AMPK-mediated vasomotor effects are impaired in arteries that display vasomotor dysfunction and dysregulated AMPK activation.

Summary of the evidence supporting a role for AMPK in the regulation of vascular tone

Together these observations provided a compelling reason to investigate the role of AMPK in the vasomotor control of healthy and dysfunctional arteries. Although a limited number of observations relating to the association between AMPK activation and vascular tone had been generated in healthy arteries (but not the healthy models used in this thesis), no data existed investigating this relationship in

any model of vascular dysfunction. Continued support for our hypotheses have emerged in the literature over the duration of data collection for this thesis. Figure 1-6 demonstrates our basic hypotheses for the role of AMPK in mediating vasomotor function based on the evidence available when designing the thesis experiments. The two hypotheses were: 1) that acute activation of endothelial AMPK would generate endothelium-dependent relaxation mediated by NO and 2) acute activation of AMPK in the vascular smooth muscle would generate endothelium-independent relaxation, resulting the contribution of both mechanisms to relaxation of an intact artery preparation.

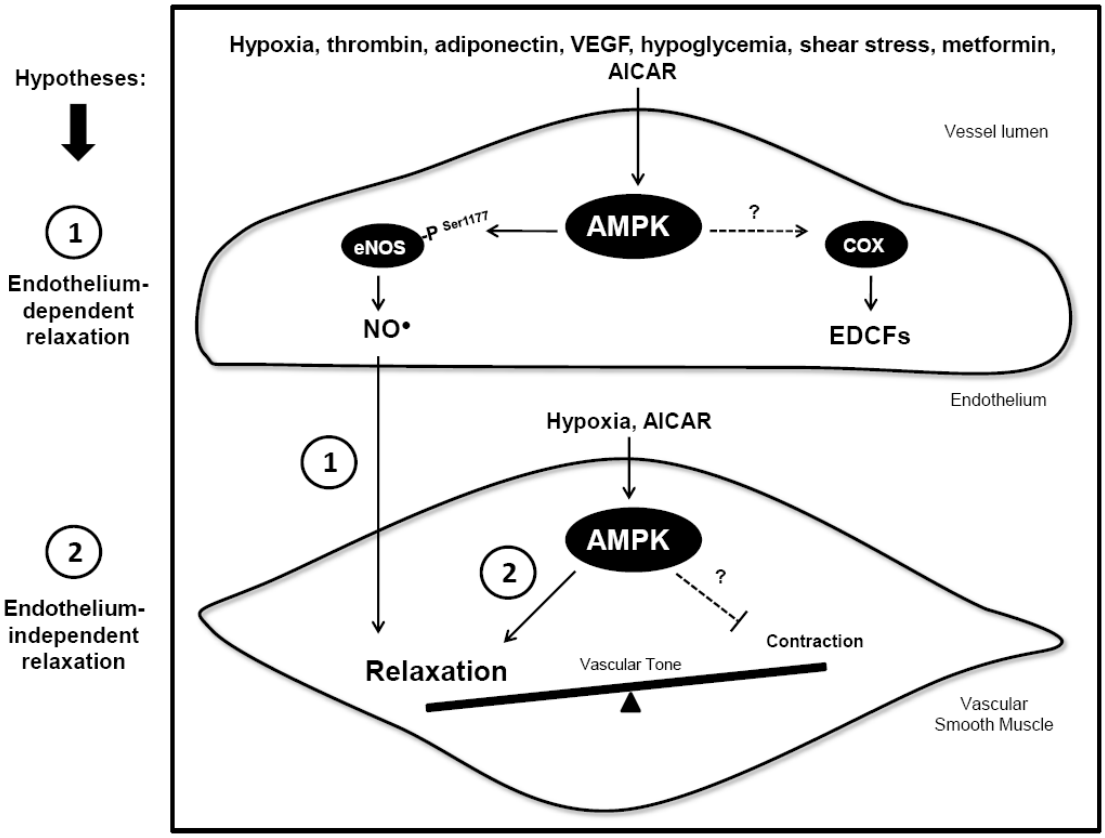


Figure 1-6. The basic hypotheses for AMPK-mediated vasomotor effects in isolated vessels based on the literature available at the time of thesis design. Based on isolated biochemical experiments and cell culture experiments, we hypothesized that activation of endothelial AMPK would generated endothelium-dependent relaxtion of isolated vessels by stimulating NO production via phosphorylation at eNOS activation site serine 1177 (indicated by circled 1). A limited number of isolated vessel experiments also suggest that direct activation of vascular smooth muscle AMPK also generates relaxation, resulting in endothelium-*in*dependent relaxation (indicated by circled 2). Thus we expected that exposure of isolated vessels to the pharmacological AMPK activator AICAR should generate vasorelaxation that has both an endothelium-dependent and an endothelium-independent component.

1.8 General purpose of the thesis and experimental approach

Based on the supporting evidence gathered above, the general purpose of this thesis was to investigate the role of AMPK in the regulation of vasomotor function in arteries from models of health and vascular dysfunction. The studies herein are designed to characterize the vasomotor responses generated by acute AMPK activation, to evaluate mechanisms mediating these responses, and to compare these components in functional arteries of healthy animals to those of dysfunctional vessels obtained from hypertensive and aged rat models. We have chosen Spontaneously Hypertensive rats (SHR) and aged Sprague-Dawley rats as models of vascular dysfunction (see rationale below, section *1.10.3 Animal models of vasomotor dysfunction*). To address these general objectives, we employed a combination of *in vitro* arterial vasomotor function, biochemical and *in vivo* hemodynamic assessments in the four distinct studies.

1.9 Specific objectives, rationale and hypotheses of the thesis studies

Overview of the relationship between thesis studies and progression through experiments

In Study 1, we investigate the basic nature of the vasodilatory response generated to acute AMPK activation using AICAR in isolated aorta of normotensive and hypertensive rats *in vitro*, the endothelium-dependent and endothelium-independency of AICAR-induced vasodilation, and potential mechanisms responsible for mediating these responses. This study begins the thesis by confirming the previous observations (i.e. the endothelium-independent vasodilatory response to AICAR in aorta of healthy rodents) and then extends this work by additionally investigating the endothelium-dependent component of relaxation in conduit aorta of normotensive WKY and hypertensive animals. A serendipidous finding of Study 1 demonstrated that AMPK may relax isolated aorta of SHR by inhibiting endothelium- and COX-dependent contraction; this finding served as the basis for developing Study 2, where we investigate the ability of AMPK to mediate prostaglandin-dependent vasoconstriction in aorta from hypertensive and

normotensive rats and dissect mechanisms involved in mediating this response. Since our observations in Study 1 showed that acute AMPK activation with AICAR relaxes conduit arteries, in Study 3 we attempt to extend these findings to resistance arteries and demonstrate parallel effects of acute AICAR injections on hemodynamic parameters *in vivo*. After observing that relaxation initiated by AMPK activator AICAR was both endothelium-, NO-dependent and endothelium-independent in arteries of WKY and SHR, we wanted to examine whether these results could be duplicated in other conditions of vasomotor function, so we performed similar experiments in aorta of young and aged rats (Study 4). The specific purpose, objectives, explicit hypotheses, and a brief statement of methods for each study are outlined below (Note: if you would prefer to read these immediately prior to reviewing the individual studies, they have been replicated identically at the beginning of each study chapter). A more focussed introduction and rationale for each study is included as part of the manuscript material in the experimental chapters that follow.

1.9.1 Study 1 (Chapter 2):

Purpose: To characterize the vasomotor responses of isolated arteries from healthy and hypertensive rats to acute AMPK activation and to investigate mechanisms involved.

Specific objectives and hypotheses:

Objective 1: To characterize the state of AMPK activation in aorta from WKY and SHR rats. **Hypothesis 1:** AMPK activation (as determined by phosphorylation of AMPK activation site threonine 172 and downstream target serine 79 of acetyl-CoA carboxylase) will be depressed in aorta of hypertensive versus normotensive rats. [Note: this data is actually included in the manuscript for Study 3, Chapter 4]

Objective 2: To investigate whether relaxation to AICAR is comprised of both an endothelium-dependent and an endothelium-independent component in WKY aortic rings. **Hypothesis 2:** Relaxation to AICAR will be only partly abolished in the absence of functional endothelium, demonstrating partial endothelium- and partially vascular smooth muscle dependence of relaxation to AICAR.

Objective 3: To assess if endothelium-dependent and –independent relaxation responses to AICAR are intact or altered in aortic rings from SHR compared to those of WKY. **Hypothesis 3:** The magnitude of relaxation to AICAR may be depressed in aorta from hypertensive rats. This hypothesis is based on 1) pilot work showing that basal AMPK activation is depressed in aorta of SHR versus WKY and 2) that vasomotor dysfunction exists in arteries from these animals; therefore it is reasonable to expect that vasomotor signalling through these pathways would be compromised.

Objective 4: To confirm uptake of AICAR into aortic rings of WKY and SHR by assessing its intracellular metabolite ZMP, and adenine nucleotide content (ATP, AMP and ADP) in control versus AICAR treated rings by high performance liquid chromatography. **Hypothesis 4:** ZMP will be elevated in rings treated with AICAR but undetectable in those receiving no drug, and that ZMP content will be similar in aortic rings of WKY and SHR. AICAR treatment will not affect ATP and AMP content of WKY and SHR aortic rings.

Objective 5: To evaluate dose-dependent AMPK activation in aortic rings of WKY and SHR over the increasing concentrations of AICAR used in the vasomotor function experiments. **Hypothesis 5:** AICAR will increase P(Thr172)-AMPK and P(Ser79)-ACC phosphorylation in a dose-dependent manner that mirrors the relaxation responses observed in the functional experiments.

Objective 6: To pharmacologically dissect potential mechanisms responsible for AICAR-induced functional outcomes in WKY and SHR aortic rings. **Hypothesis 6:** The endothelium-dependent component of relaxation to AICAR will be NO-dependent in WKY and SHR aortic rings.

Objective 7: To evaluate phosphorylation of eNOS at Ser1177 in aortic rings of WKY and SHR exposed to increasing doses of AICAR to determine if this may be a mechanism of enhanced NO-dependent relaxation. **Hypothesis 7:** AMPK will dose-dependently increase phosphorylation at eNOS serine 1177 and therefore present a possible mechanism by which active AMPK generates NO-dependent relaxation.

1.9.2 Study 2 (Chapter 3):

Purpose: To evaluate the influence of AMPK activation status on endothelium-dependent contractions in dysfunctional arteries of hypertensive rats and to elucidate mechanisms responsible for this interaction.

Specific objectives and hypotheses:

Objective 1: To verify that endothelium-dependent contractions to ACh are enhanced in quiescent (non-precontracted) aortic rings from SHR versus those of WKY, and that these contractions are COX-dependent (as has been shown by others and previously in our own lab). **Hypothesis 1:** Contractions to ACh will be enhanced in aortic rings of SHR versus WKY, and will be abolished by inhibition of COX or removal of the endothelium.

Objective 2: To determine the effect of the AMPK activator AICAR on endothelium-dependent contractions in SHR aortic rings. **Hypotheses:** Endothelium-dependent contractions to ACh will be blunted by AICAR pre-incubation in aortic rings of both WKY and SHR.

Objective 3: To demonstrate reversibility the inhibitory effect of AICAR on endothelium-dependent contractions by co-incubating rings with the AMPK inhibitor Compound C. **Hypothesis 3:** Co-incubation of WKY and SHR aortic rings with Compound C will preserve the magnitude of endothelium-dependent contraction generated in these vessels to ACh (contractions to ACh will be no different from those in no drug control rings).

Objective 4: To verify that AMPK is activated by AICAR but inhibited by AICAR-Compound C co-incubation in aortic rings. **Hypothesis 4:** AMPK activation (P(Thr172)-AMPK and P(Ser79)-ACC) will be elevated in rings treated with AICAR, but unaltered in rings treated with AICAR and CC compared to controls in vessels of both WKY and SHR.

Objective 5: To evaluate the effect of the presence of AICAR on contraction to increasing doses of TP-receptor agonists U46619 and PGH_2 to evaluate vascular smooth muscle responsiveness to TP-receptor

stimulation. **Hypothesis 5:** Contraction to increasing concentrations of TP-receptor agonists U46619 and PGH_2 will be blunted in aortic rings of WKY and SHR in the presence of AICAR

Objective 6: To investigate the production/release of 6-keto-prostaglandin $F_{1\alpha}$ (a stable metabolite of prostacyclin) by aortic rings of WKY and SHR stimulated with acetylcholine **Hypothesis 6:** 6-keto-prostaglandin $F_{1\alpha}$ levels will be blunted in buffer bathing aortic rings of WKY and SHR treated with AICAR, but unaltered in the presence of AICAR together with Compound C.

Objective 7: To determine the effect of AICAR on dose-dependent relaxation/re-contraction of phenylphrine pre-contracted SHR aortic rings to increasing [ACh]. Since the vasomotor response of phenylephrine pre-contracted SHR aorta to increasing [ACh] is biphasic (relaxation at lower [ACh] is NO-dependent followed by COX-dependent recontraction at higher concentrations, this experiment will afford the opportunity to confirm the effect of AICAR on endothelium- and COX-dependent contraction, and also on NO-dependent relaxation. **Hypothesis 7:** The endothelium- and COX-dependent re-contraction at higher concentrations will be abolished in SHR aortic rings pre-treated with AICAR, but NO-dependent relaxation will be enhanced with AICAR.

1.9.3 Study 3 (Chapter 4)

Purpose: To determine the effects of AMPK activation by an acute injection of AICAR on *in vivo* hemodynamic responses of normotensive and hypertensive rats, and to evaluate whether parallel relaxation responses occurs in isolated, pre-contracted resistance arteries of normotensive and hypertensive rats exposed to AICAR acutely *in vitro.*

Specific objectives and hypotheses:

Objective 1: To investigate the effect of acute AICAR injection on blood pressure responses of SHR and WKY *in vivo*, and to determine the NO-dependency of these effects via carotid arterial catheter pressure

transducers. **Hypothesis 1:** Acute injections of AICAR will lower blood pressure in WKY and SHR, and that this effect will be partly NO-dependent.

Objective 2: To investigate the effect of acute AICAR injection on heart rate response of SHR and WKY *in vivo* by carotid arterial catheter. **Hypothesis 2:** AICAR will have minimal to no influence on heart rate of WKY or SHR.

Objective 3: To verify the efficacy of the AICAR treatment during the *in vivo* experiments by assessing AMPK activation in vascular tissue from these animals by immunoblotting. **Hypothesis 3:** Acute injections of AICAR will elevate P(Thr172)-AMPK levels in aortas of WKY and SHR rats.

Objective 4: To characterize basal levels of AMPK activation and expression in mesenteric artery sections from SHR and WKY by immunoblotting. **Hypothesis 4:** AMPK activation (P(Thr172)-AMPK) will be depressed in mesenteric arteries of SHR versus those of WKY.

Objective 5: To evaluate the vasomotor response of pre-contracted isolated mesenteric resistance artery segments to acute AICAR exposure, to determine the NO-dependency of this response, and to compare the responses generated in SHR versus WKY vessels using vascular myography. **Hypothesis 5:** Relaxation to AICAR will occur in mesenteric arteries of both WKY and SHR and will be partly NO-dependent.

Objective 6: To verify AMPK activation in mesenteric artery segments from SHR and WKY incubated with AICAR by immunoblotting. **Hypothesis 6:** AMPK activation (P(Thr172)-AMPK) will be elevated in mesenteric arteries of WKY and SHR following incubation with AICAR**.**

Objective 7: To compare baseline levels of P(Ser1177)-eNOS and total eNOS protein content in aortas and mesenteric arteries of WKY and SHR rats. **Hypothesis 7:** P(Ser1177)-eNOS will be depressed in aorta and mesenteric arteries of SHR versus those of WKY, and total eNOS protein content will be elevated in these vessel types in SHR versus WKY.

Objective 8: To evaluate the effect of AICAR injections *in vivo* and AICAR incubation *in vitro* on P(Ser1177)-eNOS (and total eNOS protein content) by immunoblotting in aortas and mesenteric arteries respectively of WKY and SHR. **Hypothesis 8:** Acute exposure to AICAR will increase P(Ser1177)-eNOS in both types of experiments (aorta and mesenteric arteries) but total eNOS protein content will not be altered by AICAR.

1.9.4 Study 4 (Chapter 5)

Purpose: To characterize the vasomotor responses of isolated arteries from young and aged rats to pharmacological AMPK activators and to investigate the mechanisms involved.

Specific objectives and hypotheses:

Objective 1: To assess basal AMPK activation in aorta of aged versus young male Sprague-Dawley rats. **Hypothesis 1:** Basal AMPK activation (P(Thr172)-AMPK and P(Ser79)-ACC) will be depressed in arteries of aged versus those of young rats.

Objective 2: To determine if vasorelaxation to AICAR is intact in isolated aortic rings from aged rats compared to those of young rats. **Hypothesis 2:** AMPK-mediated relaxation will be impaired in arteries of aged versus young rats.

Objective 3: To evaluate the endothelium-dependency and/or –independency of vasodilatory responses to AICAR in aortic rings young and aged rats. **Hypothesis 3:** AICAR will generate relaxation in aortic rings of young and aged rats that is partly endothelium-dependent and partly endothelium-independent.

Objective 4: To pharmacologically dissect the mechanisms responsible for AMPK-mediated vasodilation in aortic rings of young and aged rats (i.e. NO- and COX-dependency). **Hypothesis 4:** The endothelium-dependent component of relaxation to AICAR will be primarily NO-dependent in aortic rings of young rats but will be NO- and COX-mediated in aortic rings of aged rats. Since NO-bioavailability and NO-

dependent functional responses are typically reduced in arteries of aged rats, we expect NO-dependent relaxation to AICAR may be reduced in aged versus young aorta. Responsiveness to vasoactive agents is often impaired in arteries of aged rats, so endothelium-independent relaxation to AICAR may also be impaired in arteries of aged animals versus those of young.

Objective 5: To biochemically confirm AMPK activation by AICAR in aortic rings of young and aged rats. **Hypothesis 5:** AMPK activation (P(Thr172)-AMPK and P(Ser79)-ACC) will be elevated by treatment with AICAR to a similar extent in rings of young and aged animals.

Objective 6: To investigate whether or not vasorelaxation of young and aged pre-contracted aortic rings also occurs in response to other known AMPK activators, metformin and resveratrol, and to compare these responses in vessels of young versus aged animals. **Hypothesis 6:** Metformin and resveratrol will each generate relaxation in aortic rings of young and aged rats and relaxation to both of these agonists will be impaired in aortic rings of aged versus young animals.

Objective 7: To biochemically confirm AMPK activation by metformin and resveratrol in aortic rings. **Hypothesis 7:** AMPK activation (P(Thr172)-AMPK and P(Ser79)-ACC) will be elevated by treatment of rings with either metformin or resveratrol.

Objective 8: To evaluate total eNOS protein content in aortic rings of young and aged rats, and the effect of AICAR on P(Ser1177)-eNOS of these vessels *in vitro*. **Hypothesis 8:** Total eNOS content will be depressed in aortic rings of aged versus young rats, and P(Ser1177)-eNOS will be enhanced in aorta rings of young and aged rats treated with AICAR.

1.10 Overview of the experimental approaches and models used in the thesis studies

The following section overviews the main experimental method used across the thesis studies to assess vasomotor function (vascular myography), and provides additional rationale for the choice of animal and artery models used in this thesis. Although each research chapter contains an introduction and

methods specific to the experiments described therein, this general orientation is provided to appreciate the overall experimental approach.

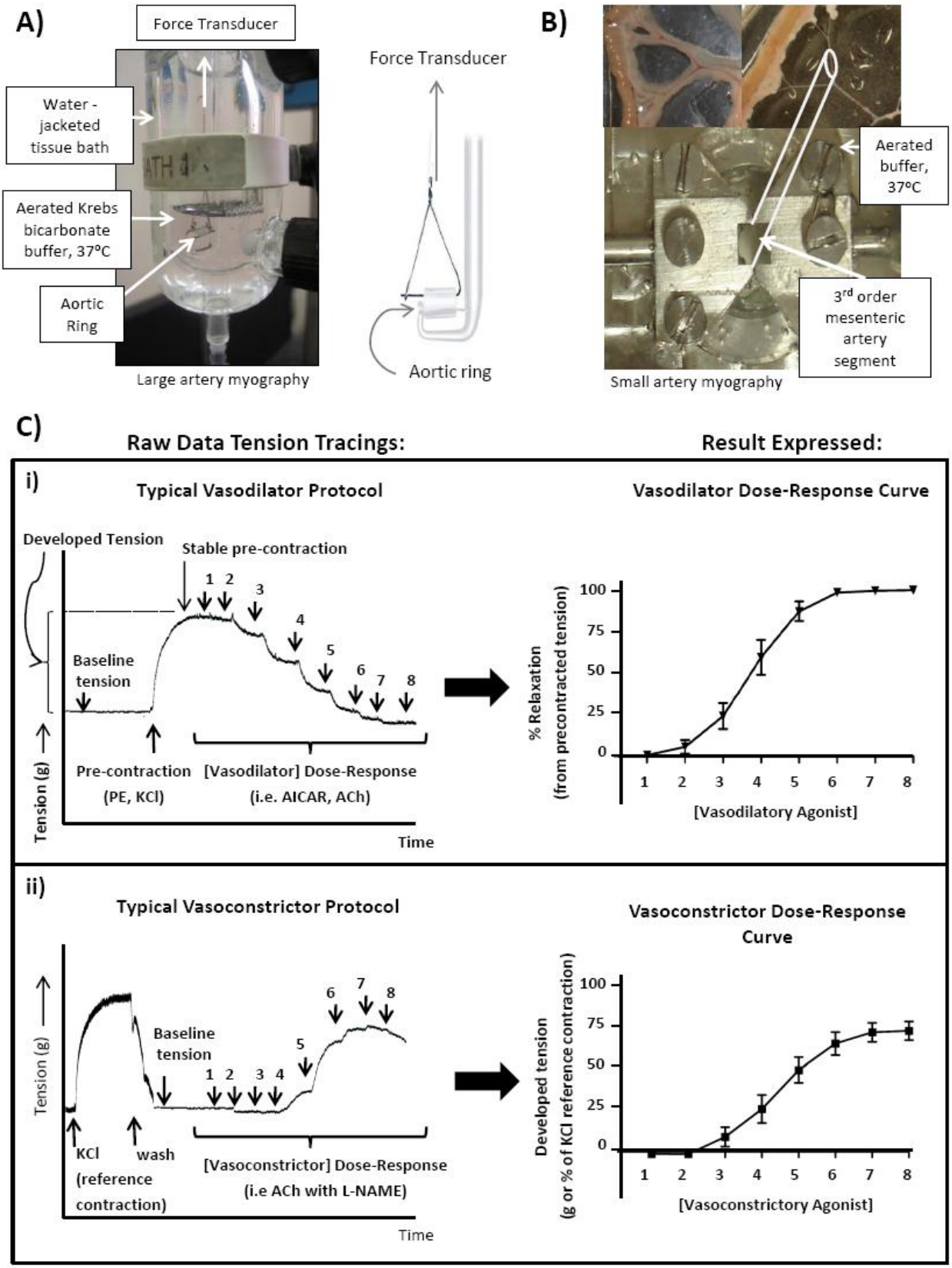
1.10.1 Isolated vessel preparations to assess vasomotor function in vitro

Vascular myography is a technique commonly used to assess vasomotor responses of isolated vessel preparations to vasoactive agents in the context of a controlled environment (i.e. in the absence of of multiple vasoactive influences present *in vivo* like mechanical, neural input etc.). This approach enables clear dissection of vasomotor pathways and mechanisms through pharmacological manipulation of signalling systems of interest. It also affords the opportunity to perform experiments where the endothelium is removed from the interior of the vessel lumen (see *Chapter 2, Study 1, 2.4 Materials and Methods* for description), and thus the capacity to distinguish between responses originating from the endothelium (endothelium-dependent) versus those mediated by the VSM (endothelium-independent).

In vascular myography experiments, isolated vessel segments are carefully mounted between two metal wires, the bottom of which is held stationary, and the top attached to a force transducer, allowing the experimenter to evaluate changes in isometric tension as the vascular ring dilates or contracts in response to the introduction of pharmacological stimuli during experimental protocols (Figure 1-7A & B). In order to observe vasodilatory responses, vessels must first be pre-contracted with a vasoconstrictory agent to generate a state of active tone from which the vessels can relax (the resting or baseline tension set by the experimenter at the beginning of the protocol is passive and theoretically does not contain any active vessel tone, and therefore vasodilatory agents to not produce an effect in vessels in this state). The ability to generate contraction is assessed in vessels from a quiescent state (non-precontracted, at baseline/optimal resting tension) that are then exposed to constrictor agonists. Endothelial dysfunction is observed by comparing the relative inability of arteries to relax to endothelium-dependent vasodilators (such as acetylcholine in pre-contracted arteries), or by the presence of enhanced contractions to endothelium-dependent vasoconstrictors (i.e. to acetylcholine in quiescent arteries). VSM vasomotor

dysfunction can also be identified by observing altered responses of VSM to endothelium-independent vasoactive agents (i.e. phenylephrine, potassium chloride, TP-receptor agonists, etc.). The isolated tissue bath apparatus used for evaluation of large conduit arteries and small resistance arteries of rats, and the general type of results obtained by typical wire myograph protocols are depicted in Figure 1-7.

Figure 1-7 (adjacent page). The vascular myography isolated vessel set-ups and protocols used for evaluation of arterial vasomotor responses *in vitro*. A) The tissue bath set-up utilized for large conduit artery (aorta) experiments and a schematic diagram of an aortic ring mounted on the apparatus that is immersed in the tissue bath during experimental protocols **B)** The dissection of 3rd order mesenteric arteries, and small artery wire myograph apparatus used for experiments in Study 3. **C)** The derivation of data from raw tension tracings generated during a typical experimental protocol using **i)** vasodilatory and **ii)** vasoconstrictory agonists. The aortic ring schematic diagram in A) was obtained from http://www.adinstruments.com/products/hardware/corporate/product/158817/ on September 27, 2011.


A)
Force Transducer
Water - jacketed tissue bath
Aerated Krebs bicarbonate buffer, 37°C
Aortic Ring
Large artery myography
Force Transducer
Aortic ring
B)
Aerated buffer, 37°C
3rd order mesenteric artery segment
Small artery myography
C)
Raw Data Tension Tracings:
Result Expressed:
i)
Typical Vasodilator Protocol
Vasodilator Dose-Response Curve
Developed Tension
Stable pre-contraction
Baseline tension
Pre-contraction (PE, KCl)
[Vasodilator] Dose-Response (i.e. AICAR, ACh)
Tension (g)
Time
% Relaxation (from precontracted tension)
[Vasodilatory Agonist]
ii)
Typical Vasoconstrictor Protocol
Vasoconstrictor Dose-Response Curve
Baseline tension
KCl (reference contraction)
wash
[Vasoconstrictor] Dose-Response (i.e ACh with L-NAME)
Tension (g)
Time
Developed tension (g or % of KCl reference contraction)
[Vasoconstrictory Agonist]

1.10.2 Rationale for the choice of vessel types studied

Conduit thoracic aortae were used for most of the experiments performed in this thesis. We chose to study these vessels for several reasons: 1) the aorta is excellent model of vasomotor dysfunction in many disease models, and its dysfunctional characteristics have been well documented in SHR 2) since one of our main hypotheses was regarding the potential influence of AMPK on vascular signalling involving NO, conduit arteries provide a good model for investigating NO-dependent effects as vasorelaxation in these vessels is mainly NO-mediated (as opposed to many resistance arteries where the contribution of NO to vasodilation is much less and other mechanisms play a larger role), 3) for practical purposes, aorta provide a larger volume of tissue on which to perform biochemical analyses than small arteries and 4) large conduit artery dysfunction has implications for vascular pathologies such as atherosclerosis and coronary artery disease in animals and humans [45,66,107].

Resistance mesenteric arteries are used in Study 3 in order to expand our characterization of the role of AMPK to include arteries from another vascular bed, and to determine if responses to AMPK manipulation are conserved across different artery types (since a substantial amount of heterogeneity exists in vasomotor regulation of arteries across different vascular beds). Mesenteric arteries are readily accessible and are available in considerable quantity relative to other resistance vessels, thereby providing a convenient source of tissue that serves the purpose of our experiments and is viable for analysis using our biochemical techniques. Given a possible role for AMPK in blood pressure modulation, examining resistance arteries may also provide insight into potential mechanisms for AMPK-mediated regulation of vascular resistance, which could influence blood pressure.

1.10.3 Animal models of vasomotor dysfunction

Spontaneously Hypertensive Rats

Spontaneously hypertensive rats (SHR) are an inbred genetic model of essential hypertension (defined as high blood pressure without obvious cause[9,37]) that were initially generated in the 1960's by

selectively breeding for high blood pressure in Wistar Kyoto rats (WKY; the normotensive control strain)[165,166]. SHR begin to exhibit elevated blood pressure as early as 2 weeks of age[87], which progresses to severe hypertension (i.e. mean arterial pressures reaching ~200 mmHg) by 12-14 weeks of age and persists for the duration of their lifespan[58,114]. Most importantly for the purposes of this thesis, arteries of SHR progressively develop impaired vasomotor function (a condition that parallels vasomotor dysfunction characteristic of human essential hypertension) that is consistently well-developed compared to WKY (that display normal vasomotor function) by 16 weeks of age [47,62,65,85,114,233].

Aged Sprague-Dawley Rats

Aged male Sprague-Dawley rats (80-95 weeks) were used as a second model of vasomotor dysfunction in the thesis experiments of Study 4 (Chapter 5) in conjunction with young male Sprague-Dawley controls (~ 10 weeks of age). Vasomotor dysfunction has been observed in aorta of aged male Sprague-Dawley rats ~80-90 weeks of age compared to young male Sprague-Dawley rats that display normal vasomotor function[16,26,49,96,156]. Since vascular dysfunction escalates with the progression of age[1,129], examining arteries of animals at this stage of maturation allows adequate time to maximize vasomotor dysfunction before the spontaneous mortality rate of the animal group becomes too high (the spontaneous mortality rate for rats of this age is ~ 20%, data provided by Harlan Laboratories, Indianapolis, IN).

1.10.4 Pharmacological manipulation of AMPK activation

AICAR (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside) is the most commonly used pharmacological activator of AMPK. It is imported into the cell by adenosine transporters (and importantly, is not an adenosine receptor agonist)[77], where it is phosphorylated by adenosine kinase[184] to form the AMP analogue ZMP[36,99]. ZMP binds to the AMP/ATP Bateman binding sites on AMPK to induce activation[124]. AICAR is a favourable method of studying of AMPK because it effectively activates AMPK without incurring detectable changes in the composition of the intracellular nucleotide

pool[36]. Compound C is currently the only available pharmacological inhibitor of AMPK, and acts competitively and reversibly by competing with AMP/ATP for the nucleotide binding sites on the gamma subunit[266]. Although Compound C can exert non-AMPK mediated effects in some circumstances (and therefore should not be used to study AMPK in isolation), its efficacy as a pharmacological AMPK inhibitor is acceptable if it is utilized in conjunction with other tools (i.e. AMPK activator AICAR), and results obtained are carefully examined along with other indications that the effects generated are AMPK-mediated (i.e. identification of the co-existence of AMPK activation/inhibition, complete reversal of AICAR-mediated effects, appropriate control experiments performed with Compound C alone, etc.).

That concludes the general introduction section of the thesis. The experimental chapters that follow provide a focussed overview, introduction, methods, results and discussion section for each of the four studies.

Chapter 2

Thesis Study 1

Endothelium-dependent vasorelaxation to the AMPK activator AICAR is enhanced in aorta from hypertensive rats and is NO- and EDCF-dependent

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2.1 Review of Study 1 Objectives and Hypotheses

The specific objectives and hypotheses for this chapter have already been stated in the introduction section, but are reproduced identically here solely for the reader's convenience.

Purpose: To characterize the vasomotor responses of isolated arteries from healthy and hypertensive rats to acute AMPK activation and to investigate mechanisms involved.

Specific objectives and hypotheses:

Objective 1: To characterize the state of AMPK activation in aorta from WKY and SHR rats. **Hypothesis 1:** AMPK activation (as determined by phosphorylation of AMPK activation site threonine 172 and downstream target serine 79 of acetyl-CoA carboxylase) will be depressed in aorta of hypertensive versus normotensive rats. [Note: this data is actually included in the manuscript for Study 3, Chapter 4]

Objective 2: To investigate whether relaxation to AICAR is comprised of both an endothelium-dependent and an endothelium-independent component in WKY aortic rings. **Hypothesis 2:** Relaxation to AICAR will be only partly abolished in the absence of functional endothelium, demonstrating partial endothelium- and partially vascular smooth muscle dependence of relaxation to AICAR.

Objective 3: To assess if endothelium-dependent and –independent relaxation responses to AICAR are intact or altered in aortic rings from SHR compared to those of WKY. **Hypothesis 3:** The magnitude of relaxation to AICAR may be depressed in aorta from hypertensive rats. This hypothesis is based on 1) pilot work showing that basal AMPK activation is depressed in aorta of SHR versus WKY and 2) that vasomotor dysfunction exists in arteries from these animals; therefore it is reasonable to expect that vasomotor signalling through these pathways would be compromised.

Objective 4: To confirm uptake of AICAR into aortic rings of WKY and SHR by assessing its intracellular metabolite ZMP, and adenine nucleotide content (ATP, AMP and ADP) in control versus AICAR treated rings by high performance liquid chromatography. **Hypothesis 4:** ZMP will be elevated in

rings treated with AICAR but undetectable in those receiving no drug, and that ZMP content will be similar in aortic rings of WKY and SHR. AICAR treatment will not affect ATP and AMP content of WKY and SHR aortic rings.

Objective 5: To evaluate dose-dependent AMPK activation in aortic rings of WKY and SHR over the increasing concentrations of AICAR used in the vasomotor function experiments. **Hypothesis 5:** AICAR will increase P(Thr172)-AMPK and P(Ser79)-ACC phosphorylation in a dose-dependent manner that mirrors the relaxation responses observed in the functional experiments.

Objective 6: To pharmacologically dissect potential mechanisms responsible for AICAR-induced functional outcomes in WKY and SHR aortic rings. **Hypothesis 6:** The endothelium-dependent component of relaxation to AICAR will be NO-dependent in WKY and SHR aortic rings.

Objective 7: To evaluate phosphorylation of eNOS at Ser1177 in aortic rings of WKY and SHR exposed to increasing doses of AICAR to determine if this may be a mechanism of enhanced NO-dependent relaxation. **Hypothesis 7:** AMPK will dose-dependently increase phosphorylation at eNOS serine 1177 and therefore present a possible mechanism by which active AMPK generates NO-dependent relaxation.

2.2 Overview of Study 1

Activation of AMP-activated protein kinase (AMPK) induces vasorelaxation in arteries from healthy animals, but the mechanisms coordinating this affect are unclear and the integrity of this response has not been investigated in dysfunctional arteries of hypertensive animals. Here we investigate the mechanisms of relaxation to the AMPK activator 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) in isolated thoracic aorta rings from Spontaneously Hypertensive (SHR) and normotensive Wistar-Kyoto (WKY) rats. Although AICAR generated dose-dependent (10^{-6}-10^{-2} M) relaxation in pre-contracted WKY and SHR aortic rings with (E+) or without (E-) endothelium, relaxation was enhanced in E+ rings. Relaxation in SHR E+ rings was also enhanced at low [AICAR] (10^{-6} M) compared to that of

WKY (57±8% vs 3±2% relaxation in SHR vs WKY E+), but was similar and near 100% in both groups at high [AICAR]. Pharmacological dissection showed that the mechanisms responsible for the endothelium-dependent component of relaxation across the dose range of AICAR are exclusively NO-mediated in WKY rings, but partly NO- and partly cyclooxygenase-dependent in SHR vessels. Further investigation revealed that acetylcholine-stimulated COX-EDCF-mediated contractions were suppressed by AICAR, and this effect was reversed in the presence of the AMPK inhibitor compound C in quiescent E+ SHR aortic rings. Western blots demonstrated that P(Thr^{172})-AMPK and P(Ser^{79})-ACC (indices of AMPK activation) were elevated is SHR vs WKY E+ rings at low AICAR (~2 fold). Together these findings suggest that AMPK-mediated inhibition of EDCF-dependent contraction and elevated AMPK activation may contribute to the enhanced sensitivity of SHR E+ rings to AICAR. These results demonstrate AMPK-mediated vasorelaxation is present and enhanced in arteries of SHR, and suggest that activation of AMPK may be a potential strategy to improve vasomotor dysfunction by suppressing enhanced endoperoxide-mediated contraction and enhancing NO-mediated relaxation.

2.3 Introduction

AMPK is emerging as a potential regulator of vascular function. Although recognized primarily as a modulator of cellular energy status and metabolism[99,247], the identification of its existence in vascular cells and of its ability to be stimulated by numerous physical[31,69,265], energetic[60], hormonal[29,31,155], and chemical[31,33,39,44,100,136,145,220] mediators of vasomotor function suggest a potential role for AMPK in the regulation of vascular control. Of particular importance to the modulation of vasomotor tone, cell culture and *in vitro* biochemical experiments have shown that activated endothelial AMPK is able to increase phosphorylation and activation of endothelial nitric oxide synthase (eNOS) at Ser^{1177} [27,29,31,44,100,145,220] and Ser^{633} [30], and to increase nitric oxide (NO) availability[29,30,44,145]. While the potential for AMPK to generate endothelium-dependent, NO-mediated relaxation *in vivo* and in intact blood vessels is implied by these data, very few observations have been made to verify this hypothesis in

isolated vessels, or *in vivo*. In addition, endothelium-*in*dependent relaxation has been documented in isolated porcine, mouse and rat conduit arteries from healthy animals in response to AMPK activators such as hypoxia[181], AICAR[82] and metformin[139], suggesting that vasorelaxation can be generated directly by activation of vascular smooth muscle AMPK. Biochemical experiments have shown that AMPK can directly regulate myosin light chain kinase (MLCK) by decreasing its sensitivity to intracellular calcium[116] and thus presumably reduce vascular tone. Collectively, these observations suggest that AMPK may modulate vasomotor function through both endothelium-dependent and –independent mechanisms.

Hypertension is associated with vasomotor dysfunction, which manifests in part as impaired endothelium-dependent vasorelaxation and also as altered vascular smooth muscle function [62,85,93,95,148,233]. Impaired endothelium-dependent vasomotor function in hypertensive models has been attributed to both reduced NO-bioavailability and enhanced endothelium-dependent vasoconstriction [48,62,85,93,95,142,233]. Endothelium-derived contracting factors (EDCFs) are products of cyclooxygenase (COX), and have been identified as prostaglandins that exert contraction through TP-receptor mediated signalling in the vascular smooth muscle[48,62,233]. Recent work suggests that activation of AMPK may be able to interact with the COX vasomotor signalling axis to suppress the EDCF-mediated vasoconstriction in dysfunctional vessels[141]. Since AMPK may also play a role in facilitating the generation of NO and vasodilation of vascular smooth muscle, it is conceivable that exogenous activation of AMPK may be able to improve vasomotor function by stimulating processes that are otherwise impaired in diseased arteries. Interestingly, depressed AMPK activation, or AMPK dysregulation, has been observed in arteries of rodent models where vascular dysfunction exists, including in streptozotocin-induced diabetes [243], Zucker diabetic fatty rats [10], aged rats [179] and Otsuka Long Evans Tokushima Fatty (OLETF) rats[135]. In pilot work, we determined that basal AMPK activation is also blunted in aorta of Spontaneously Hypertensive Rats (SHR; a genetic model of essential hypertension and vasomotor dysfunction) compared to levels in normotenisve control Wistar Kyoto (WKY) rats. However, it is unknown if the

ability to activate AMPK is impaired in arteries of hypertensive animals, and whether pharmacological activation of AMPK in dysfunctional arteries is able to generate relaxation in a similar manner to that observed in healthy vessels.

The objectives of this study therefore are: to characterize the endothelial-dependency and –independency of AMPK-mediated vasorelaxation and AMPK activation in aortic rings from normotensive (WKY) rats; to determine if these responses are altered in aortic rings from hypertensive (SHR) animals; and to elucidate potential mechanisms responsible for AMPK-mediated vasomotor outcomes. Our data: illustrate that AMPK-mediated relaxation in response to exogenous AMPK activation occurs through both endothelium-dependent and –independent mechanisms in arteries from normotensive animals; demonstrate for the first time that these responses also occur and are actually *enhanced* in arteries from hypertensive animals despite depressed basal AMPK activation; and suggest that the mechanisms responsible for endothelium-dependent relaxation are exclusively NO-mediated in WKY, but both NO- and COX-, EDCF-dependent in SHR aorta. Activation of AMPK suppresses the exaggerated EDCF-dependent contractions observed in SHR compared to WKY arteries, and this effect likely contributes to the enhanced endothelium-dependent, AICAR-mediated relaxation observed in SHR.

2.4 Materials and Methods

Animal care and procedures. The University of Waterloo Animal Care Committee approved all animal-related procedures in this study. Experiments were performed using a total of 50 male WKY and 50 male SHR obtained from Harlan (Indianapolis, IN). Animals were group-housed at a constant air temperature (20-21^{o}C) and humidity (~50%) in a 12 h:12 h reverse light:dark cycle. Rats had free access to standard 22/5 Rodent Diet (W) lab chow (Harlan) and tap water. Prior to all experiments, body mass was recorded and rats were anesthetised by sodium pentobarbital injection (50-65 mg/kg, i.p.; Vetoquinol N.-A. Inc., Lavaltrie, QC, Canada). In order to confirm blood pressure levels in SHR and WKY, a subset of anesthetised animals from each group were instrumented for mean arterial pressure (MAP) and heart rate

(HR) measurements by inserting a Mikro-Tip Pressure Transducer catheter (Millar Instruments, Houston, TX) into the left common carotid artery[47,86]. Data were collected as previously described[47,86].

