Influence of AMPK and ROCK Inhibition on Contractile Responses in Isolated Carotid Arteries in Adult and Aged WKY and SHR Rats

by

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

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ABSTRACT

AMP-activated protein kinase (AMPK) is emerging as an important regulator of vascular function and is proven to affect vascular tone. Here we investigate how AMPK inhibition in arteries from both young and aged normotensive Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats affects contraction in response to different receptor agonists. In study 1, isolated common carotid artery (CCA) segments (denuded of endothelium) from WKY and SHR were used to determine vasomotor dose-responses to the alpha-adrenergic agonist phenylephrine (PE: $10^{-9.0}$ - $10^{-4.5}$ M) and to the thromboxane-prostanoid receptor agonist U46619 ($10^{-9.0}$ – $10^{-6.0}$ M) after incubation with no drug (CON); the AMPK inhibitor compound C (CC; 20 μM); the rho-assosiated protein kinase (ROCK) inhibitor Y27632 (1 μM); or, a combination of CC and Y27632 at the same concentration. PE contraction was suppressed in all groups for all treatment conditions (CC, Y, CC+Y; $P<0.05$) with the combination condition (CC + Y) being significantly greater than either individual drug effect in both WKY and SHR CCA, though this effect was not completely additive in all groups. Vasomotor responses of CCA segments exposed to U46619 under the same incubation conditions exhibited significant increase in EC_{50} when compared to the CON within their respective groups, but no significant differences were found in the maximum developed tension (MAX; $P<0.05$). The greatest differences were found between the receptor-mediated responses to contraction, with CCA segments of all groups having a higher sensitivity to the TPr agonist U46619 than to the alpha-adrenergic agonist PE. Fold increase in EC_{50} was significantly greater in groups subject to PE-induced contraction compared to the same responses in U46619 treated groups, with the greatest increase being present in the young

WKY (CC+Y; 13.0 ±3.5 fold increase vs. CON). Vasomotor responses were relatively unaffected by hypertension and age. In study 2, the vasomotor constriction response to separate and combined AMPK and/or HMG-CoA reductase inhibition was measured via dose-response curves to PE. Four curves were generated: CON; CC (20 μM); Simvastatin (SIM; 5 μM); and, CC + SIM. PE contraction was suppressed in all groups for all treatment conditions (CC, SIM, CC+SIM; $P<0.05$) with the combination condition being significantly greater than either individual drug effect. The results suggest that AMPK may contribute to modulating the PE contraction response in denuded CCA via RhoA/ROCK-dependent and –independent mechanisms.

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List of Abbreviations

ACh	Acetylcholine (muscarinic receptor agonist)
AUC	Area under the curve
BCA	Bicinchoninic acid
Ca^{2+}_i	Intracellular calcium
CaM	Calmodulin
CC	Compound C (AMPK inhibitor)
CCA	Common carotid artery
CVD	Cardiovascular disease
DAG	Diacylglycerol
DBP	Diastolic blood pressure
DMSO	Dimethyl sulfoxide (organic solvent)
EC_{50}	Concentration of drug that elicits 50% of the maximum amplitude of the drug stimulated response
ECM	Extra-cellular matrix
EDCF	Endothelium-derived contracting factor
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factor
Endo-	Endothelium-denuded
eNOS	Endothelial nitric oxide synthase (NOS)
GPCR	G-protein coupled receptor
HR	Heart rate

HRP	Horseradish peroxidase
IP_3	Inositol triphosphate
Max Amp	Maximum amplitude
MLC	Myosin light-chain
MLCK	Myosin light-chain kinase
MLCP	Myosin light-chain phosphatase
MPYT1	Myosin targeting subunit of MLCP
PE	Phenylephrine (a1-adrenergic receptor agonist)
PG	Prostaglandin
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLA	Phospholipase A
PLC	Phospholipase C
ROCK	Rho kinase
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SR	Sarcoplasmic reticulum
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
SHR	Spontaneously hypertensive rat
TBS-T	Tris-buffered saline with Tween-20
TP	Thromboxane-prostanoid
TPr	Thromboxane-prostanoid receptor

U46619	TPr agonist
VSM	Vascular smooth muscle
VSMC	Vascular smooth muscle cell
WKY	Wistar-Kyoto rat
Y27632 or Y	Selective ROCK inhibitor

INTRODUCTION

Thesis Background

Smooth muscle is an integral part of the cardiovascular system that is responsible for the maintenance and control of vascular tone. The magnitude of this tone has global effects on the cardiovascular system and is highly involved in the regulation of blood pressure, blood flow, tissue oxygenation and nutrient delivery. Regulation of vascular tone relies on a complex integration of mechanical and chemical processes the smooth muscle cell itself in response to neural, endocrine, paracrine and mechanical signals. Pathologies of the smooth muscle, such as aging and hypertension, result in profound changes to cellular signaling that can lead to dysfunctional regulation of vascular tone.

This thesis aims to examine the subcellular mechanisms regulating vascular smooth muscle contraction, particularly the possible interaction between two vascular smooth muscle (VSM) enzymatic signaling pathways involving AMP-activated protein kinase (AMPK) and rho-associated protein kinase (ROCK). This will be examined under normal conditions, in both aging and hypertensive models. The following provides an introduction to help aid in understanding the rationale behind the experiments performed in this thesis.

VASCULAR SMOOTH MUSCLE

Vascular Smooth Muscle Background

Vascular smooth muscle (VSM) plays a pivotal role in the complex processes regulating blood pressure and flow. In addition to discovery of fundamental understanding of vascular function, observing and understanding the mechanisms of VSM contraction may elucidate specific changes occurring in disease states and provide insight into specific interventions. The latter is particularly important with respect to the health of Canadians as, with the exception of cancer, cardiovascular disease accounts for the highest cause of mortality (Statistics Canada, 2012).

The regulation and control of vascular tone is managed by the dynamic ability for VSM to change its contractile state; an integral part of accommodating changes in blood flow as well as blood pressure[1]. VSM's dynamic ability to accommodate such changes is based on its ability to regulate the concentration of and sensitivity to intracellular calcium. The initiation of contraction begins either with depolarized-induced contraction or the binding of agonists to specific G-protein coupled receptors (GPCRs), which are coupled to ion channels and enzymes that can modify specific pathways involved in VSM contraction[2].

Calcium influx is the dominant pathway to increase intracellular calcium concentration ($[Ca^{2+}]_i$), the facilitator of smooth muscle contraction[1]. Influx is facilitated by a variety of channels including L-type calcium channels[3], nonselective cation channels[4], the sodium-calcium exchanger (NCX) [4] and the sarcoplasmic reticulum[4] of the smooth muscle[5]. Second messengers inositol-triphosphate (IP_3), diacylglycerol (DAG),

cyclic adenosine monophosphate (cAMP), and cyclic guanosine monophosphate (cGMP) also affect intracellular calcium levels by modulating these mechanisms[5]. Increased [Ca^{2+}_i] results in the formation of the Ca^{2+}-Calmodulin (Ca-CaM) complex, activating Ca-CaM-dependent myosin light-chain kinase (MLCK) via phosphorylation of its regulatory 20 kDa light chain[6]. This is followed by a rise in myosin ATPase activity which then, in turn, leads to crossbridge cycling and ultimately, VSM contraction[2].

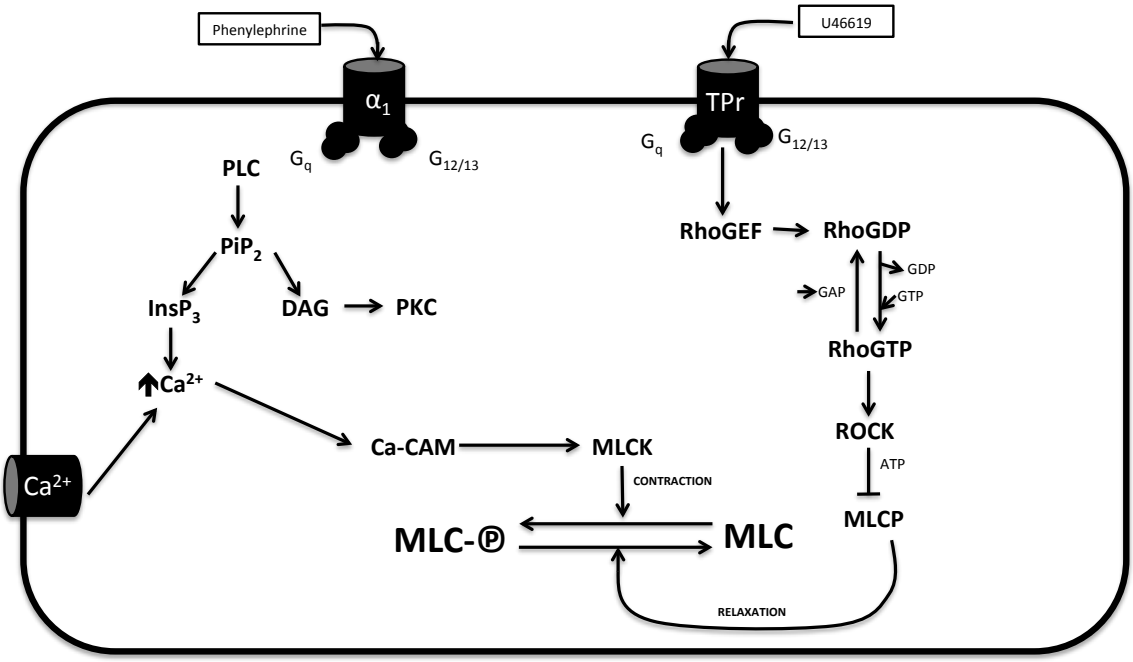


Diagram 1: *Vascular smooth muscle cell.* VSM contraction is initiated via Ca^{2+}-dependent or independent mechanisms that lead to the phosphorylation of the regulatory myosin light-chain, resulting in contraction.

Though VSM contraction is regulated by [Ca^{2+}_i] and its affinity for calmodulin, sensitivity to calcium is also modulated by GPCR activity[7]. GPCR activity is also linked to the Rho-mediated modulation of the balance between MLCK/MLCP activity[6], an important downstream pathway involved in the regulation of vascular tone.

A variety of agonists can initiate calcium sensitization via RhoA and its downstream effectors. Agonist-induced activation of thromboxane-prostanoid (TPr) GPCR requires active guanosine exchange factors (GEFs) [8], which are responsible for catalyzing the exchange from inactive RhoA•GDP to RhoA•GTP[9]. The subsequent result is the binding of RhoA•GTP to rho-kinase (ROCK), inducing a conformational change and activation of the ROCK enzyme[10]. ROCK phosphorylates specific site threonine sites (Thr696,853) on MLCP[7], resulting in its inhibition and decreased MLCP activity, increasing the relative MLCK/MLCP activity ratio[7], thus increasing the magnitude of contraction.

GPCRs can also initiate contraction independent of RhoA activation. For instance, the α-adrenergic receptor can also initiate signaling cascades that ultimately result in smooth muscle contraction (Diagram 1). Stimulation of α-adrenergic receptors leads to stimulation of G_q GPCR, activating phospholipase C (PLC), leading to the production of phosphatidylinositol-4,5-bisphosphate (PIP_2) [11]. PIP_2 then stimulates the production of IP_3, resulting in the influx of calcium which subsequently initiates formation of Ca-CaM complex, leading to MLCK activation, increased RLC phosphorylation, and enhanced VSM contraction[12].

Following the light-chain phosphorylation peak between 30-60 seconds, we observe a drop in phosphorylation while tone is maintained[1]. This is an indication that elevation in myosin light-chain phosphorylation is not essential for sustained, tonic VSM contraction and the magnitude of tone is variable, depending on the activation of kinase and phosphates acting on MLC[2]. One such kinase, AMPK, has been implicated in having a direct role in the regulation of MLC activity[13].

AMP-Activated Protein Kinase (AMPK)

AMP-activated Protein Kinase

In addition to the specific pathways described in the previous section, a variety of enzymes can affect the signaling pathways converging on MLCK/MLCP-mediated contraction. Adenosine monophosphate-activated protein kinase, or AMPK, is classically recognized as an enzyme responsible for maintaining cellular energy homeostasis across a variety of eukaryotic cell types[14]. It was first identified in 1973 as a kinase of both acetyl-CoA carboxylase[15] and HMG-CoA reductase[16] before being described as the allosteric kinase "AMP-activated protein kinase" [17].

Historically, AMPK was studied for its dynamic ability to detect alterations in cellular energy levels, mainly changes in the AMP:ATP ratio[17-19]. This kinase, which is highly sensitive to levels of AMP and ATP, swiftly responds to such changes in cellular energy levels by activating energy production pathways and/or inhibiting energy consumption pathways[19]. In addition to nucleotide balance, there are several stimuli that influence AMPK activity including intracellular calcium[20], hormones[21] and cytokines[21], with additional influence of chronic mechanisms controlling AMPK transcription and protein synthesis[22].

AMPK Structure and Function

The structure of AMPK can be a main determinant of its activity during cellular stress[20]. AMPK is composed of three subunits; the catalytic α subunit in addition to its regulatory β and γ subunits[14]. Both the α and β subunits have 2 isoforms while the γ subunit has 3 isoforms, allowing a total of 12 possible subunit conformations[23].

Distinct combinations of these subunits affords the enzyme its dynamic ability to accommodate changes in cellular energy.

AMPK activity can be controlled, in part, by covalent mechanisms that phosphorylate its catalytic α subunit (which must occur for enzyme activation) or via the allosteric binding of AMP to the β subunit[24]. Allosteric binding results in a conformational change of the protein that not only increases its affinity for phosphorylation by upstream kinases[20] but also decreases its potential for dephosphorylation via PP2C[23,25]. Though the allosteric role of AMP is important to AMPK activity, the bulk of the enzyme's activation results from phosphorylation of the α subunit at its Thr[172] residue[19]. This phosphorylation is achieved through activation of upstream kinases LKB1and CaMKK β [23], both of which transfer a phosphate group from ATP to AMPK, though it should be noted that AMP itself does not directly activate these upstream kinases[26].

AMPK in Research

During periods of increased energy demands, vasodilation is an essential mechanism that regulates systemic blood flow[27]. As an energy sensing kinase, AMPK could be involved in management of vascular tone. AMPK was first demonstrated to have a role in the *in vitro* regulation of vascular tone by Rubin et all in 2005[28]. It was found that hypoxia-induced AMPK activation was associated with vasodilation in endothelium-denuded isolated porcine coronary arteries[28]. Goriand et al in 2007[29] then observed AICAR (AMPK activator) dose-dependent vasodilation in the isolated aortic rings of mice, an effect that was severely blunted in the $AMPK_{\alpha 1}^{-/-}$ animal model[29]. This

demonstrated that AMPK activation of the α_1 subunit induces vasorelaxation in the aortic smooth muscle of mice. In 2011, Ford and Rush[30] showed that endothelium-dependent vasorelaxation to AICAR is enhanced in SHR, an animal model of hypertension, and is NO and EDCF dependent. One of the most interesting observations with respect to AMPK research is the novel finding that AMPK activity has been linked to specific GPCR's[31,32]. This provides powerful evidence to suggest that AMPK plays an important role in the maintenance and regulation of vascular tone, and the influence of AMPK could be altered in cardiovascular disease states, making it a treatment target in specific disease models.

AMPK in Pathophysiology

Documentation shows that a variety of vasoactive factors modulate the activity of AMPK in vascular smooth muscle. Such factors include hypoxia, free radicals, bradykinin, adiponectin, thrombin, metformin, resveratrol and AICAR[33,34]. With so many influences on activity, these vasoactive factors could play a role in the manner in which AMPK is involved in regulating contraction in VSM.

Impairment of AMPK activation, whether it be diminished responsiveness of AMPK to signaling cascades or changes in basal activation, occurs in a variety of disease states, particularly metabolic and cardiovascular disease, and in the arteries of aged rats[35]. Zucker diabetic fatty rats and aged rats both have depressed AMPK activation, indicating dysregulation of AMPK function[36]. AMPK basal activity is also blunted in SHR rats compared to WKY rats, though it is unknown whether or not the ability of these rats to activate AMPK is compromised[30]. Characterizing this enzyme and its mechanism of

action with respect to downstream signaling pathways (like Rho-mediated MLCP inhibition) involved in the regulation of vascular tone would provide great insight towards the role AMPK plays in regulating contraction in disease states like hypertension and aging.

SALICYLATES

In addition to modulating vascular reactivity in disease states, AMPK is also influenced by a variety of pharmacological agents. One group of compounds, salicylates, an acid derived from the bark of a willow tree, was of particular interest, with it having several proven effects and uses in medicine and health[37]. Initially, a paper describing the effect of salicylates on AMPK in HEK and mouse liver cells[38] peaked my interest in salicylates and their possible influence on AMPK activity in VSM. The literature clearly demonstrated that increasing doses of salicylate were directly correlated to an increase in AMPK was activity[38]. With salicylates having a dose dependent effect on AMPK activity in cultured cells, investigation of this effect in VSM could provide valuable insight towards another potential method of pharmacologically modulating AMPK activity. Pilot work using isolated rat CCA segments examined the effect of increasing doses of salicylates and their effect on relieving agonist-induced contraction (see appendix, figures S1 and S2). After demonstrating that increasing doses of salicylates led to vasorelaxation in these CCA segments, our results were excitingly promising. Further research was conducted to follow up on this curiosity, with examination of any potential effects salicylates may be having on the VSM contraction taking priority. It became evident that salicylates were already found to act through the RhoA/ROCK pathway[39], an important

downstream regulator of vascular tone that is highly involved in VSM contraction[11]. The realization that salicylates work through RhoA/ROCK during VSM contraction and relaxation, coupled with their potential ability to activate AMPK led to the inception of an idea; could AMPK and ROCK activity be linked to one another during the regulation of VSM contraction? If an effect was present, was the effect of one enzyme –dependent or –independent of the other? These curiosities eventually led to this thesis project examining the subcellular mechanisms responsible for influence of AMPK activity on the control and regulation of vascular tone and the potential involvement of ROCK activity.

RHOA AND RHO KINASE

RhoA and Rho-kinase Function

RhoA and it's downstream effector, Rho associated protein kinase (ROCK), have been identified to play crucial roles in regulating vascular smooth muscle activity[6]. The presence of specific G-protein receptors found on vascular smooth muscle membranes are directly correlated to smooth muscle contraction. Such receptor proteins are $G\alpha_q$, $G\alpha_{12,13}$ and $G\alpha_{1,2}$, all of which are linked to RhoA/ROCK activity[40-42].

Stimulation of the $G\alpha_{12,13}$ in smooth muscle results in RhoA activation via RhoGEF's that phosphorylate Rho-GDP to form RhoA-GTP[11]. RhoA-GTP then activates ROCK, a protein serine/threonine kinase that is highly involved in smooth muscle contraction[43]. Specific functional motifs are essential to function of the enzyme, including a rho-binding carboxyl terminal domain that forms an autoinhibitory region that reduces the activity of the kinase[11]. This kinase activity can be enhanced by rho binding but only in an activated GTP bound form. GTP binding subsequently leads to ROCK activation

and the phosphorylation of the regulatory subunit of MLCP, inhibiting its ability to dephosphorylate MLC and relieve contraction[44]. Regulation of ROCK activity is essential to the regulation of MLC activity and thus contraction.

RhoA/ROCK in Pathophysiology

Research has implicated that variations in the RhoA/ROCK activity and signaling are responsible for increased peripheral vascular resistance in hypertension[12,45]. Increased RhoA activity is associated with enhanced ROCK activation, leading to hypertension[12]. Animal studies using spontaneously hypertensive rats (SHR) showed that Rhoa/ROCK-mediated Ca^{2+} sensitization is elevated in hypertension and when compared to smaller, resistance arteries, conduit arteries appear to have a greater contribution from RhoA/ROCK signaling pathways[46]. Analysis of gene expression in stroke-prone SHR (SPSHR) animals has also implicated upregulated RhoA/ROCK activity as a consequence, not cause, of hypertension[44]. Dysfunction in protein synthesis, transcription signaling and calcium handling have all been linked to RhoA/ROCK in aging conditions[47-49], though it is relatively unknown how RhoA/ROCK function is affected by aging in the VSM of conduit arteries of WKY and SHR animals. Understanding the potential dysfunction of ROCK signaling is essential when considering the effect AMPK inhibition may have on the RhoA/ROCK signaling pathway in normal and disease states.

STATINS

Statins inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme reductase (HMG-CoA reductase), a rate-limiting enzyme essential to cholesterol biosynthesis in the

mevalonate pathway[50]. Statins are classically used to treat cardiovascular disease through manipulation of LDL, as reduced cholesterol synthesis forces hepatocytes to upregulate expression of LDL receptors leading to enhanced clearance of LDL from circulating plasma[51].

Statins have been implicated in a variety of cellular signalling mechanisms in vascular smooth muscle including reduction of reactive oxygen species[52], reduction of inflammatory signaling hormones[53] and anti-thrombotic effects[54]. However, little research regarding the role of statins in modulating vascular smooth muscle contraction is known. Uhiara et al. in 2012 demonstrated that simvastatin modulated β-adrenoreceptor-mediated vasodilation in porcine coronary arteries[55]. Seto et al. later demonstrated that simvastatin also inhibits potassium ATPase channels in porcine coronary arteries leading to impaired vasorelaxation[56]. Although limited, research suggests that the signaling mechanisms involved in vascular smooth muscle HMG-CoA reductase inhibition are involved in the regulation of contraction[56]. Pilot work in our laboratory has demonstrated that simvastatin (an HMG-CoA reductase inhibitor) modulates VSM α_1-adrenergic induced contraction. Whether these effects are specific to HMG-CoA reductase activity or whether they are pleiotropic in nature is yet to be seen.

PATHOLOGY OF VASCULAR SMOOTH MUSCLE

Aging and the Cardiovascular System

Studies have shown that age is the leading risk factor for the development of cardiovascular disease[57-59]. With normal function, the heart acts as a reciprocating pump that forces blood into the vasculature, which, in turn, is responsible for adequately

distributing blood to specific tissues of the body[60]. Continuous blood flow is facilitated by the elastic conduit arteries that expand and store the elastic energy generated by the left ventrical during systole. After the aortic valve closes, the artery recoils and releases its elastic energy, pumping the remaining arterial blood through the cardiovascular system during diastole. As age progresses, arteries become stiffer and less compliant[49], hindering their ability to furnish the continuous blood flow during diastole as a result of their decreased capacity to store the energy generated during systole. This loss of function has the potential to evoke serious complications, such as hypertension and atherosclerosis, and can exacerbate already existing conditions[59,61].

Age-related changes in the elasticity and compliance of arteries results from changes in the structural composition of the vascular wall[60]. Normal arteries are made up of three distinct layers: the intima, media, and adventitia. The intima is the innermost layer and contains the endothelium and a basement membrane that is mostly collagen in its composition[62]. Consequently, the intima has little to do with the structurally elastic properties of the blood vessel. The media and intima are composed of collagen, elastin and vascular smooth muscle cells. The elastic fibres found in the media are comprised of up to 90% elastin in concert with at least 19 other proteins[63]. Variations of elastin-collagen composition, along with muscularity/VSM content determine the overall non-linear elastic properties of the artery. Farther away from the heart, a decrease in elastin content and an increase in muscularity is observed when capacitance vessels transition towards becoming resistance vessels[64]. This concept is essential in the case of this study, as the focus is on the common carotid artery, a capacitance vessel responsible for approximately 80% of the brain's blood flow[65].

In aging and vascular disease, the intima thickens as an accompanying increase in lumen diameter also occurs. A decrease in elastin content and increase in collagen content has a compounded effect and ultimately results in decreased large artery compliance and increase in stiffness with age[49,66]. These pathological effects can also lead to more severe conditions like left ventricular hypertrophy and hypertension.

HYPERTENSION

Systemic hypertension is characterized by increased vascular resistance resulting from increased contractility, thrombosis, and structural remodeling of the arterial wall, mainly smooth muscle cell proliferation[2,44,61]. Such characteristics suggest an imbalance between signaling factors that are responsible for maintaining the normal relationship between vasodilation and vasoconstriction as well as growth inhibitors and mitogenic factors[67]. These imbalances are usually the consequence of endothelial dysfunction or injury to the vascular wall, with imbalance between vasoactive signaling factors causing much of the dysfunction[68].

The balance between two of these important vasoactive factors, prostacyclin and thromboxane, is essential for proper regulation of the VSM contractile state. Both are metabolites of aracadonic acid but have opposing effects with prostacyclin initiating vasodilation and thromboxane being a potent vasoconstrictor[69]. Increased levels of circulating thromboxane metabolites and decreased production of prostacyclin synthase shifts the balance in favour of vasoconstriction, an effect that is more prevalent in hypertension[70].

On a molecular level, VSM cells undergo a variety of changes in signaling cascades during aging that eventually lead to altered responses to agonist-induced contractile stimuli. Age provokes a loss of receptors in vascular smooth muscle cells[71] and can result in decreased agonist potency. In rat aorta, a decreased maximal contractile response is observed in aging, which can be attributed to a decrease in receptor density with specificity to α_1-adrenoreceptors[72]. Aging has also been associated with inhibition of matrix metallo-proteases, proteins responsible for maintaining the structural integrity of the vessel wall[60]. This inhibition is accompanied by increased levels of circulating pro-inflammatory molecules, like IL-6 along with other leukocyte stimulating factors[73]. This leads to increases in ECM matrix protein synthesis and can result in intimal hypertrophy[74]. VSM hypertrophy is also observed in aging, stemming from continuously elevated pressure, indicating that mechanical stress can also act a as a regulator of VSM cell function and structure[75]. With a variety of age related factors acting on VSM, observing changes in the contraction profile of isolated WKY/SHR CCA segments could provide valuable insight regarding the pathological significance of age-related changes in contraction.

ENDOTHELIAL INFLUENCE ON VASCULAR TONE

The vascular endothelium has demonstrated a variety of effects on signaling mechanisms responsible for the maintenance and control of vascular tone and has swiftly emerged as an interesting target for current vascular research[2,30,76]. Endothelial dysfunction resulting from aging, hypertension and a variety of other disease states makes the endothelium an ideal target for pharmacological intervention. However, for all intents

and purposes of this project, ALL carotid artery segments were excised and denuded of an endothelium. This allowed us to attribute any of the observed changes to the vascular smooth muscle alone, as the potentially confounding influence of the endothelium was not present during experimentation.

SUMMARY

In summary, the effect of AMPK inhibition either separately, or combined with ROCK inhibition, is currently unknown in isolated rat CCA segments. Examining the vasomotor response of these isolated segments after incubation with specific enzyme inhibitors could provide valuable insight regarding the regulation of VSM contraction, particularly the possible interaction between two VSM enzymatic signaling pathways involving AMPK and ROCK (see diagram 2). Discerning the role of AMPK in normal, hypertensive and aged models would aid in not only developing a sound understanding of their independent function, but it would also develop an understanding of their function with respect to each other.

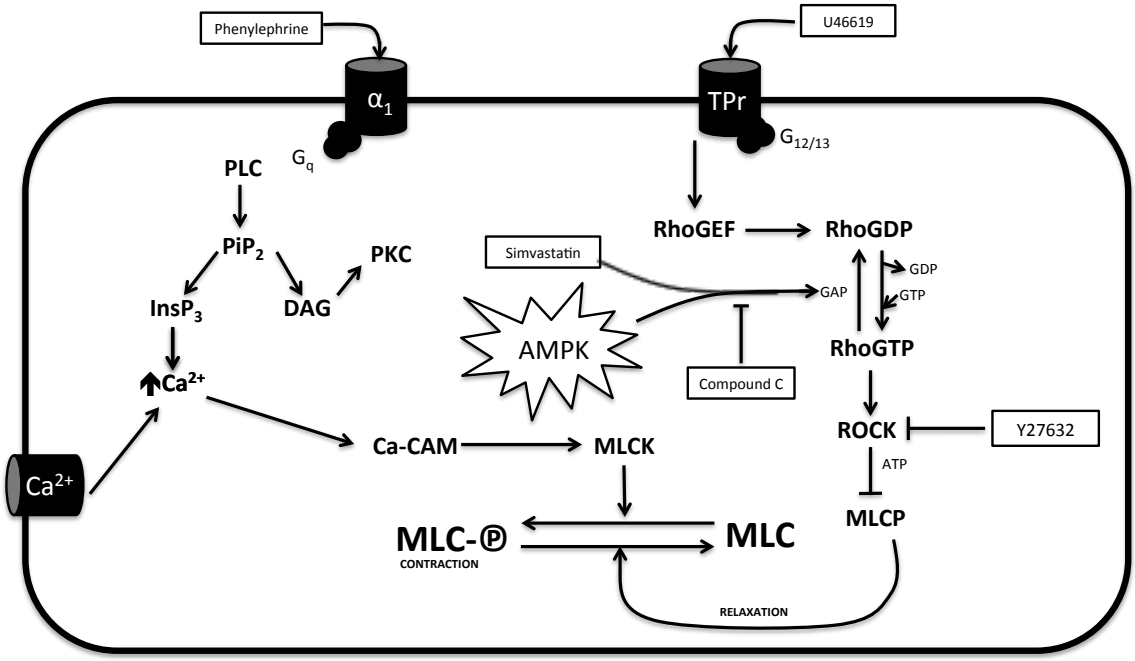


Diagram 2*: Potential mechanisms of action for AMPK*. Vascular smooth muscle cell describing the potential sites of action for AMPK and the HMG-CoA reductase inhibitor Simvastatin in the regulation of contraction.

EXPERIMENTAL DESIGN

Study 1 – Investigation of CCA vasomotor contractile responses to separate or combined AMPK and ROCK inhibition

Rationale

Pilot work from my original thesis topic of salicylates (see supplemental material; S1, S2) indicated that AMPK activity may play a role in mediating the influence of salicylates on the regulation of the RhoA/ROCK contractile pathway. Examining these effects in isolated CCA WKY segments led to the current work examining the effect of combined, direct AMPK and ROCK inhibition.

Receptor mediated contraction can be regulated by RhoA activation and downstream ROCK-mediated MYPT1 phosphorylation, resulting in MLCP inhibition[76]. If AMPK plays a role in mediating RhoA/ROCK activity, then AMPK's inhibition should affect the VSM response to RhoA/ROCK inhibition as well. AMPK and ROCK activity have been linked to GPCR activity in the regulation of vascular tone[11], suggesting some sort of cross-talk between the modulation of vascular tone by these kinases. Whether AMPK acts independently or dependently on ROCK is yet to be established, though it can be hypothesized that AMPK does modulate ROCK activity in response to pharmacologically induced contraction. The use of two separate agonists (the alpha-adrenergic agonist PE and the thromboxane-prostanoid receptor agonist U46619) will aid in examining any receptor mediated differences present.

We also know that age and hypertension are associated with decreased levels of basal AMPK activity and could also be involved in modulating the vascular response to

contraction. These two conditions are also associated with increased ROCK signaling activity. Examining their effect on modulating contraction could also be beneficial for characterizing the role of AMPK in the regulation of VSM contraction.

Objectives

The objective of thesis study 1 was to confirm the mechanical vasomotor response of isolated WKY carotid artery segments to AMPK and/or ROCK inhibition. This study was also conducted to further expand on that knowledge and elucidate whether or not AMPK played a role in ROCK and it's ability to mediate vascular tone in response to agonist-induced contraction. The second objective of thesis study 1 was to examine the effect of aging and hypertension on the vasomotor response of isolated SHR and WKY CCA segments to AMPK and/or ROCK inhibition. The final objective of study 1 was to examine whether or not any receptor mediated differences were present during the vascular response to separate or combined AMPK and ROCK inhibition. Comparison of response to the α_1-adrenergic contractile agonist PE and the thromboxane-prostanoid receptor agonist U46619 was the approach used to elucidate the presence of any receptor-mediated differences. Investigation using these objectives would aid in determining the role of AMPK and RhoA/ROCK in modulating contraction in response to vascular agonists.

Anticipated results and importance of study 1

The completion of thesis study 1 would clarify: 1) the influence of AMPK inhibition and its potential to modulate RhoA/ROCK activity in pharmacologically-

induced VSM contraction and 2) how age and hypertension can affect the ability of AMPK to modulate a the downstream RhoA/ROCK activity pathway, either separately or in combination and 3) any receptor-mediated differences that may be present in purposes (1) and (2).

Study 2 – Investigation of CCA vasomotor contractile responses to separate or combined AMPK and HMG-CoA reductase inhibition

Rationale

AMPK was initially discovered as a kinase of HMG-CoA reductase and has been shown to activate and phosphorylate HMG-CoA reductase, inhibiting its activity[77]. HMG-CoA inhibition has also been shown to downregulate Angiotensin II-induced Rho activation, and consequently, MYPT1 phosphorylation, in cultured VSM cells[78]. With implications in both the energy-sensing role of AMPK and the contraction mediating role of ROCK, HMG-CoA reductase is a strong candidate for pharmacological manipulation of agonist-induced contraction in VSM.

Using wire myography, this study aims to examine the potential effect that AMPK activation has on HMG-CoA reductase activity in VSM. Through the use of the specific AMPK inhibitor compound C (CC), and the HMG-CoA reductase inhibitor simvastatin (SIM), this study will observe and investigate the mechanical responses to pharmacologically-induced contraction.

Objectives

The objective of thesis study 2 was to examine vasomotor response of isolated WKY CCA to HMG-CoA reductase inhibition using the HMG-CoA reductase inhibitor simvastatin.

Anticipated results and importance of study 2

The completion of this study would clarify: 1) the mechanical vascular response to HMG-CoA reductase inhibition in smooth muscle alone and 2) the influence of AMPK inhibition on HMG-CoA reductase activity in smooth muscle.

HYPOTHESES

Study 1:

1. Combined and separate AMPK and ROCK inhibition will increase the EC_{50} and decrease the maximum tension developed in the contractile responses to the VSM agonists PE and U46619 across all treatment groups.

2. Hypertension and age will enhance the EC_{50} and MAX tension VSM contractile response to agonist-induced contraction with a greater magnitude of difference in the old SHR compared to young WKY.