Vasomotor responses in isolated vessels. Animals were killed by rapidly removing the heart which was dissected and weighed. The thoracic aorta was excised and 2mm aortic rings were prepared for vascular myography as previously described[74]. In some experiments, the endothelium was removed by inserting a 256 μm diameter titanium wire through the vessel lumen and rolling it on Whatman blotting paper (Whatman, Maidstone, England) soaked with 4°C Krebs-bicarbonate buffer. Consistent removal of the endothelium by this method was verified functionally by non-responsiveness to a maximal dose of the endothelium-dependent vasodilatory agent acetylcholine in phenylephrine pre-constricted aortic rings, and biochemically by substantial removal of endothelial-nitric oxide synthase (eNOS) protein content (i.e. < 10-15% residual eNOS compared with endothelium-intact aortic rings from the same animals) assessed by western blotting (Figure A2-1). Rings were mounted onto a vascular myography apparatus (Radnotti, Monrovia, CA) where they were immersed in 37°C Krebs-bicarbonate buffer continuously aerated with 95% O_2/5% CO_2, and data collected as already described[74]. Gradual stretching to a pre-determined optimal resting tension of 7 g[85] was achieved by increasing the tension by 0.5g increments from 1g every 5 minutes, and rings were equilibrated at optimal resting tension for 30 minutes. Each vessel was then contracted by two consecutive exposures to 60mM potassium chloride (KCl, with washouts in between) to ensure contractile integrity of all aortic rings.

Dose-dependent vasorelaxation to AICAR. Following washout of KCl and return to baseline tension, rings were allowed to equilibrate for 30 minutes and then pre-contracted with phenylephrine (PE; $10^{-6.5}$ M). When a stable plateau in tension was achieved, each ring was exposed to increasing concentrations of the AMPK activator 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR; 10^{-6} to 10^{-2} M) to generate dose-dependent relaxation responses. In some experiments, some rings with and without endothelium were pre-incubated with the NOS inhibitor N_ω-nitro-L-arginine methyl ester (L-NAME; 10^{-4} M), the cyclooxygenase inhibitor indomethacin (INDO; 10^{-5} M), or both L-NAME and

INDO for 30 minutes prior to PE pre-constriction. The dose range for AICAR was based on prior experiments examining rodent aorta[82] and on our own pilot work. The functional viability of aortic rings following exposure to this dose and duration of AICAR was also tested in pilot experiments by evaluating the ability of rings to generate tension to KCl and PE following the AICAR dose-response curve (DRC) protocol. Tension generated to KCL or PE post AICAR DRC was not significantly different from values obtained prior to the AICAR DRC in rings both with and without endothelium (KCL: E+ 83±7%, E- 103±9% of KCl exposure pre-AICAR and PE: E+ 108±2%, E- 92±1% of PE pre-contracted tension pre-AICAR in *n* =3 rats) when rings under-went extensive wash out (i.e. 6 consecutive exchanges of buffer bath) and a one hour recovery period post AICAR DRC (Figure A2-2). This is consistent with previous reports that it is possible to wash out AICAR[36,60], and that AICAR does not produce non-specific toxic effects under these conditions.

Functional index of NO bioavailability. In other experiments, rings were pre-incubated with no drug, L-NAME (10^{-4}M), AICAR (2mM), or AICAR + L-NAME for 30 minutes (following KCl exposures) and then contracted with PE ($10^{-6.5}$M). The difference in tension generated to PE was compared across groups to assess the interactive effects of L-NAME and AICAR on NO bioavailability.

Endothelium-dependent contractions. Quiescent aortic rings (not pre-contracted) were pre-incubated with L-NAME (10^{-4}M, to block the NO-mediated component of the response to ACh) and with either no drug, AICAR (500µM, 30 minutes), the AMPK inhibitor Compound C (CC; 20µM, 45 minutes), or AICAR + CC. The lowest dosages of AICAR and CC capable of generating maximal functional effects were chosen based on pilot experiments (data not shown). Rings were then exposed to increasing concentrations of acetylcholine to elicit endothelium-dependent, COX-EDCF-mediated contractions as previously described[47,48].

ZMP and adenine nucleotide content in aortic rings. Using other WKY and SHR animals, aortic rings were prepared either with or without endothelium, mounted on the vascular myography apparatus as

described above, and subjected to the same set-up and equilibration procotol. Following incubation with or without AICAR (2mM) for 30 minutes, rings were quickly removed from the apparatus, snap frozen in liquid nitrogen and stored at -80^{o}C. Samples were freeze-dried under vacuum, weighed, cut into small pieces and extracted for analysis of ZMP (the intracellular metabolite of AICAR responsible for AMPK activation[36]) and adenine nucleotide content by high performance liquid chromatography (HPLC) as previously described[88,120]. ZMP (Sigma) was included in the methodological calibration standards, and was clearly resolved from other peaks in standards and samples.

Immunoblot analysis of protein content and phosphorylation levels. Aortic rings from a different set of WKY and SHR animals were prepared with and without endothelium and mounted on the vascular myography apparatus as described above. Rings were exposed to the protocol used above to obtain the AICAR dose-response functional measures (with all rings being assigned to the 'no drug' condition) and were removed before and during the AICAR DRC to be snap frozen for Western blotting. Some rings were removed immediately prior to administration of the first AICAR dose (following PE precontraction) for baseline measures, and when stable responses were obtained at each consecutive AICAR dose. Some rings were also snap frozen as time controls (pre-contracted with PE and allowed to remain contracted for the duration of the AICAR DRC protocol, but not exposed to AICAR) to ensure that AMPK activation did not occur as a function of time during the collection period of the DRC. Rings were rapidly removed from the apparatus at appropriate points (care was taken not to damage the endothelium), snap frozen in liquid nitrogen, and stored at -80^{o}C. The time from removal of the rings from the buffer to immersion in liquid nitrogen was < 5 seconds. One ring was removed for each control point and AICAR dose, and all rings (controls and AICAR doses) were obtained from a single rat for each E+ or E- experiment. Sample preparation and immunoblotting procedures were performed as described previously[74]. Briefly, 30µg of protein was loaded per well, and membranes were ponceau stained to confirm consistent protein loading across lanes. All blots were normalized to a thoracic aorta standard (aorta from young male Sprague-Dawley rats) that was run on all gels.

Drugs, Chemicals and Antibodies. All drugs and chemicals were purchased from either Sigma-Aldrich (St. Louis, MO) or Bioshop Canada Inc. (Burlington, ON, Canada), with the exception of AICAR which was purchased from Toronto Research Chemicals (Toronto, ON, Canada). For immunoblotting experiments, primary antibodies specific for the α subunit of AMPK (recognizing both α1 and α2 subunit isoforms, 1:500 dilution) and P(Thr^{172})-AMPK (1:1000 dilution), acetyl-CoA carboxylase (1:250), P(Ser^{79})-ACC (1:500), and P(Ser^{1177})-eNOS (1:3000) were obtained from Cell Signaling Technology (via New England Biolabs, Pickering, ON, Canada). eNOS primary antibody (1:750) was obtained from BD Biosciences (Mississauga, ON, Canada) and horse-radish-peroxide conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Data Analyses. Values are reported as mean ± standard error, with *n* referring to the number of animals per group. For comparisons consisting of more than two treatment conditions or groups, one- or two-way ANOVAs with Tukey's or Bonferroni post-hoc analyses were conducted to evaluate within or between group comparisons. A two-tailed Student's *t*-test was used for comparisons involving only two groups. Differences were considered significant if $P < 0.05$. All statistical analyses were performed using SAS (SAS Institute, Cary, NC) or GraphPad Prism 4 analysis software (La Jolla, CA).

2.5 Results

Animal Characteristics. Animals were 20-24 weeks of age at the time of experiment (Table 2-1). Hypertension was confirmed in SHR by measuring mean arterial pressure in a sub-set of animals prior to removal of the aorta for *in vitro* experiments (Table 2-1). Left ventricular hypertrophy, apparent in the greater left ventricle/BM and heart weight/BM ratios, also confirms typical cardiovascular pathology in SHR versus WKY (Table 2-1).

Endothelium-dependent and –independent vasomotor responses to AICAR.

AICAR generates both endothelium-dependent and endothelium-independent relaxation in aortic rings from WKY rats. Figure 2-1A displays representative tracings of responses to AICAR for both WKY E+ (middle tracing) and WKY E- (bottom tracing) rings, and for a PE-contracted time control (WKY E+ ring, no AICAR; top tracing). Pre-contracted tension to $10^{-6.5}$M PE was not different between WKY rings with or without endothelium (E+: 2.39 ± 0.32g, E-: 2.31 ± 0.12g, P = NS). WKY rings with intact endothelium relaxed from PE pre-constriction in an AICAR dose-dependent manner to a maximum of 99±7 % in response to 10^{-2}M AICAR (Figure 2-1C). Removal of the endothelium blunted relaxation to AICAR at all concentration points, with relaxation reaching only 60±1% at 10^{-2}M ($P < 0.0001$ vs. WKY E+; Figure 2-1D). Relaxation was not detectable in either E+ or E- WKY rings until 10^{-3}M AICAR (Figure 2-1C and 1D).

Endothelium-dependent and –independent relaxation to AICAR is enhanced in aortic rings from SHR compared to those from WKY. Representative tracings for SHR E+ (middle), E- (bottom) and time control (top) are shown in Figure 2-1B. Since SHR E+ rings unexpectedly relaxed ~ 50% of pre-contracted tension at the lowest AICAR concentration (10^{-6}M) used in our experimental protocol, we retrospectively performed a small number of experiments, in SHR E+ rings only, at lower concentrations of AICAR in order to demonstrate the full dose-response relationship in this group. A sample tracing over the expanded [AICAR] range (i.e. beginning 10^{-9}M AICAR) has been included in Figure 2-1B (middle tracing) for SHR E+ rings. Relaxation to AICAR in SHR rings with endothelium intact was elevated above that observed in WKY aorta at 10^{-6}M AICAR and remained higher at every concentration to 10^{-2}M AICAR (Figure 2-1C). In rings with endothelium removed, SHR rings relaxed more than rings from WKY beginning at 10^{-4}M AICAR and at each increasing concentration to 10^{-2}M (Figure 2-1D). PE pre-contraction was 1.88 ± 0.25g in SHR E+ rings and 1.84 ± 0.25g in SHR E- rings and these values were not different from each other, or from those of the respective WKY E+ and E- rings.

ZMP and adenine nucleotide content following incubation with AICAR.

ZMP and adenine nucleotide content is not different in SHR versus WKY aortic rings following AICAR treatment. ZMP was not detectable in control rings from either WKY or SHR (Table 2-2). Following exposure to AICAR, ZMP content was not different in WKY or SHR rings either in the presence or absence of the endothelium (Table 2-2). Similarly, ATP, AMP and the AMP to ATP ratio were not different in WKY or SHR rings with or without endothelium following AICAR treatment, and AICAR-treated values for these parameters were not different from those of the non-treated controls (Table 2-2).

AMPK protein content and activation status in SHR and WKY aortic rings before and during the AICAR dose-response protocol.

Basal AMPK activation is blunted in SHR versus WKY aortic rings with endothelium prior to AICAR exposure. Phosphorylation of AMPK at Thr^{172}, a mandatory modification required for enzyme activation[99], and phosphorylation of acetyl-CoA carboxylase (ACC) at Ser^{79}, a downstream target and well established marker of AMPK activity[99], were evaluated as surrogate markers of AMPK activation. AMPK phosphorylation was expressed as the phosphorylated-to-total protein ratio to account for slightly higher levels of AMPK total protein expression in SHR versus WKY rings with endothelium (total AMPK: 1.31±0.04 vs 1.00±0.13 in SHR and WKY E+ rings respectively, P = 0.0317). Prior to AICAR exposure, the P(Thr^{172})-AMPK/AMPK ratio in SHR rings with endothelium was only ~39% of that observed in WKY rings (Figure 2-2A; 0.39±0.07 vs 1.00±0.11 respectively at baseline, P = 0.0008). P(Ser^{79})-ACC was similarily depressed in E+ rings from SHR vs WKY rings prior to AICAR exposure (Figure 2-3A; 0.33±0.10 vs 1.00±0.20 respectively at baseline, P = 0.0185), although total ACC protein content was not different in SHR vs WKY rings with endothelium (1.00±0.26 vs 0.93±0.16 respectively, P = 0.8304). In contrast, in rings lacking endothelium, neither P(Thr^{172})-AMPK/AMPK and P(Ser^{79})-ACC (Figure 2-2B and 2-3B), nor total AMPK and ACC protein levels, were different in SHR versus WKY (AMPK: 1.05±0.17 vs 1.00±0.24 in SHR and WKY E- rings, P = 0.8583; ACC: 1.09±0.37 vs 1.00±0.26

in SHR and WKY E- rings respectively, P = 0.8601). Thus, the depressed AMPK activation in SHR versus WKY aorta appears to be localized to the endothelium rather than the smooth muscle.

AMPK activation is enhanced in SHR versus WKY aortic rings with endothelium at low AICAR doses, but is similar in SHR and WKY aortic rings without endothelium. The levels of P(Thr^{172})-AMPK (Figure 2-2) and P(Ser^{79})-ACC (Figure 2-3) were increased significantly from baseline with increasing concentrations of AICAR in E+ and E- rings from WKY and SHR. Time controls, pre-contracted with PE, but not exposed to any AICAR doses (designate "TC" in Figure 2-2A/B, 2-3A/B), showed no changes in P(Thr^{172})-AMPK/AMPK or P(Ser^{79})-ACC from baseline in SHR or WKY rings with (Figure 2-2A and 2-3A) or without (Figure 2-2B and 2-3B) endothelium, demonstrating that no changes in AMPK activation occurred as a function of time over the duration of the protocol in the absence of AICAR. When data were expressed relative to WKY baseline values (Figure 2-2A/B), P(Thr^{172})-AMPK/AMPK in SHR E+ was either not different, or was lower (at 10^{-4} P = 0.0041 and 10^{-3}M P = 0.0029) than in WKY E+ rings in response to AICAR (Figure 2-2A), and was not different at any concentration in SHR versus WKY E- rings (Figure 2-2B). Similarly, P(Ser^{79})-ACC was not significantly different at any AICAR concentrations in SHR versus WKY rings either with (Figure 2-3A) or without (Figure 2-3B) endothelium. However, when values were expressed as fold increase from their *respective* baseline values, the increase in P(Thr^{172})-AMPK/AMPK over baseline was significantly greater in SHR than in WKY E+ rings at 10^{-6}M (P = 0.0430) and 10^{-5}M (P = 0.0065) (Figure 2-2E), and P(Ser^{79})-ACC was increased in SHR over WKY at 10^{-5}M (P = 0.0137) and 10^{-4}M (P = 0.0250) in rings with endothelium (Figure 2-3E). In contrast, no differences were observed between P(Thr^{172})-AMPK/AMPK and P(Ser^{79})-ACC fold increases from baseline between SHR and WKY in E- rings at any AICAR dose (Figure 2-2F and 2-3F).

Pharmacological dissection of mechanisms responsible for endothelium-dependent relaxation to AICAR.

Endothelium-dependent relaxation to AICAR in WKY aortic rings is NO-mediated. Aortic rings were incubated with L-NAME, indomethacin (INDO), or L-NAME + INDO to determine the involvement of NO- and cyclooxygenase-dependent signalling in the vasodilatory responses to AICAR. Relaxation was blunted in WKY E+ aortic rings treated with either L-NAME or L-NAME + INDO compared the untreated controls (Figure 2-4A), to levels resembling those of E- rings (P = NS vs. E- CON; Figure 2-4C). Indomethacin alone, however, did not alter relaxation compared to the E+ control condition at any AICAR concentration (Figure 2-4A). In WKY E- rings, relaxation to AICAR was not affected by L-NAME, INDO or L-NAME + INDO (Figure 2-4C). Pre-contraction to PE was not affected by drug incubation and was not significantly different across groups (WKY E+ CON: 2.39±0.32g, E+ LN: 2.72±0.27g, E+ INDO: 2.13±0.24g, E+ LN INDO: 2.82±0.20g, E- CON: 2.31±0.12g, E- LN: 2.42±0.28g, E- INDO: 2.62±0.36g, E- LN INDO: 2.55±0.10g).

Endothelium-dependent relaxation to AICAR in SHR aortic rings is NO- and endoperoxide-mediated. Across the lowest three concentrations of the AICAR dose response curve (10^{-6}, 10^{-5}, and 10^{-4}M), aortic rings with endothelium intact and pre-incubated with INDO relaxed only ~20-40% from pre-contraction while no drug controls relaxed 57-71% (P < 0.0001 E+ INDO vs. CON at each of the three lowest concentrations; Figure 2-4B). E+ rings treated with L-NAME relaxed only ~15-25% (P < 0.0001 vs. E+ CON at 10^{-6}, 10^{-5}, and 10^{-4}M; Figure 2-4B). Relaxation was absent across the lowest three AICAR concentrations in E+ rings pre-incubated with both L-NAME + INDO (P < 0.0001 vs. E+ CON at 10^{-6}, 10^{-5}, and 10^{-4}M; Figure 2-4B) and was not different from responses observed in SHR E- CON rings (P = NS vs. E- CON; Figure 2-4D). L-NAME and INDO groups were also each significantly different from the L-NAME + INDO group across the first three doses of AICAR (P < 0.0001 for both L-NAME and INDO vs. L-NAME + INDO at 10^{-6}, 10^{-5}, and 10^{-4}M; Figure 2-4B). In contrast, pre-incubation with L-NAME, INDO or L-NAME + INDO did not alter relaxation responses to AICAR in any of in E- rings compared

to E- CON (Figure 2-4D). In general, tension generated to PE was not affected by drug pre-incubation and was not different from the respective WKY treatment groups, except that SHR E+ LN pre-contracted tension was greater than SHR E+ CON (P=0.0321), and pre-contracted tension in SHR E+ INDO was lower than that of WKY E+ INDO (P=0.0043) and of SHR E+ CON (P = 0.0163). All other pre-contracted tension values were not significantly different from each other (SHR E+ CON: 1.88±0.25g, E+ LN: 2.98±0.15g, E+ INDO: 0.95±0.16g, E+ LN INDO: 1.91±0.15g, E- CON: 1.84±0.25g, E- LN: 1.77±0.14g, E- INDO: 2.17±0.20g, E- LN INDO: 2.05±0.23g). Vasorelaxation responses to the endothelium-independent vasodilator sodium nitroprusside in rings pre-contracted with phenylephrine were not altered between drug conditions where pre-contraction values were different (as determined by comparison of EC_{50} and maximal response in each group, data not shown), confirming that drug-dependent variations in pre-contraction values *per se* did not significantly influence relaxation responses.

Phosphorylation of eNOS at activation site Ser^{1177} was not increased in aortic rings over the AICAR dose-response curve. To determine if AMPK-mediated phosphorylation of eNOS activation site Ser^{1177} could account for the NO-dependent vasomotor responses to AICAR (a potential mechanism suggested by previous work of others), P(Ser^{1177})-eNOS was assessed in the homogenates of aortic rings collected over the functional AICAR dose-response curve. P(Ser^{1177})-eNOS was not significantly different between SHR and WKY aortic rings at baseline, and was not significantly affected by AICAR over the AICAR dose-response curve protocol (Figure 2-5A). *Total* eNOS protein content also did not change over the AICAR dose response curve in either SHR or WKY (data not shown), although total eNOS content was greater in SHR versus WKY (SHR 1.56 ± 0.07 vs. WKY 1.00 ± 0.05, P < 0.0001).

NO bioactivity is increased with AICAR incubation in SHR and WKY aortic rings as determined by the influence of AICAR on phenylephrine contraction in the presence or absence of L-NAME. The additional tension generated to PE in the presence versus the absence of NOS inhibition (L-NAME) is an established functional indicator of NO bioavailability in *in vitro* pharmacological studies[53,93]. Greater NO bioavailability is revealed by a larger increment in PE-generated tension (L-NAME vs no L-NAME) in

one condition compared to that in another condition. Since the functional data in Figure 4 indicate a role for NO in AICAR-induced relaxation generated in WKY and SHR aortic rings despite a lack of increase in eNOS Ser^{1177} phosphorylation with AICAR treatment (Figure 2-5), we evaluated the influence of AICAR on PE contraction in the presence versus absence of L-NAME as an additional functional index of the effect of AICAR on NO bioavailability[53,93] (Figure 2-6). As expected, pre-incubation of aortic rings with L-NAME alone resulted in greater PE-generated tension compared to the no L-NAME control group in both WKY and SHR, demonstrating the influence of NO on net PE contraction. Pre-incubation with AICAR significantly decreased the contraction generated to PE compared to the no drug condition in both SHR and WKY aortic rings. This effect was fully prevented in WKY and partially prevented in SHR rings that had been pre-treated with AICAR and L-NAME (Figure 2-6C and D). The resulting differential in PE generated tension comparing L-NAME to no L-NAME groups is thus much greater in rings treated with AICAR than in the respective control rings, demonstrating that AICAR enhances NO bioavailability in both WKY and SHR groups.

Endothelium-dependent, EDCF-mediated contraction is blunted in the presence of AICAR but recovered with AMPK inhibitor Compound C in both SHR and WKY aortic rings. In addition to producing NO-dependent relaxation, acetylcholine (ACh) also generates COX-, EDCF-dependent contraction that is augmented in conduit arteries of SHR versus WKY and is considered to be a major contributor to the vascular dysfunction in this and other models of cardiovascular disease[48,62,233]. As expected based on previous work in SHR and WKY conduit arteries[48,62,233], endothelium-dependent contractions generated to increasing concentrations of acetylcholine in quiescent vessel preparations were robust in aortic rings from SHR (Figure 2-7A) but were of a much lower magnitude in those from WKY (Figure 2-7B). Both removal of the endothelium, and pre-incubation of rings with the cyclooxygenase inhibitor indomethacin, abolished contractions in SHR and WKY aortic rings (Figure 2-7A and B), confirming the endothelium- and cyclooxygenase dependency of these responses respectively as has been previously reported[48,233]. Pre-incubation with AICAR similarly blunted the COX-EDCF-mediated

contraction to ACh in both SHR and WKY compared to the E+ CONs (Figure 2-7C and D). Compound C almost completely reversed the effect of AICAR in WKY (Figure 2-7D) and SHR rings (Figure 2-7C), although contractions were not completely restored in SHR at lower acetylcholine concentrations. Responses in rings pre-incubated with CC alone were not different from those treated with AICAR + CC in either SHR or WKY (data not shown) confirming the AMPK-specificity of the AICAR effect. Collectively these data imply that AMPK suppressed the COX-EDCF-mediated contractile response that is more robust in SHR than in WKY aortic rings.

Table 2-1. ***Animal Characteristics***

	WKY	SHR	*P*
Age, wks	21.0±2.0	21.9±2.5	0.332
Whole body mass (BM), g	308±19	334±12	0.004
Tissue Masses			
Left Ventricle, mg	857±65	1111±56	<0.001
Left Ventricle/BM (mg/g BM)	2.81±10	3.31±14	<0.001
Right Ventricle, mg	200±12	220±11	0.007
Right Ventricle/BM (mg/g BM)	0.659±0.034	0.657±0.025	0.657
Heart, mg	1058±72	1331±61	<0.001
Heart/BM, (mg/g)	3.47±0.10	3.97±0.14	<0.001
Mean Blood Pressure, mmHg	77±6	172±10	0.003
Heart Rate, beats/min	323±21	414±34	0.096

Hemodynamic parameters were assessed in the left common carotid artery of anesthetized WKY and SHR using a Millar pressure probe (n = 3). n=12 for all other measures. Due to differences in body weight between strains, values were expressed both in absolute terms and relative to body weight. Data represents means ± sem.

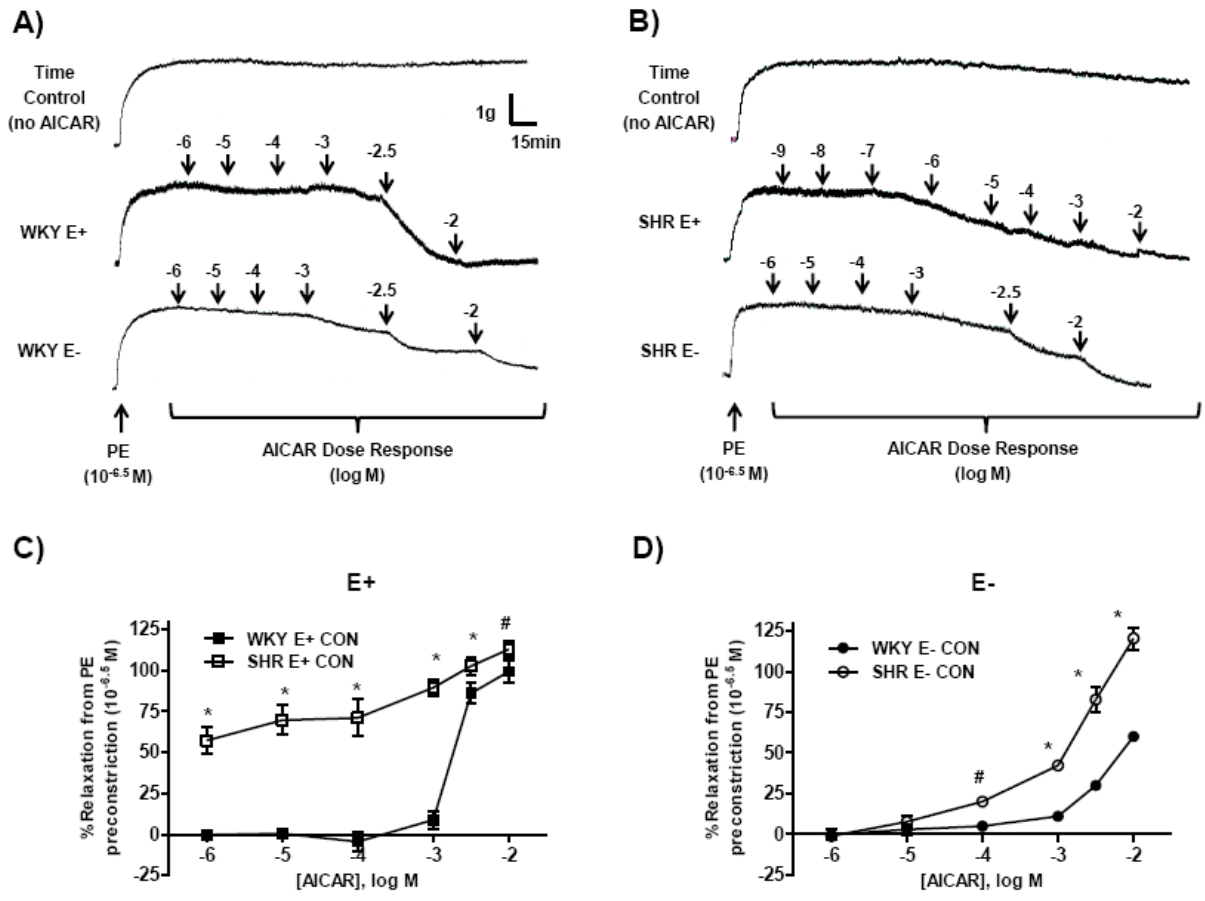

Figure 2-1. Vasorelaxation to AICAR is both endothelium-dependent and –independent and is enhanced in aorta from SHR. Representative tracings demonstrate the relaxation responses to increasing concentrations of AICAR in aortic rings from **A)** WKY and **B)** SHR rats with (E+; middle tracings) and without (E-; bottom tracings) endothelium following phenylephrine ($10^{-6.5}$M) pre-contraction. Time control tracings (top; rings pre-contracted with PE but not exposed to AICAR doses) demonstrate the ability of rings to maintain contraction over the duration of the AICAR dose-response protocol. Relaxation of WKY and SHR aortic rings **C)** with and **D)** without endothelium to increasing concentrations of AICAR. Responses are expressed as mean percent relaxation from phenylephrine pre-contraction (PE $10^{-6.5}$M). *n* = 4-6 rings per group from different animals. # $P < 0.05$, * $P < 0.01$ versus WKY.

Table 2-2. ***ZMP and Adenine Nucleotide Content of SHR and WKY aortic rings***

	E+		E-	
	WKY	SHR	WKY	SHR
ZMP				
CON	ND	ND	ND	ND
AICAR	4.0 ± 0.9	4.0 ± 0.3	3.4 ± 0.2	3.7 ± 0.3
ATP				
CON	0.89 ± 0.15	1.32 ± 0.26	0.82 ± 0.12	0.78 ± 0.17
AICAR	0.79 ± 0.13	1.12 ± 0.19	0.62 ± 0.13	0.69 ± 0.17
AMP				
CON	0.67 ± 0.06	0.46 ± 0.10	0.53 ± 0.08	0.60 ± 0.15
AICAR	0.72 ± 0.04	0.47 ± 0.09	0.72 ± 0.15	0.75 ± 0.17
AMP/ATP				
CON	0.87 ± 0.15	0.56 ± 0.24	0.77 ± 0.18	1.02 ± 0.37
AICAR	1.03 ± 0.19	0.55 ± 0.19	1.00 ± 0.27	0.99 ± 0.31

All values are expressed as µmol/g dry weight except the AMP/ATP ratio which is unitless. Aortic rings were loaded on the myography apparatus, subjected the same start and equilibration protocol as used in collection of functional measures and then incubated with 2mM AICAR or no drug (CON) for 30 minutes. Rings were snap frozen and then extracted for analysis by high performance liquid chromatography. No significant differences were present between groups following AICAR incubation or compared to control rings. n = 5-6 rings per group from different animals. Data represents means ± sem. ND; not detectable, WKY; Wistar Kyoto rats, SHR; Spontaneously Hypertensive rats.

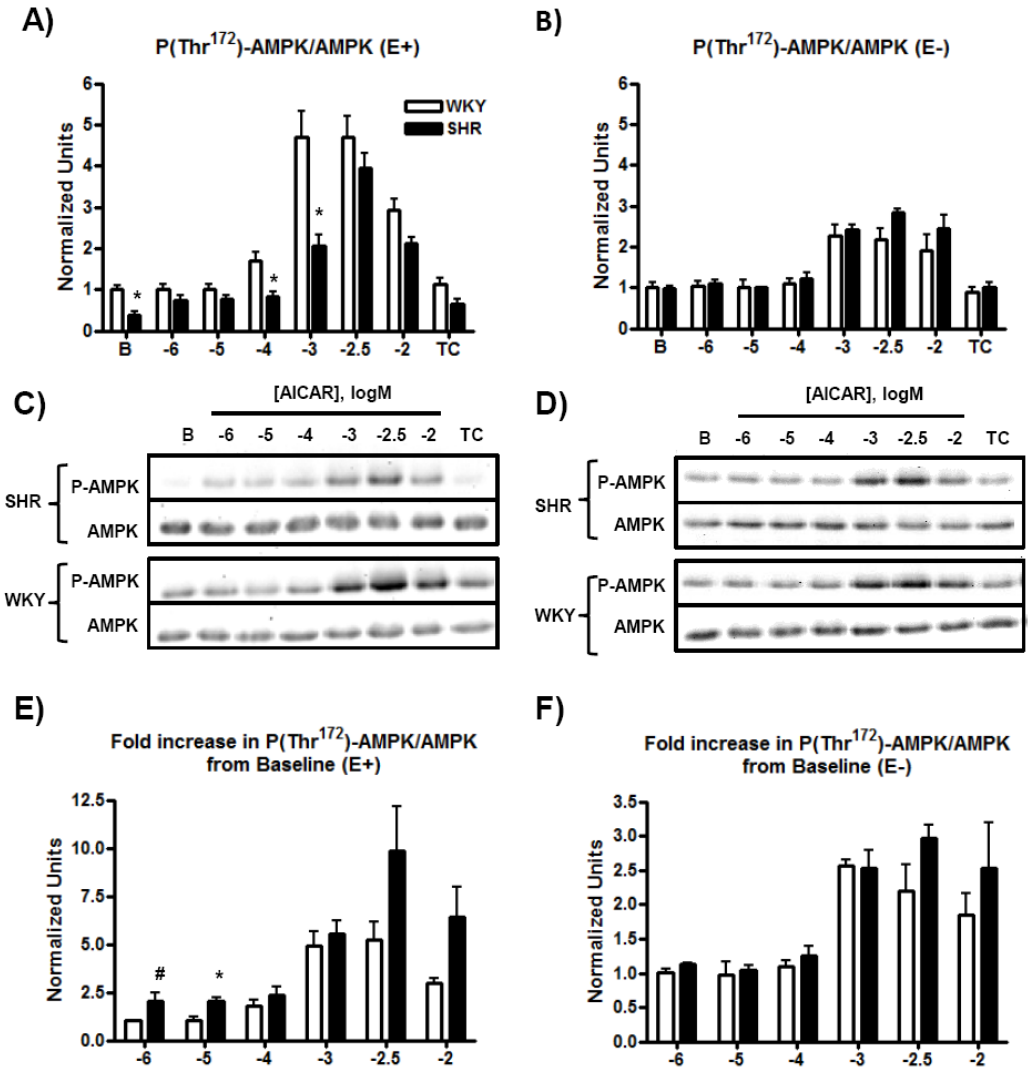

Figure 2-2. Phosphorylation of AMPK activation site Thr^{172} during AICAR dose-response curves in SHR and WKY aortic rings. P(Thr^{172})-AMPK and total protein content of the AMPKα subunit were assessed by western blotting in homogenates of aortic rings that were removed during the AICAR dose-response curve protocol. The ratio of phosphorylated to total AMPK protein is expressed in rings **A)** with (E+) and **B)** without (E-) endothelium immediately prior to AICAR exposure (baseline; B, PE pre-contraction only), at each AICAR concentration during the dose-response curve (expressed in log M) and in time controls (TC; PE precontraction for the duration of the dose-response curve with no AICAR exposure). Representative blots are shown for p-AMPK and AMPK in SHR and WKY aortic rings **C)** with and **D)** without endothelium. The bottom two panels show the same data expressed as fold increase normalized to the respective WKY or SHR baseline in **E)** E+ and **F)** E- rings to account for initial baseline differences between WKY and SHR. Legend conventions are conserved across panels. Results represent data collected from 3 independent experiments where aortic rings from a single animal were used to generate all treatment conditions for each experiment. Blots were run in duplicate for each set of samples. Data is expressed as mean ± sem of densitometry analyses. # $P < 0.05$, * $P < 0.01$ versus WKY.

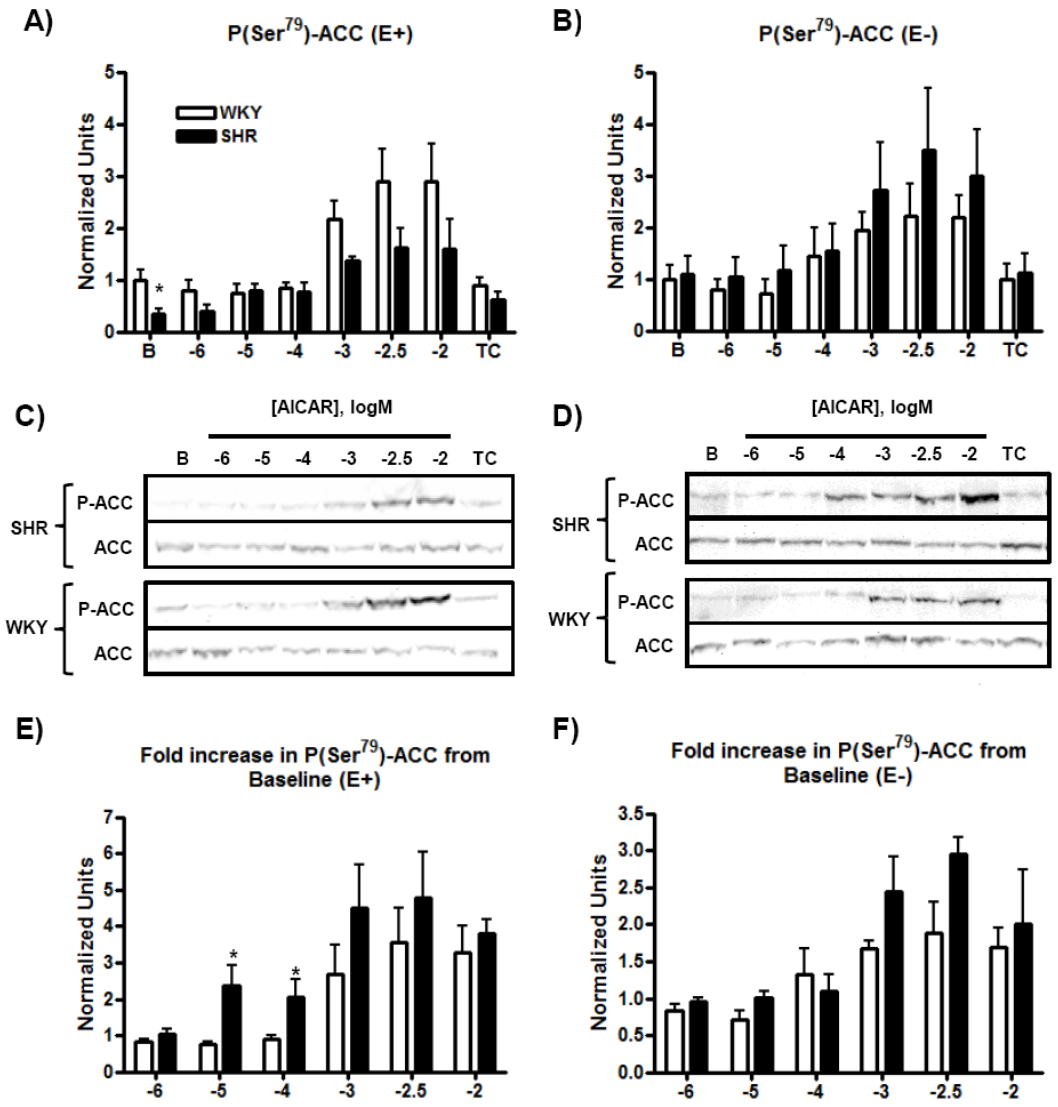

Figure 2-3. Phosphorylation of AMPK downstream target acetyl-CoA carboxylase during AICAR dose-response curves in SHR and WKY aortic rings. P(Ser^{79})-ACC and total ACC protein content were assessed by western blotting in aortic ring homogenates prepared from vessels frozen during the AICAR dose-response curve protocol. P(Ser^{79})-ACC is expressed in rings **A)** with (E+) and **B)** without (E-) endothelium either immediately prior to AICAR exposure (baseline; B, PE pre-contraction only), at each AICAR concentration during the dose-response curve (expressed using log M nomenclature) and in time controls (TC; PE precontraction for the duration of the dose-response curve with no AICAR exposure). Representative blots are shown for p-ACC and ACC in SHR and WKY aortic rings **C)** with and **D)** without endothelium. The bottom two panels show the same data expressed as fold increase normalized to the respective WKY or SHR baseline in **E)** E+ and **F)** E- rings to account for initial baseline differences between WKY and SHR. Results represent data collected from 3 independent experiments where aortic rings from a single animal were used to generate all treatment conditions for each experiment. Blots were run in duplicate for each set of samples. Legend conventions are conserved across panels. Data is means ± sem of densitometry analyses. * $P < 0.01$ versus WKY.

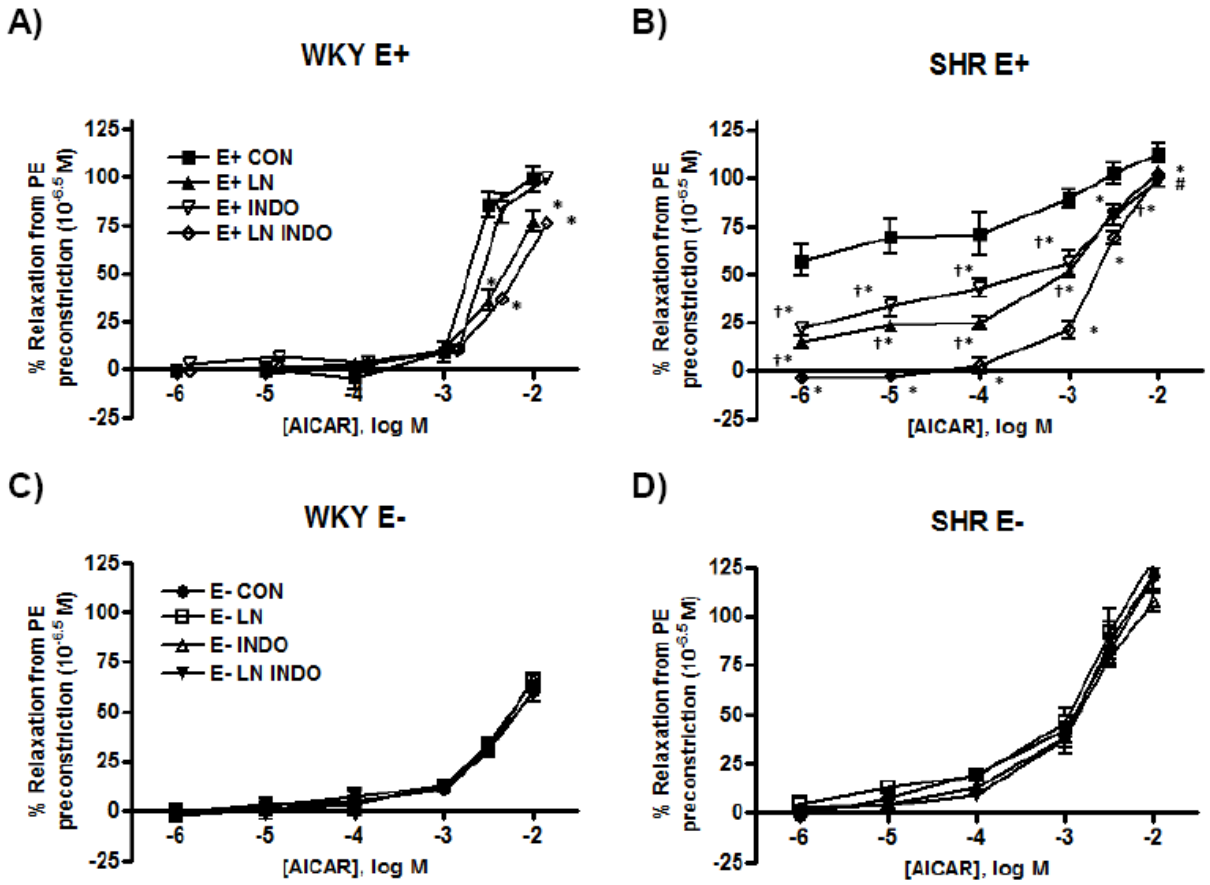

Figure 2-4. Mechanisms of AICAR-mediated endothelium-dependent relaxation in SHR and WKY aortic rings. Aortic rings from SHR and WKY rats were incubated with either NOS inhibitor L-NAME, cyclooxygenase inhibitor indomethacin (INDO), or L-NAME + INDO to determine the NO- and endoperoxide-dependency of relaxation responses to AICAR. Percent relaxation from phenylephrine pre-constriction is displayed in WKY and SHR rings with (**A** and **B**) and without (**C** and **D**) endothelium. Legend conventions are conserved across E+ and E- panels. SHR and WKY E+ CON and E- CON data has been repeated in the figure above (from previous figures) for comparison. Each treatment was performed in aortic rings from each animal. *n* = 3-6 rings per group from different animals. * $P < 0.01$, # $P < 0.05$ versus E+ CON, † $P < 0.01$ vs E+ LN INDO.

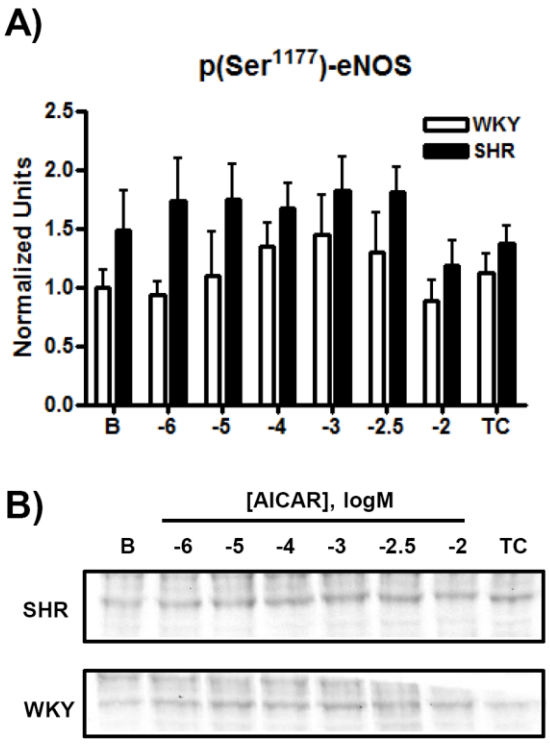

Figure 2-5. Phosphorylation of eNOS at Ser^{1177} was not increased in response to AICAR in SHR or WKY aortic rings. P(Ser^{1177})-eNOS content was assessed by western blotting in aortic rings removed during the AICAR dose-response curve protocol. **A)** P(Ser^{1177})-eNOS is expressed in rings with endothelium either immediately prior to AICAR exposure (baseline; B, PE pre-contraction only), at each AICAR concentration during the dose-response curve (expressed using log M nomenclature) and in time controls (TC; PE precontraction for the duration of the dose-response curve with no AICAR exposure). Representative blots depict **B)** p-eNOS for all conditions in the figures above. Results represent data collected from 3 independent experiments where aortic rings from a single animal were used to generate all treatment conditions for each experiment. Blots were run in duplicate for each set of samples. Legend conventions are conserved across panels. Data represents means ± sem. No significant differences were observed with AICAR treatment.

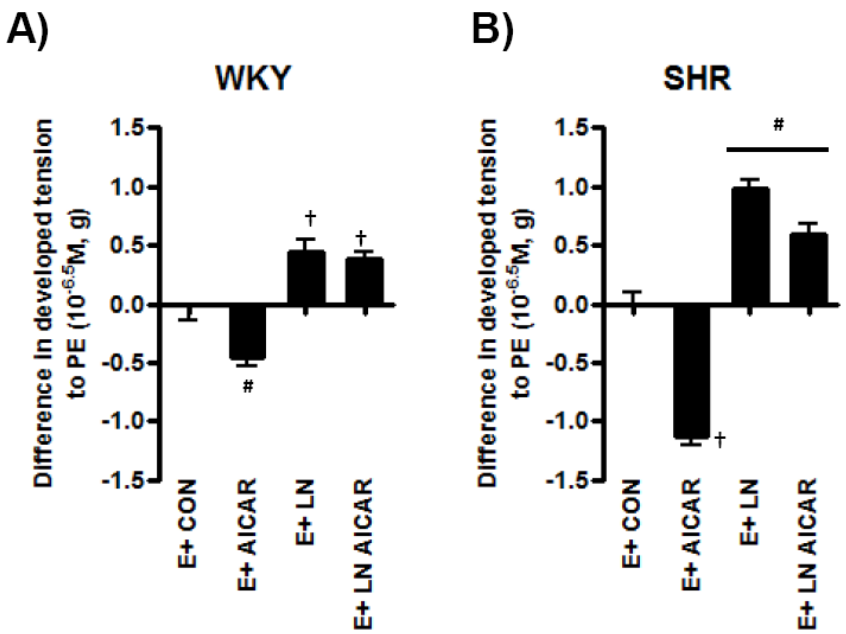

Figure 2-6. AICAR pre-incubation blunts contraction to phenylephrine in an NO-dependent manner demonstrating an AICAR-mediated increase in NO-bioavailability. Aortic rings from **A)** WKY and **B)** SHR were pre-incubated with no drug (CON), AICAR (2mM), L-NAME (LN; 10^{-4}M) or AICAR + L-NAME for 30 minutes and then contracted with phenylephrine (PE; $10^{-6.5}$M). Values are expressed as the mean difference in developed tension from the no drug control group (E+ CON). *n* = 5-15 rings per group from different animals. # $P < 0.05$, † $P < 0.001$ versus E+ CON or versus an adjacent group where indicated.

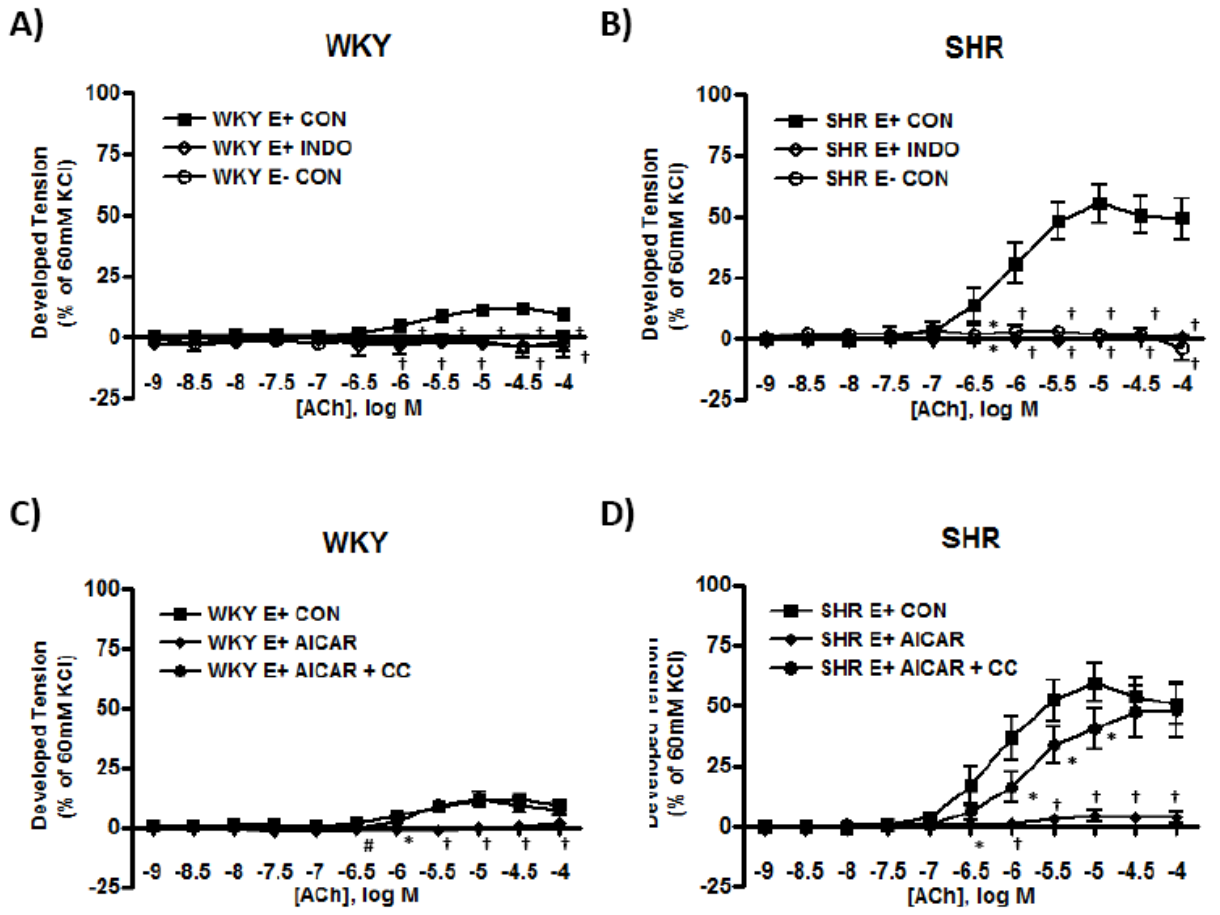

Figure 2-7. EDCF-mediated contractions are blunted in aortic rings from SHR and WKY rats pre-incubated with AICAR. Endothelium-dependent contractions were observed by exposing rings to increasing concentrations of acetylcholine (ACh) in the presence of NOS inhibitor L-NAME and in the absence of pre-contraction (quiescent rings). The endothelium- and COX-dependency of these responses was verified in **A)** SHR and **B)** WKY aortic rings by removal of the endothelium (E-) and by pre-incubation with the COX inhibitor indomethacin (INDO), respectively. In some experiments rings from **C)** SHR or **D)** WKY were pre-incubated with either no drug (CON), AICAR (500µM), or AICAR + AMPK inhibitor Compound C (CC; 20µM) to determine the influence of AMPK on this response. Some rings were also pre-incubated with CC alone; these responses were not different from the AICAR + CC response in SHR or WKY and have therefore not been illustrated in the figure for clarity. Values are expressed as mean percent of tension developed to 60mM potassium chloride (KCl). Each treatment was performed in aortic rings from each animal. n = 3-10 rings per group from different animals. # $P < 0.05$, * $P < 0.01$, † $P < 0.001$ versus E+ CON.

2.6 Discussion

The main findings of our study are: that AICAR generates both endothelium-dependent and –independent relaxation in arteries from normotensive rats, that these responses also occur and are actually enhanced in arteries from hypertensive animals despite depressed basal AMPK activation, and that the mechanisms responsible for endothelium-dependent relaxation are exclusively NO-mediated in WKY but NO- and COX-dependent in SHR aorta. Further investigation into the mechanisms responsible for the enhanced endothelium-dependent relaxation to AICAR in SHR, revealed that AMPK-mediated suppression of downstream COX-EDCF-dependent vasoconstrictory signalling pathways and enhanced AMPK activation at low AICAR doses likely account for a significant portion of the augmented sensitivity of SHR versus WKY E+ aortic rings. These results are novel and add significantly to our understanding of AMPK-mediated vasomotor function by being the first to investigate endothelium-dependent and –independent functional responses to AICAR in arteries of hypertensive animals and by elucidating mechanisms responsible for the endothelium-dependent functional response to AICAR in a conduit artery from WKY and SHR.

Endothelium-independent relaxation to AICAR and other AMPK activators (i.e. deoxy-glucose/ hypoxia and metformin) has been observed by others in isolated arteries from several animal models and vessel types, including murine aorta[82], porcine carotid arteries[181] and rat aorta[139]. During the preparation of this manuscript, observations were also published showing that endothelium-dependent dilation of isolated resistance arteries and increased flow in an isolated vessel bed *in situ* occur following exposure to AICAR[15]. While our findings partly confirm these observations made in other models, they also provide the first evidence for the co-existence of endothelium-dependent and independent relaxation to AICAR in isolated conduit arteries from SHR and WKY. Consistent with the dose-dependent functional relaxation to AICAR observed in SHR and WKY aortic rings during our experiments (Figure 2-1), markers of AMPK activation (phosphorylation at both AMPK activation site Thr^{172} and AMPK downstream target Ser^{79} on acetyl-CoA carboxylase) responded in a dose-dependent manner over the

AICAR dose-response curve protocol in SHR and WKY rings with and without endothelium during our experiments (although responses in E+ rings were several fold higher and began at lower doses than those of E- rings, Figures 2-2 and 2-3), supporting the involvement of AMPK in mediating the dose-dependent functional responses to AICAR.

We were interested in investigating mechanisms responsible for the endothelium-dependent component of the relaxation generated by AICAR in SHR and WKY aortic rings. A number of cell culture and *in vitro* biochemical experiments have demonstrated that AMPK can phosphorylate eNOS at activation site Ser^{1177} [27,29,31,44,100,145], and that AMPK activation has been associated with increased NO release from endothelial cells[29,31,44,145], establishing NO as a potential mediator of endothelium-dependent effects of AMPK. However, AMPK activation does not result in eNOS phosphorylation under some conditions[69,147], suggesting that AMPK activity and eNOS phosphorylation can be dissociated and that the influence of AMPK on NO-mediated vasomotor function may also involve other mechanisms in addition to the Ser^{1177} phosphorylation-mediated activation of eNOS. While some studies infer that NO-dependent relaxation stimulated by AMPK-mediated activation of eNOS may be possible, there is limited evidence from previous studies that acute activation of AMPK is capable of generating functional vasodilatory outcomes by this mechanism in an intact blood vessel, or in the context of an *in vivo* vascular system[13,15]. Our data, along with the recently published work of Bradley et al[15], are some of the first to our knowledge to demonstrate an association between endothelium, NO-dependent relaxation and the activation of AMPK in isolated intact vessels. Pre-incubation with L-NAME completely removed the endothelial component of AICAR-mediated relaxation in WKY and partially blocked the endothelium-dependent component in SHR aortic rings (Figure 2-4), suggesting that AICAR-mediated endothelium-dependent relaxation is completely NO-dependent in WKY and only partially NO-dependent in SHR. Our observations indicate no AICAR-dependent increases in phosphorylation of eNOS activation site Ser^{1177} in SHR or WKY aortic rings (Figure 2-5), but further experiments verified that functional NO bioavailability is increased with AICAR nonetheless by demonstrating that pre-incubation with AICAR

blunts contraction to phenylephrine in rings from WKY and SHR in an NO-dependent manner (Figure 2-6). Therefore we conclude that AMPK must be facilitating the eNOS-mediated production of NO through mechanisms other than Ser^{1177} phosphorylation such as: phosphorylation of eNOS Ser^{633} [31], assisting in eNOS association with heat shock protein 90[44,189], promoting the deacetylation of eNOS[30] to assemble active eNOS complexes, etc. Alternatively, AMPK may increase NO bioavailability independently of NO production *per se* via possible effects on NO destruction or the sensitivity of vascular smooth muscle to NO (i.e. the other determinants of NO bioavailability). For example, activation of AMPK has been associated with decreased oxidative stress in human aortic endothelial cells and mouse aorta[137,241], and could thus potentially increase NO bioavailability by reducing the interaction of NO with reactive oxygen species. However, the mechanisms attributed to the AMPK-mediated reduction in oxidative stress in these studies involved AMPK-mediated modifications in protein transcription, translation, etc., processes which require a time frame of hours to days to produce effects, and therefore such mechanisms do not likely contribute to the acute functional responses observed here. Further studies will be required to determine if oxidative stress is involved in mediating acute effects of AMPK activiation on vasomotor function.

Interestingly, a portion of the endothelium-dependent component of the relaxation generated by AICAR was COX-dependent in SHR but not WKY aortic rings, as the COX 1 and 2 inhibitor indomethacin partially blocked endothelium-dependent relaxation in SHR, but not in WKY vessels (Figure 2-4). Since a portion of the tension generated by PE is EDCF-mediated in SHR aorta from our experiments, (see PE pre-contracted tension values in results above, as also observed previously by our lab[86] and others[2,142]), it appeared that AICAR was causing endothelium-dependent relaxation of pre-contracted SHR aortic rings by inhibiting the enhanced COX-EDCF-mediated contraction that is present in SHR (an effect that would not be expected to occur in WKY since the contribution of EDCFs to PE-mediated contraction in WKY rings is minimal[2,86,142]). In order to test this hypothesis, we assessed ACh-induced contraction in quiescent rings in the presence of L-NAME according to an established protocol that has previously shown these contractions to be endothelium-, COX-, and TP receptor-dependent, and

to be greatly enhanced in SHR vs WKY[48,62,233]. Indeed, our results herein confirm the endothelium- and COX-dependency of the ACh-induced contractions and that the magnitude of these contractions is greater in SHR versus WKY aorta. We found that these responses were robustly blunted with AICAR and that the effect of AICAR was largely prevented by pre-incubation of rings with the AMPK inhibitor compound C, confirming AMPK specificity of this observation (Figure 2-7). Therefore, these novel results demonstrate that AMPK is suppressing COX-EDCF-mediated contractions in SHR aortic rings, through inhibition of either COX-EDCF production/release, or TP-receptor signalling in the vascular smooth muscle. These results are consistent with related work in other models from other investigations that have reported that AICAR and metformin both blunt endothelium-dependent contraction in mesenteric arteries from OLETF rats (another model of enhanced endothelium-derived contraction) likely by suppressing the release of both prostacyclin and thromboxane A_2 [141]. These findings also support the hypothesis that AICAR-mediated dilation of pre-contracted SHR aortic rings was achieved in part through suppression of COX-EDCF-mediated contraction.

The relaxation responses to AICAR in SHR and WKY aortic rings were also partially endothelium-*in*dependent (Figure 2-1). Although we did not investigate intracellular signalling mechanisms within the vascular smooth muscle that could contribute to AICAR-mediated endothelium-independent relaxation in the present study, previous *in vitro* biochemical experiments demonstrate that AMPK is able to phosphorylate and desensitize MLCK, and thus could presumably induce relaxation in the context of an intact vascular system [116]. Endothelium-independent relaxation to AICAR may also occur due to effects of AMPK on HMG-CoA reductase [36,59,131,157], which could involve modulation of Rho-kinase and L-type channel extracellular calcium entry[170], thus potentially exerting influence over vascular smooth muscle mechanisms of calcium-sensitization and calcium-mediated contraction. As indicated, however, we did not undertake to study these mechanisms in the current study, and additional cellular and physiological studies will be required to elucidate mechanisms of AMPK-mediated relaxation in vascular smooth muscle.

Both the endothelium-dependent and –independent components of the relaxation response to AICAR were enhanced in SHR aortic rings compared to those of WKY (Figure 2-1). Initially it was tempting to speculate that functional responses in SHR vessels would be less responsive to AICAR than those of WKY, as our own pilot work indicated depressed activation of AMPK in SHR aortas prior to AICAR treatment (P(Thr^{172})-AMPK and P(Ser^{79})-ACC in SHR vs. WKY E+ rings, Figures 2-2A and 2-3A). However, studies performed in tissues from diseased or aging models have shown that the ability to activate AMPK pharmacologically is not necessarily compromised when AMPK activation is depressed in the basal state[176,182,261], and thus there does not appear to be dysfunction in the capacity to activate the AMPK protein *per se* under these conditions. In order to help explain the different functional sensitivities of SHR and WKY rings to AICAR, we assessed the intracellular stimulus for AMPK activation and the activation of AMPK over the AICAR dose-response curve protocol in SHR and WKY vessels. The stimulus for AMPK activation was not different between SHR and WKY rings, as all rings were similarly capable of uptake and conversion of AICAR to ZMP (Table 2-2). ZMP is the intracellular metabolite of AICAR, and acts as an AMP analogue to activate AMPK both allosterically and by rendering it a poorer substrate to upstream phosphatases[186,208]. However, both P(Thr^{172})-AMPK and P(Ser^{79})-ACC fold increases from baseline were ~ 2 fold greater in SHR versus WKY E+ rings at the lower concentrations of AICAR (Figure 2-2E and 2-3E), but P(Thr^{172})-AMPK and P(Ser^{79})-ACC were similar in SHR versus WKY E- rings over all AICAR doses (Figures 2-2F and 2-3F). It is unknown whether expression and/or activity of upstream kinases (i.e. LKB1, CaMKKβ, TAK-1) and phosphatases (i.e. PP2C), that phosphorylate and dephosphorylate AMPK at Thr172, respectively[68], are altered in SHR versus WKY vascular tissue. Thus, it is difficult to speculate on how these might contribute to the SHR/WKY differences observed here. While slightly elevated AMPK activation may have contributed to greater relaxation in SHR E+ rings than those from WKY (although this does not appear to be the case for SHR and WKY E- rings), it is likely that enhanced sensitivity of downstream signalling targets, rather than differences in the activation of AMPK *per se*, is the main contributor to the differential responses of SHR

versus WKY E+ and E- rings to AICAR. Indeed, as already discussed, dissection of the mechanisms responsible for the endothelium-dependent component of the relaxation response to AICAR provided insight into the interpretation of differences in tissue sensitivity by revealing that a COX-dependent component of the vasodilatory response to AICAR in SHR but not WKY (Figure 2-7), contributing to the responsiveness of SHR E+ rings at lower AICAR concentrations. Furthermore, conduit vessels from SHR exhibit higher levels of eNOS protein content expression than those from WKY (see results discussed above[47,85]), presenting a more abundant target for AMPK stimulation and NO generation and thus possibly contributing to greater sensitivity and enhanced NO-mediated relaxation observed in SHR aortic rings. In the vascular smooth muscle, signalling through mechanisms such as the Ca^{2+} sensitization that are responsible for regulating contraction is up-regulated in aorta from SHR versus WKY[110,148]. Any or all of these mechanisms may contribute to the SHR versus WKY differences observed in relaxation to AICAR, and further investigation will be necessary to elucidate the contribution of these and other mechanisms to vascular smooth muscle responses in SHR and WKY aortic rings.

A limitation of the present study is that we cannot rule out the potential for non-specific activity of AICAR[36,247], and caution should be exercised in interpreting results generated using only pharmacological tools. However, two features of the current study help mitigate against this limitation: first, the consistency between the functional responses to AICAR and the biochemical indices of AMPK activation under control and pharmacological dissection conditions (L-NAME, INDO) across a wide range of AICAR concentrations, and secondly, the reversibility of the AICAR-mediated effects demonstrated by that AMPK inhibitor Compound C. While there has been some concern regarding toxicity of AICAR in cell and tissue preparations at high doses, we determined in pilot work that the contractile viability of vessels was not compromised following the AICAR dose response curve, as tension generated in response to KCl and PE was not different from that obtained prior to AICAR exposure (see discussion of pilot work in methods). Thus relaxation to AICAR in our study was not due to a decreased viability of our tissue preparations or an artefact of AICAR toxicity in our aortic rings.

In summary, AICAR-mediated relaxation is comprised of both endothelium-dependent and – independent components in aortic rings from WKY and SHR rats. Endothelium-dependent relaxation is NO-mediated in WKY, but NO- *and* COX-mediated in SHR. Greater activation of AMPK in vessels with intact endothelium as well as the suppression of COX-, EDCF-mediated vasoconstriction that is enhanced in SHR versus WKY are likely the main contributors to the enhanced endothelium-dependent relaxation respectively of SHR versus WKY aortic rings to AICAR. These results suggest AMPK may be a useful target for improving vasorelaxation in arteries from hypertensive rats and demonstrate that these vessels maintain their ability to respond in spite of lower basal AMPK activation. Activation of AMPK in the vasculature may be a potential strategy for improving the vascular dysfunction that exists in cardiovascular disease states such as hypertension or other disease states exhibiting vasomotor dysfunction due to impaired NO-dependent relaxation and/or enhanced COX-mediated contraction.

2.7 Addendum to Study 1:

The addendum contains data collected in conjunction with this study but not published as part of the American Journal of Physiology Heart and Circulatory Physiology manuscript. These items include:

Figure A2-1: Functional and biochemical validation for removal of the endothelium from aortic rings by mechanical denudation (stated as data not shown in manuscript publication).

Figure A2-2: Functional viability of aortic rings is not compromised following the AICAR dose response curve protocol (stated in the methods section of the manuscript publication).

Figure A2-3: AMPK activation over time in rat aortic rings following treatment with AICAR. This data demonstrates the consistency between the time it takes an aortic ring to achieve full relaxation to a single dose of AICAR (i.e. ~ 30 minutes at high doses, Figure 2-1) and AMPK activation over time to a single high dose of AICAR (i.e. it takes ~ 30 minutes to maximally activate AMPK in aortic rings).

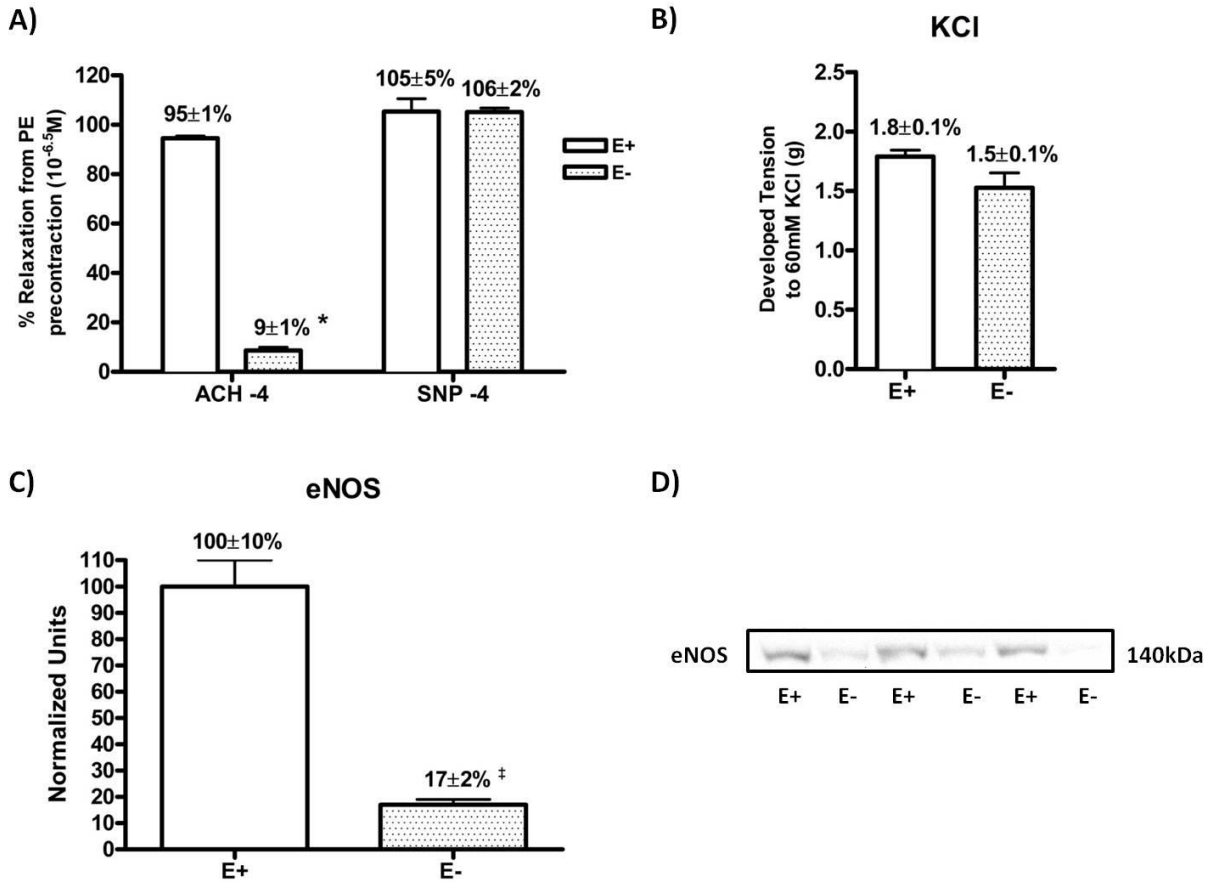

Figure A2-1. Functional and biochemical validation for removal of the endothelium from aortic rings by mechanical denudation. Aortic rings isolated were prepared either with (E+) or without (E-) endothelium. **A)** Functional removal of the endothelium was verified by exposing pre-contracted rings to a maximal dose of the endothelium-dependent vasodilator aceylcholine (10^{-4}M). Vascular smooth muscle vasodilatory and vasoconstrictory capacities were tested in the same rings by exposure to a maximal dose of endothelium-independent vasodilator sodium nitroprusside (**A**; 10^{-4}M, following pre-contraction with PE) and **B)** vasoconstrictor potassium chloride (B, 60mM) respectively. **C)** Biochemical verification of removal of the endothelium was assessed by immunoblotting for total endothelial nitric oxide synthase (eNOS) protein content in E+ and E- rings. **D)** Representative blot pics are shown for eNOS content in E+ and E- rings. n = 4 for E+ vasomotor function data, n = 8-10 for E- vasomotor function data, n = 6 for western blot data. Values are expressed as means ± sem.

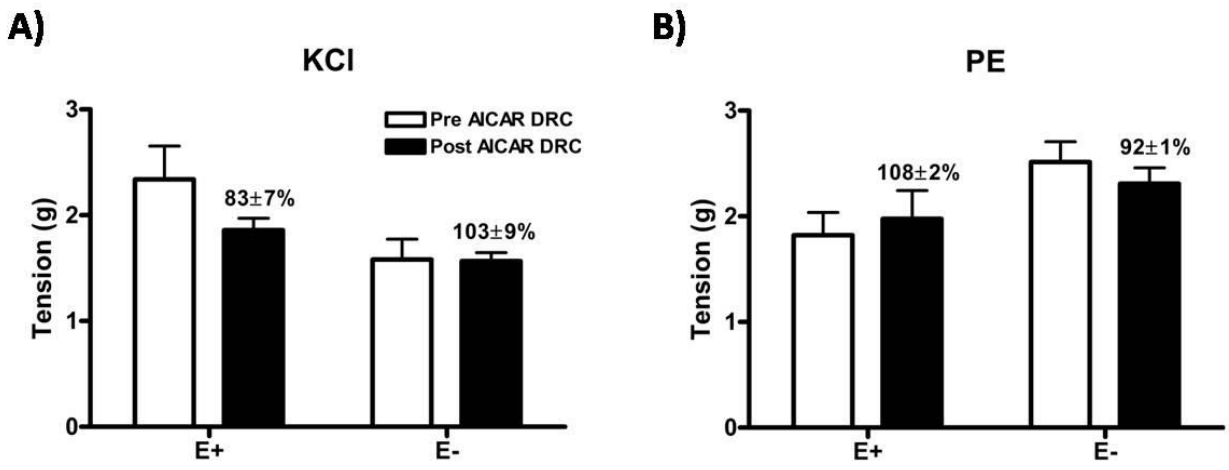

Figure A2-2. Functional viability of aortic rings is not compromised following the AICAR dose response curve protocol. Tension generated to potassium chloride (KCl; 40mM) or phenylephrine (PE; $10^{-6.5}$M) before and after execution of the AICAR dose-response curve protocol were compared and used as indices of functional viability following exposure to AICAR. Rings were contracted with either KCl or PE prior to exposure to AICAR (Pre AICAR DRC) to obtain initial values. Following the AICAR dose-response curve procotol, rings under-went extensive wash out (i.e. 6 consecutive exchanges of buffer bath) and a one hour recovery period before being contracted by the second KCl or PE exposure (Post AICAR DRC). The numerical values displayed over the Post AICAR DRC data columns represent tension generated Post AICAR DRC as a mean percentage of the Pre AICAR DRC values. All data is expressed as means ± sem. n = 3 rings per group for PE experiments and n = 5 rings per group for KCl experiments. There were no significant differences between tension values Pre versus Post AICAR DRC for any groups. The observations above are consistent with previous reports that it is possible to wash out AICAR [36,60], and suggest that AICAR under these conditions does not produce non-specific toxic effects.

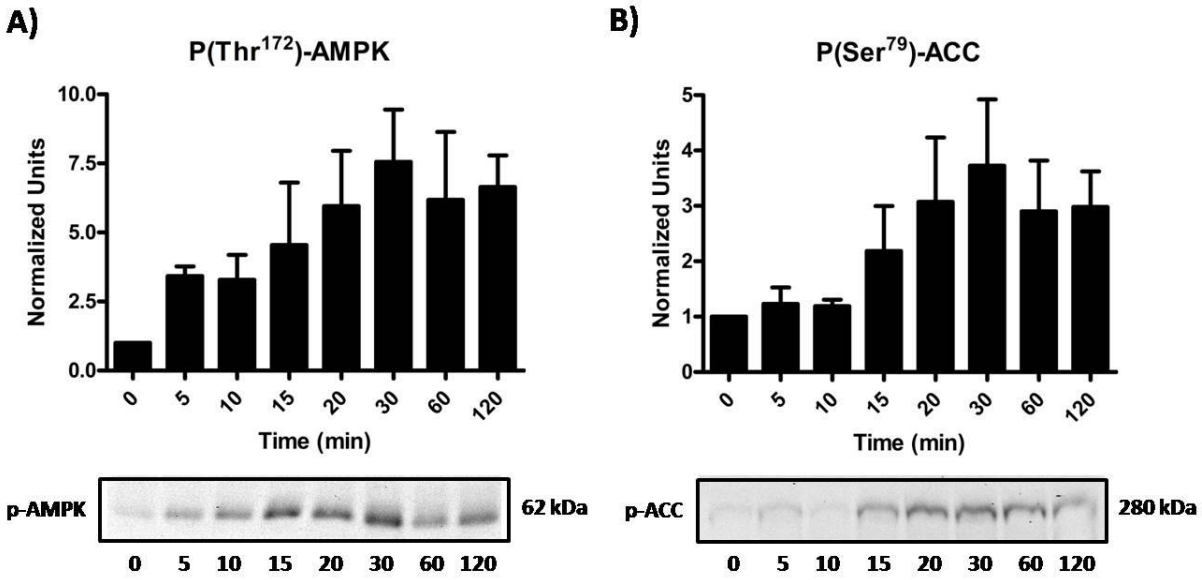

Figure A2-3. AMPK activation over time in rat aortic rings following treatment with AICAR. Aortic rings were loaded on the myography apparatus, subjected to a standard start and equilibration protocol and then incubated with 2mM AICAR. At the specified time points beginning at time 0 (addition of AICAR to the tissue bath), rings were quickly removed and snap frozen for western blots of **A)** P(Thr172)-AMPK and **B)** P(Ser79)-ACC. All time points were collected in rings from the same rat. Data represents results from three independent experiments. Values from densitometry analyses were normalized such that the time 0 value is equal to 1 normalized unit. Data is expressed as mean ± sem.

Chapter 3

Thesis Study 2

AMP-activated protein kinase blunts acetylcholine-induced contractions by endothelium-dependent mechanisms in aorta of normotensive and hypertensive rats

Rebecca J. Ford, Christopher S. Smith and James W.E. Rush

This chapter is presented in manuscript form.

Acknowledgements: Thank you to Andrew Levy, Steven Denniss and Dawn McCutcheon for assistance.

3.1 Review of Study 2 Objectives and Hypotheses

The specific objectives and hypotheses for this chapter have already been stated in the introduction section, but are reproduced identically here for the reader's convenience.

Purpose: To evaluate the influence of AMPK activation status on endothelium-dependent contractions in dysfunctional arteries of hypertensive rats and to elucidate mechanisms responsible for this interaction.

Specific objectives and hypotheses:

Objective 1: To verify that endothelium-dependent contractions to ACh are enhanced in quiescent (non-precontracted) aortic rings from SHR versus those of WKY, and that these contractions are COX-dependent (as has been shown by others and previously in our own lab). **Hypothesis 1:** Contractions to ACh will be enhanced in aortic rings of SHR versus WKY, and will be abolished by inhibition of COX or removal of the endothelium.

Objective 2: To determine the effect of the AMPK activator AICAR on endothelium-dependent contractions in SHR aortic rings. **Hypotheses:** Endothelium-dependent contractions to ACh will be blunted by AICAR pre-incubation in aortic rings of both WKY and SHR.

Objective 3: To demonstrate reversibility the inhibitory effect of AICAR on endothelium-dependent contractions by co-incubating rings with the AMPK inhibitor Compound C. **Hypothesis 3:** Co-incubation of WKY and SHR aortic rings with Compound C will preserve the magnitude of endothelium-dependent contraction generated in these vessels to ACh (contractions to ACh will be no different from those in no drug control rings).

Objective 4: To verify that AMPK is activated by AICAR but inhibited by AICAR-Compound C co-incubation in aortic rings. **Hypothesis 4:** AMPK activation (P(Thr172)-AMPK and P(Ser79)-ACC) will be elevated in rings treated with AICAR, but unaltered in rings treated with AICAR and CC compared to controls in vessels of both WKY and SHR.

Objective 5: To evaluate the effect of the presence of AICAR on contraction to increasing doses of TP-receptor agonists U46619 and PGH_2 to evaluate vascular smooth muscle responsiveness to TP-receptor stimulation. **Hypothesis 5:** Contraction to increasing concentrations of TP-receptor agonists U46619 and PGH_2 will be blunted in aortic rings of WKY and SHR in the presence of AICAR

Objective 6: To investigate the production/release of 6-keto-prostaglandin $F_{1\alpha}$ (a stable metabolite of prostacyclin) by aortic rings of WKY and SHR stimulated with acetylcholine **Hypothesis 6:** 6-keto-prostaglandin $F_{1\alpha}$ levels will be blunted in buffer bathing aortic rings of WKY and SHR treated with AICAR, but unaltered in the presence of AICAR together with Compound C.

Objective 7: To determine the effect of AICAR on dose-dependent relaxation/re-contraction of phenylephrine pre-contracted SHR aortic rings to increasing [ACh]. Since the vasomotor response of phenylephrine pre-contracted SHR aorta to increasing [ACh] is biphasic (relaxation at lower [ACh] is NO-dependent followed by COX-dependent recontraction at higher concentrations, this experiment will afford the opportunity to confirm the effect of AICAR on endothelium- and COX-dependent contraction, and also on NO-dependent relaxation. **Hypothesis 7:** The endothelium- and COX-dependent re-contraction at higher concentrations will be abolished in SHR aortic rings pre-treated with AICAR, but NO-dependent relaxation will be enhanced with AICAR.

3.2 Overview of Study 2

Enhanced endothelium-dependent contractions contribute to vasomotor dysfunction in hypertension. Activation of vascular AMP-activated protein kinase (AMPK) blunts endothelium-dependent contractions generated by acetylcholine (ACh) in isolated arteries. The purpose of this study was to dissect mechanisms responsible for this interaction in isolated aortic rings of Spontaneously Hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY). In vascular myography experiments, endothelium- and cyclooxygenase-dependent contractions to ACh were blunted in quiescent

aortic rings of SHR and WKY pre-treated with the AMPK activator AICAR (500μM) compared to controls, but the contractions were fully preserved in rings treated with both AICAR and the AMPK competitive inhibitor Compound C (CC; 20μM). In contrast, contractions to vascular smooth muscle TP-receptor agonists U46619 and prostaglandin H_2 were not altered by AICAR in aortic rings of either SHR or WKY. ACh-stimulated production/release of 6-keto-$PGF_{1\alpha}$ (the main product of prostacyclin) was blunted in WKY and SHR rings treated with AICAR, but not with AICAR + CC or with CC alone. In precontracted SHR rings, NO-dependent relaxation at low [ACh] was preserved while COX-dependent recontraction/blunted relaxation at high [ACh] was abolished with AICAR. These results suggest that active AMPK attenuates endothelium-, and COX-dependent contractions in WKY and SHR aorta by reducing endothelial prostanoid production, but not via alteration of VSM TP-receptor sensitivity or signalling. AMPK may be as a useful target to improve vasomotor function under conditions of enhanced endothelium-dependent contraction.

3.3 Introduction

The vascular endothelium is an important regulator of vessel tone through the production and release of both endothelium-derived relaxing and contracting factors, the balance of which is important for maintaining healthy vascular function. In arteries of hypertensive, diabetic and aged rats, endothelium-dependent relaxation is reduced and endothelium-dependent contraction is enhanced [47,85,230,232], upsetting the homeostatic regulation of vessel tone and resulting in vasomotor dysfunction. Endothelium-dependent contractions are generated by endothelium-derived contracting factors (EDCFs) that are released from the endothelium in response to vasoactive stimuli such as acetylcholine (ACh), adenine nucleotides (ATP, ADP), endothelin-1, nicotine and calcium ionophore A23187 [65,232]. Although a variety of compounds can act as EDCFs depending on disease condition, artery bed, and agonist present [65,216,230,232], ample evidence has been provided delineating cyclooxygenase (COX)-derived endoperoxides and prostaglandins as

primary contributors to endothelium-dependent contractions in conduit arteries of Spontaneously Hypertensive rats (SHR).