3. The α_1-adrenergic receptor agonist phenylephrine (PE) and the thromboxane receptor agonist U46619 will exhibit receptor-mediated differences between all treatment groups (SHR/WKY, old/young).

4. The influence of AMPK inhibition and ROCK inhibition will be more pronounced in response to the TPr agonist-induced contraction since this signaling pathway directly affects ROCK activity.

Study 2

1. AMPK activity in vascular smooth muscle modulates RhoA/ROCK activation via HmG-CoA reductase inhibition.

2. HMG-CoA reductase inhibition cause a blunted response to agonist-induced contraction.

MATERIALS AND METHODS

Animal Model

In this study, Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) will be used as a model for studying the effects of essential hypertension.

WKY rats were first bred in 1963 by Okamotao and Aoki[79] and commonly serve as control strain animals, especially when compared to SHR animals. The strain was further developed at the National Institute of Health in the United States of America via inbreeding within the colony using brother and sister rats[67].

SHR rats are genetic descendants of the WKY animals with substantial phenotypic variation resulting in elevated blood pressure. These animals typically reach hypertensive plateau at 16-24 weeks of age[67]. Male animals develop hypertension more rapidly than females and experience hypertrophic vasculature resulting in an increase in total peripheral resistance[80]. Within the strain, intra-individual changes are rare and

relatively uniform when they do occur, making SHR animals ideal candidates for modeling human essential hypertension.

Animal Characteristics

Male WKY (n=56) and SHR (n=45) rats were obtained from the University of Waterloo breeding colony. Animals were classified as either adult (18-26 weeks of age) or old (>52 weeks) and housed in a temperature and humidity controlled environment with access to standard chow (Harlan Laboratories) and tap water ad libitium. Animals were acclimated to a twelve-hour reverse light cycle. Before tissue was collected for measurement and analysis, animals were anesthetized via intraperitoneal injection of sodium pentobarbital (50-65 mg/kg of body weight; Bimeda-MYC, Cambridge, Ontario). Sedation level was gauged based on withdrawal reflex in response to a toe-pinch, with a complete lack of withdrawal indicating an appropriate level of sedation. Prior to assessment of vasomotor responses in isolated vessels, animals were sacrificed via exsanguination. All procedures involving rats were approved by the University of Waterloo Animal Care Committee and were in accordance with the guidelines of the Canadian Council on Animal Care.

Vasomotor Response Assessment in Isolated Vessels

For this study, vasomotor responses were collected and measured using wire myography as the primary means of investigation. A general procedure for conducting wire myography in our integrative vascular biology lab was followed[30,76,81]. Specifics of the pharmacological treatment of the isolated vessels was the main difference between

each individual protocol. Following sedation and exsanguination, excised CCA segments were placed in a 4°C solution of Krebs Bicarbonate buffer (concentration (mmol/L): 131.5 NaCl, 5 KCl, 25 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 0.025 EDTA, 13.5 NaHCO, 11.2 Glucose). Cleaned arteries were cut into 2-mm segments using a surgical blade under a dissecting microscope (Zeiss; VWR, Mississauga, Ontario) before being isometrically mounted on a wire myography unit (vascular myography unit, Radnoti Glass Technology Inc., Monrovia, California). In all instances, the endothelium was removed by inserting a thin, tungsten wire through the 2-mm vessel segment and rolling it on Whatman blotting paper soaked with 4°C Krebs Bicarbonate buffer. This was confirmed by observing a dose-dependent relaxation response to the endothelium-dependent vasodilator, ACh. If <5% vasodilation, compared to maximum PE tension developed, was observed at any concentration of ACh, the vessel was considered denuded (see appendix for data supporting the efficacy of this technique). Segments were then threaded onto a wire triangle and suspended from a force transducer (model MLT0201/D, ADI Instruments) before being threaded through a fixed wire foot and submersed in Krebs-bicarbonate buffer at 37°C bubbled with 95% O_2 and 5% CO_2. Passive tension was applied and vessels were equilibrated via washing in fresh buffer before being ramped to 2.85 grams (optimal tension previously determined by Denniss and Rush, 2009[76]) of tension for 10 minutes. This process of washing with fresh buffer and ramping to 2.85 grams of tension was performed 3 times. Viability was assessed using the addition of 60mM of KCl to generate a depolarization-dependent contraction, which was assessed for 30 minutes. After this 30-minute period, vessels were washed with fresh buffer 3 times with a 5 minute waiting period following each wash. This 30 minute KCl contraction protocol,

followed by washout to baseline tension, was repeated one more time before vessels were subjected to one of the specific experimental protocols as detailed in the following section.

Measurement of AMPK and ROCK contributions to agonist-induced VSM contraction

After the equilibration period, vessels were subject to four separate incubation conditions; No drug (control); 30 minute incubation with AMPK inhibitor compound C (CC; 20 μM); 30 minute incubation with the ROCK inhibitor Y27632 (1μM); or a 30 minute incubation with both CC(20 μM) and Y27632 (1 μM). The concentration of 20 μM for compound C was based on the work of R.J. Ford and J.W. Rush[30], where the same concentration was found to have a maximal inhibition effect on AMPK. Pilot work examining the effect of Y27632 incubation on VSM contraction led to the use of a concentration of 1 μM (see appendix for data).

Following the incubation period, vessels were subjected to one of two different contractile agonists: the α_1-adrenergic receptor agonist phenylephrine (PE) with concentrations ranging from $10^{-9.0}$ to $10^{-4.5}$ mmol/L or the thromboxane receptor agonist U46619 with concentrations ranging from $10^{-9.0}$ to $10^{-6.0}$ mmol/L. Both contractile agonists were administered in a stepwise fashion, with concentration increasing by a half log per dose until a plateau in constriction was achieved.

Measurement of HMG-CoA reductase contribution to agonist-induced VSM contraction

Wire myography was used to examine the potential modulating effect statins may have on the VSM constriction response and its potential interaction with either the

AMPK or ROCK contractile pathways. After equilibration, vessels were subjected to four separate conditions; No drug (control); 30 minute incubation with AMPK inhibitor compound C (CC; 20 μM); 30 minute incubation with the statin simvastatin (SIM; 5 μM); or a 30 minute incubation with both CC(20 μM) and SIM (5 μM). Pilot work determined 5 μM as the optimal incubation concentration of simvastatin, with doses greater than 5 μM having no significantly greater effect to PE-induced contraction (see appendix for data).

After this incubation period, vessels were subjected to 2 different contractile agonists: the α_1-adrenergic receptor agonist phenylephrine (PE) with concentrations ranging from 10^{-9} to $10^{-4.5}$ mmol/L and the thromboxane receptor agonist U-46619 with concentrations ranging from $10^{-9.0}$ to $10^{-6.0}$ mmol/L. Both contractile agonists were administered in a stepwise fashion, with concentration increasing by a half log per dose until a plateau in constriction was achieved.

Statistics

Contraction dose-response curve data was expressed as developed tension (peak tension – resting tension) in milligrams or as a percentage relative to the peak tension developed during the administration of 60 mM KCl ((dose tension – resting tension)/(KCl peak tension – resting tension)). Dose-response cruves were fit into a sigmoidal model with a bottom boundary of 0 (GraphPad Prism version 6.0b, San Diego, CA). Curve characteristics (EC_{50}, maximum amplitude of tension, area under the curve) were generated for each CCA segment based on their respective dose-response curves. All 3

parameters were measured for all treatment groups in both thesis study 1 and thesis study 2.

Comparisons between curves were assessed with 3-way ANOVA analysis (incubation*strain, incubation*age, age*strain, incubation*strain*age) using SPSS software (version 11.5.1) to examine treatment effects within groups (aging or hypertension groups). Groups were compared statistically in singular comparison conditions (age comparison, hypertension comparison, control vs. separate inhibition, control vs. combined inhibition) using multiple and unpaired t-tests ($P<0.05$; GraphPad Prism version 6.0b). All values are expressed as a ± SEM.

RESULTS

For the reader, the results section will be organized in the following way:

Study 1

First, the effects of PE-induced contraction in isolated CCA for study 1 will be established by examining the effects observed in young WKY CCA segments. This will provide a "normal" reference point to compare other group. Following the response to PE-induced contraction in young WKY, any potential hypertensive effects will be described. This will include a comparison of both the young and old cohorts (WKY vs. SHR in young and old groups). Next, an age comparison will be made in both WKY and SHR groups, completing the comparisons for the PE-induced contraction portion of the study.

The results from the U46619-induced contraction will be presented in the exact same fashion using the exact same comparisons as the PE-induced study with the only exception being the use of U46619 as a contractile agonist instead of PE.

Following the descriptive statements explaining the results of the agonist-induced contraction components of the study, a comparison explaining receptor-mediated differences will be made. This comparison will examine the changes in EC_{50} compared to the respective controls as well as the changes in MAX developed tension compared to the controls.

Study 2:

With PE being the only agonist and old WKY being the only treatment group, the presentation of these results will be a straightforward analysis of the EC_{50}, MAX and AUC response to PE-induced contraction in response to separate or combined AMPK and/or ROCK inhibition.

Rat characteristics and classification

Based on availability, both WKY and SHR animals were classified into two different age groups; young (20-30 weeks) and old (48+ weeks) (see tables 1-3 for average ages and body weight).

VASOMOTOR RESPONSE TO ALPHA-ADRENERGIC AGONIST PE

The vasomotor contraction response to separate and combined AMPK and/or ROCK inhibition was measured via dose-response curves to the α_1-adrenergic receptor specific agonist PE in the presence or absence of pharmacological antagonists. Four curves were generated: CON; CC; Y; and, CC + Y. Each curve was characterized using three parameters: half of the maximal effective concentration (EC_{50}); maximum vasoconstriction (MAX) and the area under the curve (AUC).

Vasoconstriction in young WKY

The EC_{50} for all three drug incubation conditions (CC, Y, CC+Y) was significantly greater than that of the control (CON; $p<0.05$) with both the separate CC and Y conditions being significantly lower compared to the combined CC+Y inhibition

condition ($p<0.05$)(table 4). There was no significant difference between the separate CC and Y incubation conditions.

The MAX developed tension in grams was also measured, with the Y and CC+Y drug incubation conditions being significantly lower than the control ($p<0.05$)(table 4). The separate CC and Y incubation conditions produced significantly greater tension developed compared to the combined CC+Y condition ($p<0.05$)(table 4). There was no significant difference between the separate CC and Y incubation conditions.

The AUC for the Y incubation condition and the combined CC+Y condition was significantly lower than that of the control condition (CON; $p<0.05$)(table 4). The separate CC and Y incubation conditions had significantly greater AUC compared to the combined CC+Y condition ($p<0.05$)(table 4). There was no significant difference between the separate CC and Y incubation conditions.

HYPERTENSION VASOMOTOR RESPONSE TO THE ALPHA-ADRENERGIC RECEPTOR SPECIFIC AGONIST PE

Young WKY vs SHR

In comparison to their age-matched WKY, the young SHR group exhibited a similar increased EC_{50} response compared to control, with separate incubation conditions being significantly different from the control but not to each other and the combined condition being significantly different from the separate conditions (table 4). This was also true of the observed AUC changes, but not the MAX in young SHR (table 4)

In the EC_{50} response of young WKY and SHR, only the CON incubation condition was significantly lower in SHR compared to WKY (SHR: 14.5±1.1 nM vs. WKY: 21.7±3.2 nM, $p<0.05$; table 4). The SHR also developed significantly greater MAX tension in the separate Y incubation condition (SHR: 1.3±0.1 grams vs. WKY: 1.1±0.0 grams, $p<0.05$; table 4) as well as the combined CC+Y incubation condition (SHR: 1.2±0.0 vs. WKY: 0.9±0.1, $p<0.05$; table 4). AUC was significantly greater in SHR for only the combined CC+Y incubation condition (SHR: 184.2±5.6 units vs. WKY: 157.4±8.2 units, $p<0.05$; table 4).

No significant differences between old WKY and SHR in EC_{50}, MAX or AUC were present for any incubation condition (table 5).

AGING VASOMOTOR RESPONSE TO THE ALPHA-ADRENERGIC SPECIFIC AGONIST PE

WKY

In the EC_{50} response of WKY, the CON, CC and Y incubation conditions were significantly lower in young WKY compared to old WKY (table 6). No significant differences in MAX or AUC were present between young and old WKY.

SHR

In the EC_{50} response of SHR, all incubation conditions (CON, CC, Y, CC+Y) were significantly lower in young SHR compared to old SHR (table 7). AUC was significantly lower in the old SHR for all incubation conditions (table 7).

3-WAY ANOVA ANALYSIS OF CURVE PARAMETERS IN RESPONSE TO PE-INDUCED CONTRACTION

EC_{50}

3-Way ANOVA analysis revealed that only incubation (separate or combined AMPK and/or ROCK inhibition) and aging had significant effects on the EC_{50} response to PE-induced contraction (table 8).

MAX

3-Way ANOVA analysis revealed that MAX tension in response to PE-induced contraction significantly was significantly affected by incubation and aging. It also revealed that there was an interaction effect present between aging and hypertension (table 9).

AUC

3-Way ANOVA analysis revealed that PE-induced contraction was significantly affected by both incubation and aging. There was also an interaction effect present between aging and hypertension (table 10).

VASOMOTOR RESPONSE TO THE THROMBOXANE PROSTANOID RECEPTOR AGONIST U46619

The vasomotor constriction response to separate and combined AMPK and/or ROCK inhibition was measured via dose-response curves to thromboxane-prostanoid receptor specific agonist U46619. Four curves were generated, one per treatment

incubation; CON, CC, Y and CC + Y. Each curve was characterized using three parameters: EC_{50}, MAX and AUC.

Vasomotor response to U46619 in young WKY

The EC_{50} for all three drug incubation conditions (CC, Y, CC+Y) was significantly greater than that of the control ($p<0.05$; table 11). There was no significant difference between the separate incubation conditions.

No significant differences in MAX or AUC were present between any conditions (table 11).

HYPERTENSION RESPONSE TO U46619-INDUCED CONTRACTION

Young WKY vs SHR

Similar to the response observed in young WKY, young SHR showed significantly greater EC_{50} for all three incubation conditions compared to their respective control ($p<0.05$; table 11). There was no significant difference present between separate incubation conditions in young SHR.

In the EC_{50} response of young WKY vs. SHR, the CON and CC incubation conditions were significantly lower in SHR compared to WKY ($p<0.05$; table 11). The SHR also developed significantly greater MAX tension in the separate CC, Y and combined CC+Y incubation conditions ($p<0.05$; table 11). AUC was significantly greater in SHR for only the combined Y incubation condition ($p<0.05$; table 11).

In the EC_{50} response of old WKY and SHR, only the separate Y incubation condition was significantly different, with the EC_{50} being higher in WKY compared to

SHR ($p<0.05$; table 12). No significant differences in maximum developed tension or AUC were present for any incubation condition.

AGING REPONSE TO U46619-INDUCED CONTRACTION

WKY

No significant differences in the EC_{50} response between old and young WKY were present. The MAX of the CON and separate CC incubation condition were both significantly greater in older WKY compared to young WKY ($p<0.05$; table 13). The AUC for the combined CC+Y incubation condition was significantly lower in the old WKY compared to the young WKY ($p<0.05$; table 13).

SHR

No significant differences were present between the EC_{50} response, MAX or AUC of young SHR compared to old SHR, with the exception of the AUC of the older SHR, which was significantly greater in the Y incubation condition ($p<0.05$; table 14).

3-WAY ANOVA ANALYSIS OF CURVE PARAMETERS IN RESPONSE TO U46619-INDUCED CONTRACTION

EC_{50}

3-Way ANOVA analysis revealed that EC_{50} in response to U46619-induced contraction was significantly affected by drug incubation and hypertension ($p<0.05$; table 15). An interaction effect was also present between aging and hypertension ($p<0.05$; table 15).

MAX

3-Way ANOVA analysis revealed that MAX tension in response to U46619-induced contraction significantly was significantly affected by hypertension and aging ($P<0.05$; table 16). It also revealed that there was an interaction effect present between aging and hypertension ($P<0.05$; table 16).

AUC

3-Way ANOVA analysis revealed that PE-induced contraction was significantly affected by incubation, hypertension and aging ($P<0.05$; table 17). There was also an interaction effect present between incubation and aging ($P<0.05$; table 17).

RECEPTOR MEDIATED DIFFERENCES TO PHARMACOLOGICALLY-INDUCED CONTRACTION

In the CCA segments of all groups, the EC_{50} (nM) of PE was significantly lower than the sensitivity to U46619 ($p<0.05$; table 18, 19, 20, 21). This was confirmed when examining the change in the EC_{50} compared to the control for both the separate CC and Y conditions as well as the combined CC+Y condition (table 22). MAX was also affected by the contractile agonist used after incubation, with the response to PE-induced contraction having a much greater effect than U46619-induced contraction (table 23). Receptor-mediated differences were most pronounced in the young WKY group for both the EC_{50} and MAX responses to agonist-induced contraction (table 22, table 23)

VASOMOTOR RESPONSE TO COMBINED AND SEPARATE AMPK AND HMG-CoA REDUCTASE INHIBITION

The vasomotor constriction response to separate and combined AMPK and/or HMG-CoA inhibition was measured via dose-response curves to the α_1-adrenergic receptor specific agonist PE. Four curves were generated: CON; CC; SIM; and, CC + SIM. Each curve was characterized using three parameters: half of the maximal effective concentration (EC_{50}), maximum vasoconstriction (MAX) and the area under the curve (AUC) (table 24).