The production of COX-derived EDCFs is initiated by elevatations in endothelial cytosolic calcium concentrations ($[Ca^{2+}]_i$) induced by agonists mentioned above. Recent findings[250] suggest that agonist-mediated reductions in ER Ca^{2+} content result in activation of Ca^{2+}-independent phospholipase A_2 β ($iPLA_2$, presumably via the release of Ca^{2+} influx factor from the ER[177], which displaces calmodulin from its inhibitory association with $iPLA_2$)[12,169,194]. Activated $iPLA_2$ then releases lysophospholipids from membrane lipids that interact with store-operated Ca^{2+} channels on the endothelial cell membrane to permit the entrance of extracellular Ca^{2+} into the cytoplasm [12,63,195,250]. This influx of Ca^{2+} actives cytosolic phospholipase A_2 α ($cPLA_2$; a Ca^{2+}-dependent isoform) to release arachondonic acid from lipid membranes [63,250]. Arachadonic acid is converted by COX isoforms 1 and 2 (mainly by COX-1 in SHR aorta) to the endoperoxide prostaglandin H_2 (PGH_2)[63], which is subsequently converted by a family of prostanoid synthases into prostanoid by-products such as thromboxane A_2, prostaglandin E_2, prostaglandin $F_{2\alpha}$, and, prostacyclin (PGI_2)[65,230,233]. Prostanoids and endoperoxides initiate contraction by acting on underlying vascular smooth muscle (VSM) thromboxane prostanoid (TP)-receptors, that activate the calcium-sensitization signalling axis to inhibit myosin light chain phosphatase and effectively increase net phosphorylation of the myosin light chain 20 regulatory subunit of VSM myosin to promote contraction [25,48]. Although under normal, healthy conditions PGI_2 acts as a vasodilator via activation of VSM IP-receptors, it paradoxically stimulates TP-receptor mediated contraction in dysfunctional arteries of SHR, and is the prostanoid primarily responsible for enhanced endothelium- and COX-dependent contraction in aorta of SHR to ACh[65,79,80]. In SHR aorta, endothelium-dependent contractions are amplified by pathophysiological alterations such as enhanced $[Ca^{2+}]_i$ release to agonists, increased expression of COX-1 and PGI_2 synthase, increased production and release of PGI_2, elevated ROS levels, impaired IP-receptor signalling, enhanced TP-receptor sensitivity, and reduced NO bioavailability (NO

functionally antagonizes EDCF contraction) [64,65,217,230]. Together these factors synergistically contribute to enhanced endothelium-dependent contraction and contribute to the vasomotor dysfunction.

AMP-activated protein kinase (AMPK) is a novel regulator of vascular tone, and can interact with a number of vasoactive signalling pathways in both the endothelium [27,30,31,44,73,141] and VSM [73,82,116,181,242]. Recently we and others have shown that acute activation of AMPK generates both endothelium-dependent [13,15,73] and *in*dependent relaxation[73,82,181] in pre-contracted arteries, the former occurring partly by suppression of endothelium-derived contractions [73]. Although we have previously demonstrated that AMPK suppresses endothelium-dependent contractions [73], mechanisms responsible for this observation have not been defined in arteries of hypertensive rats. Depressed basal AMPK activation co-exists with enhanced endothelium-dependent contraction in arteries of hypertensive[73], diabetic[10,135] and aged[179] animals, presenting an intriguing question of whether or not enhancing AMPK activation may help remedy the vasomotor dysfunction in conditions where endothelium-dependent contractions are major contributors to vascular pathology. Thus, since the AMPK-EDCF interaction could have important implications for vasomotor function in hypertension, the specific mechanisms involved in this relationship warrant attention.

In this investigation, we explore mechanisms involved in the interaction between AMPK and endothelium-dependent contractions by pharmacologically manipulating AMPK activation in isolated artery preparations *in vitro*. The primary objectives of this investigation are: 1) to evaluate the effects of AMPK activation on endothelium- and COX-dependent contractions in isolated aortic rings of normotensive and hypertensive rats, 2) to investigate the influence of AMPK on TP-receptor mediated contraction of the VSM, 3) to determine the influence of AMPK on the production/release PGI_2, the main EDCF responsible for enhanced endothelium-dependent contraction in SHR aorta. We hypothesize that activation of AMPK will suppress endothelium-dependent contractions, lower production/release of PGI_2, and alter VSM responsiveness to TP-receptor agonists. The findings reveal novel mechanisms involved in

AMPK-mediated suppression of endothelium-, COX-dependent contractions (in arteries from a model of hypertension), and broaden the current understanding of the AMPK and COX-EDCF-TP receptor-mediated signalling axis interaction and the general involvement of AMPK in vasomotor signalling processes.

3.4 Materials and Methods

Animal care and procedures. The University of Waterloo Animal Care Committee approved all animal-related procedures in these experiments. A total of 50 male Wistar-Kyoto rats (WKY) and 50 male Spontaneously Hypertensive rats (SHR) (all 20-22 weeks of age) obtained from Harlan Laboratories (Indianapolis, IN) were used for this study. Animals were maintained on a 22/5 Rodent Diet (W) and tap water *ad libitum*, and group-housed at constant air temperature (20-21^{o}C) and humidity (~50%) on a 12 h:12 h reverse light-dark cycle. On the day of experiment, animals were weighed to obtain body mass, anesthetized by pentobarbital sodium injection (50-65 mg/kg ip; Vetoquinol N.-A., Lavaltrie, QC, Canada) and then euthanized by rapid removal of the heart. Hearts were dissected and weighed. Blood pressure was confirmed in a cohort of animals by inserting a Mikro-Tip Pressure Transducer catheter (Millar Instruments, Houston TX) inserted into the carotid artery prior to removal of the heart (as previously described)[73].

Vasomotor responses of isolated vessels. Isolated vessel experiments were performed as already described [73]. Briefly, aortic rings 2mm in axial length were prepared, mounted on a myography apparatus and maintained in 37^{o}C Krebs-bicarbonate buffer aerated with 95%O_2/5%CO_2. For some experiments, the endothelium was mechanically removed (E-) from rings prior to mounting by gently rolling a titanium wire over the vessel lumen (as previously described [73]). After step-wise adjustment to a pre-determined optimal tension of 7g [85], rings were contracted with two consecutive doses of 60 mM potassium chloride (KCl, with washout back to baseline tension following each). To evaluate endothelium-dependent contractions, quiescent rings (not pre-contracted, at baseline tension) with intact endothelium (E+) were pre-incubated with either no drug, the AMPK activator 5-aminoimidazole-4-carboxamide 1-β-D-

ribofuranoside [36] (AICAR; 500µM, 30 min), the AMPK inhibitor Compound C [266] (CC; 20µM, 45 min), AICAR + CC, or the cyclooxygenase (COX) inhibitor indomethacin (INDO; inhibits both COX1 and COX2 isoforms; 10^{-5}M, 30 min), and then exposed to increasing concentrations of muscarinic receptor agonist acetylcholine (ACh; 10^{-9}M to 10^{-4}M). VSM responses to increasing concentrations of TP-receptor agonist U46619 (10^{-10} M to 10^{-6} M) or prostaglandin H_2 (PGH_2; 10^{-9} M to 10^{-6} M) were evaluated in rings pre-incubated using the same drug conditions as above. All of these experiments were performed in the presence of nitric oxide synthase inhibitor N_ω-nitro-L-arginine methyl ester (L-NAME; 10^{-4}M) to optimize observation of the contractile responses (i.e. by removing the functional antagonistic vasodilatory influence of NO, net contraction is enhanced). All contractions are expressed as a percentage of the tension generated by 60mM KCl (2nd exposure) in the same ring. Concentrations and timing for AICAR and CC pre-incubation were chosen based on our own pilot (data not shown) and previous work [73]. In other experiments, rings were pre-incubated with no drug or with AICAR (2mM) for 30 minutes, then pre-contracted with the α-adrenergic receptor agonist phenyephrine (PE; $10^{-6.5}$M), followed by a dose-response relaxation to increasing concentrations of ACh (10^{-9}M to 10^{-4}M). These data are expressed as percent relaxation from PE pre-contracted tension.

Immunoblot analyses. Immunoblotting was performed as previously described [73,74]. Briefly, aortic rings were carefully removed from the myography apparatus at peak contraction after exposure to the last concentration of the ACh dose response protocol (10^{-4}M, during the quiescent vessel functional experiments), snap frozen in liquid nitrogen, and stored at -80°C until time of analysis. Rings were homogenized in urea extraction buffer containing protease (Complete Protease Inhibitor, Roche, Mannheim, Germany) and phosphatase inhibitors (Halt Phosphatase Inhibitor, Thermo Scientific, Nepean, Canada). Samples were prepared at 1ug/ul and wells of polyacrylamide gels were loaded with 30ug protein/lane. After detection, membranes were stained with ponceau red to confirm equal protein loading. All data were normalized to the E+ CON group run on each gel.

Prostanoid release from aortic rings. Aortic rings from a different set of animals were prepared for vascular myography as described above, pre-incubated with L-NAME (10^{-4}M) and either no drug, AICAR (500µM, 30 min), CC (20µM, 45 min), or AICAR + CC, and then exposed to increasing concentrations of ACh (10^{-9}M to 10^{-4}M). Additionally, some rings from the no drug condition were not exposed to ACh in order to evaluate unstimulated, basal prostanoid release, and to act as time controls. At peak contraction following addition of the final ACh concentration (10^{-4}M), the Krebs-bicarbonate buffer bathing the aortic rings (5mL) was collected, snap frozen in liquid nitrogen and stored at -80^{o}C. 6-keto-$PGF_{1\alpha}$, the primary metabolite of prostacyclin, was measured in thawed buffer samples using competitive EIA kits according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbour, MI).

Drugs, Chemicals and Antibodies. All drugs and chemicals were purchased from either Sigma-Aldrich (St. Louis, MO) or Bioshop Canada (Burlington, ON, Canada) unless otherwise stated. AICAR was obtained from Toronto Research Chemicals (Toronto, ON, Canada) and Compound C from Calbiochem (EMD Biosciences, La Jolla, CA). Prostaglandin H_2 and U46619 were purchased from Cayman Chemical Company (Ann Arbour, MI). Primary antibodies specific for P(Thr172)-AMPK (1:1000 dilution), acetyl-CoA carboxylase (ACC; 1:250), P(Ser79)-ACC (1:500), and the α-subunit of AMPK (recognizing both α1 and α2 isoforms) were obtained from Cell Signaling Technology (via New England Biolabs, Pickering, ON, Canada), and horse-radish-peroxidase conjugated secondary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA).

Data Analyses. All data is reported as means ± s.e.m., with *n* referring to the number of animals per group. One- or two-way ANOVAs were used for comparisons consisting of more than two groups, with Tukey's or Bonferonni post hoc tests to evaluate within or between group comparisons. A two-tailed Student's *t*-test was utilized for comparisons involving only two groups. All statistical analyses were performed using either SAS (SAS Institute, Cary NC), or GraphPad Prism 4 analysis software (La Jolla, CA). Differences were considered significant if $P < 0.05$.

3.5 Results

Animal Characteristics. SHR displayed traits associated with cardiovascular pathology including greater left ventricle mass, total heart mass, left ventricle-to-body mass ratio and heart-to-body mass ratio compared to WKY (Table 3-1). Mean body mass of SHR was also slightly greater than WKY of the same age (Table 3-1). Blood pressure measurements confirmed the presence of hypertension in SHR and normotension in WKY (mean arterial pressure WKY: 91 ± 4 mmHg vs SHR: 192 ± 4 mmHg, n=4, $P < 0.0001$).

Contractions to acetylcholine are blunted by AMPK activation in SHR and WKY aortic rings. As expected and previously observed in dysfunctional arteries of hypertensive animals [47,48,138], contraction to increasing [ACh] was enhanced in aortic rings of SHR versus WKY ($P < 0.001$ at all concentrations > $10^{-6.5}$M), with SHR rings reaching peak contraction of 76±6% of the tension level generated by 60mM KCl (Figure 3-1B), and WKY only 19±3% of KCl-generated tension (Figure 3-1A). These contractions were completely abolished by either pre-incubation with indomethacin or removal of the endothelium in both WKY (Figure 3-1A) and SHR rings (Figure 3-1B), confirming COX- and endothelium-dependency of these responses, as previously reported [47,48,138]. Examination of the AMPK-mediated influence on this response revealed that pre-incubation of rings with AICAR blunted ACh-induced contractions in both WKY (peak contraction: 4±1%, $P < 0.0001$ vs E+ CON, Figure 3-1C) and SHR (peak contraction: 31±9%, $P < 0.0001$ vs E+ CON, Figure 3-1D). Co-incubation of rings with AICAR and CC completely prevented the blunting of contractions induced by AICAR alone (contractions with AICAR + CC were not different from respective WKY and SHR E+ CON at any [ACh], Figures 3-1C and 3-D). Contractions were slightly greater in WKY and SHR rings pre-incubated with CC alone at high [ACh] (10^{-5}M, $10^{-4.5}$M, 10^{-4}M) compared to those in the respective E+ CON rings (Figure 3-1C and 3-1D).

AMPK activation by AICAR and inhibition by Compound C in SHR and WKY aortic rings following ACh dose-response curves. To confirm the efficacy of the AICAR and CC treatments on AMPK

activation/inhibition, phosphorylation at AMPK activation site threonine 172 (P(Thr172)-AMPK; a modification both necessary and sufficient for AMPK activation) [99,104], and phosphorylation of serine 79 on acetyl-CoA carboxylase (P(Ser79)-ACC; a well known downstream target of AMPK) [99], were analyzed by immunoblotting in homogenates of aortic rings collected following the ACh dose-response functional experiments. AICAR is taken up by cells and converted to the AMP analogue ZMP, which activates AMPK in an AMP-like fashion by binding to the nucleotide binding sites of the λ subunit to make it a poorer substrate to upstream kinases, and thus facilitating increased phosphorylation of Thr172 [36,208]. Compound C is an ATP-competitive inhibitor of AMPK [266] by presumably interfering with the nucleotide binding sites. P(Thr172)-AMPK (Figure 3-2A & B) and P(Ser79)-ACC (Figure 3-2C & D) were elevated ~3-4 fold in aortic rings of both WKY and SHR rats in the presence of AICAR alone versus no drug controls (E+ CON). Co-incubation of AICAR and CC fully prevented elevation of P(Thr172)-AMPK (Figure 3-2B) and P(Ser79)-ACC (Figure 3-2D) in SHR aortic rings and partially prevented elevation of P(Thr172)-AMPK (Figure 3-2A) and P(Ser79)-ACC (Figure 3-2C) in aortic rings from WKY (values remained slightly elevated relative to E+ CON, but were significantly lower than AICAR alone). Phosphorylation at both sites was not different from E+CON in all other treatment conditions (CC alone, INDO, E-) following the ACh curve. Exposure of aortic rings to ACh alone did not generate detectable differences in P(Thr172)-AMPK compared with un-stimulated time controls (E+ TC, not exposed to ACh) in either WKY (E+ CON (ACh): 1.00 ± 0.15 vs E+ TC:1.37 ± 0.15, P = 0.1976) or SHR (E+ CON (ACh): 1.00 ± 0.14 vs E+ TC: 1.10 ± 0.03, P = 0.4318).

Contractions to vascular smooth muscle TP-receptor agonists U46619 and prostaglandin H_2 are unaffected by AICAR. To determine if blunting of ACh-mediated contractions with AMPK activation originated from changes in VSM sensitivity to vasoconstrictor prostanoids, we evaluated VSM responsiveness to increasing concentrations of TP-receptor agonist U46619 in SHR and WKY aortic rings. Contraction to U46619 was enhanced in aortic rings of SHR versus WKY at 10^{-9}M and $10^{-8.5}$M U46619 (P < 0.0001 vs. WKY, Figures 3-3A and 3B), consistent with previous observations in SHR and

WKY aortic rings[79]. Pre-treatment with AICAR, AICAR + CC or CC alone did not alter contractions to U46619 compared to the no drug controls in WKY or SHR aortic rings (Figure 3-3A & B). Results generated using U46619 (a synthetic TP-receptor agonist) sometimes vary compared to those obtained using endoperoxides in WKY and SHR aorta (i.e. U46619 is a more potent activator of TP-receptors than endoperoxides, and may mask subtle differences in VSM responses under some circumstances) [78]; therefore we also investigated responses using prostaglandin H_2 (PGH_2) as a second TP-receptor agonist. However, similar to the results obtained using U46619, pre-incubation with AICAR did not affect contractions to increasing [PGH_2] in WKY (Figure 3-3C) and SHR (Figure 3-3D) vessels.

Pre-incubation with AICAR suppresses ACh-induced production/release of 6-keto-prostaglandin $F_{1\alpha}$ by aortic rings of WKY and SHR. We investigated whether 6-keto-$PGF_{1\alpha}$ (the primary metabolite of PGI_2) levels were altered in buffer obtained from aortic rings stimulated with ACh in the presence of AICAR. PGI_2 is the main COX-derived EDCF released by the endothelium in response to stimulation with ACh in WKY and SHR aorta, and is the primary contributor to endothelium-dependent contractions in aorta of WKY and SHR to this agonist (PGI_2 release by these vessels in response to ACh is overwhelmingly greater than other prostanoids[79], originates primarily from the endothelium[80], and paradoxically generates contraction rather than vasodilation in aorta of SHR and WKY[79]; the authors refer readers to Félétou et al. 2009 [65] for an excellent review of the evidence supporting the primary role of PGI_2 as an EDCF in SHR and WKY conduit arteries stimulated with ACh). These considerations allow us to assume that major changes in 6-keto-$PGF_{1\alpha}$ using this assay can be reasonably attributed to alterations in PGI_2 production/release and not to other prostanoid /endoperoxide mediators. As expected and previously observed [65,79], stimulated (E+ CON, exposed to ACh) and unstimulated (E+ TC, no ACh exposure) aortic rings of SHR produced/released greater levels 6-keto-$PGF_{1\alpha}$ than those of WKY (ACh-stimulated E+ CON WKY: 1862±144 vs SHR: 8754±1509 pg/mL, $P < 0.001$, unstimulated E+ TC WKY: 520±70 vs SHR: 2831±830 pg/mL, $P < 0.05$, Figure 3-4A & B). Evaluation of the ability of AMPK to influence PGI_2 production/release revealed that pre-incubation of WKY and SHR aortic rings with

AICAR reduced 6-keto-$PGF_{1\alpha}$ to levels nearing those of un-stimulated WKY and SHR E+ time controls respectively (E+ AICAR WKY: 799±68 pg/mL, Figure 4A, SHR: 3533±1061 pg/mL, Figure 3-4B). 6-keto-$PGF_{1\alpha}$ levels in buffer of rings co-incubated with AICAR + CC were not different from those pre-incubated with CC alone in either WKY (Figure 3-4A) or SHR (Figure 3-4B), but were elevated by treatment with AICAR + CC in WKY, and CC and AICAR + CC in SHR above their respective E+ CON groups (Figure 3-4A & B).

Endothelium- and COX-dependent re-contraction/blunted relaxation to acetylcholine is inhibited by AICAR in pre-contracted aortic rings of SHR. Exposure of pre-contracted arteries to increasing concentrations of ACh generates a biphasic response in dysfunctional vessels of SHR, where endothelium-, NO-dependent relaxation is observed at lower [ACh], and COX-dependent re-contraction (or blunted relaxation) occurs at higher [ACh] [47,86] (Figure 3-6B). Therefore exposure of pre-contracted SHR aortic rings to this protocol afforded the opportunity to confirm the effects of AICAR on endothelium- and COX-dependent contractions using a different protocol/experimental approach, and to also investigate potential effects of AICAR on NO-mediated vasodilatory effects to ACh. Re-contraction/blunted relaxation is not observed in WKY aortic rings at higher [ACh] (an effect observed only in SHR arteries using this protocol due to the presence of enhanced endothelium-dependent contractions in these vessels), and therefore we did not perform these experiments in WKY aortic rings. As expected, relaxation to ACh was blocked by L-NAME, re-contraction/blunting of relaxation was reversed by indomethacin, and both responses were completely abolished by the combination of L-NAME + INDO, confirming the NOS- and COX-dependency of the relaxation and contraction/blunted relaxation phases of the responses to ACh, respectively, in SHR aorta (Figure 3-6A). Recontraction/blunted relaxation to high concentrations of ACh was blocked by AICAR in SHR aorta (Figure 3-6B, in a manner similar to that observed in SHR aortic rings treated with indomethacin (Figure 3-6A), confirming the ability of AMPK to blunt endothelium- and COX-dependent contraction as

observed in the quiescent vessel experiments (Figure 3-1). NO-dependent relaxation to lower concentrations of ACh was unaffected by AICAR treatment in these vessels (Figure 3-6B).

Table 3-1. ***Physical Characteristics of WKY and SHR***

	WKY	SHR
Whole body mass (BM), g	321 ± 4	354 ± 4‡
Tissue Masses		
Left Ventricle, mg	651 ± 13	848 ± 16‡
Left Ventricle/BM (mg/g BM)	2.02 ± 0.02	2.44 ± 0.03‡
Right Ventricle, mg	177 ± 5	195 ± 4†
Right Ventricle/BM (mg/g BM)	0.55 ± 0.01	0.56 ± 0.01
Heart, mg	828 ± 17	1043 ± 18‡
Heart/BM, (mg/g)	2.56 ± 0.02	3.01 ± 0.03‡
Kidney (mg)	1033 ± 22	1117 ± 17†
Kidney/BM (mg/g)	3.20 ± 0.03	3.22 ± 0.03

Due to differences in body weight between strains, values were expressed both in absolute terms and relative to body weight. Data represents means ± sem. n = 25 for body mass, n=8-9 for all other measures.

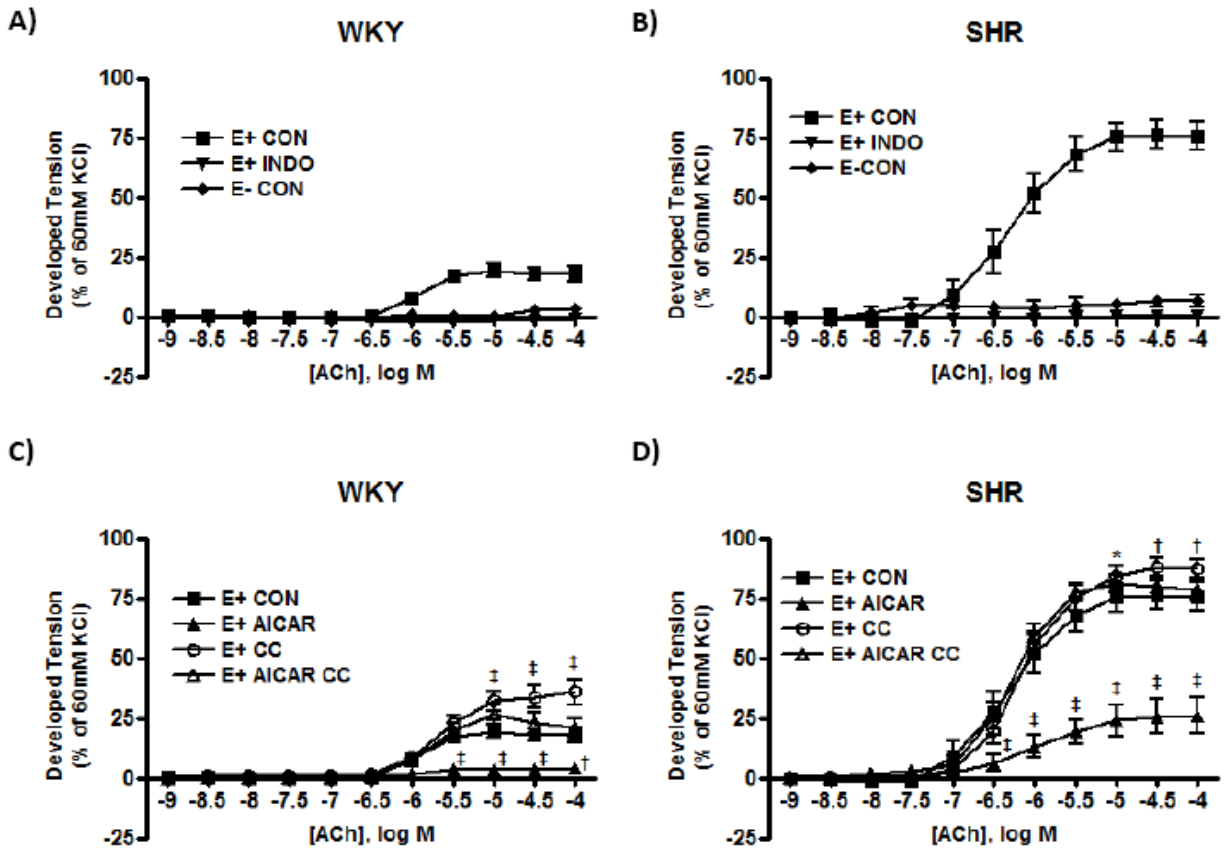

Figure 3-1. Activation of AMPK blunts endothelium-dependent contractions generated by acetylcholine in WKY and SHR aortic rings. Endothelium-dependent contractions were generated by exposing quiescent (non-precontracted) aortic rings to increasing concentrations of acetylcholine (ACh). The endothelium- and COX-dependency of these responses is demonstrated in WKY (**A**) and SHR (**B**) aortic rings by removal of the endothelium (E-) or pre-incubation with the COX-inhibitor indomethacin (INDO) respectively. In some experiments, rings from WKY (**C**) and SHR (**D**) were pre-incubated with either no drug (CON), AMPK activator AICAR (500µM), AMPK competitive inhibitor Compound C (CC; 20µM), or AICAR + CC to investigate the influence of AMPK on endothelium-, COX-dependent contractions. All experiments were performed in the presence of NOS-inhibitor L-NAME (10^{-4}M). Values are expressed as mean percentage of tension developed by individual rings to 60mM potassium chloride (KCl). Each treatment was performed in aortic rings from a single animal. *n* = 8-12 rats per group. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ vs. E+ CON. In panels A and B, E+ INDO and E-CON are all $P < 0.0001$ vs. E+ CON for concentrations greater than $10^{-6.5}$M in SHR and 10^{-6}M in WKY, but significance symbols have been excluded for clarity.

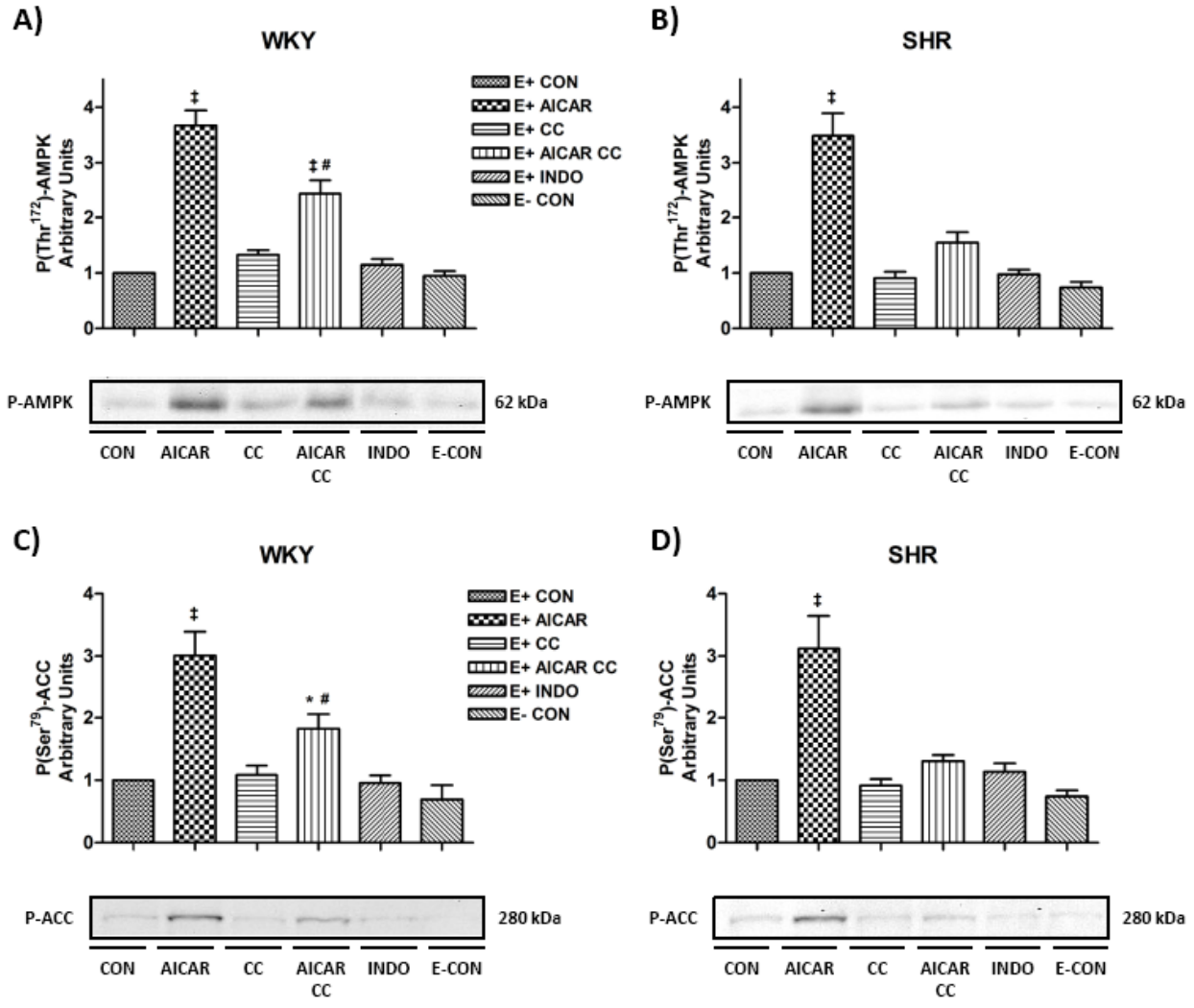

Figure 3-2. AMPK activation by AICAR and inhibition by Compound C is confirmed in aortic rings of WKY and SHR following the functional acetylcholine dose response curve protocol. After attaining a stable functional response at the last dose of ACh in the functional protocol used to generate data in Figure 1, rings were carefully and rapidly removed from the apparatus, snap frozen in liquid nitrogen and stored at -80^{o}C until they were processed for immunoblotting. The status of AMPK activation was assessed by immunoblotting for phosphorylation of AMPK activation site Thr172 (**A**) and AMPK downstream target Ser79 on acetyl-CoA carboxylase (ACC; **B**). Values are expressed as the mean ± s.e.m. of the densitometry analyses (arbitrary units), and all values are normalized such as that the mean of the WKY E+ CON or SHR E+ CON is equal to 1.00. Representative blot images for WKY and SHR P(Thr172)-AMPK or P(Ser79)-ACC display 1 lane from each group. *n* = 6 animals for each group (samples run in duplicate). * $P < 0.05$, ‡ $P < 0.001$ vs WKY E+ CON or SHR E+ CON, # $P < 0.05$ vs WKY E+ AICAR. INDO; cyclooxygenase 1 and 2 inhibitor indomethacin, E-; aortic rings with endothelium mechanically removed, TC, time control (no ACh exposure).

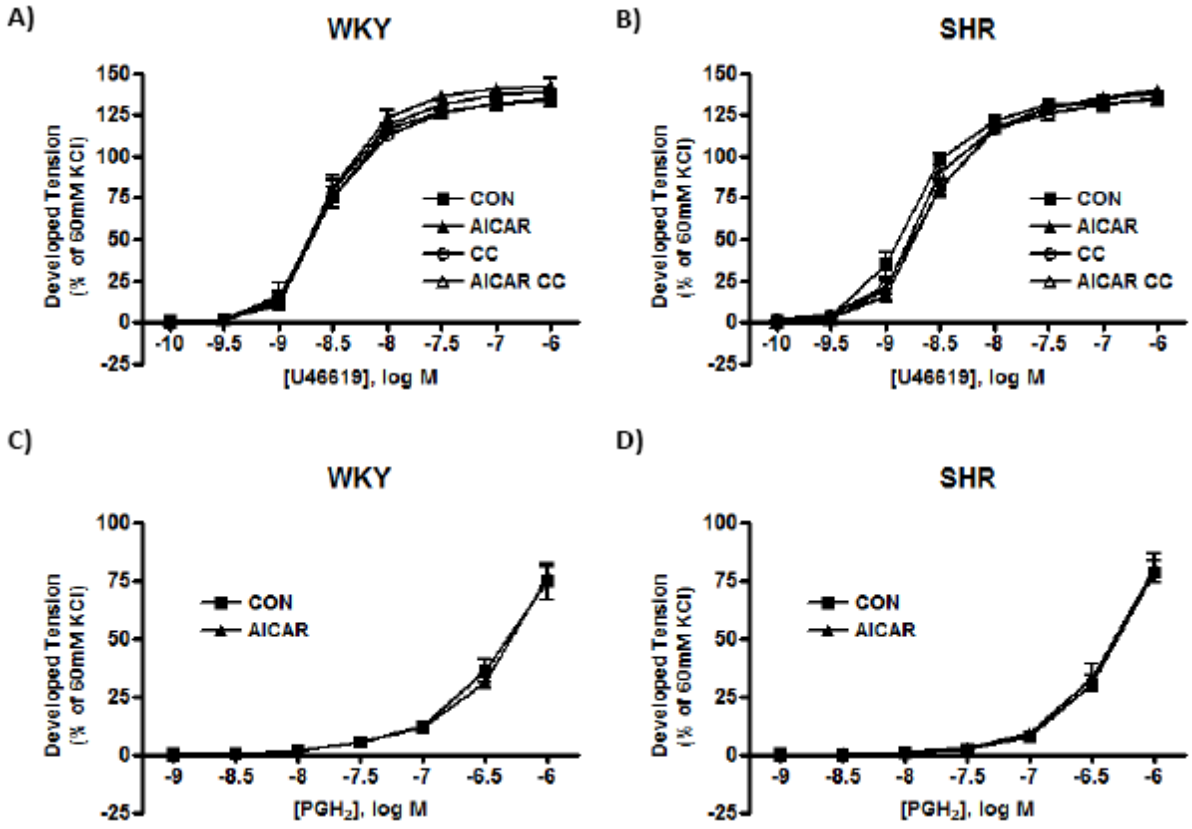

Figure 3-3. Contraction to vascular smooth muscle TP-receptor agonists is unaltered by AMPK activation or inhibition. Endothelium-independent contractions were generated by exposing WKY and SHR aortic rings to increasing concentrations of TP-receptor agonists U46619 (**A,B**) or prostaglandin H_2 (**C,D**) in the presence of either no drug (CON), AMPK activator AICAR (500µM), AMPK competitive inhibitor Compound C (CC; 20µM), or AICAR + CC to investigate the influence of AMPK on VSM sensitivity to TP-receptor stimulation. All experiments were performed in the presence of NOS-inhibitor L-NAME (10^{-4}M). Values are expressed as mean percentage of tension developed to 60mM potassium chloride (KCl) by individual rings. Each treatment was performed in aortic rings from a single animal. *n* = 8-12 rats per group.

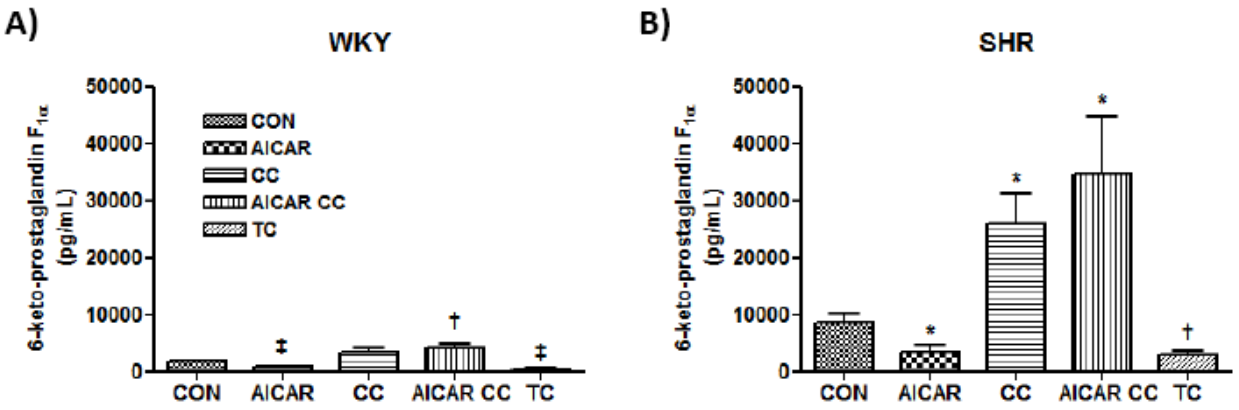

Figure 3-4. Pre-incubation with AICAR suppresses the production/release of 6-keto-PGF$_{1\alpha}$ by aortic rings of SHR and WKY rats. 6-keto-prostaglandin F$_{1\alpha}$ (6-keto-PGF$_{1\alpha}$) a stable metabolite of prostacyclin, was assessed in the Kreb's bicarbonate buffer bathing aortic rings over the duration of the functional ACh dose-response curve data generated in Figure 1. After attaining a stable functional response at the last dose of ACh, buffer was collected, snap frozen in liquid nitrogen and stored at -80^{o}C until processed using an EIA kit. Buffer was also collected from a time control (TC) ring, that present for the duration of the experiment but not exposed to ACh, in order to assess basal, unstimulated prostacyclin production/release by the rings. Values are expressed as means ± s.e.m. in picograms per millilitre. *n* = 6-8 animals per group (samples run in duplicate). * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ vs. CON.

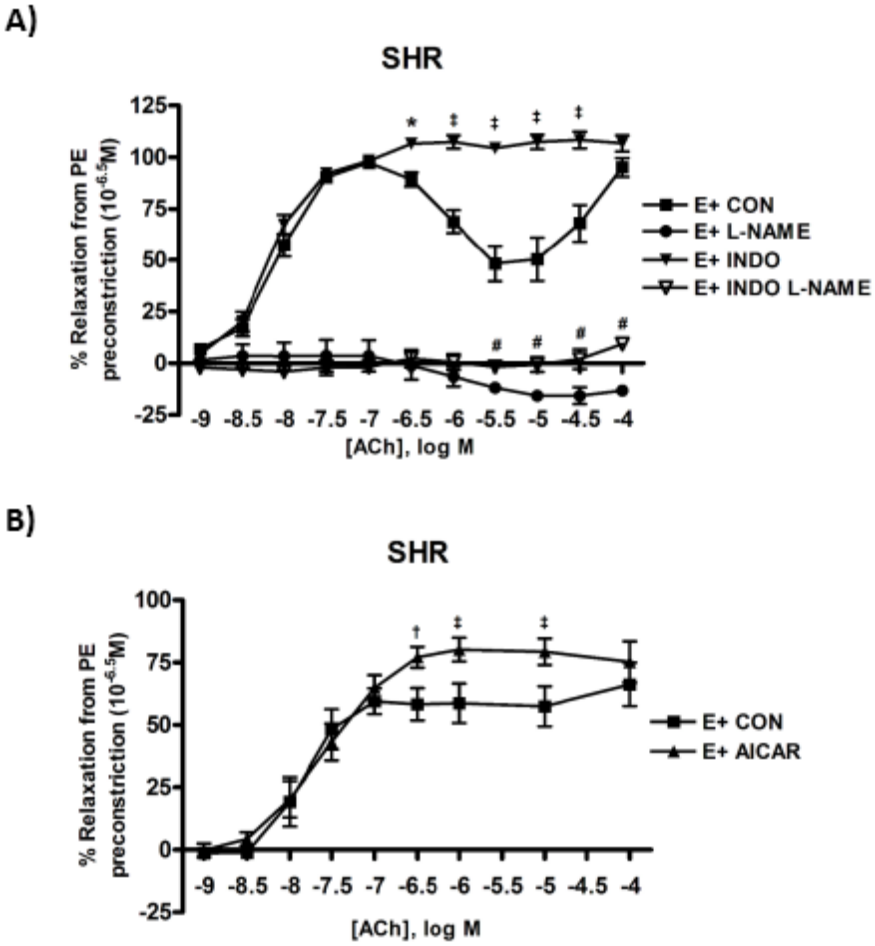

Figure 3-5. Endothelium- and COX-dependent recontraction/blunted relaxation to acetylchloline is inhibited by AICAR in precontracted aortic rings of SHR. Relaxation to increasing concentrations of acetylcholine (ACh) was generated in SHR aortic rings pre-contracted with phenylephrine (PE). Panel **A)** demonstrates the NO-dependency of vasodilation to lower [ACh] and COX-dependency of re-contraction/blunted relaxation at higher [ACh] by pre-incubating rings with endothelium (E+) for 30 minutes with either no drug (CON), NOS-inhibitor L-NAME (10^{-4}M), COX inhibitor indomethacin (INDO; 10^{-5}M), INDO + L-NAME prior to precontraction with PE. In panel **B),** rings were pre-incubated with either no drug (CON) or AICAR (2mM) to evaluate the effect of AMPK activation on the vasomotor responses of to ACh. Values are expressed as mean percent relaxation of tension developed to PE. n = 4-12 rings per group from different animals. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ vs. E+ CON, # $P < 0.05$ vs E+ L-NAME.

3.6 Discussion

In this study, we investigate the interaction of AMPK with endothelium-, COX-dependent contraction in isolated aortic rings of normotensive and hypertensive rats. The major findings are: 1) AMPK activator AICAR blunts ACh-induced contractions in SHR and WKY aorta, an effect that is prevented in the presence of AMPK inhibitor Compound C, 2) contractions initiated by direct stimulation of VSM TP-receptors are unaffected by pharmacological manipulation of AMPK activity, and 3) AICAR blunts ACh-stimulated production/release of 6-keto-$PGF_{1\alpha}$ (the major metabolite of PGI_2 that is primarily responsible for endothelium-dependent contractions in SHR and WKY aorta to ACh). This is the first description of the mechanisms involved in AMPK-mediated inhibition of endothelium-dependent contractions in arteries of hypertensive animals.

We begin by showing that ACh-induced endothelium-dependent contractions are blunted in the presence of AMPK activation with AICAR (Figure 3-1). These results are consistent with our previous results[73] and with those of Matsumoto et al. who have shown that pharmacological activation of AMPK with AICAR and metformin (an anti-diabetic drug that also activates AMPK [266]) inhibits endothelium-dependent contractions to ACh in mesenteric arteries of diabetic rats [141]. Together our observations and those of Matsumoto and colleagues are the first reports to our knowledge of an interaction of AMPK with endothelium-dependent contractions, and ours are the first reported in arteries from a model of hypertension.

Collectively, the observations of this study indicate that the blunted endothelium-dependent contractions to ACh originate from AMPK-mediated effects on the endothelium rather than the VSM of WKY and SHR aorta. Sensitivity of VSM to TP-receptor agonists U46619 and PGH_2 was unaltered by the presence of AICAR in WKY or SHR aorta (Figure 3-3), and thus blunted ACh-induced contractions by AMPK activation cannot be explained by altered responsiveness of the VSM to EDCFs. These results were somewhat unexpected, as a number of findings from other work suggest that activation of VSM

AMPK can alter vascular responsiveness to contractile agonists. However, since some agonists (i.e. KCl and PE) are more reliant on increases in intracellular Ca^{2+} where as others operate more predominantly via increased Ca^{2+} sensitization (i.e. TP-receptor agonists)[197,198], it is conceivable that the effects of AICAR could vary depending on the constrictor agonist applied if the effects of AMPK were mediated through either one or the other of these pathways. Contraction to other stimuli such as membrane hyperpolarizing agent KCl and α-adrenergic receptor agonist PE is enhanced in aorta of AMPKα1$^{-/-}$ mice [116], and correspondingly, we have also observed that AMPK activation with AICAR blunts endothelium-independent contraction of rat aorta to these two agonists (Figure A3-1 and data not shown). However, Wang et al. have recently reported enhanced contraction to PE and U46619 in aorta and mesenteric arteries of AMPKα1$^{-/-}$ and AMPKα2$^{-/-}$ mice, along with depressed contraction to these agonists in mouse aorta with AICAR pre-incubation [242]. Pre-incubation of rings with a higher dose of AICAR may have elicited effects on VSM responsiveness in our experiments with TP-receptor agonists, as the observations discussed above were generated from arteries pre-incubated with 2mM AICAR (rather than 500μM used to generate our data) [242]. Nonetheless, altered VSM TP-receptor sensitivity at AICAR concentrations used here do not explain AMPK-mediated blunting of contraction to ACh in this study. Further work will be required to resolve discrepancies in the currently available data.

ACh-induced 6-keto-$PGF_{1\alpha}$ production/release by WKY and SHR aortic rings is also reduced in the presence of AICAR (Figure 3-4). Gluais et al. (2006) have shown that ACh-induced 6-keto-$PGF_{1\alpha}$ release originates almost exclusively from endothelial sources[80], and thus we can assume that major alterations in 6-keto-$PGF_{1\alpha}$ production/release observed here originate from direct effects on the endothelium. This is the first report of AMPK-dependent suppression of PGI_2 production/release from arteries of hypertensive rats, and is consistent with the ability of AICAR and metformin to inhibit the production/release of prostanoids in diabetic rat mesenteric arteries [141]. Although we only investigated the effect of AICAR on PGI_2 (6-keto-$PGF_{1\alpha}$) production/release, Matsumoto et al. demonstrated that incubation of mesenteric arteries with AICAR also blunts the production/release of PGE_2 and

thromboxane B_2 (stable metabolite of thromboxane A_2), other prostanoids derived from PGH_2 by their respective synthases[141]. Considering these data collectively, it seems more reasonable that AICAR/AMPK likely acts upstream of the prostanoid synthases to inhibit the production of their common substrate (PGH_2), and that the blunted PGI_2 levels observed in our study in the presence of AICAR probably did not result from a direct effect of prostacyclin synthase specifically.

Examining vasomotor responses of precontracted aortic rings to ACh afforded the opportunity to confirm the inhibitory effects of AICAR on endothelium- and COX-dependent contractions and also to evaluate the effect of AMPK activation on the NO-dependent component of relaxation to ACh. Although AICAR pre-incubation abolished COX-dependent re-contraction/blunted relaxation to high [ACh] (in accordance with observations on the endothelium-dependent contractions quiescent vessel experiments in SHR vessels), the NO-dependent vasorelaxation portion of the response to lower [ACh] was unaffected by AMPK activation in SHR aorta (Figure 3-5B). NO-dependent relaxation was also maintained in similar experiments using AMPK activators in diabetic rat mesenteric arteries [141], and ACh-mediated relaxation is also unaltered in arteries from AMPK α1-/- or AMPKα2 -/- mice [68,86]. Since the production of NO and COX-derived prostanoids both require elevated $[Ca^{2+}]_i$, it is unlikely that AMPK is acting upstream of Ca^{2+} release by the intracellular ER stores in these experiments (if this were the case, both COX-dependent contraction *and* NO-dependent relaxation would be expected to be inhibited by AMPK activation); thus it is more probable that AMPK acts downstream of SR Ca^{2+} release on a signalling process specific to COX-dependent contraction.

Perspectives

These experiments are the first to investigate mechanisms of AMPK-mediated suppression of endothelium-derived contractions in arteries of hypertensive animals, and some of the first data to suggest a link between AMPK and the COX-EDCF signalling axis. Our data demonstrate that activation of AMPK reduces endothelium-dependent contractions through suppression of endothelial PGI_2 production.

These results highlight the potentially important therapeutic utility of AMPK, as the ability to suppress COX-dependent contractions, but not NO-dependent relaxation, would be favourable for treating endothelial dysfunction where endothelium-dependent contraction is enhanced. In future experiments it will be important to investigate whether chronic improvements in AMPK activation can reduce endothelium-dependent contractions and improve vascular function by an AMPK-dependent mechanism *in vivo*. The link between AMPK activation and reduced endothelium-dependent contraction detailed here provides support for targeting AMPK as a therapeutic strategy for interventions aiming to improve vasomotor function in essential hypertension and in other conditions of vascular dysfunction where endothelium-dependent contractions are enhanced.

3.7 Addendum to Study 2:

The addendum includes data collected in conjunction with this study that is not part of the manuscript.

Figure A3-1: Contraction to potassium chloride is blunted in aortic rings of WKY and SHR pre-treated with AICAR.

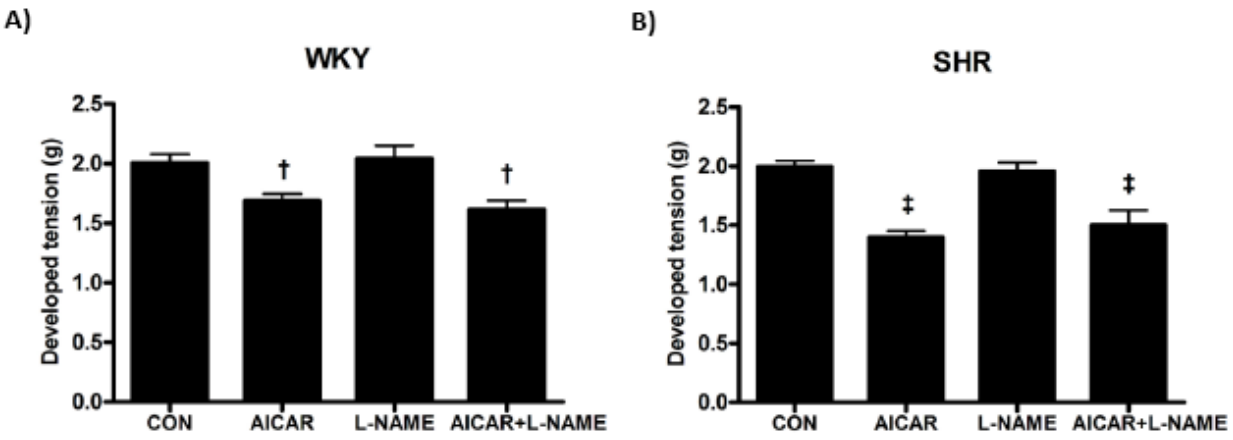

Figure A3-1. Contraction to potassium chloride is blunted in aortic rings of WKY and SHR pre-treated with AICAR. Aortic rings of **A)** WKY and **B)** SHR without functional endothelium were pre-incubated with no drug (CON), AMPK activator AICAR (2mM), NOS inhibitor L-NAME (10^{-4}M), or AICAR + L-NAME for 30 minutes, and then exposed to 60 mM potassium chloride (KCl). Values are expressed as tension developed to KCl in grams. *n* = 10-18 rings per group from different animals. † $P < 0.01$, ‡ $P < 0.001$ vs. CON.

Chapter 4

Thesis Study 3

AMPK activator AICAR acutely lowers blood pressure and relaxes resistance arteries of hypertensive rats.

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This chapter is presented in manuscript form, and has been submitted for publication.

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4.1 Review of Study 3 Objectives and Hypotheses

The specific objectives and hypotheses for this chapter have already been stated in the introduction section, but are reproduced identically here for the reader's convenience.

Purpose: To determine the effects of AMPK activation by an acute injection of AICAR on *in vivo* hemodynamic responses of normotensive and hypertensive rats, and to evaluate whether parallel relaxation responses occurs in isolated, pre-contracted resistance arteries of normotensive and hypertensive rats exposed to AICAR acutely *in vitro.*

Specific objectives and hypotheses:

Objective 1: To investigate the effect of acute AICAR injection on blood pressure responses of SHR and WKT *in vivo,* and to determine the NO-dependency of these effects via carotid arterial catheter pressure transducers. **Hypothesis 1:** Acute injections of AICAR will lower blood pressure in WKY and SHR, and that this effect will be partly NO-dependent.

Objective 2: To investigate the effect of acute AICAR injection on heart rate response of SHR and WKY *in vivo* by carotid arterial catheter. **Hypothesis 2:** AICAR will have minimal to no influence on heart rate of WKY or SHR.

Objective 3: To verify the efficacy of the AICAR treatment during the *in vivo* experiments by assessing AMPK activation in vascular tissue from these animals by immunoblotting. **Hypothesis 3:** Acute injections of AICAR will elevate P(Thr172)-AMPK levels in aortas of WKY and SHR rats.

Objective 4: To characterize basal levels of AMPK activation and expression in mesenteric artery sections from SHR and WKY by immunoblotting. **Hypothesis 4:** AMPK activation (P(Thr172)-AMPK) will be depressed in mesenteric arteries of SHR versus those of WKY.

Objective 5: To evaluate the vasomotor response of pre-contracted isolated mesenteric resistance artery segments to acute AICAR exposure, to determine the endothelium- and NO-dependency of this response, and to compare the responses generated in SHR versus WKY vessels using vascular myography. **Hypothesis 5:** Relaxation to AICAR will occur in mesenteric arteries of both WKY and SHR and will be partly endothelium-, NO-dependent.

Objective 6: To verify AMPK activation in mesenteric artery segments from SHR and WKY incubated with AICAR by immunoblotting. **Hypothesis 6:** AMPK activation (P(Thr172)-AMPK) will be elevated in mesenteric arteries of WKY and SHR following incubation with AICAR**.**

Objective 7: To compare baseline levels of P(Ser1177)-eNOS and total eNOS protein content in aortas and mesenteric arteries of WKY and SHR rats. **Hypothesis 7:** P(Ser1177)-eNOS will be depressed in aorta and mesenteric arteries of SHR versus those of WKY, and total eNOS protein content will be elevated in these vessel types in SHR versus WKY.

Objective 8: To evaluate the effect of AICAR injections *in vivo* and AICAR incubation *in vitro* on P(Ser1177)-eNOS (and total eNOS protein content) by immunoblotting in aortas and mesenteric arteries respectively of WKY and SHR. **Hypothesis 8:** Acute exposure to AICAR will increase P(Ser1177)-eNOS in both types of experiments (aorta and mesenteric arteries) but total eNOS protein content will not be altered by AICAR.

4.2 Overview of Study 3

Activated AMPK may alter blood pressure by directly influencing vascular tone. The purpose of this study is to examine if these effects occur acutely in a model of hypertension. Using distinct groups of Wistar-Kyoto (WKY) and Spontaneously Hypertensive Rats (SHR) we compare 1) baseline aortic and mesenteric artery AMPK activation (by immunoblotting), 2) hemodynamic (blood pressure and heart rate via carotid catheter) and biochemical responses to an acute injection of AMPK activator 5-

aminoimidazole-4-carboxyamide-1-β-D-ribofuranoside (AICAR) *in vivo*, and 3) vasomotor responses of isolated mesenteric vessels to AICAR exposure *in vitro* using myography. Mean arterial pressure (MAP) decreased from 196±3 to 122±15 mmHg ($P < 0.001$) during the 30 minutes following AICAR injection in SHR (an effect partially prevented by NOS inhibitor L-NAME), but in WKY MAP was unaffected by AICAR. Basal AMPK activation (phosphorylation of AMPK activation site threonine 172) was reduced ~50% in aorta of SHR versus WKY (0.49±0.1 vs 1.0±0.1 arbitrary units, $P < 0.001$), and was improved ~1.6 fold in SHR but not WKY aorta 30 minutes following AICAR injection. In isolated vessel experiments, dose-dependent vasorelaxation to AICAR was similar in mesenteric arteries of SHR and WKY, although responses were more reliant on NO in SHR than in WKY. The ability of AICAR to improve vascular AMPK activation, and to generate parallel reductions in blood pressure and relaxation of SHR resistance vasculature, highlights the potential importance of AMPK in the regulation of blood pressure and vascular tone.

4.3 Introduction

A potential role for AMP-activated protein kinase (AMPK) in blood pressure (BP) regulation is suggested from incidental observations that 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), a compound that results in the activation of AMPK[124], has hypotensive effects when administered acutely in normotensive rats [71] and humans[14]. AMPK has also been identified a regulator of vascular tone by experiments showing that activation of AMPK generates vasorelaxation in conduit arteries of healthy murine[82], rat [73] and porcine [181] models and in rat [15] and human [13] resistance vasculature. The potential hemodynamic effects of AMPK-activation, coupled with acute AMPK-mediated vasodilation in isolated vessels, make it is reasonable to suggest that AMPK activation may exert BP lowering effects by directing vasorelaxation responses in resistance arteries. The hemodynamic effects of acute AICAR/AMPK activation have not been studied in models of essential hypertension, in which systemic artery endothelium-dependent dilation is impaired[62,236]. Furthermore, there is no

available data investigating vasomotor responses to AMPK activation in resistance arteries of a hypertension model.

AMPK increases nitric oxide (NO) bioavailability in isolated protein experiments [27] in cultured bovine [44] and human [145] endothelial cells, and in mouse aorta *in vivo*[44] via mechanisms that could include phosphorylation of eNOS at activation sites serine 1177[145] [27,44] or serine 633[31], or by facilitation of active eNOS complexes[30,44]. We and others have provided evidence to suggest that vasorelaxation of conduit arteries evoked by activation of vascular AMPK with AICAR is partially endothelium-, NO-dependent [13,15,73] and partly endothelium-*in*dependent via direct vasorelaxation of vascular smooth muscle[73,82,181]. Therefore it is plausible that activation of AMPK in resistance vasculature is also partly NO-dependent and that this response could translate into an NO-dependent BP response to acute AMPK activation *in vivo.*

The main purposes of this study were: 1) to determine the BP responses to acute *in vivo* injections of the AMPK activator AICAR in WKY and SHR, and the NOS-dependency and -independency of these responses and 2) to investigate the influence of AICAR-induced AMPK activation on vasorelaxation of isolated resistance vasculature *in vitro* using mesenteric arteries of normotensive Wistar Kyoto Rats (WKY) and Spontaneously Hypertensive Rats (SHR). We hypothesized that acute administration of AICAR *in vivo* will lower BP in hypertensive and normotensive animals in a NOS-dependent manner, and that parallel AICAR-dependent vasorelaxation of isolated resistance vessels will support a potential role for AMPK-mediated control of vascular resistance in the acute regulation of BP in hypertensive animals.

4.4 Methods

Animal care procedures. The University of Waterloo Animal Care Committee approved all animal-related procedures in this study. Experiments were performed with a total of 64 male WKY and 64 male

SHR all aged 20-24 weeks and obtained from Harlan (Indianapolis, IN). Animals were group-housed at a constant air temperature (20-21^{o}C) and humidity (~50%) in a 12 h:12 h reverse light:dark cycle. Rats had free access to standard 22/5 Rodent Diet (W) lab chow (Harlan) and tap water. Prior to all experiments, body mass was recorded and rats were anesthetised with pentobarbital sodium injection (50-65 mg/kg, i.p.; Vetoquinol N.-A. Inc., Lavaltrie, QC, Canada).

Hemodynamic responses following acute injections of AICAR in vivo. Anesthetized animals were instrumented for mean arterial pressure (MAP) and heart rate (HR) measurements by inserting a heparinized saline-filled PE-50 cannula into the left common carotid artery, and data were collected as previously described[74]. Baseline MAP was analyzed during the final 5 minutes of an initial 15 minute equilibration period. AMPK activator AICAR and NOS inhibitor N_{ω}-Nitro-L-arginine monoethyl ester (L-NAME; L-arginine analog and competitive NOS inhibitor) were then administered acutely according to the protocol outlined in Figure 4-2A to evaluate AMPK- and NOS-dependent hemodynamic responses. L-NAME (30 mg/kg body weight in 200µl of 37^{o}C saline) or saline only (200µl) was administered via the carotid artery catheter (slowly over 1 minute), and AICAR (0.5mg AICAR/g body weight in 2mL 37^{o}C saline) or saline alone (2 ml) was injected intraperitoneally according to doses previously reported[19].

Vasomotor responses of isolated WKY and SHR thoracic aorta and mesenteric artery rings to acute AICAR exposure. Animals were anesthetized as outlined above and killed by rapid removal of the heart. For thoracic aorta experiments, aortic rings 2mm in axial length were prepared for the collection of vasomotor data using vascular myography as previously described [74]. Following a standard set-up protocol, rings were pre-contracted with phenylephrine (PE, α-adrenergic receptor agonist, $10^{-6.5}$M) then exposed to a single dose of 2mM AICAR to observe vasodilation (concentration selected based on previous experiments in WKY and SHR aorta[73]). For the mesenteric artery experiments, the superior mesenteric artery from SHR and WKY rats was exposed and the mesentery was excised from ~2cm distal to the pylorus to the beginning of the large intestine. Tissue was immediately placed in 4°C, aerated (95%O_2/5%CO_2) physiologic salt solution (PSS, pH 7.4, composition in mM: 118.99 NaCl, 4.69 KCl,

1.17 $MgSO_4 \circ 7H_2O$, 1.18 KH_2PO_4, 2.50 $CaCl_2 \circ 2H_2O$, 25.00 $NaHCO_3$, 0.03 EDTA, 5.50 glucose). The 3rd order arteries were cleaned of connective tissue and the venous branches were carefully removed under a dissecting microscope (Zeiss; VWR, Mississauga, ON). A single section of 3rd order mesenteric resistance artery was divided into two segments which were placed into aerated PSS at room temperature (22°C). The vessel segments were then placed in baths filled with 10mL of 37°C aerated PSS and mounted onto a calibrated wire myograph (DMT, Denmark) as outlined previously[61,151,159]. Artery segments were washed and incubated for 30min in 37°C PSS prior to normalization according to previous protocols[151]. Ninety percent of the normalized diameter was used as the experimental diameter based on previous studies[61], as well as our own pilot work. The experimental diameter was set for each vessel for the remainder of the protocol. Mean normalized mesenteric resistance artery diameters were not significantly different between groups (256±10 μm in SHR and 310±12 μm in WKY). All vessels were incubated for another 30 minute equilibration period following the standard start protocol[159], and were then incubated for 30 minutes with propranolol (3μmol/L) to block β-adrenergic receptor-mediated signaling, which remained present during all subsequent experiments. In addition to propranolol, rings were incubated with either no drug or L-NAME (10^{-4}M) to determine the NOS-dependency of the responses. All vessels were then pre-constricted using norepinephrine (NEPI; 10^{-5} M), and after achieving a stable plateau, exposed to increasing concentrations of AICAR (10^{-6}M, 10^{-4}M, 10^{-2}M). These concentrations of AICAR were chosen based on pilot work and previous experiments in WKY and SHR arteries [73]. Responses were expressed as percentage relaxation from pre-contracted tension in each ring. The functional viability of the endothelium in our preparations was tested by pre-constricting arterial segments with NEPI (10^{-5} M) followed by exposure to a maximal dose of acetylcholine (10^{-4} M).

Immunoblotting analyses. Tissue for immunoblotting was derived from three distinct sets of animals: first, thoracic aorta and 3rd order mesenteric arteries were harvested from untreated SHR and WKY. Secondly, thoracic aortas were retained from WKY and SHR of all treatment groups (after animals were killed by rapid removal of the heart) following the 30 minutes of hemodynamic data collection in the *in*

vivo experiments (Figure 4-2A). Thirdly, for mesenteric artery experiments, 3rd order mesenteric artery segments were isolated as for small vessel myography experiments, placed in aerated phosphate buffered saline (PSS), and warmed slowly to 37°C over 30 minutes and left to equilibrate for 1 hour. Vessels were then exposed to either AICAR (2mM, in PSS) or no AICAR (PSS only) for 30 minutes. In all experiments, arteries were quickly excised, dissected free of connective tissue in 4°C Krebs-bicarbonate buffer, snap frozen, and stored -80°C until processed for immunoblotting. Aortas and mesenteric artery segments were homogenized and samples prepared as previously described [74]. Polyacrylamide gels were loaded with 30µg total protein per lane. All membranes were stained with ponceau to confirm equal protein loading across lanes.

Drugs, Chemicals and Antibodies. All drugs and chemicals were purchased from either Sigma-Aldrich (St. Louis, MO) or Bioshop Canada Inc. (Burlington, ON, Canada) except AICAR which was purchased from Toronto Research Chemicals (Toronto, ON, Canada). For immunoblotting experiments, primary antibodies specific for the α subunit of AMPK (recognizing both α1 and α2 subunit isoforms, 1:500 dilution), P(Thr172)-AMPK (1:500) and P(Ser1177)-eNOS (1:3000) were obtained from Cell Signaling Technology (via New England Biolabs, Pickering, ON, Canada), and eNOS (1:750) was from BD Biosciences. Horse-radish-peroxide conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Data Analyses Values are reported as mean ± standard error, with *n* referring to the number of animals per group. For comparisons consisting of more than two treatment conditions or groups, one- or two-way ANOVAs with Tukey's or Bonferroni post-hoc analyses were conducted to evaluate within or between group comparisons. A two-tailed Student's *t*-test was used for comparisons where only two groups were present. Differences were considered significant if $P < 0.05$. All statistical analyses were performed using SAS (SAS Institute, Cary, NC) or Graphpad Prism 4 analysis software (La Jolla, CA).

4.5 Results

Baseline AMPK activation in SHR and WKY arteries

Phosphorylation of AMPK at activation site threonine 172 (a modification necessary and sufficient for AMPK activation [99]) was used as marker of AMPK activation in our experiments. Although relative total AMPK-α protein levels were not different between aorta of WKY and SHR, P(Thr172)-AMPK and the P(Thr172)-AMPK/AMPK-α ratio were reduced by ~50% in SHR aorta compared to WKY, demonstrating reduced basal AMPK activation in aorta of SHR vs WKY (Figure 4-1A). In mesenteric arteries, total AMPK-α protein was ~ 2 fold greater in SHR whereas P(Thr172)-AMPK was similar in SHR vs. WKY, resulting in a reduced P(Thr172)-AMPK/AMPK-α ratio (~50%) in SHR vs. WKY (Figure 4-1B).

In vivo hemodynamic responses to acute AICAR injections in WKY and SHR

MAP. Baseline MAP was 91±2 mmHg and 196±3 mmHg in WKY and SHR, respectively ($P < 0.0001$). Saline injections did not alter MAP from baseline in WKY or SHR over the 30 minutes of data collection (Figure 4-2C and D respectively). Injections of AICAR generated a progressive decrease in MAP to ~ 75 mmHg below saline values by 30 minutes post-injection in SHR (Figure 4-2D), but AICAR did not alter MAP relative to the saline group in WKY (Figure 4-2C). MAP reductions to AICAR in SHR were partially NO-dependent, as pre-injections with L-NAME partly prevented the effects of AICAR on MAP in SHR, with SHR MAP only decreasing ~40 mmHg over the 30 minutes following AICAR injection in the L-NAME + AICAR group (Figure 4-2D). Pre-injections of L-NAME alone did not alter MAP from the saline control in SHR (Figure 4-2D), but generated a ~ 35 mmHg elevation in MAP above saline values in WKY that was maintained for the duration of the experiment and was not altered by the addition of AICAR (Figure 4-2C).

Heart Rate. In SHR, HR was similar in all groups at time 0, did not change over the 30 minutes following injections of saline alone, but was depressed only modestly and transiently by a maximum of ~ 10% over the 30 minutes with AICAR treatment (429±7 vs. 378±9 bpm in saline vs. AICAR at 20

minutes, $P < 0.0001$) (Figure 4-2B). Pre-injections of L-NAME did not alter HR compared with saline only, nor did HR of SHR treated with L-NAME and AICAR differ from those treated with only AICAR at any time point (Figure 4-2B). Mean HRs in WKY groups were ~ 235-275 bpm immediately prior to saline or AICAR injection (Time 0) and remained constant in all treatment groups through the duration of data collection (Figure 4-2B).

AMPK activation in SHR and WKY aorta after AICAR treatment *in vivo*

AICAR is taken up by tissues and phosphorylation into its active form ZMP (an AMP analogue), that increases both AMPK activity allosterically and covalently by making it a more favourable substrate for upstream kinases and a poorer substrate for upstream phosphatases, (effectively increasing the net level of phosphorylation at activation site threonine 172). In SHR aortas harvested 30 minutes following AICAR injections, P(Thr172)-AMPK was increased ~1.6 fold compared to those of SHR injected with saline alone (Figure 4-3B), but was not increased in aortas of WKY 30 minutes after AICAR treatment relative to the saline controls (Figure 4-3A). As a control experiment, the efficacy of AICAR injections was confirmed in the WKY animals by examining liver tissue P(Thr172)-AMPK in WKY. Liver P(Thr172)-AMPK was 2.6 ± 0.45 fold greater in AICAR injected WKY compared to those injected with saline (1. 00 ± 0.05 arbitrary units, $P < 0.05$, Figure A4-3). L-NAME did not affect P(Thr172)-AMPK in aortas of WKY (Figure 4-3A) or SHR (Figure 4-3B) treated with saline or AICAR. Not surprisingly, AMPK-α total protein levels were not affected by any treatment condition in WKY or SHR (Figure 4-3A & B).

Functional responses of isolated conduit and resistance arteries to acute AICAR exposure *in vitro*.

Thoracic Aorta. We have previously demonstrated enhanced, dose-dependent relaxation in pre-contracted SHR versus WKY aorta over a range of AICAR concentrations (10^{-6}M -10^{-2}M) [73]. As a control experiment, we chose to replicate experiments in aorta of WKY and SHR here using a single dose of AICAR, in order to confirm the previous conduit aorta work and add value to the overall interpretation of the results of the current study. In confirmation of our previous work, in the current experiment, acute

exposure to a single submaximal dose of 2mM AICAR generated greater relaxation in aortic rings of SHR than of WKY (75±15% and 27±8% of pre-contracted tension respectively, P = 0.0133). Pre-contracted tension to phenylephrine was not different in WKY and SHR aortic rings (WKY: 1.8 ± 0.4g, SHR: 1.4 ± 0.3g, P = 0.4160).

Mesenteric arteries. AICAR generated dose-dependent relaxation that reached near 100% relaxation from pre-contracted tension at high [AICAR] (10^{-2}M) in isolated mesenteric artery segments of both WKY and SHR. In contrast to the responses observed in aorta, relaxation to AICAR did not differ between SHR and WKY mesenteric arteries at any AICAR concentration (Figure 4-4A). Relaxation of mesenteric arteries to AICAR was more NO-dependent in SHR than in WKY, as L-NAME significantly blunted relaxation in SHR rings at all AICAR concentrations, but blunted relaxation in WKY rings only at 10^{-6}M AICAR (Figure 4-4A). Pre-contraction to NEPI (10^{-5}M) was not different across treatment groups or between SHR and WKY arteries (WKY: CON 15.5±1.0mN, L-NAME 15.4±1.0mN, SHR: CON 15.6±0.9mN, L-NAME 17.1±1.6mN).

To more rigorously evaluate the contribution of NO-dependent and NO-independent mechanisms to the relaxation generated by AICAR in WKY and SHR mesenteric arteries, the proportion of the relaxation response accounted for by NO-dependent mechanisms were also expressed as at each concentration of AICAR for both WKY and SHR vessels (Figure 4-4B). Relaxation to AICAR was entirely NO-mediated at 10^{-6}M AICAR in both WKY and SHR (100% of the relaxation response was removed by L-NAME in arteries of both groups at this concentration, Figure 4-4B). This proportion was significantly decreased in both WKY and SHR as [AICAR] increased, until NO-dependent mechanisms accounted for only ~2% and 29% of the relaxation generated by WKY and SHR rings respectively at 10^{-2}M AICAR (thus at higher [AICAR] responses were predominantly NO-independent, Figure 4-4B). Although there were no differences between WKY and SHR at each [AICAR], there was a main effect of rat type over the range of AICAR doses (P = 0.0133) in favour of greater overall reliance on NO-dependent mechanisms of relaxation by vessels of SHR compared to those of WKY (Figure 4-4B).

AMPK activation in SHR and WKY mesenteric arteries after AICAR incubation *in vitro*

The observation that P(Thr172)-AMPK was elevated ~2-2.5 fold in both WKY (Figure 5A) and SHR (Figure 4-5B) mesenteric artery segments following 30 minutes of AICAR (2mM) incubation relative to the no drug controls verifies that AICAR activates AMPK in mesenteric arteries of both SHR and WKY. The fold-increase in P(Thr172)-AMPK relative to their respective no drug control groups was significantly greater in SHR than WKY arteries (fold increase from respective CON: WKY 1.99 ± 0.04 vs. SHR 2.73 ± 0.10 arbitrary units, $P < 0.01$). AMPK-α total protein content was unaltered with AICAR in either WKY (Figure 4-5A) or SHR (Figure 4-5B).

Phosphorylation of eNOS Ser1177 in SHR and WKY aorta and mesenteric arteries after AICAR exposure

Phosphorylation of eNOS at activation site Ser1177 and eNOS total protein content were unaltered in mesenteric artery segments of WKY and SHR following 30 minutes of AICAR incubation *in vitro* (Figure 4-6). P(Ser1177)-eNOS was also unaffected in aortas that were removed 30 minutes following injections of AICAR or saline during the *in vivo* experiments from WKY (saline: 1.00±0.10 versus AICAR: 1.04±0.09) and SHR (saline: 1.00±0.10 versus AICAR: 1.03±0.17) (Figure A4-2).

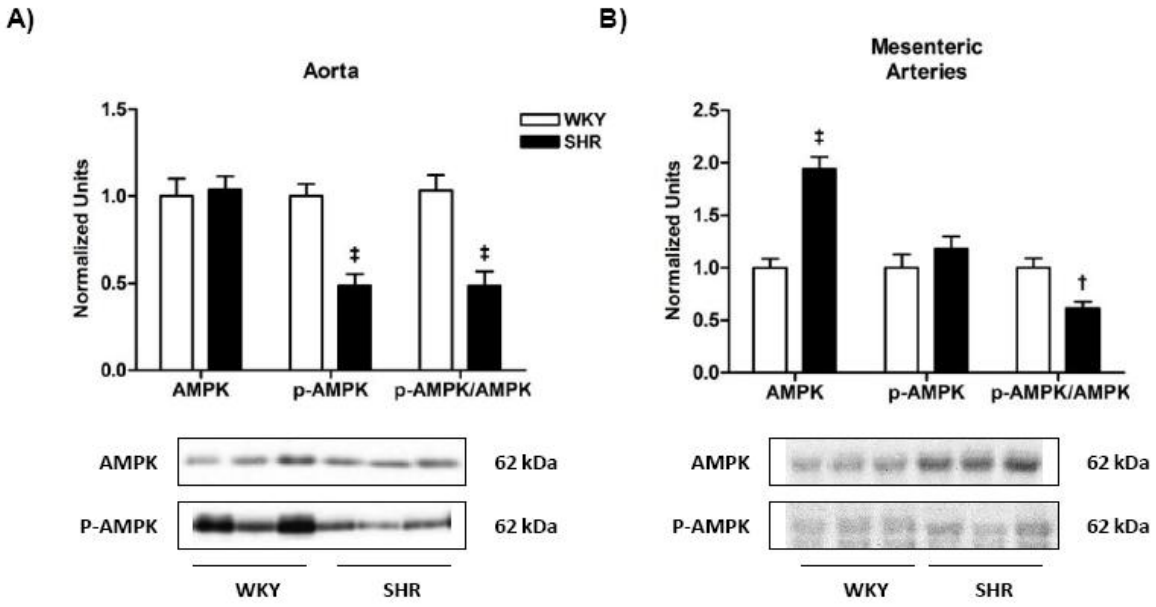

Figure 4-1. Baseline levels of AMPK total protein expression and phosphorylation in aorta and mesenteric arteries of untreated SHR versus WKY. Total AMPK-α protein content and phosphorylation of AMPK at activation site threonine 172 and were analyzed by immunoblotting in WKY and SHR **(A)** aortic and **(B)** mesenteric artery homogenates. The ratio of phosphorylated to total AMPK protein content was also calculated for both vessel types. Data are normalized such that the mean of WKY is 1.00. Representative blot images display 3 lanes from each group. $n = 6$ (samples run in duplicate). † $P < 0.01$, ‡ $P < 0.001$ all vs. WKY.

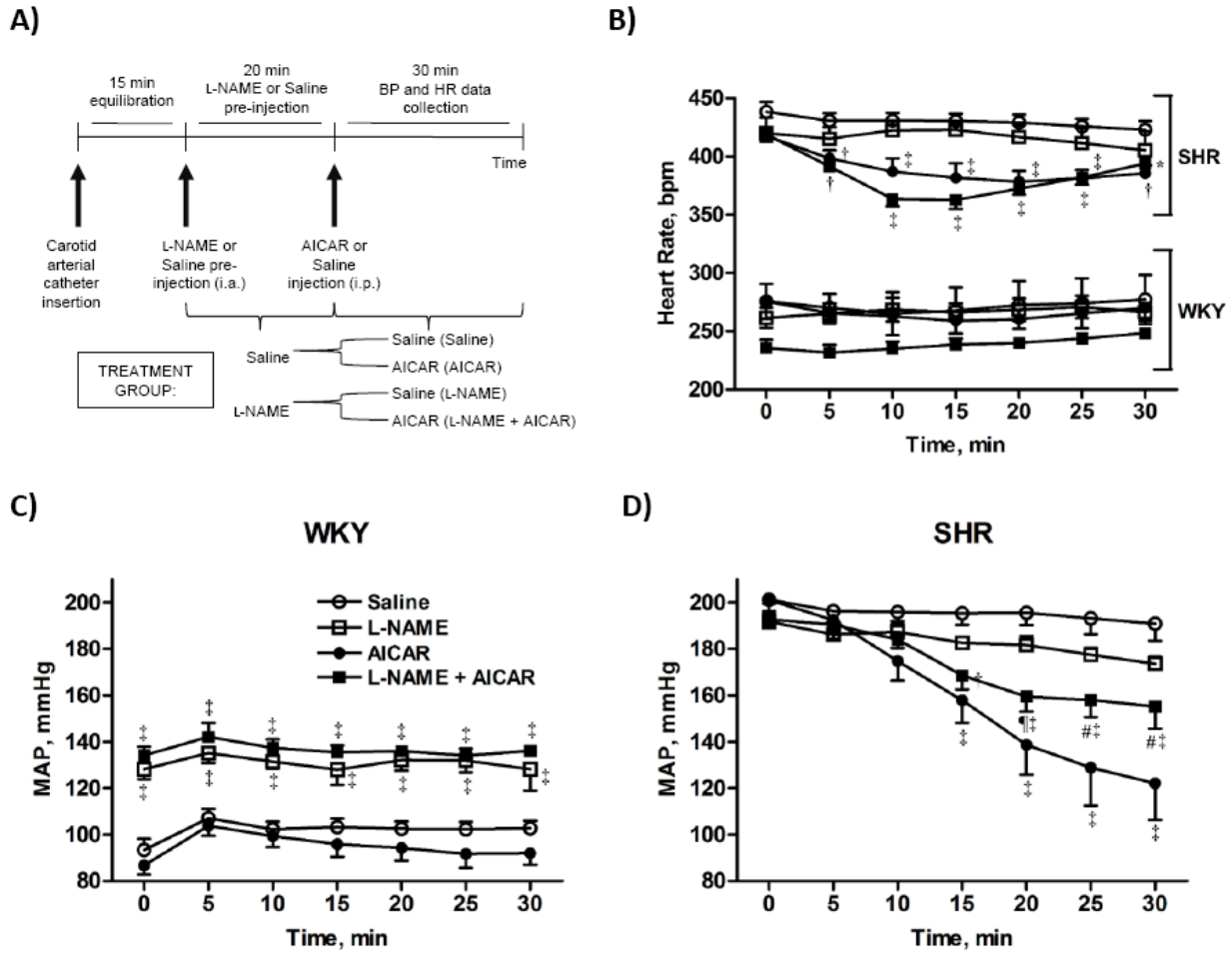

Figure 4-2. Hemodynamic responses of SHR and WKY rats to acute injections of AICAR. A) The protocol and treatment groups used to generate *in vivo* hemodynamic responses to acute AICAR injections. Following instrumentation with a carotid catheter and a period of equilibration, animals were given an intra-arterial pre-injection of L-NAME (30 mg/kg body weight) or a saline control, followed 20 minutes later by intra-peritoneal injections of either saline or AICAR (0.5 mg/g body weight) to generate four treatment groups: saline only (○), L-NAME pre-injection (□), AICAR only (●), or L-NAME pre-injection + AICAR (■). **B)** Heart rate responses and mean arterial pressure for **C)** WKY and **D)** SHR were assessed over 30 minutes following the injections of AICAR or saline for each treatment. Legend conventions are conserved across panels. n = 5-7 rats per group. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ vs. saline. ¶ $P < 0.05$, # $P < 0.01$ AICAR vs. L-NAME + AICAR.

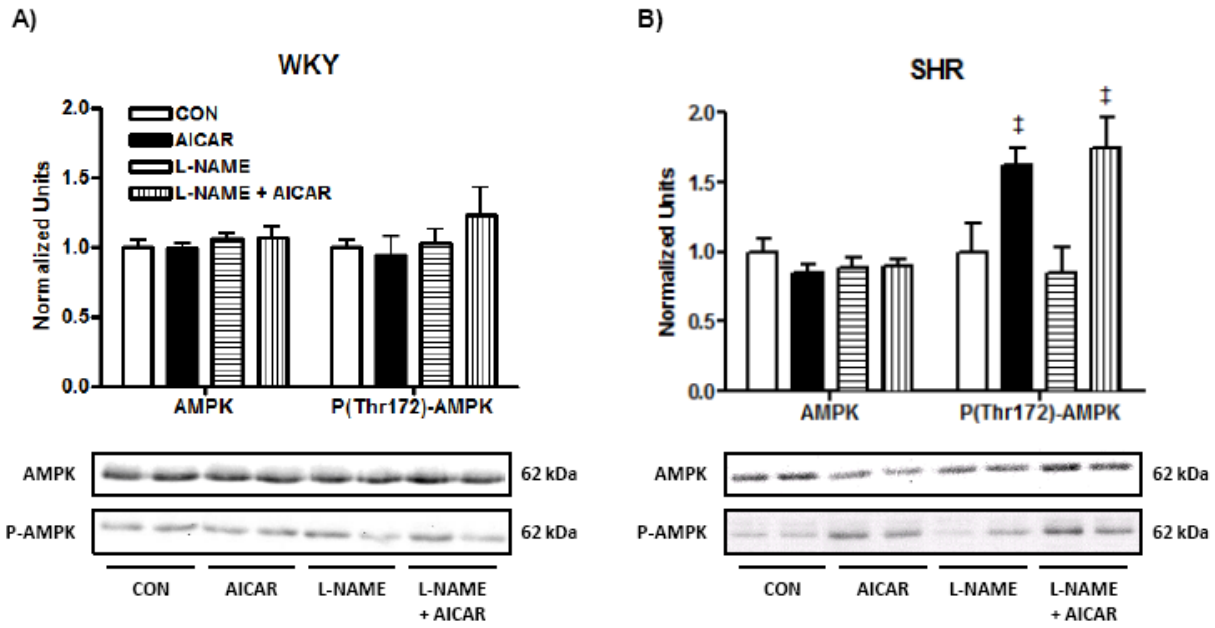

Figure 4-3. AMPK activation in WKY and SHR aorta following acute drug injection *in vivo*. Phosphorylation at AMPK activation site threonine 172 and total AMPK-α protein content were analyzed by immunoblotting in aortic homogenates of WKY (**A**) and SHR (**B**) rats treated with injections of either saline, AICAR, L-NAME or AICAR + L-NAME during the *in vivo* experiments (see Figure 1 for derivation of treatment groups). Data are normalized such that the mean of saline control is 1.00. Legend conventions are conserved across panels. Representative blot images for AMPK and P(Thr172)-AMPK display 2 lanes from each group. $n = 6$ (samples run in duplicate). ‡ $P < 0.001$ all vs. saline.