The EC_{50} for all three drug incubation conditions (CC, SIM, CC+SIM) was significantly greater than that of the control (CON; $p<0.05$; table 24) with both the separate CC and Y conditions being significantly different to the combined CC+Y inhibition condition as well as being significant different to each other ($p<0.05$; table 24).

The MAX was also measured, with the separate Y incubation condition and the combined CC+Y incubation condition being significantly lower than the control ($p<0.05$; table 24). There was no significant difference between the separate incubation conditions.

The AUC for all three drug incubation conditions (CC, SIM, CC+SIM) was significantly lower than that of the control (CON; $p<0.05$; table 24) with both the separate CC and SIM conditions being significantly different to the combined CC+SIM inhibition condition as well as being significant different to each other ($p<0.05$; table 24).

Table 1: *Study 1 WKY Rat characteristics*

Characteristic	Young	*n*	Old	*n*	*p-value*
Age (weeks)	24.9±0.8	12	58.0±1.7	10	<0.01
Body Mass (g)	395.0±5.3	12	445.8±6.9	10	<0.01

Age expressed in weeks. Body mass expressed in grams. Young, animals classified as young; Old, animals classified as aged. P-value obtained by t-test for independent means. Data are presented as means ± SEM.

Table 2: *Study 1 SHR Rat characteristics*

Characteristic	Young	*n*	Old	*n*	*p-value*
Age (weeks)	27.5±0.5	12	66.1±2.4	11	<0.01
Body Mass (g)	405.4±4.2	12	420.8±6.6	11	<0.01

Age expressed in weeks. Body mass expressed in grams. Young, animals classified as young; Old, animals classified as aged. P-value obtained by t-test for independent means. Data are presented as means ± SEM.

Table 3: *Study 2 WKY characteristics*

Characteristic	WKY rats	*n*
Age (weeks)	53.4±3.0	11
Body Mass (g)	450.9±8.2	11

Age expressed in weeks. Body mass expressed in grams. P-value obtained by t-test for independent means. Data are presented as means ± SEM.

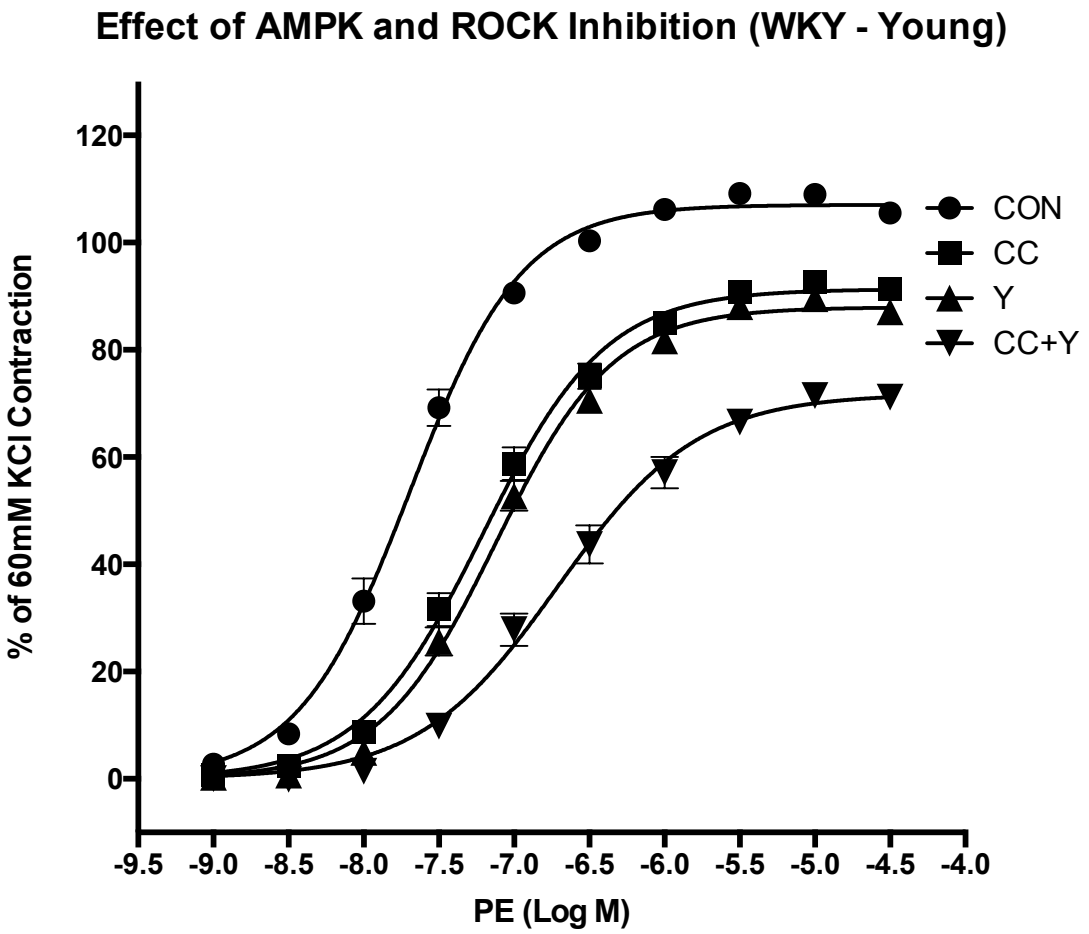


Figure 1: Vasoconstriction stimulated by phenylephrine (PE) in 2mm endothelium-denuded (E-) young WKY (n=12) CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK Y27632 (Y; $10^{-6.0}$ M) or a combination of Y27632 and CC (CC+Y; 20μM, $10^{-6.0}$ M) for 30 minutes. Contraction was stimulated following incubation using PE at concentrations from $10^{-9.0}$ to $10^{-4.5}$ M.

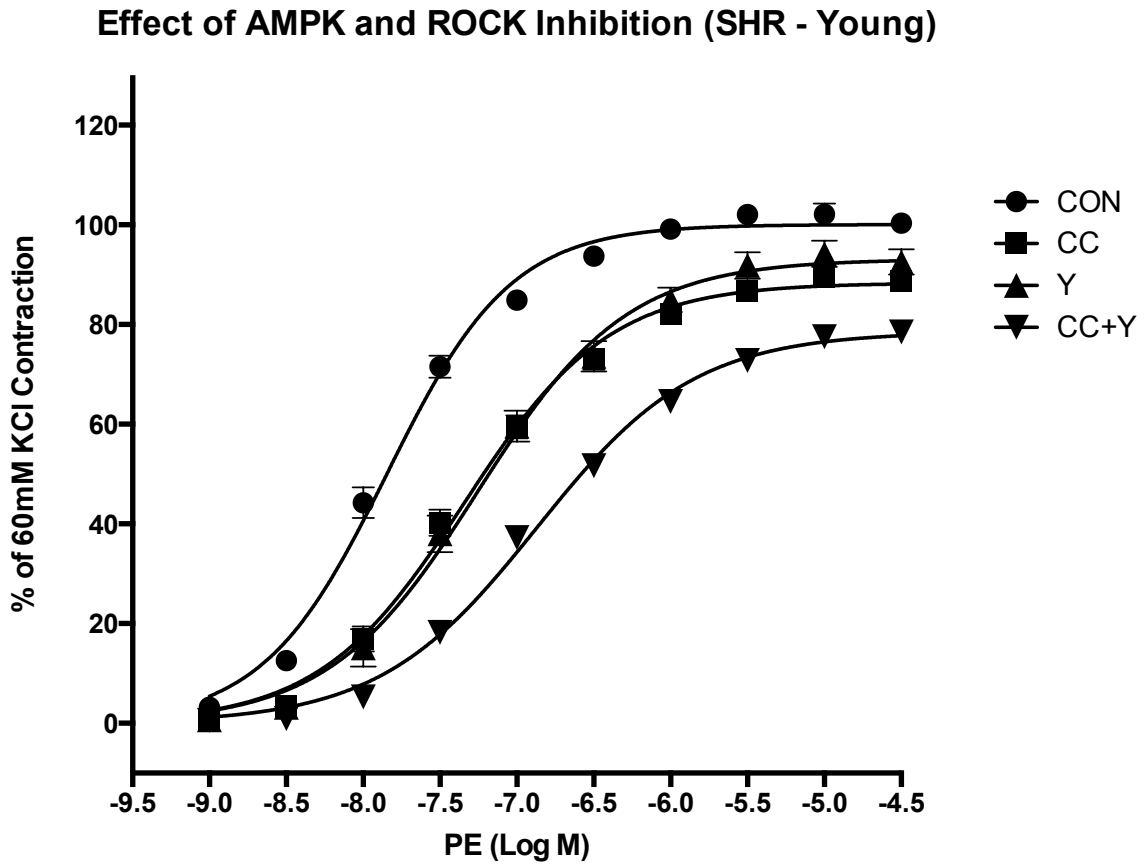


Figure 2: Vasoconstriction stimulated by phenylephrine (PE) in 2mm endothelium-denuded (E-) young SHR (n=12) CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK Y27632 (Y; $10^{-6.0}$ M) or a combination of Y27632 and CC (CC+Y; 20μM, $10^{-6.0}$ M) for 30 minutes. Contraction was stimulated following incubation using PE at concentrations from $10^{-9.0}$ to $10^{-4.5}$ M.

Table 4: *Strain comparison of PE curve parameters for young WKY and SHR*

PE - YOUNG	EC_{50} (nM)		MAX Tension (g)		AUC (Arbitrary Units)	
	WKY	SHR	WKY	SHR	WKY	SHR
CON	21.7±3.2	14.5±1.1¥	1.3±0.1	1.3±0.0	339.5±8.8	331.1±8.5
CC	72.0±13.7*‡	46.3±6.0*‡	1.2±0.0‡	1.3±0.0	245.6±8.0*‡	248.2±8.8*‡
Y	78.7±8.9*‡	67.1±8.4*‡	1.1±0.0*‡	1.3±0.1¥	230.7±7.8*‡	253.7±11.8*‡
CC+Y	234.4±42.3*§†	159.6±24.3*§†	0.9±0.1*§†	1.2±0.0*¥	157.4±8.2*§†	184.2±5.6*§†¥

EC_{50} (nM), MAX (g) and AUC (arbitrary units) of young WKY (n=12) and SHR (n=12) CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK inhibitor Y27632 (Y; 10^{-6} M) or a combination of Y27632 and CC for 30 minutes. Contraction was stimulated following incubation using PE at concentrations from $10^{-9.0}$ to $10^{-4.5}$ M. Data are presented as means ± SE. * $P < 0.05$ vs. CONTROL; § $P < 0.05$ vs. CC; † $P < 0.05$ vs. Y; ‡ $P < 0.05$ vs. CC+Y. ¥ $P < 0.05$ vs. WKY within treatment.

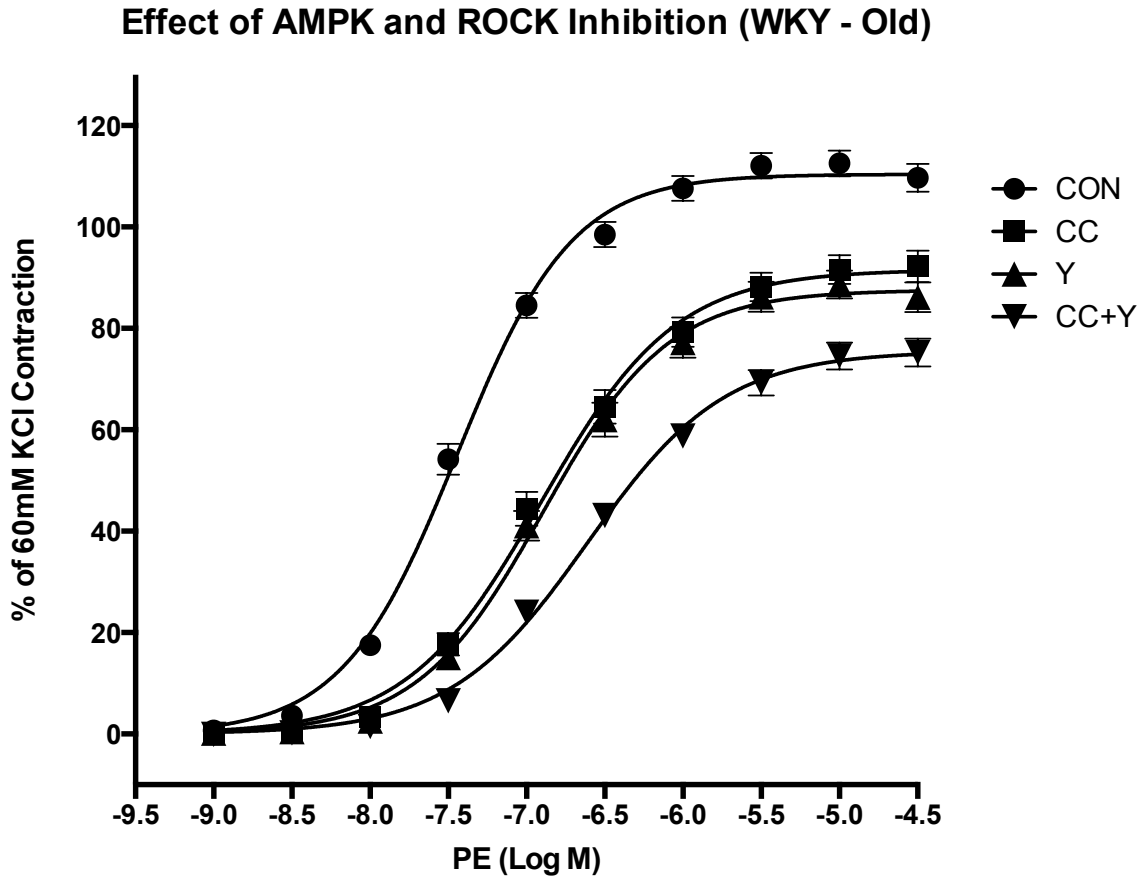


Figure 3: Vasoconstriction stimulated by phenylephrine (PE) in old WKY (n=10) CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK inhibitor Y27632 (Y; $10^{-6.0}$ M) or a combination of Y27632 and CC (CC + Y) for 30 minutes. Contraction was stimulated following a 30-minute incubation using PE at concentrations from $10^{-9.0}$ to $10^{-4.5}$ M.

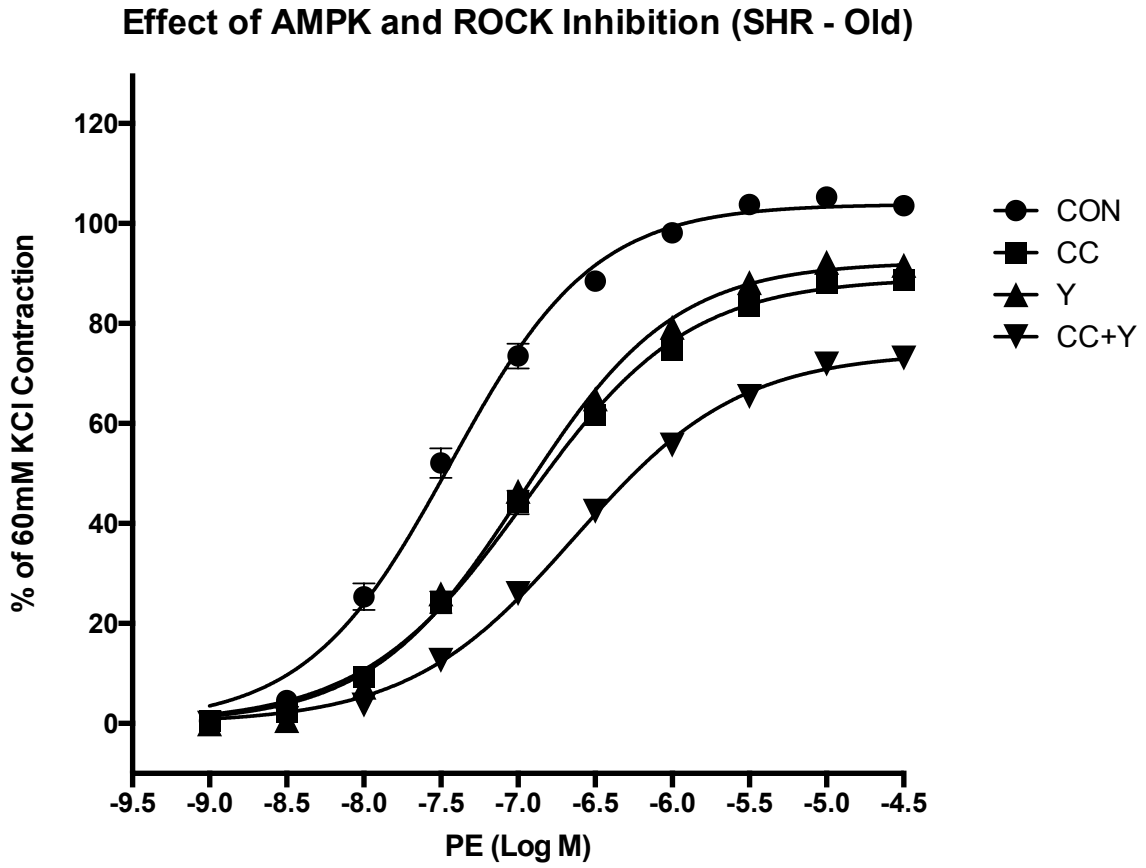


Figure 4: Vasoconstriction stimulated by phenylephrine (PE) in old SHR (n=11) CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK inhibitor Y27632 (Y; $10^{-6.0}$ M) or a combination of Y27632 and CC (CC + Y) for 30 minutes. Contraction was stimulated following a 30-minute incubation using PE at concentrations from $10^{-9.0}$ to $10^{-4.5}$ M.