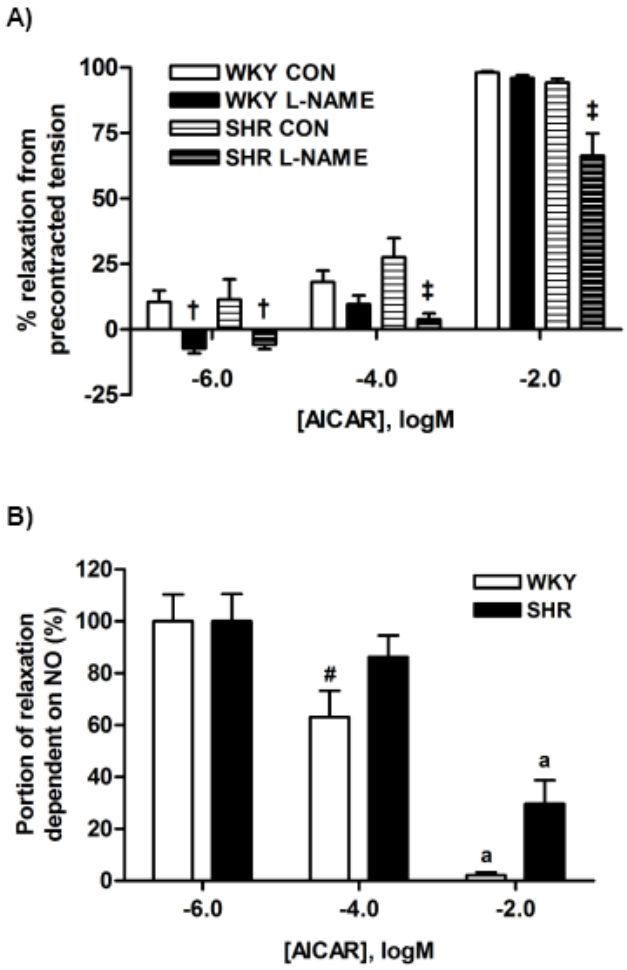

Figure 4-4. Vasorelaxation of isolated WKY and SHR mesenteric artery segments to acute AICAR exposure *in vitro*. A) Mesenteric artery segments of WKY and SHR were pre-incubated with either no drug (CON) or the NOS inhibitor L-NAME (all in the presence of β-adrenergic receptor blocker propranolol, 3μM) for 30 minutes to illustrate the NOS-dependency of the response to AICAR. Vessels were then pre-contracted with norephinephrine (NEPI, 10^{-5}M) prior to exposure to increasing AICAR concentrations, and responses are expressed as mean percent relaxation of NEPI pre-contracted tension. **B)** The portion of the relaxation response generated by NO-dependent mechanisms at each concentration of AICAR in WKY and SHR mesenteric arteries calculated from the data in panel A. *n* = 6-8 rings per group, obtained from different animals. † $P < 0.01$, ‡ $P < 0.001$ vs. respective WKY or SHR CON in panel A. # $P < 0.05$, a $P < 0.001$ versus 10^{-6}M [AICAR] in WKY and SHR respectively. There was a main effect for rat type ($P = 0.0153$), but no significant differences between WKY and SHR responses at any [AICAR] in panel B.

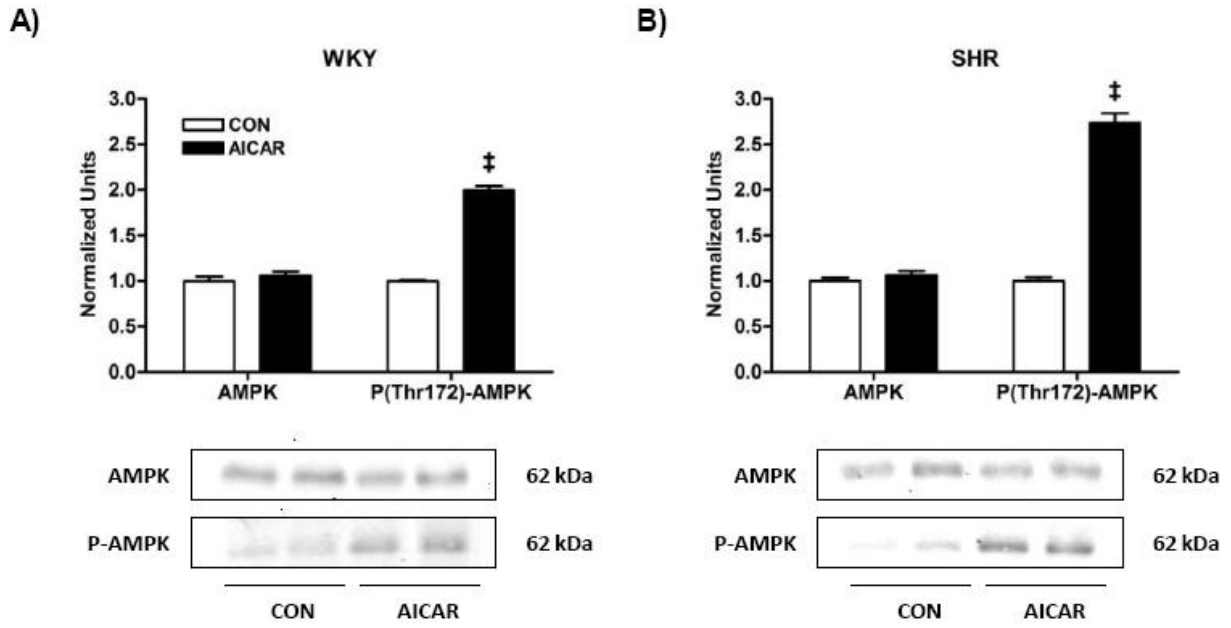

Figure 4-5. AMPK activation in isolated WKY and SHR mesenteric artery segments following acute incubation with AICAR *in vitro*. Phosphorylation at AMPK activation site threonine 172 and total AMPK protein content were analyzed by immunoblotting in mesenteric artery homogenates from WKY (**A**) and SHR (**B**) rats. Mesenteric artery segments of WKY and SHR were incubated with either no drug (PSS alone) or AICAR (2mM AICAR in PSS) *in vitro* for 30 minutes. Data are normalized such that the mean of the control group (CON) is 1.00. Representative blot images display 2 lanes from each group. n = 6 (samples run in duplicate). ‡ $P < 0.001$ vs. CON.

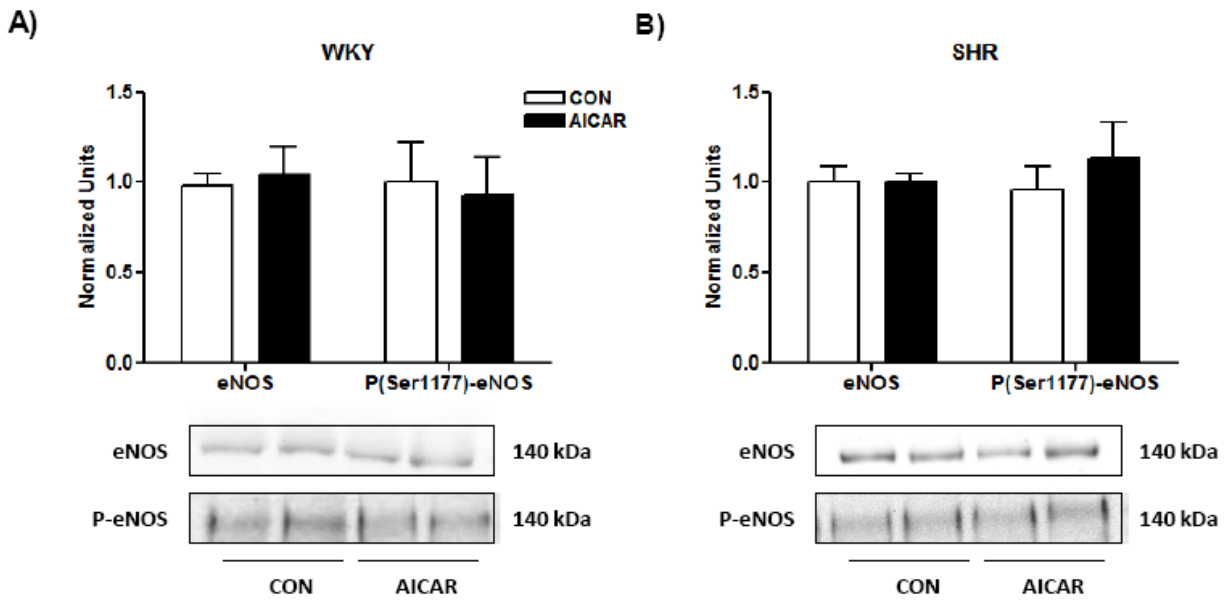

Figure 4-6. Phosphorylation of eNOS at activation site Ser1177 does not change in WKY and SHR mesenteric artery segments following acute incubation with AICAR *in vitro*. Phosphorylation at eNOS activation site serine 1177 and total eNOS protein content were analyzed by immunoblotting in mesenteric artery homogenates from WKY (**A**) and SHR (**B**) rats. Mesenteric artery segments of WKY and SHR were incubated with either no drug (PSS alone) or AICAR (2mM AICAR in PSS) *in vitro* for 30 minutes. Data are normalized such that the mean of the control group (CON) is 1.00. Representative blot images display 2 lanes from each group. $n = 6$ (samples run in duplicate). There were no significant differences between CON and AICAR in any group.

4.6 Discussion

We investigated the ability of the AMPK-activator AICAR to acutely alter BP and to cause vasorelaxation in isolated mesenteric arteries of normotensive and hypertensive rats. These data are the first to demonstrate dysregulated basal AMPK activation in conduit and resistance arteries of hypertensive versus normotensive rats, to observe the influence of acute AMPK activation on hemodynamic parameters in a model of severe hypertension, and to evaluate parallel vasorelaxation effects in resistance vasculature of hypertensive and normotensive models.

AMPK activation is dysregulated in basal, unstimulated conduit aorta and resistance mesenteric arteries of hypertensive animals, as indicated by reduced P(Thr172)-AMPK/AMPK ratios in these tissues (Figure 4-1A and B respectively). The origin of these differences is unclear (i.e. whether reduced AMPK activation is influenced by genetic factors, high blood pressure or other metabolic abnormalities in SHR), and it was not the intent of this study to provide insight in this regard. These results do not represent an impaired ability to activate AMPK *per se*, (i.e. AMPK was activated by AICAR in SHR conduit and resistance arteries, and may even be more sensitive to stimulation than those of WKY as demonstrated in aorta and mesenteric arteries here, and previously in SHR versus WKY aorta [73]), but instead suggest dysfunctional regulation of AMPK by upstream kinases and/or phosphatases. The expression and activity of AMPK upstream kinases (i.e. LKB1, CaMKKβ, TAK-1) and phosphatases (PP2A, PP2C) have not been evaluated in arteries of these animals or other cardiovascular disease models where vascular AMPK dysregulation persists; whether or not abnormal functioning of upstream regulatory systems contribute these observations is an intriguing question that warrants further investigation.

In accordance with previous indications that AMPK may modulate BP [14,19,71], we report the novel finding that single injections of AICAR generate an acute, profound ~70mmHg reduction in BP of SHR (Figure 4-2D). Although we did not observe a significant reduction in BP in our normotensive animals following acute AICAR injections, there was a trend towards a BP lowering effect of AICAR over the 30 minutes following drug injection (i.e. BP was lower by ~10 mmHg in AICAR versus saline treated

WKY). Others have reported modest BP decreases in normotensive rats and humans following acute injections of AICAR (i.e. 4-20mmHg)[14,71], although the ability of these studies to delineate significant BP reductions in normotension may be due to use of higher doses of AICAR[71], different experimental protocols[14,71], or human versus animal models [14]. Although *in vivo* hemodynamic responses can be affected by the anesthetic regime in place during experimental protocols, heart rates of the anesthetized animals in our study are comparable to those of conscious animals [4,122] and thus our rats were not likely sedated to the extent required to compromise the integrity and validity of the hemodynamic measurements.

Since we have previously observed relaxation of conduit WKY and SHR arteries in response to acute AICAR exposure[73], in the current study we wanted to investigate whether similar effects could be generated in resistance arteries, and if to parallel these results, a single injection of AICAR also acutely depresses BP in these animals *in vivo* (an effect that could originate from altered vascular resistance). Here we demonstrate for the first time that AMPK activation causes vasorelaxation of WKY and SHR mesenteric arteries (Figure 4-4), observations that support the potential activity of AICAR on vascular resistance and depressed BP in our *in vivo* experiments. Several other observations from this study and from previous reports also support this hypothesis. First of all, despite the dramatic effects of AICAR on MAP in SHR, HR was relatively well maintained (only dropping transiently by ~10% over the 30 minutes post injection) and did not mirror the pattern of continual MAP drop observed with AICAR in SHR (Figure 4-2). Others have observed that acute AICAR administration in un-anesthetized human subjects even evokes a paradoxical increase in HR with AICAR[14]. The absence of concurrent decreases in heart rate and BP suggest that reduced vascular resistance, rather than alterations in central control, contributes to the BP decrease with AICAR in SHR. Secondly, we have also demonstrated that AMPK activation causes vasorelaxation of SHR aorta and that this response is *enhanced* in conduit arteries of SHR compared to those from WKY (here as described in results section and previously[73]), an effect that could thus translate into greater reductions in vascular resistance in some parts of the vascular tree, and

consequently, in BP of SHR. Although relaxation to AICAR did not differ between SHR and WKY mesenteric arteries (where we may have expected to observe enhanced relaxation in SHR versus WKY based on the MAP responses), and collectively, resistance arteries hold the greatest capacity to influence BP, this is not to say that other conduit or resistance vascular beds do not more closely mirror responses observed in aorta of SHR versus WKY (given the heterogeneity that exists within the vascular tree) or that responsiveness of more conduit-like vasculature could not have produced some hemodynamic effect. Lastly and notably, our marker of AMPK activation was only increased in arteries of SHR *but not* WKY following *in vivo* injections of AICAR (Figure 4-3); thus if vascular resistance is a contributor to the BP effects, it is possible that the AICAR dose used here was not sufficient to exert an influence on the vasculature of WKY, as a previous report has also demonstrated that vascular AMPK can be more sensitive to activation by AICAR in blood vessels of SHR compared to those of WKY [73]. While our data provide support for the involvement of resistance vessel vasorelaxation effects of AICAR on the BP responses, *in vivo* conditions (innveration, endogenous vasoactive factors, mechanical effects, hemodynamics etc.) may generate differential effects to AICAR in comparison with those of the isolated vessel experiments, or alternatively, any decreases in vascular resistance in WKY may have been masked by compensatory BP regulatory mechanisms that are impaired in SHR. Thus, these observations suggest that altered vascular resistance (rather than mechanisms involving central BP control) is likely a main effecter of the acute AICAR-mediated hemodynamic responses observed in SHR.

Cell culture, biochemical, and *in vitro* functional experiments have associated AMPK activation with increased endothelial NO-bioavailability[27,30,31,44,145]. Consistent with these observations and implications of NO in the regulation of BP [119], pre-administration of NOS inhibitor L-NAME partially prevented the AICAR-induced MAP reduction in SHR (Figure 4-2D). We have previously demonstrated that vasorelaxation generated by acute AMPK-activation is partly NO-dependent in aortas of SHR [73] and here that relaxation of isolated SHR mesenteric artery segments to AICAR is also partly NO-mediated (Figure 4-4), contributing to the evidence supporting a role for NO-dependent vascular tone in modulation

of the blood pressure responses. Although we did not endeavor to exhaustively elucidate mechanisms responsible for the NO-dependent effects of AICAR here, we did assess P(Ser1177)-eNOS in thoracic aorta of animals injected with AICAR *in vivo* (stated in the results section, Figure A4-2) and in mesenteric arteries incubated with AICAR *in vitro* (Figure 4-6), but found no differences in P(Ser1177)-eNOS between control and AICAR-treated tissue (consistent with previous experiments where we demonstrate that phosphorylation at eNOS serine 1177 does not account for increased NO-dependent relaxation to AICAR in WKY and SHR aorta [73]). Mechanisms suggested in other work that could account for enhanced NO-bioavailability or bioactivity with AMPK activation include phosphorylation of eNOS activation site Ser633[31], formation of active eNOS complexes [30,44], increased sensitivity of downstream signaling mechanisms or other yet unidentified mechanisms. Importantly, a large portion of the BP (Figure 4-2) and vasodilatory responses of mesenteric arteries in SHR (Figure 4-4A), and most of WKY mesenteric artery vasodilation (Figure 4-4A), was not mediated by NO. These NO-*in*dependent effects are most likely accounted for by endothelium-*in*dependent, direct vasodilation of the vascular smooth muscle by AICAR/AMPK activation, as a number of former investigations have established the ability of acutely activated AMPK to relax vascular smooth muscle directly [73,82,181]. In SHR, these effects could also be due in part to suppression of endothelium-dependent vasocontraction, as we have recently elucidated[73]. Further analyses of the functional responses of WKY and SHR mesenteric arteries to AICAR revealed that at low [AICAR] (i.e. 10^{-6}M), relaxation was fully accounted for by NO-mediated mechanisms in both WKY and SHR rings, but that the contribution to relaxation was largely NO-independent at high [AICAR] (although SHR mesenteric arteries maintained greater reliance on the NO-dependent component over the range of AICAR doses than WKY, Figure 4-4B). The findings highlight the importance of considering the strength of the stimulus when examining the relative contribution of NO-mediated or other mechanisms to the vasodilatory response.

In light of the profound hypotensive affect of acute AMPK activation observed in hypertensive rats in this study, it would be interesting to determine if chronic manipulation of AMPK activation exerts

sustained BP depression in SHR or under other conditions of severe hypertension. Chronic administration of AICAR lowers BP in Zucker diabetic fatty rats (that are moderately hypertensive)[19], and other drugs associated with AMPK activation (such as metformin and thiazolidiones)[75] also have modest hypotensive[8,171,234] and vasomotor [234] effects when administered chronically to animals that exhibit hypertension. Elevated BP has also been reported recently in AMPK α2-/- mice [242], providing further evidence for a potential role of AMPK in BP regulation. Although it is not clear whether dysfunctional AMPK signalling contributes directly to hypertension or vasomotor dysfunction in SHR, an intriguing finding from a recent report by Dolinksy et al. [52]showed that short-term caloric restriction prevents development of hypertension in SHR rats, a result that was accompanied by increased AMPK phosphorylation in mesenteric arteries of these animals, highlighting the potential importance of the link between AMPK activation/dysregulation, vascular function and the regulation of BP in hypertension. Further experiments will be necessary to delineate whether or not chronic increases in AMPK activation induce persistent depressions in blood pressure of SHR and whether these alterations are sustained by vasodilatory effects. Regardless of potential chronic benefit, the acute hemodynamic and vasomotor effects observed here are intriguing and may have interesting implications for physiological regulation of blood pressure and vascular tone by acute AMPK activation in conditions of regular vascular control and in hypertension.

Perspectives

This study represents the first parallel investigation into the influence of acute AMPK activation on isolated vessel function and BP in hypertension. We have extended previous work by establishing an anti-hypertensive effect of acute AICAR *in vivo* that is partially NOS-dependent, and by delineating a vasorelaxation effect of acute AICAR exposure on resistance arteries in a hypertensive rat model. Our findings support a role for AMPK in the mediation of vasomotor responses in vessels of hypertensive animals, and provide valuable insight by suggesting the potential involvement of vascular AMPK in BP

regulation in hypertension. Continued investigation regarding the implications of chronic AMPK activation in hypertensive models may reveal a role for AMPK in the management of hypertension.

4.7 Addendum to Study 3:

The addendum contains data collected in conjunction with this study but not included as part of the manuscript submitted for publication. These items include:

Figure A4-1: As stated in the initial specific objectives for this study, we also measured P(Ser1177)-eNOS and eNOS total protein content by immunoblotting in aortic rings from WKY and SHR removed untreated animals (to assess baseline differences). Since phosphorylation of eNOS at serine 1177 was unaltered by AICAR treatment, we did not include basal eNOS and P(Ser1177)-eNOS data in the manuscript.

Figure A4-2: Phosphorylation of eNOS at activation site Ser1177 does not change in WKY and SHR aorta following acute drug injection *in vivo*. Some of this data is stated numerically in the results section of the manuscript.

Figure A4-3: AMPK activation is increased in liver of WKY animals injected with AICAR *in vivo* (discussed in the results section).

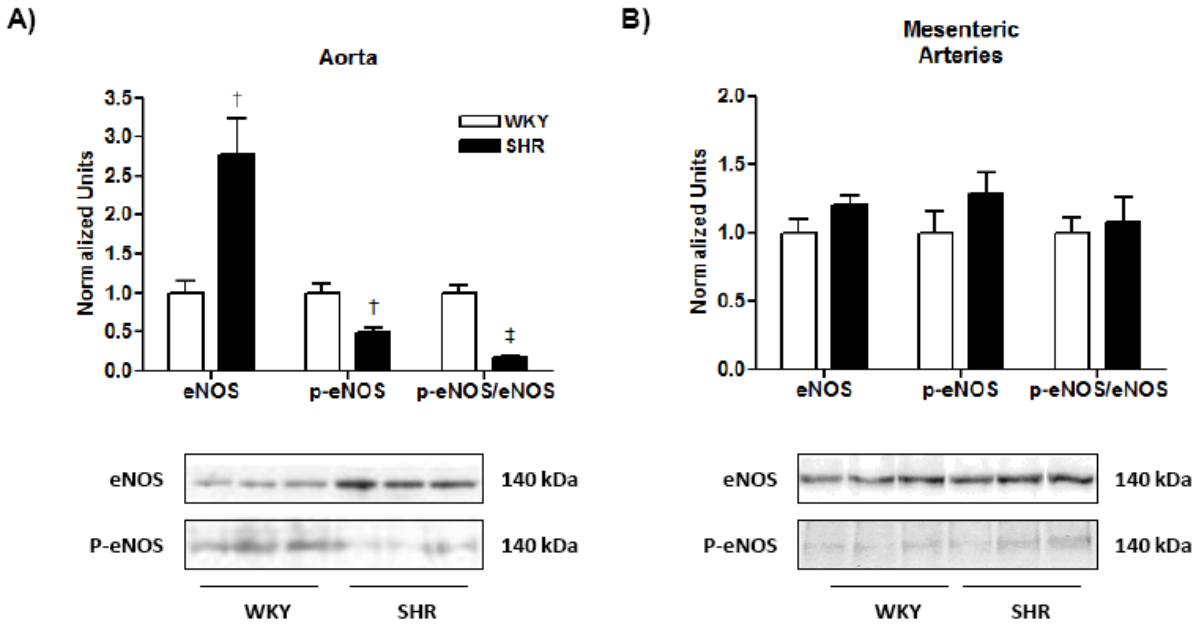

Figure A4-1. Baseline levels of eNOS total protein expression and phosphorylation in aorta and mesenteric arteries of untreated SHR versus WKY. Total eNOS-α protein content and phosphorylation of eNOS at activation site serine 1177 and were analyzed by immunoblotting in WKY and SHR **(A)** aortic and **(B)** mesenteric artery homogenates. The ratio of phosphorylated to total eNOS protein content was also calculated for both vessel types. Data are normalized such that the mean of WKY is 1.00. Representative blot images display 3 lanes from each group. $n = 6$ (samples run in duplicate). † $P < 0.01$, ‡ $P < 0.001$ all vs. WKY.

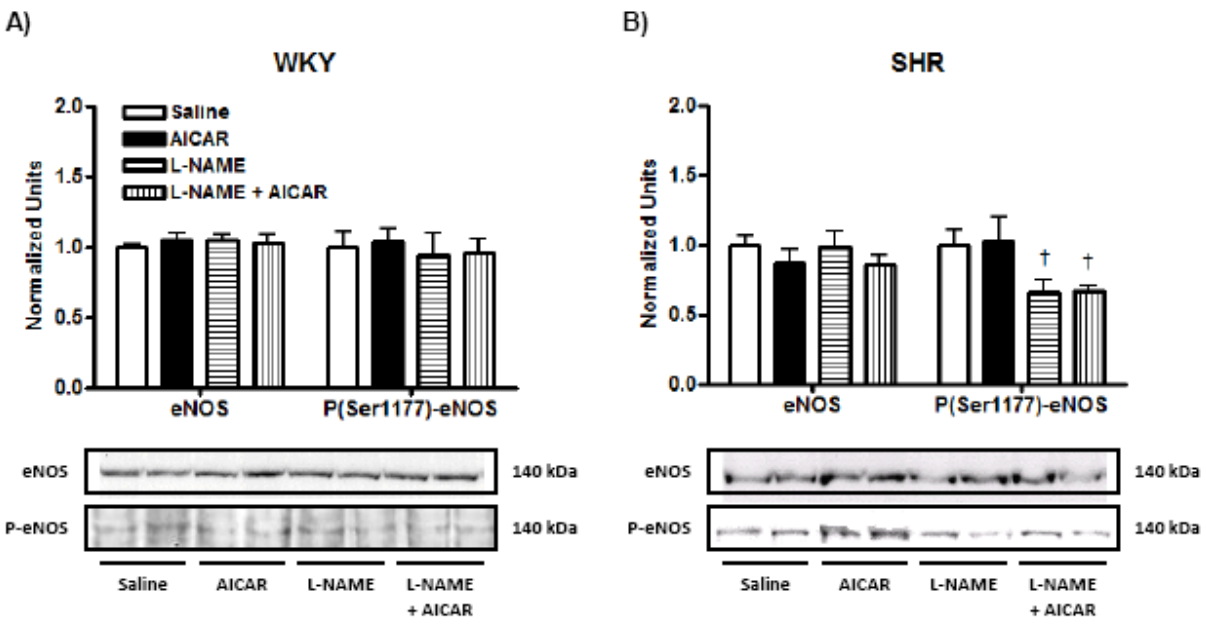

Figure A4-2. Phosphorylation of eNOS at activation site Ser1177 does not change in WKY and SHR aorta following acute drug injection *in vivo*. Phosphorylation of eNOS activation site serine 1177 and eNOS total protein content were analyzed by immunoblotting in aortic homogenates from WKY (**A**) and SHR (**B**) rats treated with injections of either saline (CON), AICAR, L-NAME or AICAR + L-NAME (see Figure 1 for derivation of treatment groups). Aortas were removed from animals immediately following 30 minutes of hemodynamic data collection, quickly dissected free of connective tissue and immediately frozen in liquid nitrogen. Data are normalized such that the mean of saline control is 1.00. Representative blot images for eNOS and P(Ser1177)-eNOS display 2 lanes from each group. $n = 6$ (samples run in duplicate). † $P < 0.01$ all vs. saline control.

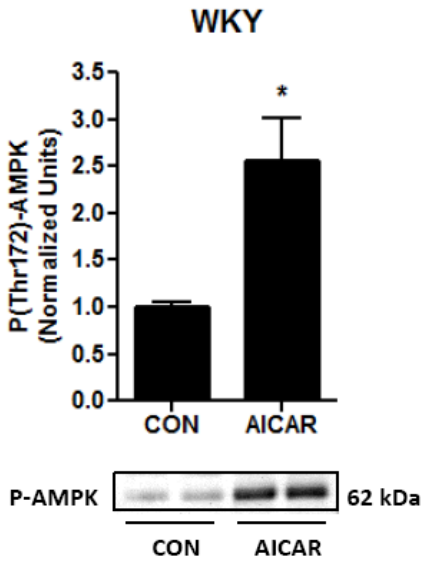

Figure A4-3. AMPK activation is increased in liver of WKY animals injected with AICAR *in vivo*. Phosphorylation at AMPK activation site threonine 172 was analyzed by immunoblotting in liver homogenates of WKY injected with saline or AICAR during the *in vivo* hemodynamic experiments. Liver samples were snap frozen and stored for immunoblotting analyses immediately following collection of hemodynamic data at 30 minutes post-injection of saline or AICAR (see Figure 4-2 for protocol). Data are normalized such that the mean of saline control is 1.00. Representative blot images display 2 lanes from each group. $n = 4$. * $P < 0.05$ vs. saline control.

Chapter 5

Thesis Study 4

Vasomotor responses generated by acute exposure to AMP-activated protein kinase activators in isolated aorta of young and aged rats

Rebecca J. Ford, Kristina K. Durham and James W. E. Rush

This chapter is presented in manuscript form.

Acknowledgements: The authors thank Andrew Levy and Dawn McCutcheon for technical assistance.

5.1 Review of Study 4 Objectives and Hypotheses

The specific objectives and hypotheses for this chapter have already been stated in the introduction section, but are reproduced identically here for the reader's convenience.

Purpose: To characterize the vasomotor responses of isolated arteries from young and aged rats to pharmacological AMPK activators and to investigate the mechanisms involved.

Specific objectives and hypotheses:

Objective 1: To assess basal AMPK activation in aorta of aged versus young male Sprague-Dawley rats. **Hypothesis 1:** Basal AMPK activation (P(Thr172)-AMPK and P(Ser79)-ACC) will be depressed in arteries of aged versus those of young rats.

Objective 2: To determine if vasorelaxation to AICAR is intact in isolated aortic rings from aged rats compared to those of young rats. **Hypothesis 2:** AMPK-mediated relaxation will be impaired in arteries of aged versus young rats.

Objective 3: To evaluate the endothelium-dependency and/or –independency of vasodilatory responses to AICAR in aortic rings young and aged rats. **Hypothesis 3:** AICAR will generate relaxation in aortic rings of young and aged rats that is partly endothelium-dependent and partly endothelium-independent.

Objective 4: To pharmacologically dissect the mechanisms responsible for AMPK-mediated vasodilation in aortic rings of young and aged rats (i.e. NO- and COX-dependency). **Hypothesis 4:** The endothelium-dependent component of relaxation to AICAR will be primarily NO-dependent in aortic rings of young rats but will be NO- and COX-mediated in aortic rings of aged rats. Since NO-bioavailability and NO-dependent functional responses are typically reduced in arteries of aged rats, we expect NO-dependent relaxation to AICAR may be reduced in aged versus young aorta. Responsiveness to vasoactive agents is often impaired in arteries of aged rats, so endothelium-independent relaxation to AICAR may also be impaired in arteries of aged animals versus those of young.

Objective 5: To biochemically confirm AMPK activation by AICAR in aortic rings of young and aged rats. **Hypothesis 5:** AMPK activation (P(Thr172)-AMPK and P(Ser79)-ACC) will be elevated by treatment with AICAR to a similar extent in rings of young and aged animals.

Objective 6: To investigate whether or not vasorelaxation of young and aged pre-contracted aortic rings also occurs in response to other known AMPK activators, metformin and resveratrol, and to compare these responses in vessels of young versus aged animals. **Hypothesis 6:** Metformin and resveratrol will each generate relaxation in aortic rings of young and aged rats and relaxation to both of these agonists will be impaired in aortic rings of aged versus young animals.

Objective 7: To biochemically confirm AMPK activation by metformin and resveratrol in aortic rings. **Hypothesis 7:** AMPK activation (P(Thr172)-AMPK and P(Ser79)-ACC) will be elevated by treatment of rings with either metformin or resveratrol.

Objective 8: To evaluate total eNOS protein content in aortic rings of young and aged rats, and the effect of AICAR on P(Ser1177)-eNOS of these vessels *in vitro*. **Hypothesis 8:** Total eNOS content will be depressed in aortic rings of aged versus young rats, and P(Ser1177)-eNOS will be enhanced in aorta rings of young and aged rats treated with AICAR.

5.2 Overview of Study 4

Acute activation of AMP-activated protein kinase (AMPK) is associated with relaxation of pre-contracted, isolated arteries via both endothelium-dependent, nitric oxide (NO)-mediated and endothelium *in*dependent direct effects on vascular smooth muscle (VSM). AMPK activation is dysregulated in arteries of aged versus young animals, and therefore vasorelaxation to acute AMPK activation may be altered with aging, but this hypothesis is untested. Here we investigate mechanisms of AMPK-mediated vasorelaxation and its associated mechanisms in isolated aortic rings of young and aged Sprague-Dawley rats. Using *in vitro* wire myography, we determined that pre-contracted aortic rings of young and aged

animals relax dose-dependently in response to increasing concentrations of the AMPK activator 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) to levels approaching 100% relaxation from pre-contracted tension. Relaxation to AICAR was partly blunted by removal of the endothelium (E-), revealing endothelium-dependent and -independent components of the response. Pre-incubation of rings with the NO synthase inhibitor L-NAME abolished the endothelium-dependent component of relaxation to AICAR in both young and aged vessels, showing that this portion of the response is NO-mediated. We also assessed the ability of the anti-diabetic drug metformin and the polyphenol resveratrol (other compounds known to activate AMPK) to relax isolated E- arteries. Relaxation, and the corresponding AMPK activation (phosphorylation of AMPK activation site threonine 172 and downstream target serine 79 on acetyl-CoA carboxylase assessed by immunoblotting) to metformin, were modest compared to the AMPK activation induced by AICAR in aged aorta, and relaxation to metformin was enhanced in aorta of aged versus young rats. Although resveratrol generated robust, dose-dependent relaxation of pre-contracted aortic rings of aged rats (that was enhanced compared to relaxation of young aorta to resveratrol), AMPK activation was not increased by resveratrol in these vessels. Therefore, these data demonstrate that AMPK-mediated vasorelaxation is intact and enhanced in arteries of aged animals, and that this response is mediated by both an endothelium-, NO-dependent component, and by endothelium-independent relaxation of the vascular smooth muscle. Although metformin induces a mild vasodilatory effect associated with mild AMPK activation, relaxation of aged arteries to resveratrol is orchestrated by mechanisms distinct from AMPK activation.

5.3 Introduction

AMP-activated protein kinase (AMPK) is a ubiquitously expressed serine-threonine protein kinase, and is well known for its role as a regulator and integrator of cell and whole body energy metabolism [21,97,205]. More recently, AMP-activated protein kinase (AMPK) has also been identified as a regulator of vascular tone. This role is supported by several lines of evidence, including observations

showing that acute activation of AMPK produces vasorelaxation of pre-contracted, isolated arteries from healthy animals [13,15,73,82,181], and that increased blood flow has been reported in vessel beds injected with AMPK activators *in situ* [13,15]. In healthy conditions, the vasodilatory response to acute AMPK activation seems to originate from direct relaxation of vascular smooth muscle (VSM) [73,82,181] and/or by endothelium-mediated signalling that is nitric oxide (NO)-dependent [13,15,73], depending on the vessel bed or model studied. In dysfunctional conduit arteries that are characterized by the presence of enhanced endothelium-dependent contractions mediated by the cyclooxygenase (COX) signalling axis (i.e. aorta of spontaneously hypertensive rats [48,73,233]), acute AMPK activation may also induce net relaxation by inhibiting this endothelium- and COX-dependent contractile response [73].

AMPK is activated covalently via phosphorylation of threonine 172 on its catalytic alpha subunit by upstream kinases, and allosterically by the binding of AMP to the Bateman domains on the gamma subunit [70,99,104,205]. Bound AMP also facilitates phosphorylation at threonine 172 by rendering AMPK an unfavourable substrate for upstream phosphatases [70,186,208], shifting the balance in kinase/phosphatase activity at this site in favour of augmented phosphorylation. AMPK can be activated pharmacologically using AICAR, a compound that is taken up by cells via adenosine transporters and phosphorylated intracellularly by adenosine kinase to form the AMP analogue ZMP [36,124].

The antidiabetic drug metformin and the red-wine polyphenol resveratrol also activate AMPK. Metformin activates AMPK in cultured endothelial [44,102,117,268] and vascular smooth muscle [160,238] cells, in aorta of mice treated with metformin *in vivo* [44,268] and in other tissues such as the heart [244], skeletal muscle [11] and adipocytes [90], and resveratrol modestly activates AMPK in cultured endothelial cells [258], the heart [24], hepatocytes [263], myotubes [168], skeletal muscle [223], epididymal fat [223] and neurons [41]. These compounds have notable clinical and health implications, as metformin is the most commonly prescribed drug for the treatment of type II diabetes [67], and resveratrol and moderate red-wine consumption are associated with numerous cardiovascular and metabolic health benefits [32,51,174].

Metformin [134,139] and resveratrol [28,83,154,258] both generate relaxation when administered directly to isolated pre-contracted arteries; however, an association between the AMPK-activating activity of these compounds and their vasodilatory capacity has not been established.

Dysregulated AMPK activation as a result of age has been observed in a variety of tissues[176,178,246], including arteries[179]. Moreover, vasomotor function is impaired progressively with age [1,129], and is characterized by reduced NO-dependent relaxation [42,199,200,228,253], enhanced endothelium- and cyclooxygenase-dependent contractions [1,42,128,129,230], and general alterations in vascular reactivity (i.e. vasomotor responsiveness can be enhanced or reduced depending on the agonist applied or vascular bed studied) [5,46,140,149]. Most investigations of AMPK-mediated vasomotor effects have been generated using functional arteries of young, healthy animals, but the vasodilatory capacity of AMPK has not been assessed in arteries of aged rats in which vasomotor dysfunction and AMPK dysregulation are known to occur. Therefore, the purpose of this study was to investigate the capacity of AMPK to generate vasorelaxation in arteries of young and aged rats, and to evaluate mechanisms underlying this response. Our main objectives were: 1) to determine the viability of the AMPK-mediated relaxation response in arteries of aged rats; 2) to assess the endothelium-dependency of the relaxation response to acute AMPK activation in pre-contracted aortic rings of young and aged rats; and 3) to elucidate mechanisms responsible for AMPK-mediated vasorelaxation using a pharmacological approach. We also tested whether metformin and resveratrol generate similar vasorelaxation effects to those of AICAR, and whether these effects are associated with AMPK activation in aorta of these animals. We hypothesize that AMPK-mediated relaxation to AICAR will occur through both endothelium-dependent and –independent mechanisms, that the endothelium-dependent component will be NO-mediated in aorta of young rats but NO- and COX-dependent in aged rat aorta, and that relaxation to AICAR will be impaired in arteries of aged animals compared to those of young controls. We also hypothesize that acute exposure of pre-contracted aortic rings to AMPK-activators metformin and resveratrol will generate vasorelaxation that is associated with AMPK activation in these vessels.

5.4 Materials and Methods

Animal care and procedures. All animal-related procedures were approved by the University of Waterloo Animal Care Committee. A total of 30 young male and 30 aged male Sprague Dawley rats obtained from Harlan (Indianapolis, IN) were used for experiments in this study. Animals were group-housed at a constant air temperature (20-21^{o}C) and humidity (~50%) in a 12 h:12 h reverse light:dark cycle. Rats had free access to standard 22/5 Rodent Diet (W) lab chow (Harlan) and tap water. Prior to all experiments, body mass was recorded, animals were anesthetised by sodium pentobarbital injection (50-65 mg/kg, i.p.; Vetoquinol N.-A. Inc., Lavaltrie, QC, Canada) and then euthanized by rapid removal of the heart. Hearts and kidneys from some young and aged animals were dissected and weighed to compare tissue masses.

Vasomotor responses in isolated vessels. Thoracic aortas were excised and 2mm aortic rings were prepared for vascular myography as previously described [74]. In some experiments, the endothelium was removed mechanically as outlined in other experiments [73]. Removal of the endothelium was confirmed by immunoblotting for endothelial nitric oxide synthase in homogenates of aortic rings removed following the functional myography protocol (Supplemental Figure A5-4; we have previously demonstrated that vasorelaxation to endothelium-dependent agonists is absent in E- aortic rings with comparable eNOS content [73]). Rings were mounted onto a vascular myography apparatus (Radnotti, Monrovia, CA), immersed in 37°C Krebs-bicarbonate buffer continuously aerated with 95% O_2/5% CO_2, and data collected as previously described [74]. Gradual stretching to a pre-determined optimal resting tension of 5 g (the resting tension predetermined to elicit maximal active tension responses to KCl in aortic rings of young and aged male Sprague Dawley rats, obtained during pilot experiments and previous studies[74]) was achieved by increasing the tension by 0.5g increments from 1g every 5 minutes, and rings were equilibrated at resting tension for 30 minutes. Two consecutive exposures to 60mM potassium chloride (KCl, with washouts in between) were then performed to ensure contractile integrity of all rings.

Dose-dependent vasorelaxation to AICAR. Following washout of KCl and return to baseline tension, rings with (E+) and without (E-) endothelium were allowed to equilibrate for 30 minutes and then pre-contracted with KCl (40mM) or phenylephrine (PE; $10^{-6.5}$ M). When a stable plateau in tension was achieved, each ring was exposed to increasing concentrations of the AMPK activator AICAR (10^{-6} to 10^{-2} M) to generate dose-dependent relaxation responses. In some experiments, rings were pre-incubated with the NOS inhibitor N_{ω}-nitro-L-arginine methyl ester (L-NAME; 10^{-4} M), the cyclooxygenase inhibitor indomethacin (INDO; 10^{-5} M; inhibits both cyclooxygenase 1 and 2), or both L-NAME and INDO for 30 minutes prior to pre-contraction in order to pharmacologically dissect mechanisms responsible for AICAR-mediated relaxation responses. The dose range for AICAR was based on prior experiments examining rodent aorta [73,82].

Dose-dependent vasorelaxation to metformin and resveratrol. After undergoing the same set-up protocol used prior to the AICAR dose-response curve experiments, E- aortic rings from young and aged rats were pre-contracted with PE ($10^{-6.5}$M), and then exposed to increasing concentrations of either metformin (10^{-6} to $10^{-1.5}$M) or resveratrol (10^{-6} to $10^{-4.5}$M) to generate dose-response curves. These dosage ranges were adapted from previous work in the literature [139] and from our own pilot work.

Immunoblot analysis of protein content and phosphorylation levels. Tissue for immunblotting was obtained from three independent experiments: 1) Aortic rings from young and aged animals were prepared and mounted on the vascular myography apparatus at optimal tension as described above. After 30 minutes of equilibration, rings were removed, snap frozen in liquid nitrogen, and stored at -80^{o}C for comparison of basal protein content and phosphorylation levels in young and aged vessels. 2) When a stable response was obtained at the final [AICAR] (10^{-2}M) in rings used for the functional protocol (pre-incubated with no drug), rings were quickly and carefully removed from the myography apparatus, snap frozen and stored at -80^{o}C for immunoblotting. To generate control rings for comparison against those treated with AICAR, rings were pre-contracted with KCL only (not exposed to AICAR) and allowed to remain contracted for the same duration as the AICAR DRC protocol before being collected and snap

frozen for immunoblotting to compare total protein and phosphorylation of rings treated with AICAR versus no AICAR. 3) To assess AMPK activation induced by metformin and resveratrol, E- rings of aged rats were collected upon completion of metformin or resveratrol functional dose-response curves for immunoblotting as described above for the AICAR experiments. Some E- rings were also collected following AICAR dose-response curves or no drug (pre-contraction with PE only, control) for comparison. Rings for all treatment groups (controls, AICAR, metformin and resveratrol) were obtained from a single rat for each experiment. Sample preparation and immunoblotting procedures were performed as described previously [74]. Briefly, 30µg of protein was loaded per well in polyacrylamide gels, and membranes were ponceau stained to confirm consistent protein loading across lanes.