Table 5: *Strain comparison of PE curve parameters for old WKY and SHR*

PE - OLD	EC_{50} (nM)		MAX Tension (g)		AUC (Arbitrary Units)	
	WKY	SHR	WKY	SHR	WKY	SHR
CON	35.5±3.0	36.5±4.6	1.3±0.1	1.3±0.0	322.5±11.0	301.7±7.6
CC	131.0±18.7*‡	114.4±13.3*‡	1.2±0.1	1.2±0.0*‡	219.7±9.2*‡	216.6±5.0*‡
Y	139.6±14.7*‡	108.1±10.1*	1.2±0.1	1.3±0.0‡	207.6±10.1*‡	225.7±4.5*‡
CC+Y	240.6±15.7*§†	234.7±18.1*§†	1.1±0.1*	1.1±0.0*§†	157.0±6.8*§†	158.2±4.8*§†

EC_{50} (nM), MAX (g) and AUC (arbitrary units) of old WKY (n=10) and SHR (n=11) CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK inhibitor Y27632 (Y; $10^{-6.0}$ M) or a combination of Y27632 and CC for 30 minutes. Contraction was stimulated following incubation using PE at concentrations from $10^{-9.0}$ to $10^{-4.5}$ M. Data are presented as means ± SE. * $P < 0.05$ vs. CONTROL; § $P < 0.05$ vs. CC; † $P < 0.05$ vs. Y; ‡ $P < 0.05$ vs. CC+Y. ¥ $P < 0.05$ vs. WKY within treatment.

Table 6: *Age comparison of PE curve parameters for WKY*

PE - WKY	EC_{50} (nM)		MAX Tension (g)		AUC (Arbitrary Units)	
	Young	Old	Young	Old	Young	Old
CON	21.7±3.2	35.5±3.0¥	1.3±0.1	1.3±0.1	339.5±8.8	322.5±11.0
CC	72.0±13.7*‡	131.0±18.7*‡¥	1.2±0.0‡	1.2±0.1	245.6±8.0*‡	219.7±9.2*‡
Y	78.7±8.9*‡	139.6±14.7*‡¥	1.1±0.0*‡	1.2±0.1	230.7±7.8*‡	207.6±10.1*‡
CC+Y	234.4±42.3*§†	240.6±15.7*§†	0.9±0.1*§†	1.1±0.1*	157.4±8.2*§†	157.0±6.8*§†

EC_{50} (nM), MAX (g) and AUC (arbitrary units) of young (n=12) and old (n=10) WKY CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK inhibitor Y27632 (Y; $10^{-6.0}$ M) or a combination of Y27632 and CC for 30 minutes (n=11). Contraction was stimulated following incubation using PE at concentrations from $10^{-9.0}$ to $10^{-4.5}$ M. Data are presented as means ± SE. * $P < 0.05$ vs. CONTROL; § $P < 0.05$ vs. CC; † $P < 0.05$ vs. Y; ‡ $P < 0.05$ vs. CC+Y. ¥ $P < 0.05$ vs. Young within treatment. *Note:* for the sake of comparison, the data from the WKY young (table 4) and WKY old (table 5) is repeated for this table.

Table 7: *Age comparison of PE curve parameters for SHR*

PE - SHR	EC_{50} (nM)		MAX Tension (g)		AUC (Arbitrary Units)	
	Young	Old	Young	Old	Young	Old
CON	14.5±1.1	36.5±4.6¥	1.3±0.0	1.3±0.0	331.1±8.5	301.7±7.6
CC	46.3±6.0*‡	114.4±13.3*‡¥	1.3±0.0	1.2±0.0*‡	248.2±8.8*‡	216.6±5.0*‡
Y	67.1±8.4*‡	108.1±10.1*¥	1.3±0.1	1.3±0.0‡	253.7±11.8*‡	225.7±4.5*‡
CC+Y	159.6±24.3*§†	234.7±18.1*§†¥	1.2±0.0*	1.1±0.0*§†	184.2±5.6*§†¥	158.2±4.8*§†

EC_{50} (nM), MAX (g) and AUC (arbitrary units) of young (n=12) and old (n=11) SHR CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK inhibitor Y27632 (Y; $10^{-6.0}$ M) or a combination of Y27632 and CC for 30 minutes (n=11). Contraction was stimulated following incubation using PE at concentrations from $10^{-9.0}$ to $10^{-4.5}$ M. Data are presented as means ± SE. * $P < 0.05$ vs. CONTROL; § $P < 0.05$ vs. CC; † $P < 0.05$ vs. Y; ‡ $P < 0.05$ vs. CC+Y. ¥ $P < 0.05$ vs. Young within treatment. *Note:* for the sake of comparison, data for the SHR young (table 4) and SHR old (table 5) is repeated for this table.

Table 8: *3-Way ANOVA table examining the EC_{50} response to PE-induced contraction*

	EC_{50} response to PE				
Effect	**Sum of squares**	**df**	**Mean Square**	**F value**	***P* value**
Incubation	777002.4	3	259000.8	89.1	0.000*
Hypertension	19281.9	1	19281.9	6.6	0.110
Age	75512.7	1	75512.7	26.0	0.000*
Incubation x Hypertension	6412.8	3	2137.6	0.7	0.532
Incubation x Age	11404.9	3	3801.6	1.3	0.274
Age x Hypertension	2680.4	1	2680.4	0.9	0.338
Incubation x HT x Age	12032.6	3	4010.9	1.4	0.251
Error	430092.2	148	2906.0		

Effects are described as follows: Incubation refers to conditions isolated CCA segments were subject to in bath for 30 minutes prior to stimulation with PE; CON (no drug); CC (AMPK Inhibitor; 20 μM); Y (ROCK inhibitor; $10^{-6.0}$ M); CC + Y (combination of CC and Y at their respective separate concentrations). Hypertension refers to any differences resulting from strain (WKY vs. SHR). Age refers to any differences resulting from age (young vs. old). *$P \leq 0.05$.

Table 9: *3-Way ANOVA table examining the MAX response to PE-induced contraction*

MAX response to PE					
Effect	**Sum of squares**	**df**	**Mean Square**	**F value**	***P* value**
Incubation	1.1	3	0.4	15.7	0.000*
Hypertension	0.2	1	0.2	10.3	0.011*
Age	0.0	1	0.0	1.6	0.208
Incubation x Hypertension	0.2	3	0.1	2.62	0.053
Incubation x Age	0.0	3	0.0	0.1	0.974
Age x Hypertension	0.2	1	0.2	8.2	0.005*
Incubation x HT x Age	0.0	3	0.0	0.6	0.603
Error	3.5	148	0.0		

Effects are described as follows: Incubation refers to conditions isolated CCA segments were subject to in bath for 30 minutes prior to stimulation with PE; CON (no drug); CC (AMPK Inhibitor; 20 μM); Y (ROCK inhibitor; $10^{-6.0}$ M); CC + Y (combination of CC and Y at their respective separate concentrations). Hypertension refers to any differences resulting from strain (WKY vs. SHR). Age refers to any differences resulting from age (young vs. old). *P ≤ 0.05.

Table 10: *3-Way ANOVA table examining the AUC response to PE-induced contraction*

AUC response to PE					
Effect	**Sum of squares**	**df**	**Mean Square**	**F value**	***P* value**
Incubation	542584.7	3	180861.6	261.1	0.000*
Hypertension	958.5	1	958.5	1.4	0.241
Age	20830.4	1	20830.4	30.1	0.000*
Incubation x Hypertension	6319.9	3	2106.6	3.0	0.031*
Incubation x Age	1504.7	3	501.6	0.7	0.539
Age x Hypertension	1627.0	1	1627.0	2.3	0.128
Incubation x HT x Age	852.1	3	284.0	0.4	0.746
Error	430092.2	148	2906.0		

Effects are described as follows: Incubation refers to conditions isolated CCA segments were subject to in bath for 30 minutes prior to stimulation with PE; CON (no drug); CC (AMPK Inhibitor; 20 μM); Y (ROCK inhibitor; $10^{-6.0}$ M); CC + Y (combination of CC and Y at their respective separate concentrations). Hypertension refers to any differences resulting from strain (WKY vs. SHR). Age refers to any differences resulting from age (young vs. old). *P ≤ 0.05.

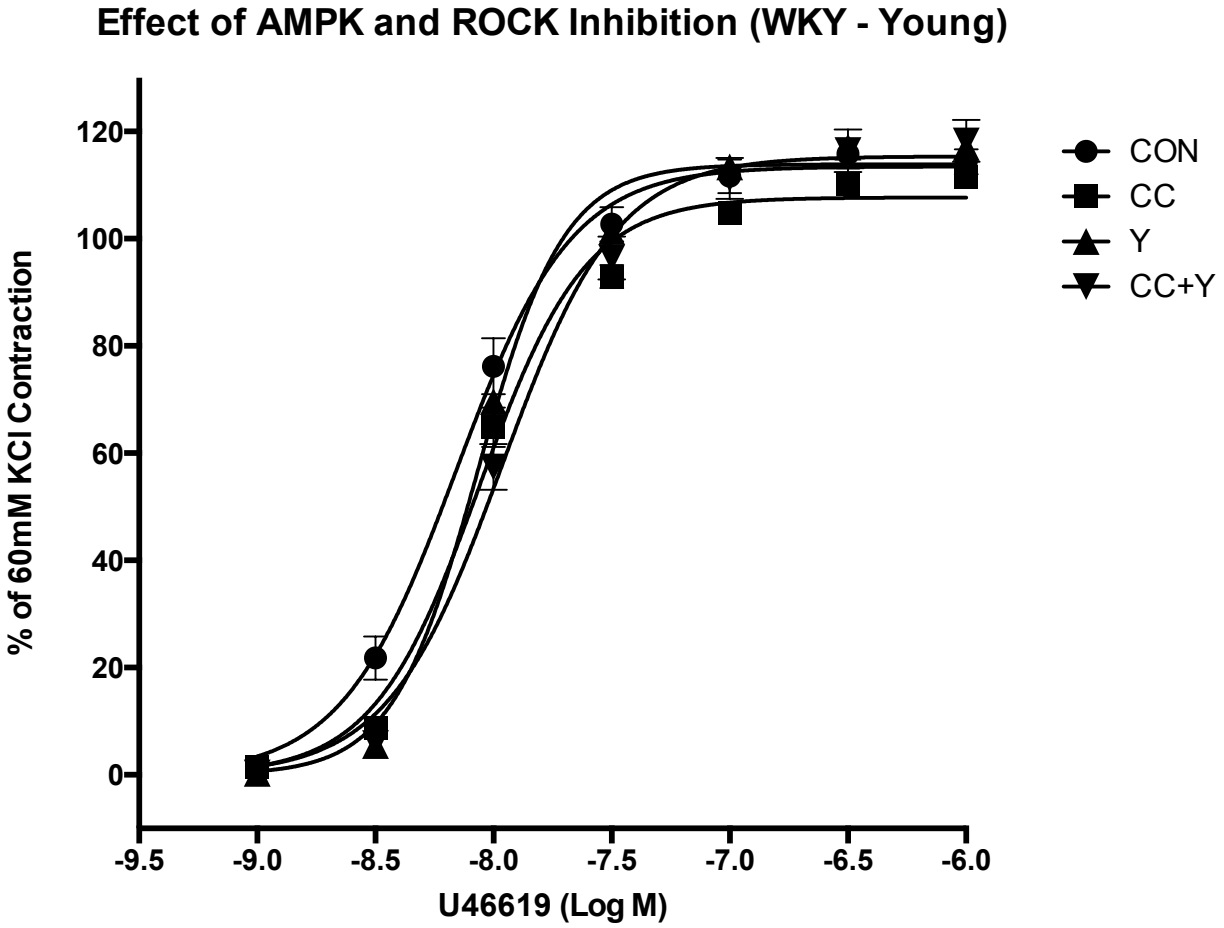


Figure 5: Vasoconstriction stimulated by the TPr agonist U46619 in young WKY (n=10) CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK inhibitor Y27632 (Y; $10^{-6.0}$ M) or a combination of Y27632 and CC (CC + Y) for 30 minutes. Contraction was stimulated following a 30-minute incubation using U46619 at concentrations from $10^{-9.0}$ to $10^{-6.0}$ M.

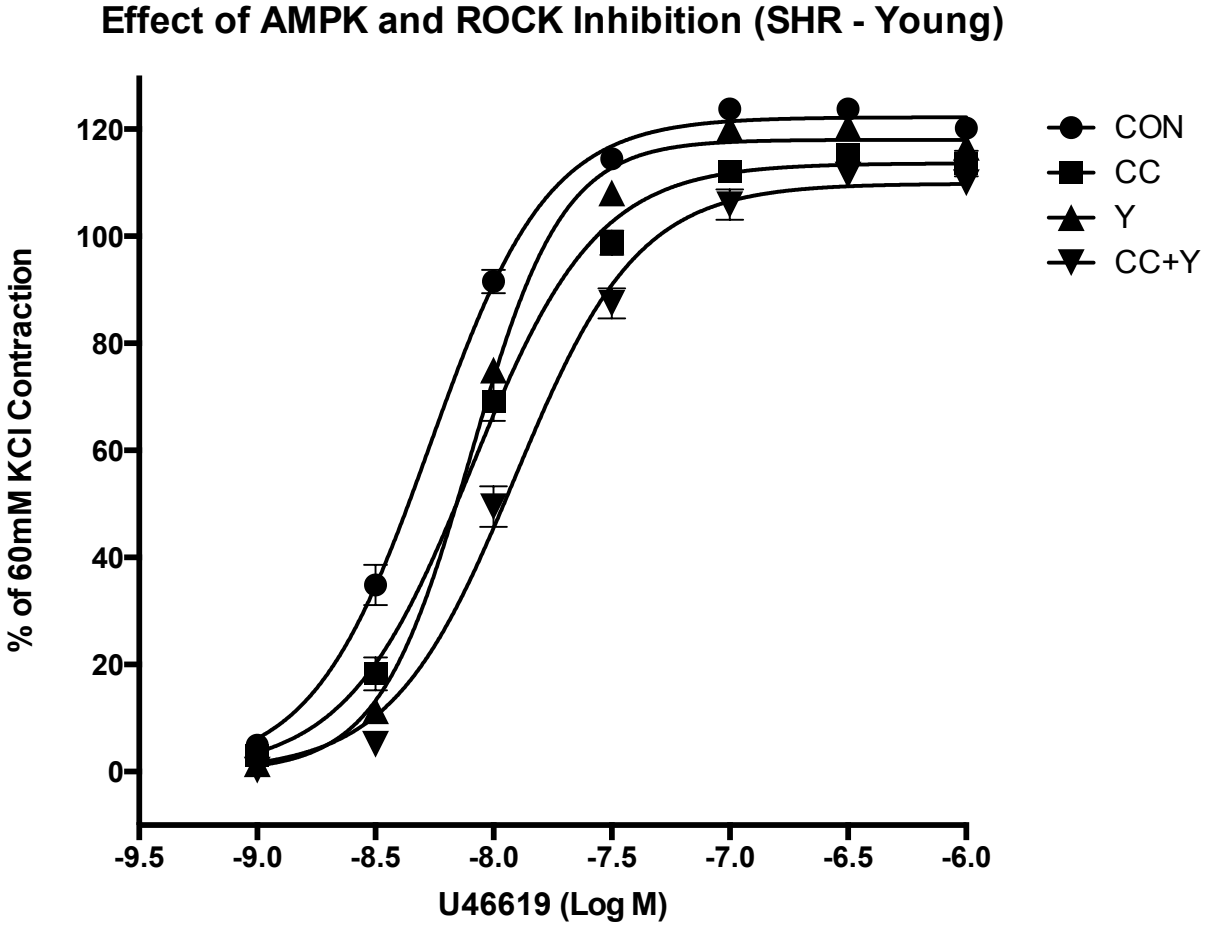


Figure 6: Vasoconstriction stimulated by the TPr agonist U46619 in young SHR (n=11) CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK inhibitor Y27632 (Y; $10^{-6.0}$ M) or a combination of Y27632 and CC (CC + Y) for 30 minutes. Contraction was stimulated following a 30-minute incubation using U46619 at concentrations from $10^{-9.0}$ to $10^{-6.0}$ M.