Drugs, Chemicals and Antibodies. All drugs and chemicals were purchased from either Sigma-Aldrich (St. Louis, MO) or Bioshop Canada Inc. (Burlington, ON, Canada), with the exception of AICAR which was purchased from Toronto Research Chemicals (Toronto, ON, Canada). For immunoblotting experiments, primary antibodies specific for the α subunit of AMPK (recognizing both α1 and α2 subunit isoforms, 1:500 dilution) and P(Thr^{172})-AMPK (1:1000 dilution), acetyl-CoA carboxylase (1:250), P(Ser^{79})-ACC (1:500) and P(Ser1177)-eNOS (1:3000) were obtained from Cell Signalling Technology (via New England Biolabs, Pickering, ON, Canada). eNOS (1:750) was from BD Biosciences (Mississauga, ON, Canada). Horse-radish-peroxide conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Data Analyses. Values are reported as mean ± s.e.m., with *n* referring to the number of animals per group. For comparisons consisting of more than two treatment conditions or groups, one- or two-way ANOVAs with Tukey's or Bonferroni post-hoc analyses were conducted to evaluate within or between group comparisons. A two-tailed Student's *t*-test was used for comparisons involving only two groups. Differences were considered significant if $P < 0.05$. All statistical analyses were performed using SAS (SAS Institute, Cary, NC) or GraphPad Prism 4 analysis software (La Jolla, CA).

5.5 Results

Animal Characteristics. The physical characteristics of the animals used in this study are reported in Table 5-1. Young and aged rats were ~ 11 and ~ 89 weeks of age respectively at the time of experiment (Table 5-1). The spontaneous mortality rate of male Sprague-Dawley rats used for the older cohort in this study is ~ 20% (Harlan). There were no differences in the heart or ventricle to body mass ratios between young versus aged animals, signifying the absence of confounding gross cardiovascular pathology. Two aged animals with abnormal heart masses (likely due to heart failure or pulmonary pathology) were excluded from the study.

Basal AMPK activation in aorta of young and aged Sprague-Dawley rats.

Basal AMPK activation is blunted in aortic rings of aged versus young rats. Phosphorylation of AMPK at threonine 172, a modification required for enzyme activation [99,103], and phosphorylation of acetyl-CoA carboxylase (ACC) at serine 79, a well-established downstream target of AMPK activity [99,152], were evaluated as surrogate markers of AMPK activation. AMPK-α and ACC total protein content did not differ in aorta of the two groups (Figure 5-1). In contrast, both P(Thr172)-AMPK and P(Ser79)-ACC were ~ 60% lower in untreated aortic rings of aged versus young rats (Figure 5-1), demonstrating depressed baseline AMPK activation in vessels of the older animals.

Functional responses of pre-contracted aortic rings from young and aged rats to AICAR.

AICAR generates both endothelium-dependent and endothelium-independent relaxation in aortic rings from young and aged rats. Aortic rings of young rats with intact endothelium relaxed from KCl pre-contracted tension in a dose-dependent manner in response to increasing [AICAR], with relaxation reaching a maximum of 95±3% of pre-contracted tension at 10^{-2}M AICAR (Figure 5-2A). Removal of the endothelium in young rings (to expose the response of vascular smooth muscle alone), blunted relaxation to AICAR at all concentrations, and maximal relaxation was only 76±4% of pre-contracted tension at 10^{-2}M (Figure 5-2A). In aortic rings of aged rats, relaxation to AICAR reached ~90 % at 10^{-2}M in E+ aortic

rings, and this response was attenuated in the absence of endothelium, with relaxation responses of E- rings blunted at 10^{-6}M, 10^{-5}M, 10^{-4}M, and 10^{-3}M AICAR compared to E+ vessels (Figure 5-2B). Relaxation to AICAR was enhanced in aortic rings of aged versus young rats both with endothelium ($P < 0.001$ at 10^{-5}M, and 10^{-4}M $P < 0.05$ at 10^{-3}M versus young) and without endothelium ($P < 0.001$ at $10^{-2.5}$M, and $P < 0.01$at 10^{-2}M versus young, Figures 5-2A and B). Maintenance of stable tension generation by time control rings (time control rings pre-contracted with 40mM KCl but *not* exposed to AICAR) is demonstrated for young (Figure 5-2A) and aged (Figure 5-2B) E+ and E- rings. Pre-contracted tension to 40mM KCl was not significantly different between rings of young versus aged rats either with or without endothelium, but E- rings of aged animals developed greater tension than those of aged E+ CON (Table 5-2). Similar functional responses to those generated by AICAR in KCl pre-contracted rings were observed across all groups when rings were pre-contracted with α-adrenergic agonist PE ($10^{-6.5}$M) and then exposed to AICAR concentrations (Figure A5-1 and Table A5-1).

To more rigorously evaluate the contribution of the endothelium and VSM to the relaxation generated by AICAR, we determined the percentage of the total relaxation response accounted for by the endothelium in both young and aged aortic rings at 10^{-4}M, 10^{-3}M, and 10^{-2}M [AICAR] (Figure 5-2C). In rings of young rats, 49 ± 8% of the total relaxation to AICAR at 10^{-4}M was endothelium-dependent, a proportion that was decreased to 22 ± 4% at 10^{-2}M AICAR ($P < 0.01$ versus 10^{-4}M AICAR; Figure 5-2C). In aged rat aorta, endothelium-dependent relaxation comprised 67 ± 2% of the total relaxation response at 10^{-4}M, but only 28 ± 2% and -5 ± 4% at 10^{-3}M and 10^{-2}M respectively (both $P < 0.001$ versus 10^{-4}M AICAR; Figure 5-2C). The endothelial contribution in aged rings was significantly greater than in those of young at 10^{-4}M AICAR, but was significantly less than in young rings at 10^{-2}M AICAR (Figure 5-2C). We also calculated the percentage of the total relaxation response dependent on the endothelium at the concentrations of AICAR that elicited 50% ($logEC_{50}$) and 100% of the maximal relaxation response in E+ rings of young and aged rings (Figure 5-2D). At the $logEC_{50}$ of E+ CON rings (young: -3.18 ± 0.08 and aged -3.68 ± 0.25, $P = 0.1837$; mean interpolated values derived from individual ring dose-response

curves), the component of relaxation accounted for by the endothelium was greater in aged versus young rings (Figure 5-2D). At maximal relaxation from pre-contracted tension in E+ CON rings (maximal relaxation for E+ rings, young: 98 ±3 % and aged 96 ± 6%, P = 0.8044), 23 ± 4% of the relaxation to AICAR was endothelium dependent in aorta from young rats, but in rings from aged rats only 4 ± 4% was attributed to the endothelium (almost entirely endothelium-independent, Figure 5-2D).

Pharmacological dissection of mechanisms mediating vasomotor responses to AICAR.

Endothelium-dependent relaxation to AICAR is NO-mediated in aortic rings of both young and aged rats. Aortic rings were pre-incubated with L-NAME, indomethacin (INDO), or L-NAME + INDO to determine the involvement of NO- and cyclooxygenase-dependent signalling in the vasodilatory responses to AICAR. Following drug pre-incubation and pre-contraction with KCL, relaxation to AICAR was blunted in E+ aortic rings of young and old rats treated with either L-NAME or L-NAME + INDO compared the no drug controls (Figure 5-3A and B), to levels not different from those of E- rings (Figure 5-3C and D). Indomethacin alone did not alter relaxation compared to the E+ control condition at any AICAR concentration in rings or young or aged rats (Figure 5-3A and B). In E- rings, relaxation to AICAR was not affected by L-NAME, INDO or L-NAME + INDO (Figure 5-3C and D). Pre-contraction to KCl was not significantly affected by any drug condition relative to the no drug controls in rings of either young or aged rats (Table 5-2). Similar results were obtained in experiments where rings were pre-contracted with PE rather that KCl (Figure A5-2, Table A5-1).

Confirmation of AMPK activation in aortic rings of young and aged rats following treatment with AICAR.

AMPK activation by AICAR was confirmed in aortic rings of young and aged rats. Both P(Thr172)-AMPK (Figure 5-4A & B) and P(Ser79)-ACC (Figure 5-4C &D) were significantly elevated following exposure to the AICAR dose-response curve protocol in E+ and E- rings from young and aged rats compared to their respective E+ controls, confirming efficacy of AICAR treatment on AMPK

activation in these vessels. The fold-increases in P(Thr172)-AMPK (Figure 5-4E) and P(Ser79)-ACC (Figure 5-4F) were not different in aortic rings of aged and young rats with or without endothelium (no main effects or individual group differences), demonstrating that the relative degree of AMPK activation by AICAR was similar between groups. When P(Thr172)-AMPK and P(Ser79)-ACC were normalized to baseline values (from Figure 5-1), and main effects were significant for age (P-AMPK: P = 0.0022, P-ACC: P = 0.0054) and drug treatment (P-AMPK: P = 0.0054, P-ACC: P = 0.0229) (Figure 5-4G and H).

Functional responses of pre-contracted aortic rings from young and aged rats to metformin and resveratrol.

Since the majority of the vasodilatory response to AICAR was endothelium-*in*dependent (involving the vascular smooth muscle only), and we were interested in testing the general vasodilatory capacity of metformin and resveratrol, we performed these experiments in E- rings only (experiments were also performed using AICAR for comparison). AICAR generated relaxation of young and aged E- rings that reached a maximum of 62±2% and 109±4% of pre-contracted tension to phenylephrine respectively at 10^{-2}M AICAR (Figure 5-5A). The time control rings pre-contracted with PE but not exposed to AICAR demonstrate the ability of these rings to maintain tension to this agonist over the experimental protocol (Figure 5-5A). In contrast to the robust relaxation response observed in E- rings of young and aged animals to increasing [AICAR], maximal relaxation to metformin was only 17±1% of pre-contracted tension in E- rings of young animals (that did not begin until $10^{-2.5}$M metformin) and 46±5% in E- vessels of aged rats (Figure 5-5B). Resveratrol generated substantial relaxation in both young and aged E- rings, with maximal relaxation reaching ~ 100% by 10^{-4}M [resveratrol] in both groups (Figure 5-5C). As with AICAR (Figure 5-5A), relaxation was enhanced to both metformin (Figure 5-5B) and resveratrol (Figure 5-5C) in rings from aged versus those of young animals.

AMPK activation in aortic rings of aged rats to metformin and resveratrol.

Based on previous experiments with metformin and resveratrol, we expected to find increased

AMPK activation following exposure to these agents. In E- rings of aged rats rings removed immediately following the functional dose-response curve protocols, exposure to metformin generated ~ 2-fold increase in P(Thr172)-AMPK (Figure 5-6A) and a concomitant increase in P(Ser79)-ACC (Figure 5-6B). Resveratrol failed to generate detectable elevations in either P(Thr172)-AMPK (Figure 5-6A) or P(Ser79)-ACC (Figure 5-6B) compared to no drug time controls. By contrast, AICAR robustly increased both P(Thr172)-AMPK (Figure 5-6A) and P(Ser79)-ACC (Figure 5-6B) in E- aortic rings of aged animals.

Table 5-1. *Physical characteristics of young and aged male Sprague-Dawley rats.*

	Young	Aged	*P*
Age, wks	10.7 ± 0.3	88.8 ± 0.7	< 0.0001
Whole body mass (BM), g	328 ± 6	552 ± 12	< 0.0001
Tissue Masses			
Left Ventricle, mg	889 ± 14	1368 ± 73	< 0.0001
Left Ventricle/BM (mg/g BM)	2.97 ± 0.06	2.67 ± 0.26	0.3358
Heart, mg	1110 ± 14	1628 ± 82	< 0.0001
Heart/BM, (mg/g)	3.71 ± 0.07	3.16 ± 0.29	0.1266
Kidney (mg)	1029 ± 54	2129 ± 99	0.0007
Kidney/BM (mg/g)	3.94 ± 0.08	4.05 ± 0.16	0.7740

Hearts and kidneys were dissected immediately following their removal from anesthetized animals to obtain tissue masses (n = 6-9). n=29 for all other measures. Due to differences in body weight with age, values were expressed both in absolute terms and relative to whole body mass. Data represents means ± s.e.m.

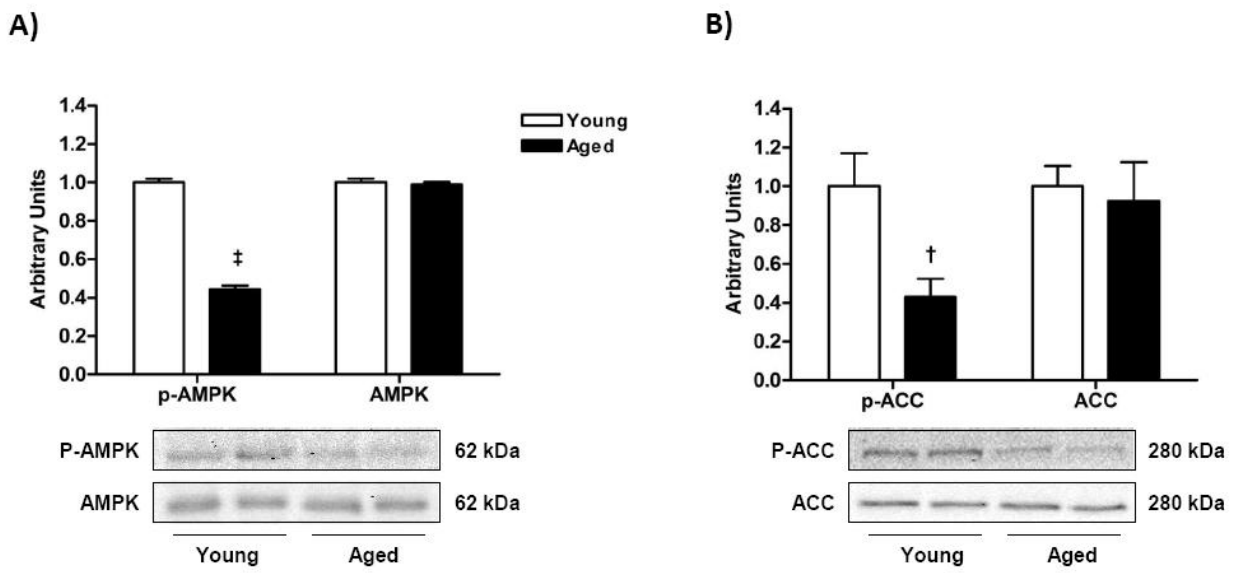

Figure 5-1. Basal AMPK activation is depressed in aorta of aged versus young male Sprague-Dawley rats. Thoracic aortas were removed from anesthetized animals, and aortic rings were prepared and mounted on a vascular myography apparatus. After undergoing a standard myography start-up protocol, rings were left to equilibrate for 30 minutes before being carefully removed from the apparatus, snap frozen and stored for immunoblotting. **A)** Phosphorylation of AMPK activation site Thr^{172} and AMPK-α total protein content and **B)** phosphorylation of AMPK downstream target Ser^{79} on acetyl-CoA carboxylase (ACC) and ACC total protein content were assessed as surrogate markers of AMPK activation. Data is expressed as mean ± sem. Values from densitometry analyses were normalized such that the mean of the young group values are equal to 1 normalized unit. All samples were run in duplicate and $n = 4$ for young and aged rats in both E+ and E- groups. † $P < 0.01$, ‡ $P < 0.001$ vs young.

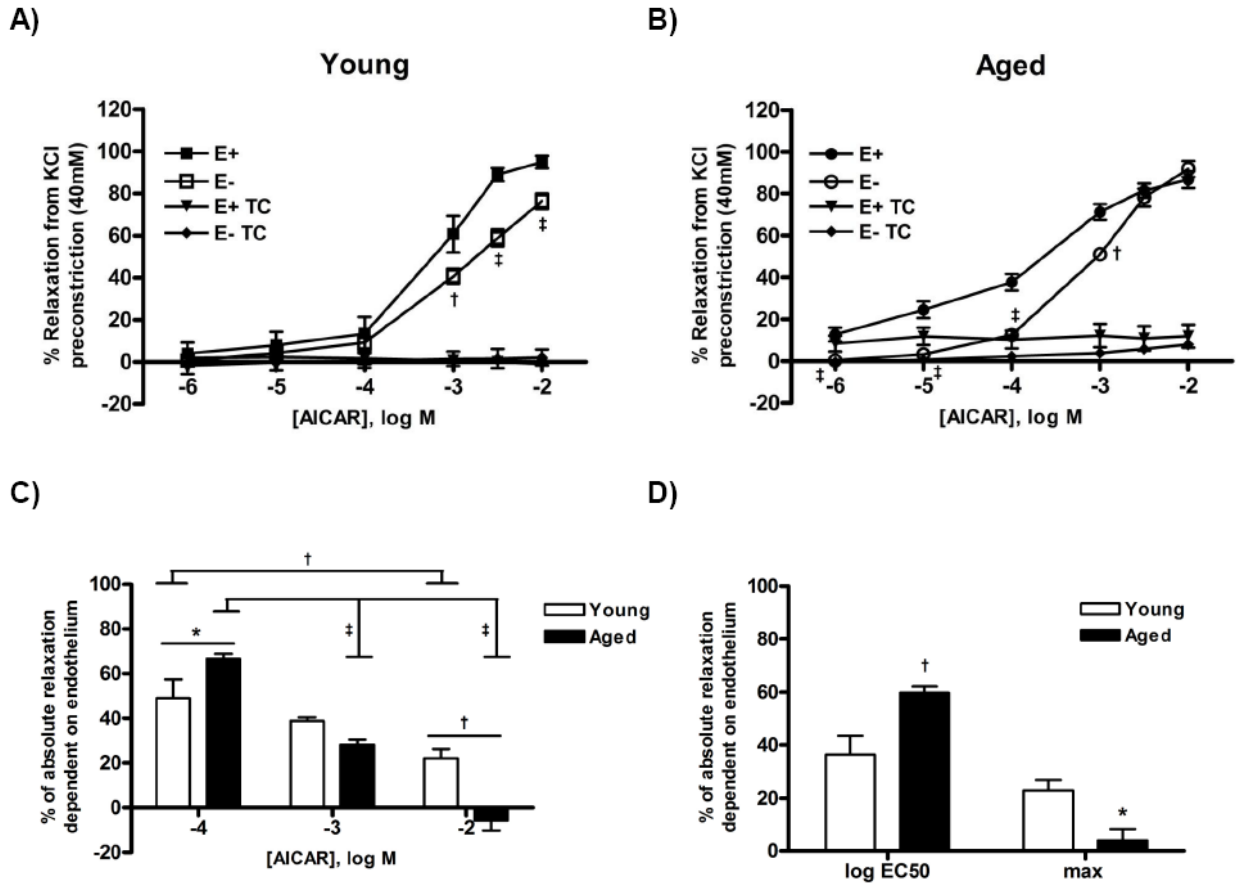

Figure 5-2. Vasorelaxation to AICAR is both endothelium-dependent and –independent in aorta of young and aged rats. Relaxation responses of aortic rings from (**A**) young and (**B**) aged rats with (E+) or without (E-) endothelium following pre-contraction with potassium chloride (KCl) to increasing concentrations of AICAR. Some rings were contracted with KCl but not exposed to AICAR (time controls; TC) to demonstrate the ability of rings to maintain stable tension for the duration of the experiments. Responses are expressed as mean percent relaxation from KCl pre-contracted tension. **C)** The proportion of the relaxation response accounted for by the endothelium-dependent component at AICAR concentrations 10^{-4}M, 10^{-3}M, and 10^{-2}M in aorta of young and aged rats. **D)** The proportion of the relaxation response accounted for by the endothelium-dependent component of relaxation at the log EC_{50} and maximal relaxation of E+ rings. *n* = 4-6 rings per group from different animals. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ versus E+ in A) and B), * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ versus [AICAR] 10^{-4}M and young in C) and D). E+ TC and E- TC are different ($P < 0.001$) from E+ CON in both young and old (symbols have been excluded above for clarity), and do not vary over the range of AICAR concentrations.

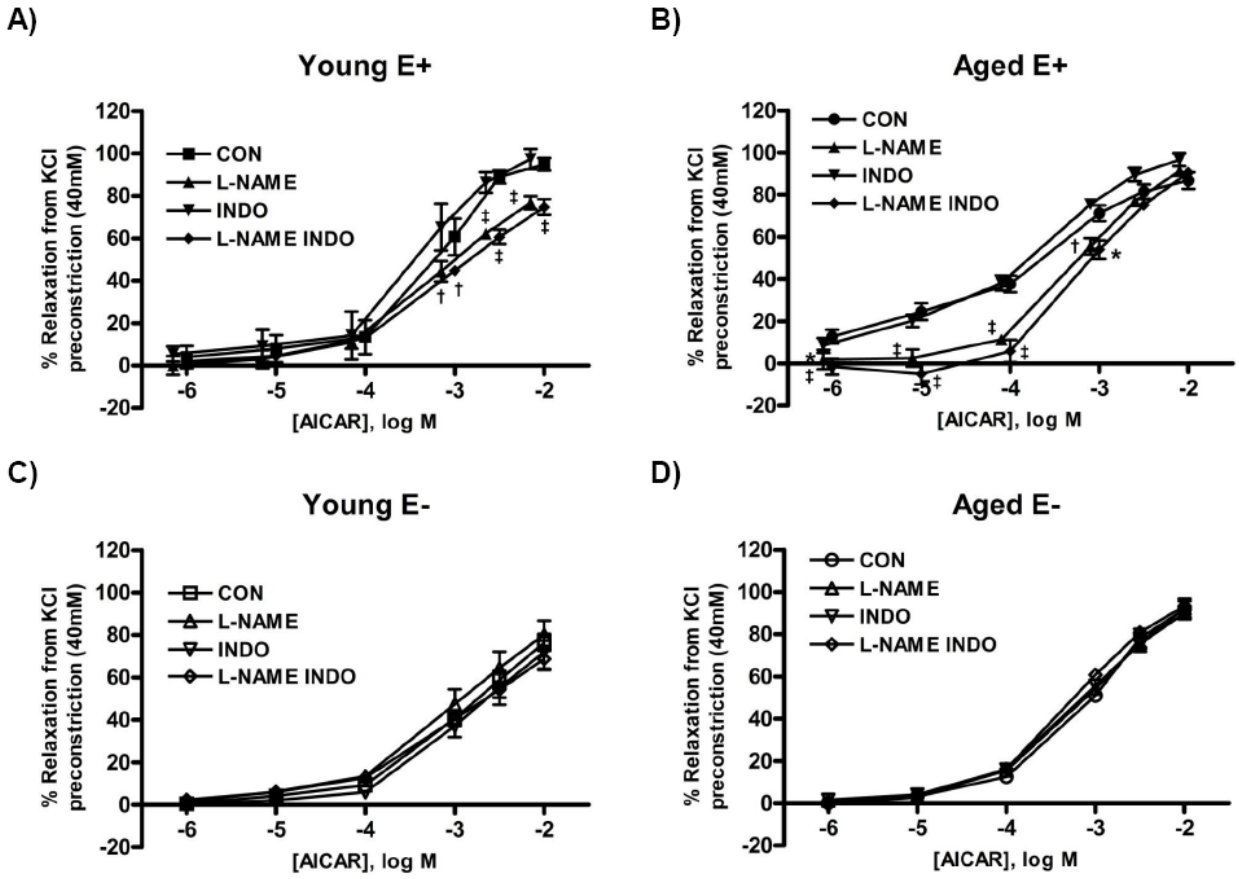

Figure 5-3. Mechanisms of AICAR-mediated relaxation in aortic rings from young and aged rats. Aortic rings from (**A,C**) young and (**B,D**) aged rats were incubated with either NOS inhibitor L-NAME, cyclooxygenase inhibitor indomethacin (INDO), or L-NAME + INDO to determine the NO- and endoperoxide-dependency of relaxation responses to AICAR. Percent relaxation from pre-contracted tension to potassium chloride (KCl) is displayed in rings from young and aged rats (**A, B**) with (E+) and (**C,D**) without (E-) endothelium. Young and Aged E+ and E- data from Figure 2 has been repeated here for comparison (no drug pre-incubation, CON groups above). Each treatment was performed in aortic rings from every animal. *n* = 4-11 rings per group from different animals. * $P < 0.05$, † $P < 0.01$ and ‡ $P < 0.001$ versus CON.

Table 5-2. *Pre-contracted tension of young and aged aortic rings to 40mM KCl*

	Young (g)	Aged (g)
E+ CON	1.8 ± 0.2	1.7 ± 0.2
E+ L-NAME	2.6 ± 0.2	2.7 ± 0.2
E+ INDO	1.7 ± 0.3	1.6 ± 0.3
E+ L-NAME INDO	2.5 ± 0.2	2.0 ± 0.2
E- CON	2.4 ± 0.2	3.3 ± 0.2‡
E- L-NAME	2.5 ± 0.2	3.0 ± 0.1†
E- INDO	2.4 ± 0.2	3.4 ± 0.3†
E- L-NAME INDO	2.4 ± 0.1	3.0 ± 0.2†

Data represents means ± s.e.m. n= 4 -11 rings per group. † $P < 0.01$, ‡ $P < 0.001$ versus E+ CON. There were no significant differences between young and aged rings of the same treatment conditions.

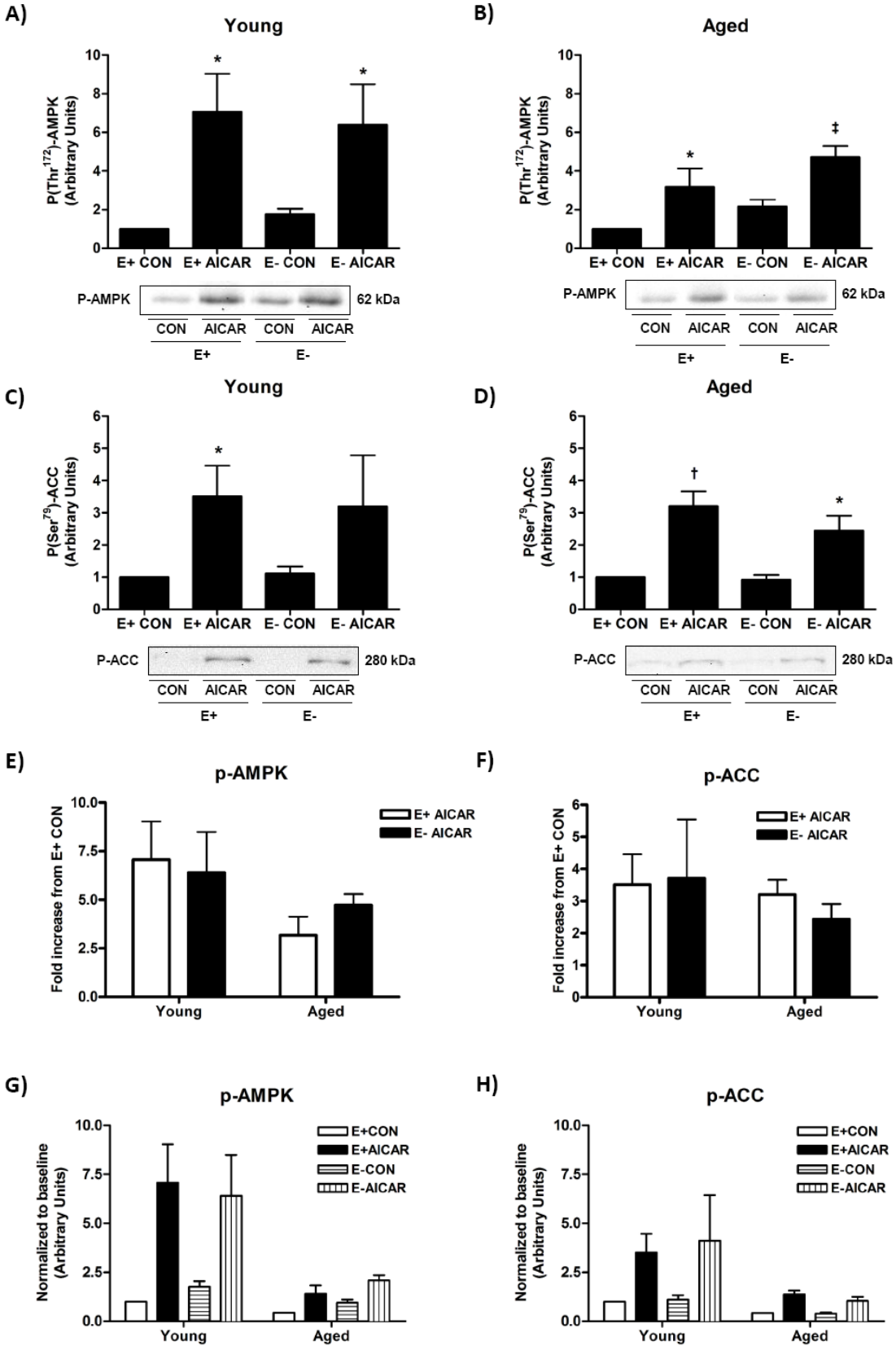
A)
Young
P(Thr172)-AMPK (Arbitrary Units)
E+ CON
E+ AICAR
E- CON
E- AICAR
P-AMPK
62 kDa
CON
AICAR
CON
AICAR
E+
E-
B)
Aged
C)
Young
P(Ser79)-ACC (Arbitrary Units)
P-ACC
280 kDa
D)
Aged
E)
p-AMPK
Fold increase from E+ CON
E+ AICAR
E- AICAR
Young
Aged
F)
p-ACC
G)
p-AMPK
Normalized to baseline (Arbitrary Units)
E+CON
E+AICAR
E-CON
E-AICAR
H)
p-ACC

Figure 5-4. AMPK activation in aortic rings of young and aged rats following the AICAR dose-response curve protocol. Aortic rings with (E+) and without (E-) endothelium were removed from the myography apparatus immediately following completion of the AICAR dose-response curve, snap frozen and stored at -80^{o}C for immunoblotting analyses. Rings pre-contracted but not treated with AICAR (time controls, CON) were also obtained for control comparisons. Phosphorylation of AMPK activation site threonine 172 (**A, B**) and phosphorylation of AMPK downstream target serine 79 on acetyl-CoA carboxylase (**C, D**) were assessed on membranes prepared using aortic ring homogenates of young and aged rats. Data is expressed as mean ± sem. Values from densitometry analyses were normalized such that the mean of the E+ CON group values are equal to 1 normalized unit. Panels **E** and **F** compare the fold increase in P(Thr172)-AMPK and P(Ser79)-ACC respectively over the E+ CON groups in E+ and E- aortic rings of young and aged animals treated with AICAR. There were no main effects or individual group differences in the fold increase of either P(Thr172)-AMPK or P(Ser79)-ACC. In Panels **G** and **H**, data were normalized to baseline P(Thr172)-AMPK and P(Ser79)-ACC (values from Figure 5-1), and main effects were significant for age (P-AMPK: P = 0.0022, P-ACC: P = 0.0054) and drug (P-AMPK: P = 0.0054, P-ACC: P = 0.0229). *n* = 6-8, for young and old rats in both E+ and E- groups (all samples run in duplictate). * P < 0.05, † P < 0.01, ‡ P < 0.001 vs E+ CON.

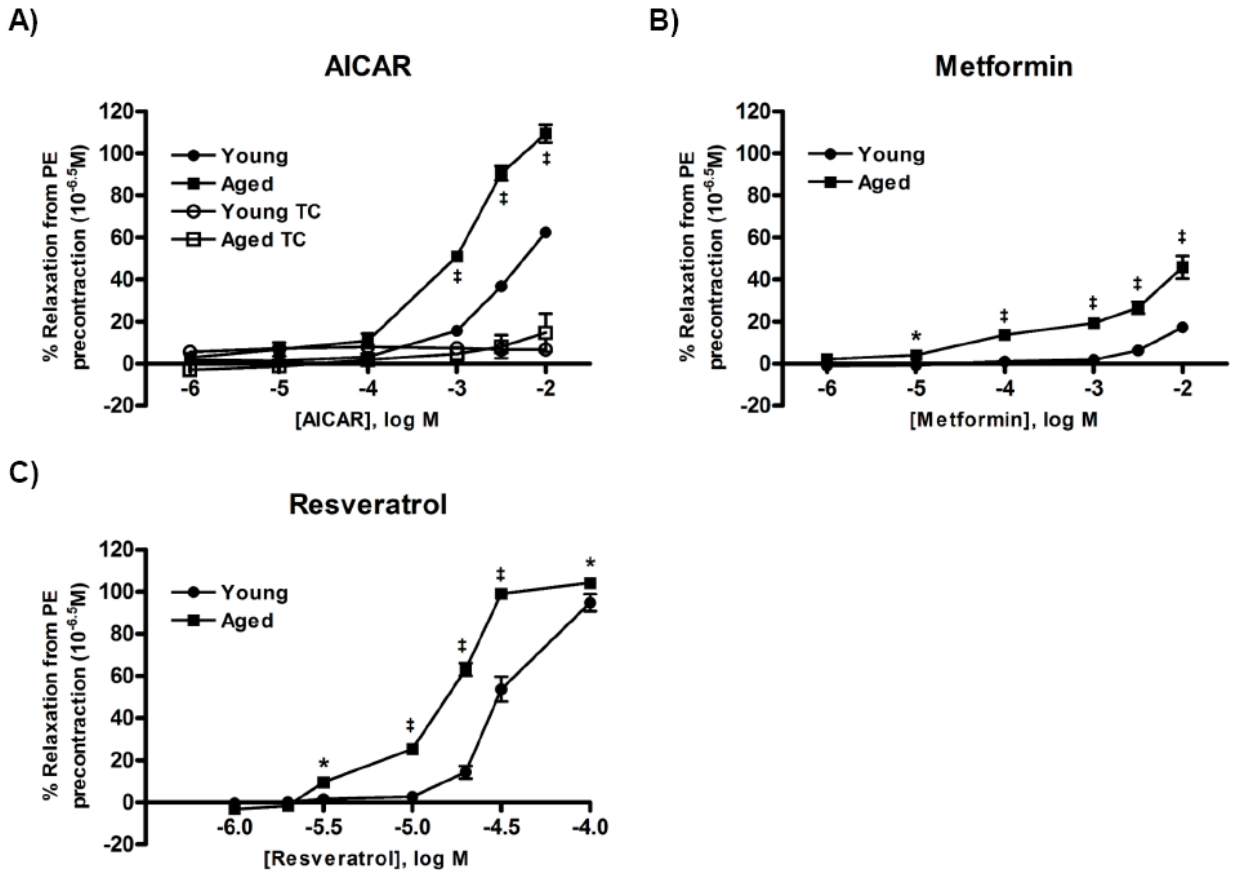

Figure 5-5. Dose-dependent vasorelaxation to AICAR, metformin or resveratrol in aortic rings of young and aged rats. Aortic rings without endothelium (E-) from young and aged rats were pre-contracted with phenylephrine (PE; $10^{-6.5}$M) and then exposed to increasing concentrations of AICAR **(A)**, metformin **(B)** or resveratrol **(C)**. Time controls (rings pre-contracted with PE but not exposed to vasodilatory agonists) are shown in **A)** to illustrate the ability of rings to maintain stable pre-contraction. All treatments elicited significant relaxation from baseline ($P < 0.0001$) by maximal concentrations of AICAR, metformin and resveratrol in aortic rings of both young and aged rats, but significance symbols have been excluded for clarity. Responses are expressed as mean percent relaxation from PE pre-contracted tension. *n* = 6-14 rings per group from different animals. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ versus Young.

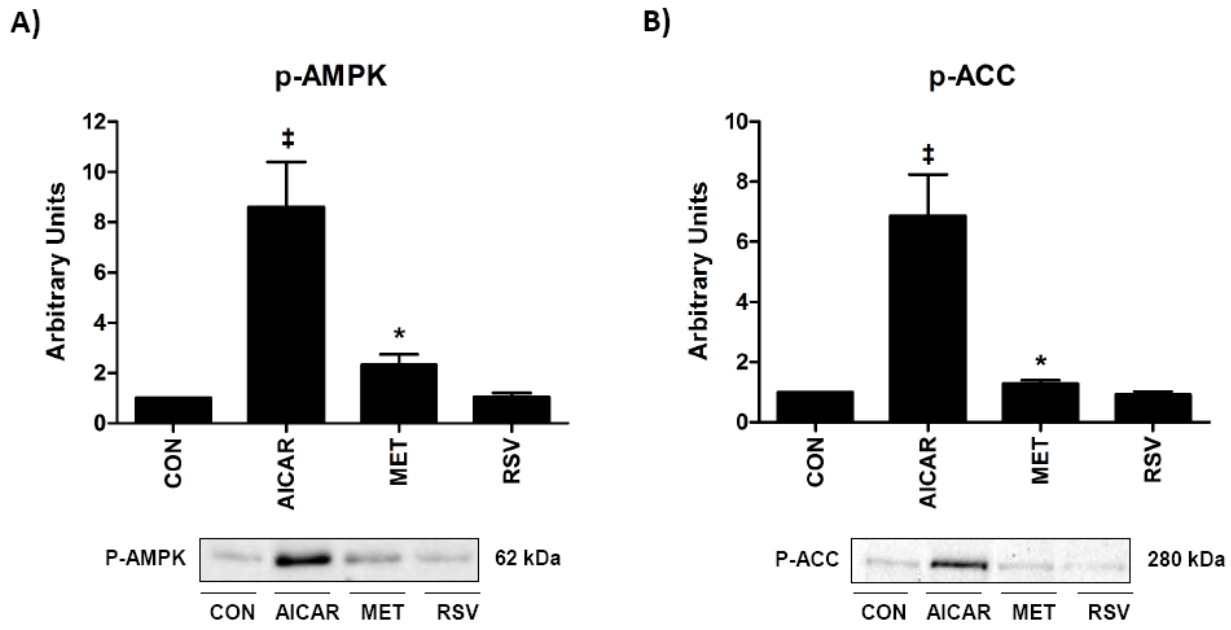

Figure 5-6. AMPK activation in aortic rings of aged rats following AICAR, metformin and resveratrol dose-response curve protocols. Aortic rings without endothelium (E-) were removed from the myography apparatus immediately following completion of the dose-response curve protocols using AICAR, metformin (MET) or resveratrol (RSV), snap frozen and stored at -80^{o}C for immunoblotting analyses. Some rings pre-contracted but not treated with AICAR, metformin or resveratrol (time controls, CON) were obtained for control comparison. **A)** Phosphorylation of AMPK activation site Thr172 and **B)** phosphorylation of AMPK downstream target Ser79 on acetyl-CoA carboxylase (ACC) were assessed in homogenates of aortic rings from young and aged rats. Data is expressed as mean ± sem. Values from densitometry analyses were normalized such that the mean of the E+ CON group values are equal to 1 normalized unit. *n* = 6-8 rats per group (all samples run in duplictate). * $P < 0.05$, ‡ $P < 0.001$ vs E+ CON.

5.6 Discussion

The main findings of this study are that: 1) AMPK-mediated relaxation to AICAR is intact and enhanced in aorta of young and aged rats, 2) that this response is partly endothelium-dependent and partly endothelium-independent, and 3) and that endothelium-dependent relaxation to AICAR is NO-dependent in aortic rings of both young and aged animals. Relaxation of pre-contracted aortic rings to metformin was not as robust as that generated to AICAR in aortic rings of aged rats, corresponding to weaker activation of AMPK in these vessels by metformin. Although relaxation of pre-contracted arteries was produced by resveratrol, this compound did not activate AMPK in aortic rings from our aged animals. This is the first examination of AMPK-mediated relaxation in aged arteries.

AMPK activation becomes dysregulated with age [150,176,178,179,246,269]. Herein we demonstrate that basal AMPK activation (P(Thr172)-AMPK and P(Ser79)-ACC) is lower in aorta of the aged animals compared to their younger counterparts (Figure 5-1). Others have also observed depressed AMPK activity in aorta [179] and gastrocnemius muscle [176] of aged rats, although this trend is not universal, and different reports have shown that AMPK activation is enhanced in murine aorta [269], liver [150] and rat plantaris muscle [219] or not changed in rat soleus [219] with age. Evaluations of the capacity to activate AMPK in aging tissue are also inconsistent, showing that the level of AMPK activation is impaired [178], enhanced [218,219], or unaltered [176,218] with age depending on the tissue and stimulus applied (i.e. AICAR, exercise). Although there was a trend towards a lower fold-increase in AMPK activation in rings of aged animals treated with AICAR versus those of young (P(Thr172)-AMPK and P(Ser79)-ACC), this effect did not reach statistical significance in our study (Figure 5-4E and F). There is no clear explanation to reconcile the discrepancies in currently available data, although factors such as tissue, stimulus and species differences or the stage of progression through the aging process likely account for some of the variation. More research will be necessary to reconcile the disparity in these findings and to provide insight regarding the consequences of AMPK dysregulation in aging tissue.

Relaxation of aortic rings from both young and aged rats to AICAR is mediated partly by the endothelium and in part by direct effects on the vascular smooth muscle (Figure 5-2A and B). These results are consistent with our previous observations in healthy and hypertensive rat aorta[73], and with observations by others that have demonstrated endothelium- [13,15] and vascular smooth muscle-dependent [82,181] relaxation to AMPK-activating stimuli. Further analyses of the relaxation responses to AICAR (Figure 5-2C & D) demonstrate that the relative contribution of the endothelium to the vasodilatory response to AMPK activation is dependent on the strength of the stimulus (i.e. relaxation at lower [AICAR] or submaximal relaxation is more endothelium-dependent than at higher [AICAR] or maximal relaxation in aorta of young and aged rats), and highlight the necessity for interpreting the endothelial and vascular smooth muscle contributions to functional responses in the context of the potency of the stimulus applied. The enhanced relaxation response to AICAR exhibited in aortic rings of aged versus young rats (both with and without endothelium) may be unmasking a compensatory adaptation of aged arteries in attempt to maintain responsiveness in the presence of other dysfunctional vasomotor pathways, or alternatively, could represent the outcome of a dysregulated vasomotor signalling process(es). The functional ramifications of this observation in a physiological context are unclear and will require further study to determine whether the enhanced vasodilation of aged arteries to AMPK stimulation is a beneficial adaptation or a manifestation of dysfunction in aging arteries.