Table 11: *Strain response to U46619-induced contraction in young animals*

U46619 - YOUNG	EC_{50} (nM)		MAX Tension (g)		AUC (Arbitrary Units)	
	WKY	SHR	WKY	SHR	WKY	SHR
CON	7.2±0.7	5.2±0.5¥	1.5±0.1	1.6±0.1	243.1±9.7	274.3±5.1
CC	11.1±1.7*	7.3±0.7*‡¥	1.4±0.1	1.6±0.1¥	219.0±5.3	236.0±6.9*‡
Y	10.7±1.4*	7.7±0.6*	1.5±0.1	1.7±0.0¥	233.0±4.4	247.1±3.6*‡¥
CC+Y	13.8±1.9*	12.6±1.4*§	1.4±0.1	1.6±0.0¥	223.4±9.3	210.3±8.5*§†

EC_{50} (nM), MAX (g) and AUC (arbitrary units) of young WKY (n=10) and SHR (n=11) CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK inhibitor Y27632 (Y;$10^{-6.0}$ M) or a combination of Y27632 and CC for 30 minutes. Contraction was stimulated following a 30-minute incubation using U46619 at concentrations from $10^{-9.0}$ to $10^{-6.0}$ M. Data are presented as means ± SE. * $P < 0.05$ vs. CONTROL; § $P < 0.05$ vs. CC; † $P < 0.05$ vs. Y; ‡ $P < 0.05$ vs. CC+Y; ¥ $P < 0.05$ vs. WKY within treatment.

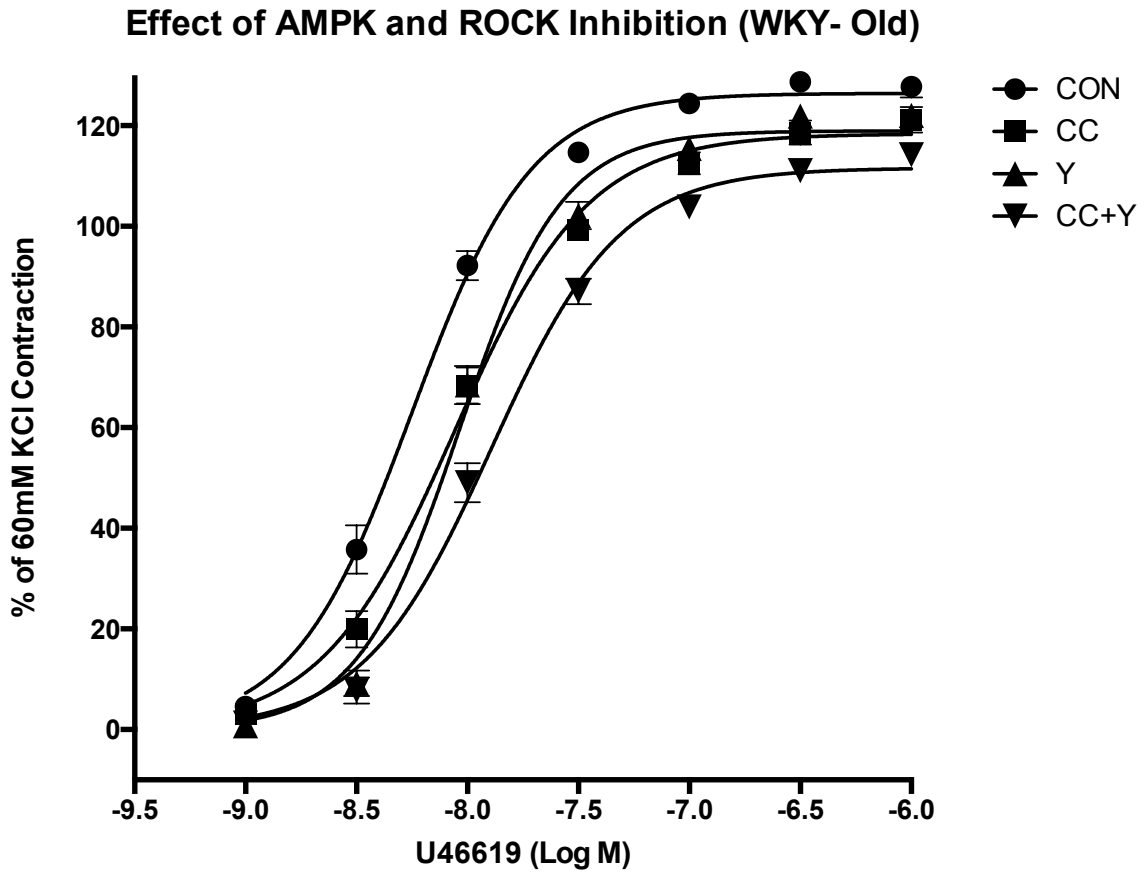


Figure 7: Vasoconstriction stimulated by the TPr agonist U46619 in old WKY (n=13) CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK inhibitor Y27632 (Y; $10^{-6.0}$ M) or a combination of Y27632 and CC (CC + Y) for 30 minutes. Contraction was stimulated following a 30-minute incubation using U46619 at concentrations from $10^{-9.0}$ to $10^{-6.0}$ M.

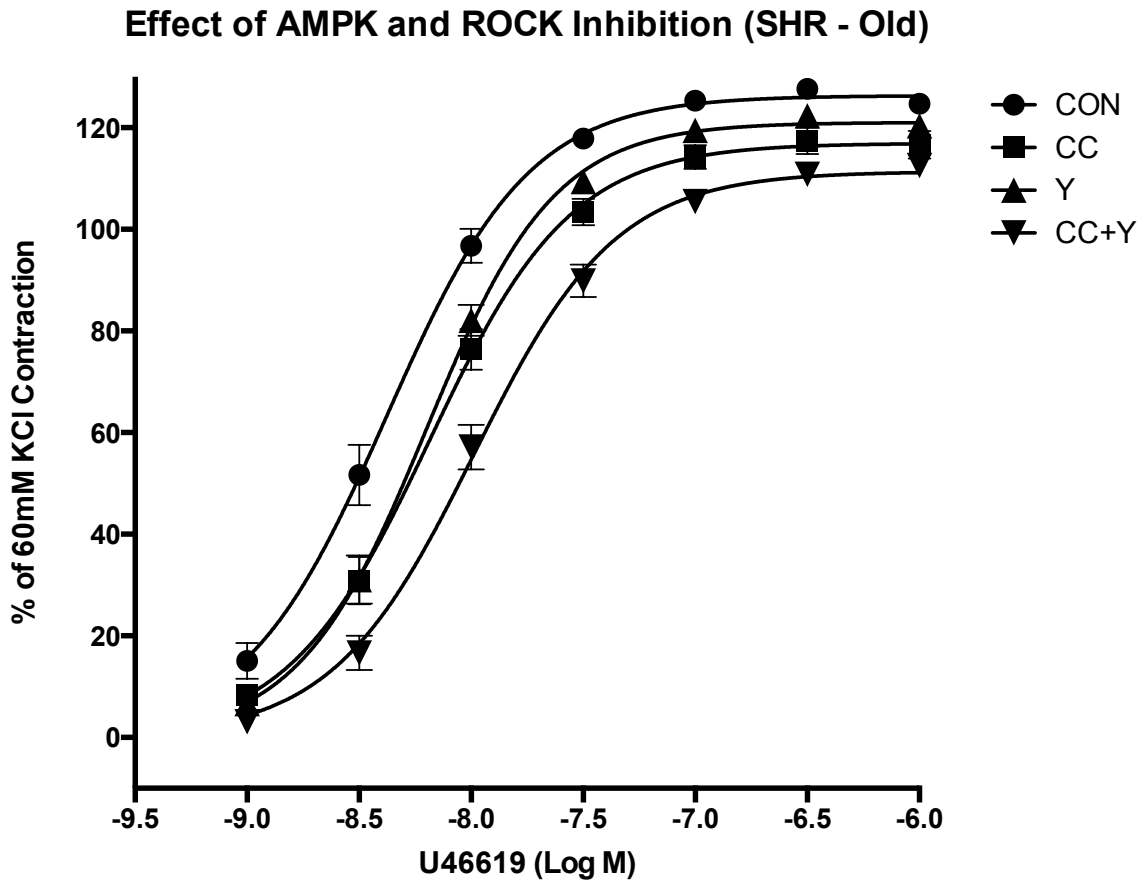


Figure 8: Vasoconstriction stimulated by the TPr agonist U46619 in old SHR (n=10) CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK inhibitor Y27632 (Y; 10-6.0 M) or a combination of Y27632 and CC (CC + Y) for 30 minutes. Contraction was stimulated following a 30-minute incubation using U46619 at concentrations from 10-9.0 to 10-6.0 M.

Table 12: *Strain response to U46619-induced contraction in old animals*

U46619 – OLD	EC_{50} (nM)		MAX Tension (g)		AUC (Arbitrary Units)	
	WKY	SHR	WKY	SHR	WKY	SHR
CON	5.9±0.7	4.5±0.8	1.7±0.1	1.7±0.1	281.0±5.3	294.6±9.3
CC	9.3±1.0*‡	7.2±1.1	1.7±0.1†	1.7±0.1	240.5±5.9*‡	251.1±8.1*‡
Y	10.1±1.1*	6.5±0.9‡¥	1.6±0.1§	1.6±0.1	240.8±6.1*‡	264.0±6.0*‡¥
CC+Y	13.9±1.8*§	11.3±1.7*†	1.6±0.1	1.6±0.1	208.3±5.6*§†	218.9±9.8*§†

EC_{50} (nM), MAX (g) AUC (arbitrary units) of old WKY (n=13) and SHR (n=10) CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK inhibitor Y27632 ($10^{-6.0}$ M) or a combination of CC + Y for 30 minutes. Contraction was stimulated following a 30-minute incubation using U46619 at concentrations from $10^{-9.0}$ to $10^{-6.0}$ M. Data are presented as means ± SE. * $P < 0.05$ vs. CONTROL; § $P < 0.05$ vs. CC; † $P < 0.05$ vs. Y; ‡ $P < 0.05$ vs. CC+Y; ¥ $P < 0.05$ vs. WKY within treatment.

Table 13: *Age response to U46619-induced contraction in WKY animals*

U46619 - WKY	EC_{50} (nM)		MAX Tension (g)		AUC (Arbitrary Units)	
	Young	Old	Young	Old	Young	Old
CON	7.2±0.7	5.9±0.7	1.5±0.1	1.7±0.1	243.1±9.7	281.0±5.3
CC	11.1±1.7*	9.3±1.0*‡	1.4±0.1	1.7±0.1†	219.0±5.3	240.5±5.9*‡
Y	10.7±1.4*	10.1±1.1*	1.5±0.1	1.6±0.1§	233.0±4.4	240.8±6.1*‡
CC+Y	13.8±1.9*	13.9±1.8*§	1.4±0.1	1.6±0.1	223.4±9.3	208.3±5.6*§†

EC_{50} (nM), MAX (g) and AUC (arbitrary units) of young (n=12) and old (n=10) WKY CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK inhibitor Y27632 (Y; $10^{-6.0}$ M) or a combination of Y27632 and CC for 30 minutes (n=11). Contraction was stimulated following incubation using U46619 at concentrations from $10^{-9.0}$ to $10^{-6.0}$ M. Data are presented as means ± SE. * $P < 0.05$ vs. CONTROL; § $P < 0.05$ vs. CC; † $P < 0.05$ vs. Y; ‡ $P < 0.05$ vs. CC+Y. ¥ $P < 0.05$ vs. Young within treatment. *Note:* for the sake of comparison, data for the WKY young (table 11) and WKY old (table 12) is repeated for this table.

Table 14: *Age response to U46619-induced contraction in SHR animals*

U46619 - SHR	EC_{50} (nM)		MAX Tension (g)		AUC (Arbitrary Units)	
	Young	Old	Young	Old	Young	Old
CON	5.2±0.5	4.5±0.8	1.6±0.1	1.7±0.1	274.3±5.1	294.6±9.3
CC	7.3±0.7*‡	7.2±1.1	1.6±0.1	1.7±0.1	236.0±6.9*‡	251.1±8.1*‡
Y	7.7±0.6*	6.5±0.9‡	1.7±0.0	1.6±0.1	247.1±3.6*‡	264.0±6.0*‡¥`
CC+Y	12.6±1.4*§	11.3±1.7*†	1.6±0.0	1.6±0.1	210.3±8.5*§†	218.9±9.6*§†

EC_{50} (nM), MAX (g) and AUC (arbitrary units) of young (n=12) and old (n=11) SHR CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the ROCK inhibitor Y27632 (Y; $10^{-6.0}$ M) or a combination of Y27632 and CC for 30 minutes (n=11). Contraction was stimulated following incubation using U46619 at concentrations from $10^{-9.0}$ to $10^{-6.0}$ M. Data are presented as means ± SE. * $P < 0.05$ vs. CONTROL; § $P < 0.05$ vs. CC; † $P < 0.05$ vs. Y; ‡ $P < 0.05$ vs. CC+Y. ¥ $P < 0.05$ vs. Young within treatment. *Note:* for the sake of comparison, data for the SHR young (table 11) and SHR old (table 12) is repeated for this table.

Table 15: *3-Way ANOVA table examining the EC_{50} response to U46619-induced contraction*

	EC_{50} response to U46619				
Effect	**Sum of squares**	**df**	**Mean Square**	**F value**	***P* value**
Incubation	921.7	3	307.2	28.8	0.000*
Hypertension	73.2	1	73.2	6.9	0.010*
Age	4.4	1	4.4	0.4	0.524
Incubation x Hypertension	17.7	3	5.9	0.6	0.648
Incubation x Age	13.8	3	4.6	0.4	0.731
Age x Hypertension	44.4	1	44.4	4.2	0.043*
Incubation x HT x Age	8.8	3	12.9	1.2	0.308
Error	1580.4	148	10.7		

Effects are described as follows: Incubation refers to conditions isolated CCA segments were subject to in bath for 30 minutes prior to stimulation with U46619; CON (no drug); CC (AMPK Inhibitor; 20 μM); Y (ROCK inhibitor; $10^{-6.0}$ M); CC + Y (combination of CC and Y at their respective separate concentrations). Hypertension refers to any differences resulting from strain (WKY vs. SHR). Age refers to any differences resulting from age (young vs. old). *P ≤ 0.05.

Table 16: *3-Way ANOVA table examining the MAX response to U46619-induced contraction*

MAX response to U46619					
Effect	**Sum of squares**	**df**	**Mean Square**	**F value**	***P* value**
Incubation	0.1	3	0.0	0.8	0.493
Hypertension	0.3	1	0.3	7.3	0.008*
Age	0.3	1	0.4	8.7	0.004*
Incubation x Hypertension	0.0	3	0.0	0.1	0.975
Incubation x Age	0.2	3	0.1	1.3	0.291
Age x Hypertension	0.3	1	0.3	6.5	0.012*
Incubation x HT x Age	0.0	3	0.0	0.3	0.808
Error	6.0	148	0.0		

Effects are described as follows: Incubation refers to conditions isolated CCA segments were subject to in bath for 30 minutes prior to stimulation with U46619; CON (no drug); CC (AMPK Inhibitor; 20 μM); Y (ROCK inhibitor; $10^{-6.0}$ M); CC + Y (combination of CC and Y at their respective separate concentrations). Hypertension refers to any differences resulting from strain (WKY vs. SHR). Age refers to any differences resulting from age (young vs. old). *P ≤ 0.05.

Table 17: *3-Way ANOVA table examining the AUC response to U46619-induced contraction*

AUC response to U46619					
Effect	**Sum of squares**	**df**	**Mean Square**	**F value**	***P* value**
Incubation	70305.4	3	23435.1	46.9	0.000*
Hypertension	7087.4	1	7087.4	14.2	0.000*
Age	7910.7	1	7910.7	15.8	0.000*
Incubation x Hypertension	3242.8	3	1080.9	2.2	0.095
Incubation x Age	5492.3	3	1830.8	3.7	0.014*
Age x Hypertension	48.6	1	48.6	0.1	0.756
Incubation x HT x Age	2460.6	3	820.2	1.6	0.182
Error	1580.4	148	10.7		

Effects are described as follows: Incubation refers to conditions isolated CCA segments were subject to in bath for 30 minutes prior to stimulation with U46619; CON (no drug); CC (AMPK Inhibitor; 20 μM); Y (ROCK inhibitor; $10^{-6.0}$ M); CC + Y (combination of CC and Y at their respective separate concentrations). Hypertension refers to any differences resulting from strain (WKY vs. SHR). Age refers to any differences resulting from age (young vs. old). *P ≤ 0.05.

Table 18: *Receptor mediated differences in the curve parameters of young WKY*

Young WKY	EC_{50} (nM)		MAX Tension (g)		AUC (Arbitrary Units)	
	PE	U46619	PE	U46619	PE	U46619
CON	21.7±3.2	7.2±0.7	1.3±0.1	1.4±0.1	339.5±8.8	243.1±9.7
CC	72.0±13.7*‡	11.1±1.7*	1.2±0.0‡	1.4±0.1	245.6±8.0*‡	219.0±5.3
Y	78.7±8.9*‡	10.7±1.4*	1.1±0.0*‡	1.5±0.1	230.7±7.8*‡	233.0±4.4
CC+Y	234.4±42.3*§†	13.8±1.9*	0.9±0.1*§†	1.4±0.1	157.4±8.2*§†	223.4±9.3

EC_{50} (nM), MAX (g) and AUC (arbitrary units) of young WKY CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the Rho-kinase inhibitor Y27632 (Y; $10^{-6.0}$ M) or a combination of Y27632 and CC (CC+Y) for 30 minutes. CCA segments were subject to two contractile agonists; PE and U46619). Data are presented as means ± SE. * $P < 0.05$ vs. CONTROL; § $P < 0.05$ vs. CC; † $P < 0.05$ vs. Y; ‡ $P < 0.05$ vs. CC+Y. *Note:* for the sake of comparison, data for the WKY young PE responses (table 4) and WKY young U46619 responses (table 11) is repeated for this table.

Table 19: *Receptor mediated differences in the curve parameters of young SHR*

Young SHR	EC_{50} (nM)		MAX Tension (g)		AUC (Arbitrary Units)	
	PE	U46619	PE	U46619	PE	U46619
CON	14.5±1.1	5.2±0.5	1.3±0.0	1.6±0.1	331.1±8.5	274.3±5.1
CC	46.3±6.0*‡	7.3±0.7*‡	1.3±0.0	1.6±0.1	248.2±8.8*‡	236.0±6.9*‡
Y	67.1±8.4*‡	7.7±0.6*	1.3±0.1	1.7±0.0	253.7±11.8*‡	247.1±3.6*‡
CC+Y	159.6±24.3*§†	12.6±1.4*§	1.2±0.0*	1.6±0.0	184.2±5.6*§†	210.3±8.5*§†

EC_{50} (nM), MAX (g) and AUC (arbitrary units) of young SHR CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the Rho-kinase inhibitor Y27632 ($10^{-6.0}$ M) or a combination of Y27632 and CC for 30 minutes. CCA segments were subject to two contractile agonists; PE and U46619). Data are presented as means ± SE. * $P < 0.05$ vs. CONTROL; § $P < 0.05$ vs. CC; † $P < 0.05$ vs. Y; ‡ $P < 0.05$ vs. CC+Y. *Note:* for the sake of comparison, data for the SHR young PE responses (table 4) and SHR young U46619 responses (table 11) is repeated for this table.

Table 20: *Receptor mediated differences in the curve parameters of old WKY*

OLD WKY	EC_{50} (nM)		MAX Tension (g)		AUC (Arbitrary Units)	
	PE	U46619	PE	U46619	PE	U46619
CON	35.5±3.0	5.9±0.7	1.3±0.1	1.7±0.1¥	322.5±11.0	281.0±5.3
CC	131.0±18.7*‡	9.3±1.0*‡	1.2±0.1	1.7±0.1†¥	219.7±9.2*‡	240.5±5.9*‡
Y	139.6±14.7*‡	10.1±1.1*	1.2±0.1	1.6±0.1§	207.6±10.1*‡	240.8±6.1*‡
CC+Y	240.6±15.7*§†	13.9±1.8*§	1.1±0.1*	1.6±0.1	157.0±6.8*§†	208.3±5.6*§†

EC_{50} (nM), MAX(g) and AUC (arbitrary units) of old WKY CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the Rho-kinase inhibitor Y27632 ($10^{-6.0}$ M) or a combination of Y27632 and CC for 30 minutes. CCA segments were subject to two contractile agonists; PE and U46619). Data are presented as means ± SE. * $P < 0.05$ vs. CONTROL; § $P < 0.05$ vs. CC; † $P < 0.05$ vs. Y; ‡ $P < 0.05$ vs. CC+Y. *Note:* for the sake of comparison, data for the WKY old PE responses (table 5) and WKY old U46619 responses (table 12) is repeated for this table.

Table 21: *Receptor mediated differences in the curve parameters of old SHR*

Old SHR	EC_{50} (nM)		MAX Tension (g)		AUC (Arbitrary Units)	
	PE	U46619	PE	U46619	PE	U46619
CON	36.5±4.6	4.5±0.8	1.3±0.0	1.7±0.1	294.6±9.3	301.7±7.6
CC	114.4±13.3*‡	7.2±1.1	1.2±0.0*‡	1.7±0.1	251.6±8.1*‡	216.6±5.0*‡
Y	108.1±10.1*	6.5±0.9‡	1.3±0.0‡	1.6±0.1	264.0±6.0*‡	225.7±4.5*‡
CC+Y	234.7±18.1*§†	11.3±1.7*†	1.1±0.0*§†	1.6±0.1	218.9±9.6*§†	158.2±4.8*§†

EC_{50} (nM), MAX (g) and AUC (arbitrary units) of old SHR CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the Rho-kinase inhibitor Y27632 ($10^{-6.0}$ M) or a combination of Y27632 and CC for 30 minutes. CCA segments were subject to two contractile agonists; PE and U46619). Data are presented as means ± SE. * $P < 0.05$ vs. CONTROL; § $P < 0.05$ vs. CC; † $P < 0.05$ vs. Y; ‡ $P < 0.05$ vs. CC+Y. *Note:* for the sake of comparison, data for the SHR old PE responses (table 5) and SHR old U46619 responses (table 12) is repeated for this table.

Table 22: *Changes in EC_{50} in response to agonist induced contraction for all groups*

Increase in EC_{50} vs. CON (fold difference)

	WKY Young		**SHR Young**		**WKY Old**		**SHR Old**	
	PE	U46619	PE	U46619	PE	U46619	PE	U46619
CC	3.4±0.3*‡	1.6±0.1*	3.2±0.3*‡	1.4±0.1*‡	3.8±0.6*‡	1.6±0.1*‡	3.5±0.4*‡	1.7±0.1
Y	4.0±0.4*‡	1.5±0.1*	4.9±0.6*‡	1.5±0.1*	4.0±0.3*‡	1.8±0.2*	3.1±0.2*	1.5±0.1‡
CC+Y	13.0±3.5*§†	1.9±0.1*	11.5±1.9*§†	2.4±0.1*§	7.3±0.9*§†	2.4±0.2*§	7.6±1.2*§†	2.6±0.2*‡

Shift in EC_{50} for ALL groups (WKY/SHR; young/old). Response to incubation with the AMPK inhibitor Compound C (CC; 20μM), the Rho-kinase inhibitor Y27632 (Y; $10^{-6.0}$ M) or a combination of CC and Y for 30 minutes. CCA segments were subject to two contractile agonists; PE and U46619). Changes are expressed as fold increase vs. CON. * $P < 0.05$ vs. CONTROL; § $P < 0.05$ vs. CC; † $P < 0.05$ vs. Y; ‡ $P < 0.05$ vs. CC+Y. Data are presented as means ± SE.

Table 23: *Changes in MAX response to agonist induced contraction in all groups*

Change in MAX tension vs. CON (% grams)

	WKY Young		**SHR Young**		**WKY Old**		**SHR Old**	
	PE	U46619	PE	U46619	PE	U46619	PE	U46619
CC	9.3±3.4‡	1.0±4.5	3.4±3.7	-2.7±3.8	7.7±3.9	-6.2±4.8†	8.3±3.5*‡	-1.8±3.2
Y	14.6±4.0*‡	0.0±4.4	0.4±2.8	-4.8±3.1	10.7±4.2	3.7±4.5§	2.2±2.9‡	0.2±4.0
CC+Y	26.2±4.1*§†	1.3±3.2	9.6±2.2*	-0.7±2.5	20.1±4.6*	4.5±3.8	16.8±2.5*§†	2.4±3.7

Changes in MAX for all groups. Response to incubation with the AMPK inhibitor Compound C (CC; 20μM), the Rho-kinase inhibitor Y27632 (Y; $10^{-6.0}$ M) or a combination of CC and Y for 30 minutes. CCA segments were subject to two contractile agonists; PE and U46619). Changes are expressed as % magnitude (grams) of CON. * $P < 0.05$ vs. CONTROL; § $P < 0.05$ vs. CC; † $P < 0.05$ vs. Y; ‡ $P < 0.05$ vs. CC+Y. Data are presented as means ± SE.

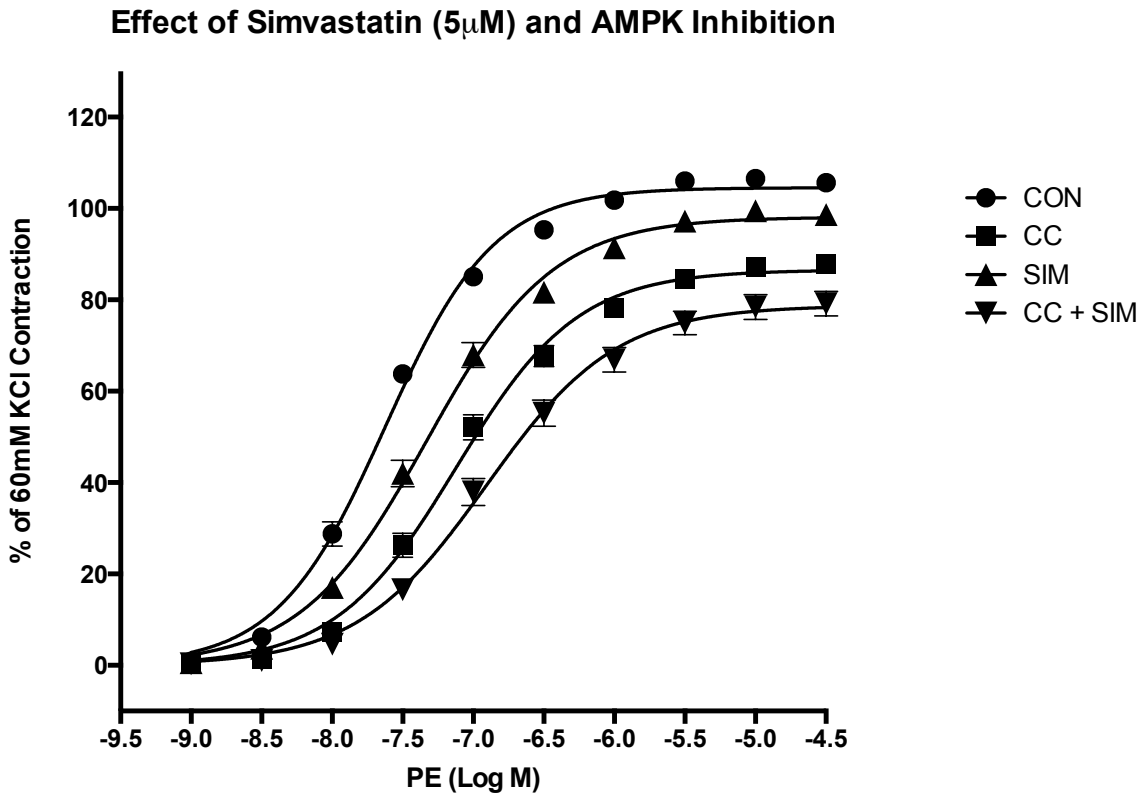


Figure 9: Vasoconstriction stimulated by phenylephrine (PE) in old WKY (n=11) CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the HMG-CoA reductase inhibitor Simvastatin (SIM; 5 μM) or a combination of CC + SIM for 30 minutes. Contraction was stimulated following a 45-minute incubation using PE at concentrations from 10-9.0 to 10-4.5 M.

Table 24: *Curve parameters for WKY response to HMG-CoA reductase inhibition*

WKY	EC_{50} (nM)	MAX Tension (g)	AUC (Arbitrary Units)
CON	24.8±2.2	1.4±0.0	322.3±7.4
CC	85.9±12.0*†‡	1.2±0.0*	224.5±8.4*†‡
SIM	49.3±4.4*§‡	1.3±0.1	273.0±8.4*†‡
CC+SIM	134.5±13.0*§†	1.2±0.1*	187.8±9.3*§†

EC_{50} (nM), MAX(g) and AUC (arbitrary units) of old WKY (n=11) CCA segments incubated with no drug (CON), the AMPK inhibitor Compound C (CC; 20μM), the HMG-CoA reductase inhibitor Simvastatin (SIM; 5μM) or a combination of CC + SIM for 45 minutes. Contraction was stimulated following incubation using PE at concentrations from $10^{-9.0}$ to $10^{-4.5}$ M. Data are presented as means ± SE. * $P < 0.05$ vs. CONTROL; § $P < 0.05$ vs. CC; † $P < 0.05$ vs. SIM; ‡ $P < 0.05$ vs. CC+Y.

DISCUSSION

With its involvement in the regulation of VSM contraction yet to be fully characterized, the potential role of AMPK activity in smooth muscle contraction is a relatively new and emerging topic in cardiovascular research. The global objective of this thesis was to examine the subcellular mechanisms regulating vascular smooth muscle contraction, particularly the possible interaction between two vascular smooth muscle signaling pathways involving AMPK and ROCK. This was examined under normal conditions, and in both aging and hypertensive models in endothelium-denuded CCA segments. The main findings of this thesis were:

1. A basal level of AMPK activation is necessary in the normal VSM response to PE-induced contraction.
2. Combined and separate AMPK and ROCK inhibition significantly increased the EC_{50} and decreased the maximum tension developed in the contractile response to PE and, to a lesser extent, U46619.
3. The contribution of TPr-agonist activity is comparatively less affected by AMPK and/or ROCK inhibition when contrasted to PE-induced contraction.
4. VSM is more sensitive to TPr stimulation compared to alpha-adrenergic stimulation, indicated by the EC_{50} comparisons made between the two agonists.
5. Hypertension and aging do significantly affect VSM smooth muscle contraction by causing a slight increase in EC_{50}, with greatest difference in curve parameter measurements present in the old SHR.
6. The HMG-CoA reductase inhibitor Simvastatin acts in a similar fashion to Y27632 in PE-induced contractile responses in isolated WKY CCA.

AMPK inhibition inhibits contraction in isolated WKY CCA

After its identification as a kinase of HMG-CoA reductase[16], AMPK quickly became the target of metabolic research. Characterization of AMPK in vascular research has been vague, as little is known about the role AMPK has in regulating vascular tone, specifically the balance between the phosphorylation of the regulatory contractile proteins MLCK and MLCP. The current study evaluated the role of AMPK in regulating pharmacologically induced contraction of isolated WKY CCA segments.

In previous work examining the role of AMPK activation in vasorelaxion, activation of AMPK in pre-contracted isolated vessels has been shown to induce vasodilation in both an endothelium –dependent and –independent fashion[30]. This activation effect of AMPK is voided by compound C and thusly; one would expect AMPK inhibition to blunt the ability of VSM to relax, increasing the relative response to agonist stimulation and thus increase contraction. We anticipated a change that would favour a decreased EC_{50} and an increased maximum developed tension at the same agonist concentration because of the inhibited ability for AMPK to induce vasodilation. However, this is not the case. Our work clearly shows that, in young WKY CCA segments, AMPK inhibition via the AMPK inhibitor Compound C (CC; 20 μM) proved effective at inhibiting the contractile response to pharmacologically induced contraction (table 4). 30-minute incubation with CC caused a marked increase in the EC_{50} of both PE-induced (~3 fold increase) and U46619-induced (~1.5 fold increase) VSM contraction (table 22). Increased EC_{50} in response to CC incubation was observed in all groups across both studies with the exception of the old SHR animals exposed to U46619 (table 14), where contraction was not significantly affected by AMPK inhibition via CC. This

increase in EC_{50} in the presence of CC alone indicates that AMPK contributes during the normal contractile response, and is thus involved in eliciting contraction in VSM. More investigation is required to determine where AMPK is acting in regulation of VSM tone.

The suppression of the VSM contraction via incubation with CC could be the consequence of modulation of the MLCK activity of the VSM. By revisiting the original work on AMPK and its identification as an "energy-sensing" kinase[82], it seems likely that AMPK is acting on specific components of the cell signaling pathways involved in energy management during VSM contraction. With known effects on regulatory MLC phosphorylation, AMPK inhibition could play a role in altering MLCK phosphorylation, possibly via decreasing calcium sensitivity of MLCK. This could indicate that AMPK activity is necessary for MLCK activity to initiate VSM contraction though more work is needed to verify if this mechanism can account for the reduction in contractile function of VSM in response to AMPK inhibition.

ROCK inhibition inhibits contraction in isolated WKY CCA

The RhoA/ROCK pathway is a major signaling pathway responsible for the management of MLCP activity and thus, the management of vascular tone[11]. Recent work has shown that in VSM, ROCK activity could be linked to AMPK activity through modulation of Rho-GEFs[13]. With ROCK partially inhibited via Y27632, it is expected that U46619 would not induce a significant contractile response. Contrary to our anticipated results, inhibition of ROCK via Y27632 caused a significant increase in the EC_{50} in all WKY CCA segments. This effect was seen during ROCK inhibition in PE-induced contraction and was also present, to a much lesser extent, during U46619-

induced contraction. Maximum developed tension was also significantly lower in young and old WKY compared to their SHR counterparts, but only for vessels exposed to PE-induced contraction. This was an unexpected result as U46619-induced contraction is initiated via thromboxane-prostanoid GPCR activity, leading to RhoA/ROCK activation and thus MLCP inhibition. This also suggests that the TPr receptor pathway responsible for initiating contraction is affected to a much lesser degree than the alpha-adrenergic GPCR contractile pathway during by AMPK and/or ROCK inhibition. This pattern is evident during the increased EC_{50} response to AMPK and/or ROCK inhibition across all groups (table 22).