Examination of the mechanisms responsible for the endothelium-dependent component of the vasodilation to AICAR revealed that this response is NO-dependent in aortic rings of both young and aged rats, as it was abolished in both young and aged E+ vessels treated with L-NAME (Figure 5-3). A number of other studies have shown that the endothelium-dependent relaxation to AMPK activation is NO-mediated [13,15,73], and this is consistent with *in vitro* cell culture and biochemical experiments that have suggested a number of different mechanisms through which AMPK may activate endothelial nitric oxide synthase and improve NO bioavailability [27,29,30,44,145]. Activated AMPK could increase NO production/bioavailability by phosphorylating eNOS at activation sites serine 1179 [27,29,44,145] or serine633

[31], improving the associating of eNOS with HSP90 [44,189], promoting deacetylation of eNOS by SIRT1[30] or through other yet unidentified mechanisms. Direct relaxation of the vascular smooth muscle by AICAR/AMPK may result from decreased sensitivity of MLCK to intracellular calcium (by phosporylation at serine 815) [116], and/or inhibition of the RhoA-Rho kinase pathway [242], both of which would presumably decrease vessel tone. The contribution of these mechanisms was not evaluated here, and more work is needed to verify which or if any of these possible mechanisms account for the endothelium,-NO-mediated and vascular smooth muscle-dependent functional responses observed *in vitro* in isolated vessels, or *in vivo*. The contribution of the endothelium, NO, and vascular smooth muscle to the vasorelaxation generated by AICAR is independent of the pre-constrictory agent used, as similar results were obtained when vessels were contracted with either KCl (vascular smooth muscle depolarizing agent; Figure 5-3) or phenylephrine (α-adrenergic receptor agonist; Supplemental Figures D1 and D2). We also evaluated the effect of the COX inhibitor indomethacin on AICAR-induced relaxation, as enhanced endothelium- and COX-dependent contractions have been observed in conduit arteries of aged animals [1,128,129], and in previous work we have shown that AICAR induces relaxation in part by suppression of the enhanced endothelium-dependent contractions in aorta of SHR [73]. Inhibition of COX did not alter relaxation to AICAR in aorta of young or aged animals pre-contracted with KCl (Figure 5-3) or phenylephrine (Supplemental Figure D2). However, endothelium- and COX-dependent contractions were not particularly robust in aorta of this cohort of aged rats (Supplemental Figure D3) which may explain the lack of COX-mediated response in aged rat aorta in this study.

Metformin and resveratrol both activate AMPK in a number of tissue types [24,44,160,223,258,263], and stimulate relaxation of isolated arteries in different studies [10,28,134,154]. Herein metformin generated modest relaxation that was associated with a small but significant increase in markers of AMPK activation, and both of these responses were lower in magnitude compared to those generated by AICAR. Modest activation of AMPK with metformin in comparison with that induced by AICAR was not unexpected, as others have observed similar results in rodent aorta [145,258]. Although resveratrol generated

robust vasodilatory responses, no indication of AMPK activation could be found in aortic rings from these experiments, and therefore alternative mechanisms must account for the vasodilation to this agonist in aorta of aged rats. While activation of AMPK by resveratrol has been previously reported in endothelial cells and other tissues, the magnitude of this effect in endothelial cells is only ~2-fold [140], and resveratrol-mediated AMPK activation has not been observed in vascular smooth muscle in any prior studies to our knowledge. In examining the functional responses to these agonists, enhanced relaxation was also observed to metformin or resveratrol in aged versus young vessels (similarly to responses generated by AICAR, Figure 5-2 and Figure 5-5). However, since the robust relaxation to resveratrol occurred in the absence of AMPK activation, it seems as though greater sensitivity of a common downstream signalling target likely accounts for the enhanced vasodilatory propensity of aged arteries rather than a relationship to AMPK activation in particular. Aged arteries often display reduced vasoreactivity in response to agonist stimulation (vasodilatory and vasoconstrictory) [46,140,149], although age-related changes in vascular reactivity are heterogeneous and responses to some agonists are unaltered [5,162] or enhanced [5,265] depending on the vascular bed studied. Further investigation will be required to decipher whether similar findings using AMPK activators are observed in other vascular beds of aged animals.

The findings of this study could have several important implications for the physiological function of arteries *in vivo* and for the vasomotor dysfunction that occurs in aging arteries. Although we activate AMPK pharmacologically *in vitro* here, vascular AMPK can be activated acutely and/or chronically by numerous chemical, physical, and metabolic stimuli that can exist *in situ* such as shear stress [29,265], hypoxia [146], VEGF [136,146], sphingosine-1-phosphate[220], thrombin [202,220], histamine [29], adiponectin [123], hydrogen peroxide [52,118], and by interventions such as exercise [20,181,264] and caloric restriction [243]. *In situ* activation of AMPK by these mechanisms may translate into acute AMPK-mediated vasodilatory events, or support a more tonic vasodilatory phenotype. Since aging arteries display vasomotor dysfunction that can be associated with reduced NO-dependent relaxation and NO

bioavailability[196], and with enhanced endothelium-dependent contractions, it is possible that the capacity to promote vasodilation via improving the activation status of AMPK acutely or chronically *in vivo*, may help offset/alleviate some of the characteristics associated with vasomotor dysfunction that manifest in arteries with age. Enhanced AMPK activation has been associated with improved vasomotor function in models where vascular dysfunction exists including in arteries of Otuska Long-Evans Tokushima fatty rats (a model of type II diabetes) [141,245] and in mouse aortas exposed to hyperglycemia [13]. Therefore, AMPK may have potential as a therapeutic target for improving vascular function, and may be one of the mechanisms by which interventions such as exercise and caloric restriction that could improve the health of aging arteries.

In summary, acute AMPK activation generates robust endothelium-dependent and endothelium-independent relaxation in aorta of both young and aged rats, the endothelial component of which is NO-mediated. Further study will be required to specify the intracellular signalling mechanisms that account for the endothelial and vascular smooth muscle relaxation to acute activation of AMPK, whether or not acute activation of AMPK is a mechanism of immediate relaxation *in vivo*, and if chronic AMPK activation will provide and sustained benefit/relief of the vasomotor dysfunction characteristic of aged arteries.

5.7 Addendum to Study 4:

The addendum includes data collected in conjunction with this study but not included as part of the manuscript. These items include:

Figure A5-1: Vasorelaxation to AICAR is both endothelium-dependent and –independent in aortic rings from young and aged rats pre-contracted with phenylephrine. These results demonstrate that vasorelaxation responses generated in the presence of phenylephrine pre-contraction are similar to those generated with potassium chloride pre-contraction.

Figure A5-2: Mechanisms of AICAR-mediated relaxation in aortic rings from young and aged rats pre-contracted with phenylephrine. These results demonstrate that vasorelaxation responses generated in the presence of phenylephrine pre-contraction are similar to those generated with potassium chloride pre-contraction.

Table A5-1: Pre-contracted tension of young and aged aortic rings to phenylephine.

Figure A5-3: Endothelium-dependent contraction to acetylcholine in aorta of the aged male Sprague-Dawley rats. These data demonstrate minimal endothelium-dependent contractions to acetylcholine in aorta of aged rats used in this study

Figure A5-4: eNOS total protein content and phosphorylation of eNOS activation site serine 1177 following exposure to AICAR in aortic rings of young and aged rats collected immediately following the AICAR dose-response vascular myography protocols. These data confirm removal of the endothelium in E- aortic rings used for the functional myography protocols. They also demonstrate (in a representative blot) that AICAR does not affect P(Ser1177)-eNOS in young and aged aortic rings.

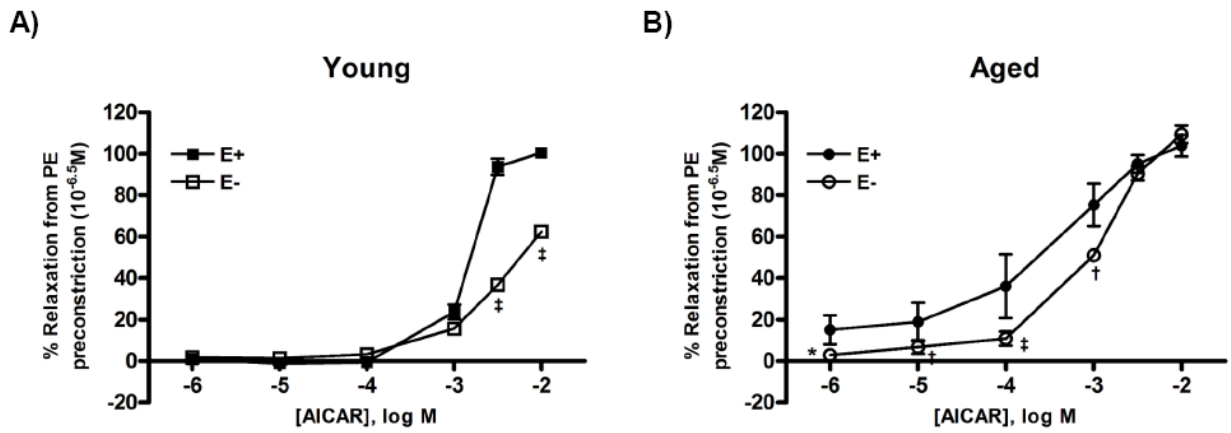

Figure A5-1. Vasorelaxation to AICAR is both endothelium-dependent and –independent in aortic rings from young and aged rats pre-contracted with phenylephrine. Relaxation responses of aortic rings from (**A**) young and (**B**) aged rats with (E+) or without (E-) endothelium following pre-contraction with phenylephrine (PE) to increasing concentrations of AICAR. Responses are expressed as mean percent relaxation from PE pre-contracted tension. *n* = 6-11 rings per group from different animals. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ versus E+.

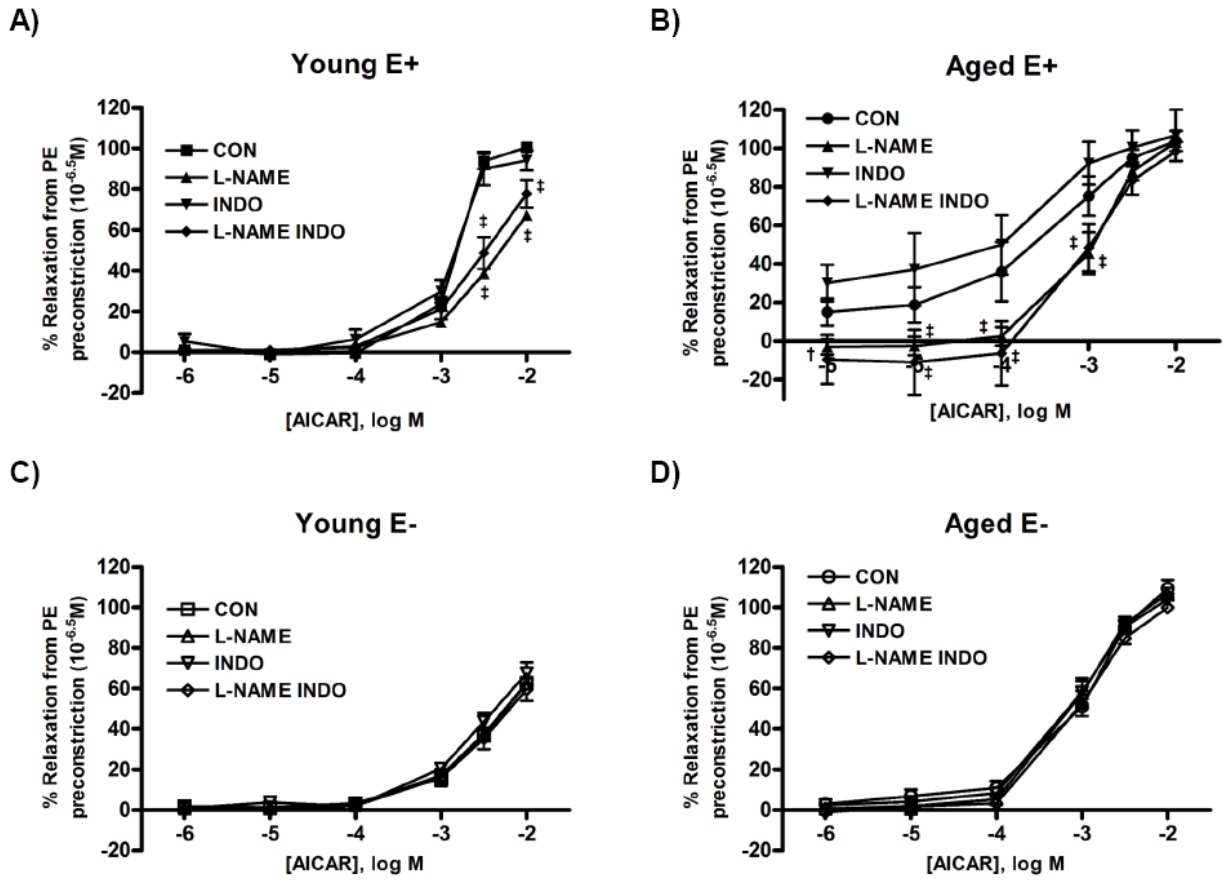

Figure A5-2. Mechanisms of AICAR-mediated relaxation in aortic rings from young and aged rats pre-contracted with phenylephrine. Aortic rings from (**A,C**) young and (**B,D**) aged rats were incubated with either NOS inhibitor L-NAME, cyclooxygenase inhibitor indomethacin (INDO), or L-NAME + INDO to determine the NO- and endoperoxide-dependency of relaxation responses to AICAR. Percent relaxation from pre-contracted tension to phenylephrine (PE) is displayed in rings from young and aged rats (**A, B**) with (E+) and (**C,D**) without (E-) endothelium. Young and Aged E+ and E- data from Figure D-1 has been repeated here for comparison (no drug pre-incubation, CON groups above). Each treatment was performed in aortic rings from every animal. *n* = 4-11 rings per group from different animals. † $P < 0.01$ and ‡ $P < 0.001$ versus CON.

Table A5-1. *Pre-contracted tension of young and aged aortic rings to $10^{-6.5}$M phenylephrine*

	Young (g)	Aged (g)
E+ CON	1.8 ± 0.2	1.4 ± 0.1
E+ L-NAME	2.4 ± 0.1	2.4 ± 0.1
E+ INDO	2.1 ± 0.2	1.0 ± 0.2#
E+ L-NAME INDO	2.5 ± 0.1	1.8 ± 0.2
E- CON	2.0 ± 0.2	3.0 ± 0.2‡#
E- L-NAME	1.8 ± 0.1	2.8 ± 0.2†#
E- INDO	1.8 ± 0.2	2.8 ± 0.1‡¶
E- L-NAME INDO	2.0 ± 0.3	2.9 ± 0.4†

Data represents means ± s.e.m. *n*= 4 -11 rings per group. † P < 0.01, ‡ P < 0.001 versus E+ CON, # P < 0.05, ¶ P < 0.01 versus Young.

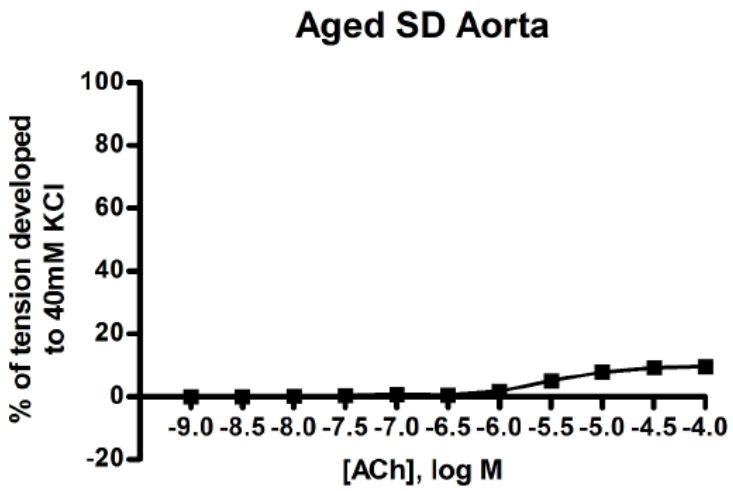

Figure A5-3. Endothelium-dependent contraction to acetylcholine in aorta of the aged male Sprague-Dawley rats used in this study. Endothelium-dependent contractions were elicited by exposing rings to increasing concentrations of acetylcholine (ACh) in the presence of NOS inhibitor L-NAME and in the absence of pre-contraction (quiescent rings). Values are expressed as mean percent of tension developed to 60mM potassium chloride (KCl). *n* = 8 rings from 3 different animals.

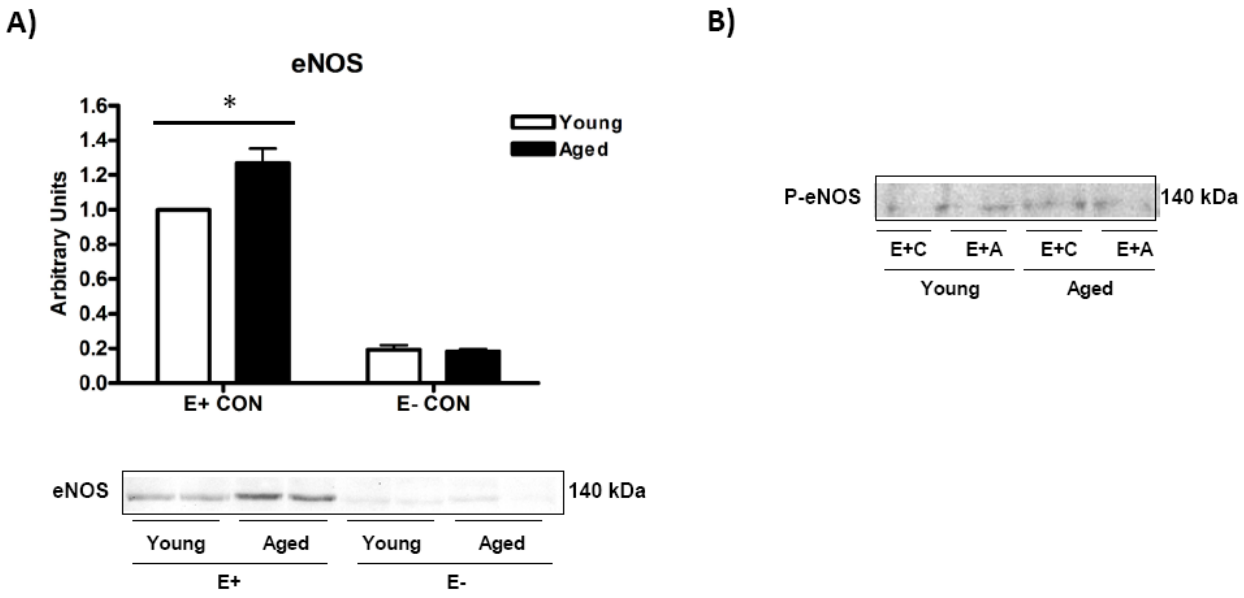

Figure A5-4. Total eNOS protein content in aortic rings of young and aged rats, and phosphorylation of eNOS activation site serine 1177 following exposure to AICAR. Aortic rings with (E+) and without (E-) endothelium were removed from the myography apparatus immediately following completion of the dose-response curve protocols, snap frozen and stored at -80^{o}C for immunoblotting analyses. In panel **A)**, eNOS total protein content was assessed in rings pre-contracted but not treated with AICAR (time controls, CON) to confirm denudation of E- rings. Panel **B)** displays a representative blot of the phosphorylation of eNOS at serine 1177 in homogenates of aortic rings that were removed from the myography apparatus immediately following completion of the AICAR dose-response curve (E+A and E-A) or in time controls not exposed to AICAR (E+C and E-C). Data is expressed as mean ± s.e.m. Values from densitometry analyses were normalized such that the mean of the Young E+ CON group values are equal to 1 normalized unit. *n* = 4-6 (all samples run in duplicate). There was a significant main effect for the presence/absence of endothelium ($P < 0.0001$) in panel A, but significance symbols have been excluded for clarity. * $P < 0.05$ vs Young E+ CON. E+C; time control with endothelium, E+A; AICAR treatment with endothelium, E-C; time control without endothelium, E-A; AICAR treatment without endothelium.

Chapter 6

General Discussion

6.1 Summary of the main thesis findings and conclusions

The main purpose of this thesis was to investigate the role of AMPK in the vasoregulation of functional arteries from health animals and dysfunctional arteries from hypertensive and aged rats. We addressed this objective by executing four studies, each with their own set of specific objectives designed to assess the effects of AMPK activation on vasomotor function, and in some cases, to investigate the mechanisms mediating these responses.

The findings of this thesis represent a significant contribution to the information that is currently available regarding the role of AMPK in regulating vascular function. Our work extends the findings of isolated biochemical and cell culture experiments detailing the relationship between AMPK and NO into the context of an intact, functional vessel system to demonstrate that these findings have functional consequences for vascular tone. While the experiments we performed in healthy arteries alone are novel and contribute substantially to the current body of knowledge (AMPK-mediated relaxation has not been assessed at all in arteries of WKY and Sprague Dawley rats), we have added an additional layer of originality to the work by exploring AMPK-mediated vasomotor regulation in arteries of hypertensive and aged animals (until our experiments, there were no data published relating to AMPK and acute vasomotor function in dysfunctional arteries, and to date our data remain the only observations of AMPK-mediated regulation of vascular tone in arteries from models of aging and essential hypertension). A summary of the key findings and conclusions from each study are presented in the section below, followed by overall conclusions and comparisons based on the thesis data collectively. Since the main findings have already been discussed in the experimental chapters, the intent of the following section is not to comprehensively cover all comparisons or re-discuss points that have already been dealt with above. However, there are several contrasts that exist between models and studies that are worth noting,

and some findings that consistently contradicted our initial hypothesis and warrant the further comments provided below.

Key Findings and Conclusions of Study 1 (Chapter 2):

- Vasorelaxation occurs following acute activation of AMPK in isolated, precontracted aortic rings of both normotensive WKY and spontaneously hypertensive rats (SHR).
- This relaxation response is comprised of both endothelium-dependent and -independent components in aorta of WKY and SHR, demonstrating that activation of AMPK in either the endothelium or vascular smooth muscle can influence relaxation.
- In WKY aorta, the endothelium-dependent component of the relaxation response to AMPK activation is solely NO-mediated. In SHR aorta, relaxation to AMPK activation is both NO- and COX-dependent. We interpret relaxation responses of this nature to mean that AMPK activation generates relaxation by enhancing NO-bioactivity and inhibiting COX-dependent endothelium-derived contractile activity.
- Enhanced NO bioavailability was not due to phosphorylation of eNOS at Ser1177 by AMPK
- Relaxation responses to AICAR are enhanced in aortic rings of SHR versus WKY. This was contrary to our original hypothesis that any vasomotor effects induced by AMPK would be depressed in arteries of SHR.

Key Findings and Conclusions of Study 2 (Chapter 3):

- Increased AMPK activation by AICAR pre-incubation suppresses endothelium-dependent contractions generated by acetylcholine in aorta of both WKY and SHR.
- AMPK activation does not alter sensitivity of the vascular smooth muscle to TP-receptor stimulation in WKY or SHR aorta, suggesting that suppressed endothelium-dependent contractions to acetylcholine in these vessels do not originate from effects on the vascular smooth muscle but rather via suppressed endothelial signalling.

- AMPK activation suppresses the production/release of PGI_2, the main vasoconstrictor prostanoid released by the endothelium in response to stimulation of endothelium-dependent contraction by acetylcholine, in aorta of both WKY and SHR.

Key Findings and Conclusions of Study 3 (Chapter 4):

- Basal AMPK activation is depressed in aorta of SHR versus WKY rats, suggesting dysregulation of AMPK signalling in conduit arteries of the hypertensive animals. Since AMPK can still be robustly activated by AICAR in SHR arteries, this depression in basal AMPK activity does not result from an inability to activate AMPK.
- An acute injection of AMPK activator AICAR generates an acute, profound decrease in blood pressure of SHR that is partly NO-dependent, but does not affect blood pressure of WKY. For a number of reasons outlined in the discussion of Chapter 3, we put forward the hypothesis that this depression is due partly to altered vascular resistance.
- Acute exposure to the AMPK activator AICAR generates dose-dependent relaxation of similar magnitude in WKY and SHR isolated mesenteric arteries, however these responses are more reliant on NO in SHR than in WKY.

Key Findings and Conclusions of Study 4 (Chapter 5):

- Acute activation of AMPK generates relaxation of pre-contracted aorta from both young and aged rats, although relaxation is enhanced in aged versus young vessels.
- Vasorelaxation is partly endothelium-dependent and partially endothelium-independent in both young and aged rat aorta.
- The endothelium-dependent component of relaxation to acute AMPK activation is solely NO-mediated in aorta of both young and aged rat aorta.
- Other known activators of AMPK, metformin and resveratrol, also generated relaxation of aged rat aorta. Relaxation to metformin was very modest compared to that of AICAR (as was the

degree of AMPK activation achieved by this drug), and although relaxation to resveratrol was robust, AMPK activation to this compound was not detected in aorta of aged rats. Therefore metformin induces modest relaxation of aged rat aorta that could be AMPK dependent, while the vasodilatory effects of resveratrol are not associated with AMPK activation.

- Relaxation responses to metformin and resveratrol are enhanced in pre-contracted aortic rings of aged versus young rats.

General conclusions, comparisons and interpretation of the collective findings of the thesis

Consistent with observations in other models of vascular dysfunction [10,135,179,243] and with our initial hypotheses, basal AMPK activation was dysregulated in arteries of SHR and aged rats compared to their respective controls. Depressed P-(Thr172)-AMPK to AMPK total protein ratios (relative to controls) were achieved differently in conduit aorta of SHR and aged rats compared to resistance mesenteric arteries of SHR (P(Thr172)-AMPK was depressed but AMPK total protein unaltered in SHR and aged Sprague-Dawley aorta, however AMPK total protein was elevated with no change in P(Thr172)-AMPK in SHR mesenteric arteries); further experiments will be required to determine if/how this heterogeneity extends to other vessel types, and to confirm the existence of AMPK dysregulation in other artery beds and models of hypertension and aging. The ability to pharmacologically activate AMPK was not different or slightly enhanced in aorta of SHR versus WKY, but tended to be impaired in aorta of aged rats compared with aorta of the young controls. More work will be needed to clarify whether these trends persist across vascular beds in hypertension and aging, and if similar responses occur to other AMPK-activating stimuli (i.e. endogenous AMPK activators, or those that activate AMPK by different mechanisms).

As expected, AMPK-mediated vasodilation was evoked in pre-contracted arteries of all models (healthy and hypertensive, young and aged rats) and artery types (conduit aorta and resistance mesenteric arteries) investigated in this thesis. The mechanisms mediating relaxation of precontracted arteries to

acute AMPK activation (partly endothelium-dependent relaxation mediated by NO and partly endothelium-independent relaxation by direct activation of the VSM) were conserved across models and vessel beds; the only exception to this convention was the additional contribution of a COX-dependent component in aorta of SHR (discussed further below). Interestingly, relaxation associated with AMPK activation (in arteries with and without functional endothelium) was consistently enhanced in conduit artery models of vasomotor dysfunction where AMPK is dysregulated (hypertension and aging). This finding was contrary to our initial hypothesis (that AMPK-mediated relaxation would be impaired in dysfunctional arteries of aged and hypertensive rats), and may represent a compensatory upregulation in sensitivity of AMPK-mediated vasorelaxation in the conduit artery vasculature to try to offset other enhanced vasoconstrictory influences that exist in dysfunctional vessels. In contrast to the conduit aorta of SHR and aged rats versus their respective controls (WKY and young rats), relaxation of precontracted mesenteric artery resistance vessels of SHR was *not* enhanced compared to those of WKY, once again highlighting the heterogeneity that exists in responses between artery types investigated in this thesis.

The initial front-running hypothesis for the potential role of AMPK in endothelium-, NO-dependent relaxation was based on cell culture experiments in which activation of AMPK was associated with increased phophorylation at eNOS activation site serine 1177 and concominant increases in NO-production/bioavailability (this was the first reported interaction of AMPK with eNOS and the mechanism initially put forward). Although our data demonstrate that AMPK activation by AICAR does generate relaxation that is NO mediated, contrary to our initial hypothesis, phosphorylation of eNOS serine 1177 does not appear to be the mechanism involved. We are not alone in our inability to observe eNOS serine 1177 phosphorylation in conjunction with AMPK activation, as others have also found the two responses to be dissociated [69,146,147,203]. Nonetheless, our immediate observations all agree with one another, and the disparate nature of observations across the literature may highlight differences that exist between isolated protein, cell culture, *in vitro,* and *in vivo* experimental conditions or arteries from different models or vascular beds, and support the hypothesis that AMPK is capable of elevating NO-

bioavailability via mechanisms other than Ser1177 phosphorylation of eNOS. These findings expose the necessity for identification of new mechanisms, and validation of other currently existing potential candidates (already discussed in the individual experimental chapters), that may be responsible for the interaction of AMPK with NO-mediated signalling pathways.

Our observations regarding the ability of AMPK to influence endothelium-dependent contractions in aorta of SHR is a particularly novel element of this thesis; only one other study to date has investigated the potential interaction between AMPK and this vascular signalling axis [141]. Our work is also novel being the first to investigate the nature of the AMPK-EDCF interaction in aorta from a model of hypertension where over-active endothelium-dependent contractions are of major importance to vascular pathology and significant contributors to vasomotor dysfunction. We also present mechanisms (i.e. inhibition of endothelium- and COX-dependent contractions by AMPK activation involves suppression of COX-derived prostanoids in the endothelium rather than by direct effects on the VSM) that will help provide direction for further dissection of the specific processes in the COX-EDCF signalling axis influenced by AMPK. The ability of AMPK to also influence contractions in aorta of normotensive WKY is intriguing, and it would also be interesting to explore the potential role for AMPK as a regulator of endothelium- and COX-derived prostanoids in healthy arteries. In contrast to the findings in SHR aorta, there was no COX-dependent component to the relaxation generated by acute AMPK activation in precontracted aorta of aged animals. Although we may have expected to observe COX-mediated relaxation in aged aorta as we did in that of SHR since enhanced COX-dependent contractions have also been reported in arteries of aged male Sprague-Dawley rats compared to their younger counterparts [49], this effect was absent in aorta of the aged animals. In retrospect, these results are reasonable, as these contractions are still much weaker in magnitude in aged Sprague-Dawley rat aorta compared with those observed in SHR (endothelium- and COX-dependent contractions are ~ 75% of tension generated by exposure to 60mM KCl in aortic rings of SHR, Figure 3-1, while in aged arteries these contractions were only ~ 15% of KCl-induced tension, Figure A5-3), but rather are comparable to the magnitude of

contraction observed in aorta of WKY (~ 20% of tension generated by KCl, Figure 3-1) where the COX-dependent component of relaxation to AICAR was also absent. Therefore, it is likely that the magnitude of the endothelium- and COX-dependent contractions in aorta of aged animals was not substantial enough to allow for an observable COX-mediated contribution of AICAR-induced relaxation.

6.2 Potential physiological relevance, clinical implications and future directions

The findings of this thesis have implications for both physiological processes, and the pathophysiology and treatment of vascular dysfunction. Our work provides insight that supports a potential role for AMPK in acute regulation of vascular tone *in situ,* a vasoprotective role for AMPK in maintaining appropriate vasomotor responsiveness in healthy vessels, and a potential therapeutic application for AMPK activation in alleviating the vasomotor dysfunction present in aging and cardiovascular disease states. Over the duration of data collection and thesis preparation, other studies have been published that, along with our findings, provide additional evidence that support the ability of AMPK to function in these capacities. Examples of potential physiological and clinical implications for AMPK-mediated regulation of vasomotor function, and supporting findings from recently published literature are provided below. Important future research questions prompted by our data, and in combination with the work of others, are also highlighted.

1) Basal AMPK activation may be vasoprotective

Studies in this thesis demonstrate that basal AMPK activation is depressed in arteries of both SHR and aged rats relative to those of WKY and young animals, respectively. Since reduced AMPK activation co-exists with vasomotor dysfunction in arteries of hypertensive and aged animals, it is conceivable that some level of basal AMPK activity assists in maintaining healthy vasomotor function and protecting against vascular insult in arteries of healthy animals, or helps attenuate the development of impairment in conditions that induce arterial dysfunction. Arteries AMPK α2 -/- mice display endothelial

dysfunction [241] and accentuated agonist-induced VSM contraction (these animals also have elevated blood pressure)[242], and the presence of AMPK α1 partially preserves endothelial function in mice chronically treated with angiotensin II (i.e. endothelial dysfunction is more pronounced in AMPK α1 -/- mice chronically treated with angiotensin II than their wild-type counterparts)[188]. These observations that may suggest that AMPK plays a vasoprotective function under healthy conditions and protects against vasomotor impairments induced by cardiovascular perturbation/stress. It is difficult to determine if vasomotor results obtained using these particular whole body knockout models of these AMPK subunit isoforms are valid, since these models also exhibit other major metabolic and physiological abnormalities that could contribute to altered vasomotor phenotypes independently of the absence of these AMPK catalytic subunit isoforms in the vasculature (i.e. elevated circulating trigylcerides, glucose intolerance, etc. [3,72,126,239]). It is also unclear to what extent compensatory upregulation of the remaining AMPK α subunit isoforms compensate for the absence of the other, and therefore potentially attenuate effects/compensate for the function of the absent isoform. Therefore, while these results do appear to support a protective role for AMPK in maintaining physiological vasomotor function, continued examination will be necessary to confirm and clarify the current findings. If AMPK does play a role in regulating physiological responses to stimuli under healthy conditions, it is possible that the depression of AMPK activation in the arteries of hypertensive and aged rats confers or contributes to the dysfunctional phenotype characteristic of these animals.

2) *A potential role for AMPK in acute physiological regulation of vascular tone in healthy arteries in situ*

The acute nature of AMPK-mediated vasodilation of isolated vessels in this thesis, demonstrates a key property of AMPK that may be relevant to regulation of vascular tone and blood flow to vasoactive stimuli under physiological conditions. As outlined in the introductory thesis rationale (section *1.7 Specific rationale for thesis topic*), AMPK can be activated by a number physiologically relevant vasoactive stimuli. One particularly intriguing example is the ability of endothelial AMPK to be activated

acutely by shear stress [30,31,69,265], a stimulus that contributes to coordinating vasomotor responses during exercise. Several recent findings demonstrate the potential involvement of vascular AMPK in shear stress and exercise-mediated vascular effects *in vivo* [20,264,265], and that AMPK, in addition to other contributing kinases PKA, PI3K and Akt, may represent a contributing and/or redundant mechanism for eNOS phosphorylation and activation in arteries during dynamic exercise [20,264]. Thus, it may be that activation of endothelial AMPK by increased shear stress during exercise is a contributing mechanism to the vasodilation and increased blood flow to exercising skeletal muscle, and this will undoubtedly be an interesting question to address in future research. AMPK may also participate in mediating vasomotor responses initiated by factors released by exercising skeletal muscle (i.e. ROS, NO etc.) that aid in satisfying exercising muscle blood flow requirements, and in doing so, assist in coupling blood flow with metabolic demand, although this has not been investigated. Further experiments will also be necessary to determine if AMPK is involved in mediating acute vasomotor responses generated by other major vascular signalling systems that are important for physiological vasomotor responses like EDHFs (an area that is completely unexplored at present), and to uncover yet unidentified factors that may influence AMPK-mediated alterations in vascular tone. Thus, the basic acute vasodilatory response and associated mechanisms of AMPK activation demonstrated here may have implications for vasoactivity of a number of agonist and stimuli under physiological circumstances, and establish an important vasoactive property of AMPK.

3) Activation of AMPK may improve vasomotor function in dysfunctional arteries of disease models

The mechanisms associated with vasoactivity of AMPK in our acute manipulations are consistent with a vasoprotective role for AMPK. Our results indicate that activation of AMPK stimulates NO-dependent relaxation, attenuates COX-dependent contractions, and relaxes VSM; all of these effects counter pathological phenotypes that contribute to dysfunction in arteries of hypertension, aging, but also in other conditions like diabetes. Thus, although our observations were generated under acute conditions, they indicate that AMPK could possess the capacity to interact with these processes in a salubrious

fashion to improve vasomotor function of dysfunctional arteries in a more chronic manner. This hypothesis is supported by a number of studies that have recently examined the vasomotor benefits of supplementing diseased animals with compounds that are associated with AMPK activation (i.e. α-lipoeic acid[135], berberine[245], metformin[141]), and the ability of these treatments to alleviate endothelial dysfunction. These studies have not investigated the ability of AMPK to directly generate changes in vascular tone *per se*, but do suggest that activation of AMPK may play a role in regulating vascular function by improving the vasomotor responses generated in response to other vasoactive agonists in dysfunctional vessels. Supplementation of obese rats with α-lipoeic acid improves endothelial dysfunction in this model by AMPK-dependent mechanisms[135], berberine treatment alleviates high-glucose mediated endothelial dysfunction and is associated with AMPK activation in mouse aortic rings[245], and administration of metformin *in vivo* improves ACh-mediated relaxation and reduces endothelium-derived contracting factor (EDCF)-mediated contraction in aortic rings isolated from OLEFT rats (a model of type II diabetes)[141]. "Lifestyle" interventions such as caloric restriction and exercise both improve vascular AMPK activation[20,57,167,206,264,265] and vasomotor function [51,85,125,183,192,200,262]; thus AMPK may be one of the mechanisms mediating the vascular benefits of these interventions, and is yet another hypothesis that should be tested in future studies. Since AMPK is also an important regulator of metabolism, and can be a target for improving metabolic abnormalities in conditions such as obesity and diabetes (it is the primary target of the most commonly used anti-diabetic drug metformin), therapeutic strategies that target AMPK hold particular promise for conditions where cardiovascular and metabolic pathologies co-exist (i.e. diabetes mellitus and obesity), and AMPK may be a useful common target for both lifestyle and pharmaceutical interventions aimed at co-ordinately improving vascular and metabolic dysregulation.

Thus, our observations that AMPK-mediated vascular signalling mechanisms are intact in dysfunctional arteries indirectly support therapeutic potential for AMPK in arterial disease. The studies cited above represent a growing body of evidence that supports a relationship between AMPK activation and vasoprotection; much more work still needs to be done, particularly in humans vasomotor

dysfunction (an area of research that virtually untouched) to evaluate the efficacy of AMPK activation on vasomotor function. It will also be important to determine an efficacious level of AMPK activation, as another line of evidence that has recently emerged in the literature demonstrating that sustained hyper-activation of AMPK could also have deleterious effects on cell and tissue function (promoting cell senescence and apoptosis) [246,269]. As is the case with many other biological signalling processes, it is likely that AMPK activation is also subject to a requirement for balance, with basal or intermittent periods of AMPK activation providing benefits, but sustained, excessive activation of AMPK leading to undesirable outcomes. This concept may be an important consideration as we continue to interpret data and investigate strategies relating to therapeutic intervention and the relationship between AMPK and vascular function.

6.3 Limitations

AMPK remains a difficult signalling system to study due to the multiplicity of its interactions in so many cell and whole body processes, and due to the limitations that exists with the research tools currently available to study this protein. Clearly new pharmacological tools with improved specificity for AMPK and tissue specific AMPK knockout models will aid in confirming and extending the results obtained using existing methods. Although models of whole body AMPK α1 or AMPK α2 deletions have been generated (AMPKα1$^{-/-}$ AMPKα2$^{-/-}$ double knockout models do not exist as they are not viable), metabolic dysregulation and other pathophysiological features may limit the use of these models for identifying a specific role for vascular AMPK in regulating vascular function (discussed already above). Recently developed AMPKβ1 knock out mice may be more useful for providing insight regarding the vascular effects of AMPK, as these animals should exhibit major reductions in endothelial AMPK activity (AMPK β1 is the predominant isoform expressed in endothelial cells) on the background of a relatively normal metabolic profile[57].

Another limitation to the overall approach of this thesis is that these experiments mostly represent pharmacological manipulation of an isolated tissue system *in vitro*, and we cannot yet conclude as to whether or not the mechanisms and responses elucidated here are intact and contribute to physiological responses *in vivo*. Thus, as is always the case with these types of experiments, on-going investigations will be necessary to continue to validate a role for AMPK in regulating vascular tone in a physiological context. However, these *in vitro* pharmacological experiments are valuable for revealing and developing potential mechanisms, interactions and responses within a controlled experimental environment that can be put forward as working hypotheses for testing *in vivo*. In the case of AMPK, which regulates multiple processes in all cells and body tissues, isolated tissue preparations provide a particular advantage over *in vivo* manipulations, as they are not clouded by the inevitable influence on other potentially confounding metabolic processes, and allow for clear resolution of basic effects and mechanisms.

6.4 Final Remarks

The findings of this thesis contribute to a greater understanding of vasomotor signalling pathways by providing insight on a novel regulator of vascular tone in both healthy and dysfunctional arteries. In the experiments of this thesis, we have made a significant contribution towards understanding how AMPK moderates vascular tone in both healthy and dysfunctional arteries, and shed new insight into the mechanisms by which AMPK exerts its function. AMPK-mediated regulation of vascular tone is a new topic in vascular biology and we are only beginning to uncover the role of this enzyme, let alone to explore the potential physiological applications of this work. It is important that we continue to study the basic signalling mechanisms that modulate vascular tone, as understanding the fundamental mechanisms of vasomotor signalling pathways and their functional outcomes will allow us to broaden our understanding of regulation of vascular tone, blood flow and hemodynamic control, as well as to develop novel therapeutic strategies to help combat vascular dysfunction, a hallmark trait of hypertension and aging.

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