Surprisingly, there was little significant difference between old SHR and WKY groups exposed to U46619 treatment. Increased basal ROCK activity is a characteristic of hypertension and aging, and thus the old SHR are most likely to exhibit the highest amount of basal ROCK activity when compared to the other animal groups. This higher proportionate basal ROCK activation in the old SHR could account for the lack of significance in the old SHR exposed to U46619-induced contraction (table 12), even in the presence of the ROCK inhibitor Y27632.

Combined AMPK and ROCK inhibition in amplifies the inhibition of contraction in isolated WKY CCA

With evidence supporting the activity of AMPK and ROCK in smooth muscle contraction[13], this thesis aimed to examine and evaluate the contribution that AMPK and ROCK would have, either separately or in combination, during VSM contraction. If the effect of AMPK on VSM contraction is ROCK-dependent during VSM contraction, then

AMPK and ROCK inhibition in combination should not result in any differences compared to separate/individual AMPK or ROCK inhibition conditions. This thesis showed that separate AMPK or ROCK inhibition significantly increased the EC_{50} of PE-induced contraction. However, the *combined* AMPK and ROCK inhibition *further* amplified the magnitude of this contraction inhibition when paired with each other. This additive effect was greatest in the WKY groups exposed to PE-induced contraction, with a similar pattern of response in the U46619-induced contraction group, but to a much lesser extent.

These results provoke an interesting scenario, one where the separate incubation conditions alter contraction response, but also where combination of the two further increases the magnitude of that response. This indicates that AMPK and ROCK activity likely modulates **PE-induced** contraction in one of two ways: either independently of one another, or, in a way that they are both partially affecting the same process that regulates contraction. This, in concert with minimal decreases in maximum developed tension, is contradicted by the relatively minimal shift in the EC_{50} in CCA segments treated with the **TPr agonist** U46619. Comparison of the fold increase in EC_{50} (table 22) provides a strong case, depicting the huge difference in sensitivity to the specific vascular agonists PE and U46619, with AMPK and/or ROCK inhibition having a much greater blunting effect on PE-induced contraction. In the young WKY response to AMPK inhibition, we observed ~3.4 fold increase in the EC_{50} for PE compared to only ~1.6 fold increase for U46619. The EC_{50} was *further increased* when AMPK and ROCK inhibition were combined for both conditions, with PE requiring a ~13 fold increase in EC_{50} (vs. CON) while U46619 only required ~2 fold increase (vs. CON)(table 22). This is a clear

indication of the receptor-mediated differences present in AMPK activity during agonist-induced VSM contraction.

Receptor mediated differences are present in the role of AMPK in VSM contraction

Significant effects of AMPK inhibition were observed in the three curve parameters from the PE-induced contraction studies compared to the minimal differences found in U46619-induced contraction. The increased EC_{50} for PE-induced contraction were greater, on average, than the shifts in EC_{50} for U46619 for the CC, Y and CC+Y incubation conditions (table 22), with the young animals having the greatest differences compared to the old animals. Hypertension was less effective at modulating receptor mediated differences compared to age (table 4/5), with aging having a greater blunting response on increasing EC_{50} (table 6). The contribution of AMPK and ROCK activity was minimal in U46619 as no significant changes in maximum developed tension were present for any group in any condition, with the exception of the CC incubation condition in the old WKY being slightly higher than that of the Y27632 incubation condition. This is significant for two reasons: 1) this demonstrates that thromboxane-prostanoid receptor signaling pathways are relatively unaffected by AMPK inhibition during agoinst-induced contraction and 2) that this TPr-specific effect is not modulated by the pathologies of age and hypertension in VSM alone.

These findings also confirm that ROCK activity is involved in GPCR alpha-adrenergic induced-contraction. PE-induced contraction likely causes an influx of calcium, leading to the formation of the Ca-CaM complex, and thus contraction. We know AMPK inhibition reduces the magnitude of contraction, and contraction is

dependent on calcium influx, whether it be from outside the VSM or from internal calcium stores. It is likely that AMPK activity is necessary for the release of calcium from intracellular stores or that it is necessary for initiating the influx of calcium from outside of the VSM. By inhibiting AMPK activity, the kinase would be rendered inactive and thus unable to phosphorylate intermediates in the calcium signaling pathways that are required for VSM contraction. Further study is required to elucidate the specific mechanisms of AMPK activity in alpha-adrenergic induced VSM contraction.

Hypertension

Significant differences were present in both the young and old groups when examining hypertension effects on PE-induced contraction, with the greatest EC_{50} effect present in the young animals, with SHR controls having a greater EC_{50} than their WKY counterparts (table 4/5). Though the young SHR also showed reduced maximum developed tension within their group, they also had a slightly greater maximum developed tension compared to the age-matched WKY (table 4/5). With the exception of the AUC for Y27632 incubation in the old SHR, there were no other significant strain differences present between old WKY and SHR, suggesting the vascular response to contraction in hypertension is unaffected by AMPK and/or ROCK inhibition.

WKY exposed to the TPr agonist U46619 showed little significant difference in EC_{50} to their SHR counterparts. A slight reduction in the EC_{50} for the CON and CC incubations in young animals (table 11), and the Y incubation in old SHR (table 12) was present with the young SHR having significantly higher maximum developed tension compared to young WKY as well, a result in accordance with previous literature. SHR

rats are known to have more active RhoA signaling[7,76] compared to age-matched WKY and are thus more sensitive to TPr stimulation, possibly explaining why the maximum developed tension was greater in the young SHR group compared to the young WKY.

The magnitude of these effects on the EC_{50} was relatively minor, indicating that the inhibition of AMPK in VSM alone is likely not involved in the pathological deficiencies associated with hypertension. This result in our hypertensive model seems logical because several attributes associated with hypertension are often associated with endothelial dysfunction. Such attributes include increased prostanoid production, reduced eNOS activity, increased inflammatory cytokines and increased EDCFs[76,83,84]. With the removal of the endothelium, confounding factors associated with hypertension were eliminated and thus could not contribute to any differences observed in our hypertensive model. More study would be required, possibly with an intact endothelium, to thoroughly examine the balance of these signaling mechanisms and their role in hypertension.

Aging

Aging is associated with several mechanical and cellular changes[48,49] that alter cardiovascular function in both WKY and SHR[60,68]. Our results regarding the aging effect are very similar to changes observed in the hypertensive state, with an increased EC_{50} in the older animals compared to the younger animals, within strain. This was only true of animals exposed to PE-induced contraction, indicating that age augmented the receptor-mediated differences present in the U46619 response. This could be the result of changes in receptor-density associated with aging, with the potential for proportionately more alpha-adrenergic receptors in older animals compared to younger animals, resulting in a

more robust contractile response to PE. This is confirmed by the fact that, with the exception of the CC+Y incubation in old SHR compared to the young SHR, there were no significant differences in maximum developed tension. With an exclusive shift in the EC_{50} occurring, we know that aging alters the sensitivity of isolated CCA vessels to alpha-adrenergic stimulation, with older animals requiring a higher concentration of the agonist to elicit the same amount of absolute tension (contractile ability is not altered, only receptor sensitivity).

U46619-induced contraction did not affect the aging SHR EC_{50} or MAX response and only showed slight significant differences between young and old WKY in the CON and CC incubation conditions for maximum developed tension. This indicates that, in aging, TPr-mediated contraction in VSM is comparative unaffected when compared to the differences exhibited in PE-induced contraction.

Statins

One of the most novel findings of this thesis was the discovery that HMG-CoA reductase inhibition via Simvastatin modulates alpha-adrenergic induced VSM contraction. Statins have been shown to alter a variety of cellular signaling mechanisms including ROS handling, reduced inflammatory signaling and anti-thrombotic effects[52-54]. It is also demonstrated that statins can inhibit potassium channel activity in porcine coronary arteries, leading to impaired vasorelaxation[85]. In isolated WKY CCA segments, it is possible that Simvastatin has a similar effect on Ca^{2+} channels, and possibly the RhoA/ROCK mediation of alpha-adrenergic induced contraction. Our results show that 45-minute incubation with simvastatin (5μM), alone, significantly lowers the EC_{50} and

maximum developed tension of denuded isolated WKY CCA segments. In combination with the AMPK inhibitor CC, the magnitude of this reduction is amplified. Simvastatin effects showed several parallels with the ROCK inhibitor responses during PE-induced contraction with respect to effects on curve parameters. Thus, simvastatin has a sort of "Y27632 mimetic" effect. In age-matched WKY CCA segments, both Y27632 and Simvastatin incubation led to comparable increases in the EC_{50}, as well as decreases in the maximum developed tension produced by PE-induced contraction (Table 5 vs. Table 28). This also confirms that signaling mechanisms involving the manipulation of HMG-CoA reductase activity in VSM can alter the manner in which VSM contraction is generated. However, it should be noted that statins have been studied to have a variety of effects in other types of tissue found outside of the vasculature. These observable effects in VSM could be pleiotropic in nature and should not be attributed to HMG-CoA reductase inhibition alone. Further investigation will be required to uncover how significant the role of VSM HMG-CoA reductase activity is during the maintenance and regulation of vascular tone.

Limitations

Animal availability sometimes restricted our ability to use animals of appropriate age. In addition to availability, it was difficult to determine an appropriate age for our "aging" criteria since most rats live to an age of about 2 years (~100 weeks) though some of our rats exhibited early-onset pathological conditions associated with aging.

Biochemical assessment of the AMPK, ROCK and HMG-CoA reductase activation state would have been ideal in order to characterize their effect during VSM

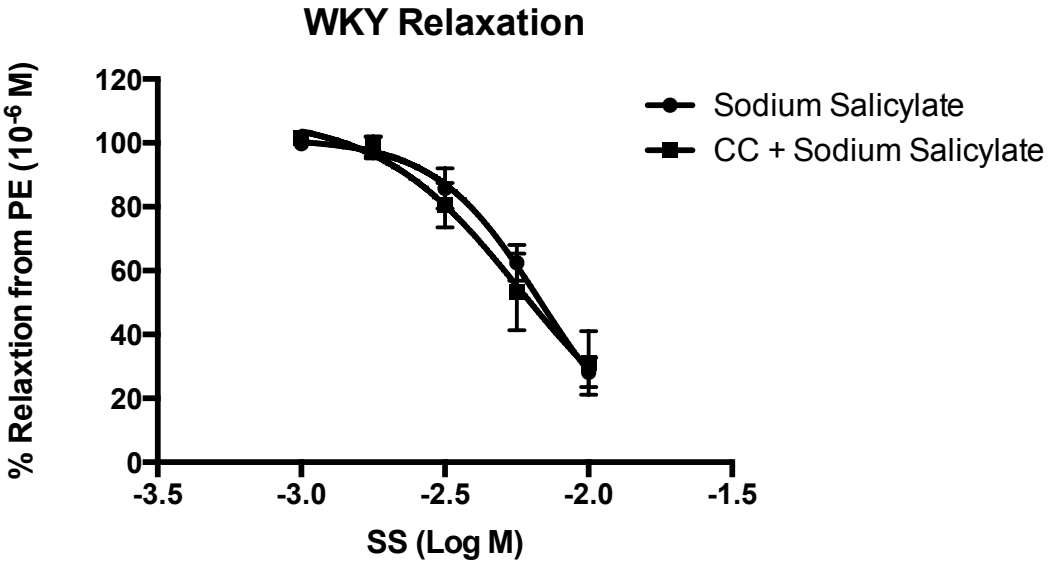
contraction in more detail. Early attempts at Western blotting for these proteins in both their normal and phosphorylated state yielded blots insufficient for use in this document.

Use of arterial segments with an intact endothelium could have greatly augmented the data set presented in this document while concurrently expanding on the characterization of AMPK in regulating vascular tone. However, preparations devoid of the endothelium were a clearer way of distinguishing how AMPK activity affects contraction at the level of the VSM alone.

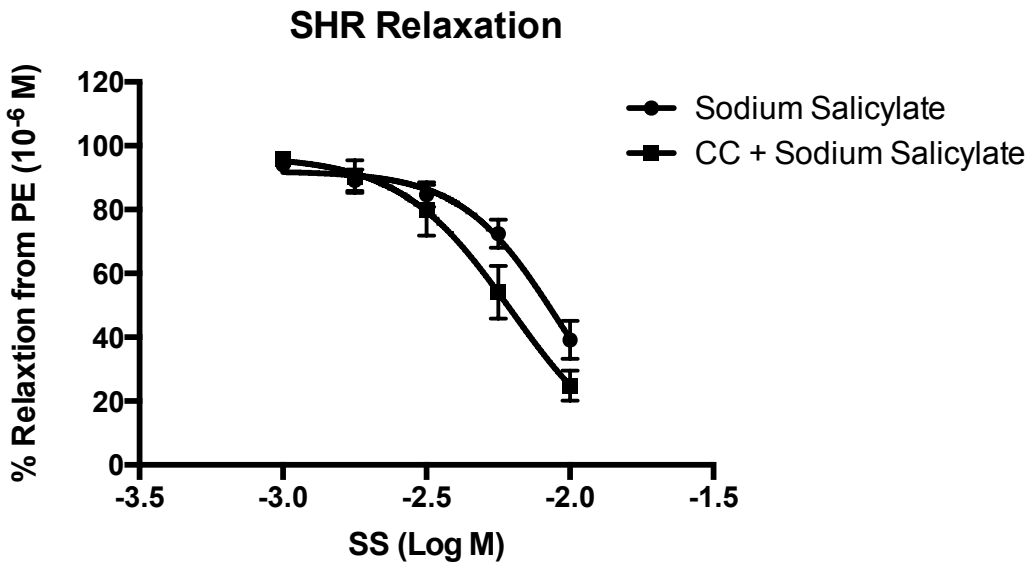
Conclusions

In summary, AMPK activity is involved and necessary for normal alpha-adrenergic induced contraction. Inhibition of this enzyme, both separately or combined with ROCK inhibition, significantly increases the EC_{50} and maximum developed tension of isolated CCA segments in both WKY and SHR, indicating a reduced sensitivity to the alpha-adrenergic agonist PE in a dose-dependent fashion. Moreover, this effect is almost exclusive to alpha-adrenergic signaling, with isolated CCA segments in the same incubation conditions eliciting a much lower degree of responsiveness to the TPr agonist U46619. HMG-CoA reductase inhibition via Simvastatin also modulates alpha-adrenergic induced contraction by acting as a Y27632 mimetic. More research will be required to identify the specific intracellular signaling mechanisms that account for these responses to AMPK inhibition in VSM and whether or not manipulation of these functional characteristics could prove beneficial in specific diseased states such as aging and hypertension.

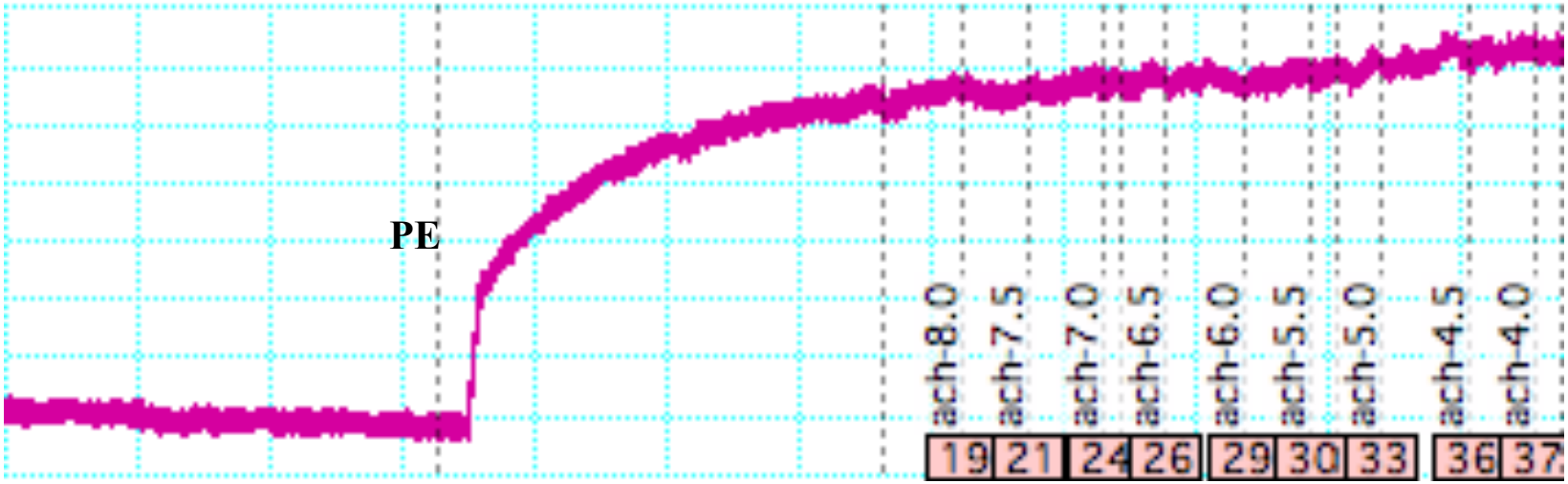
APPENDIX



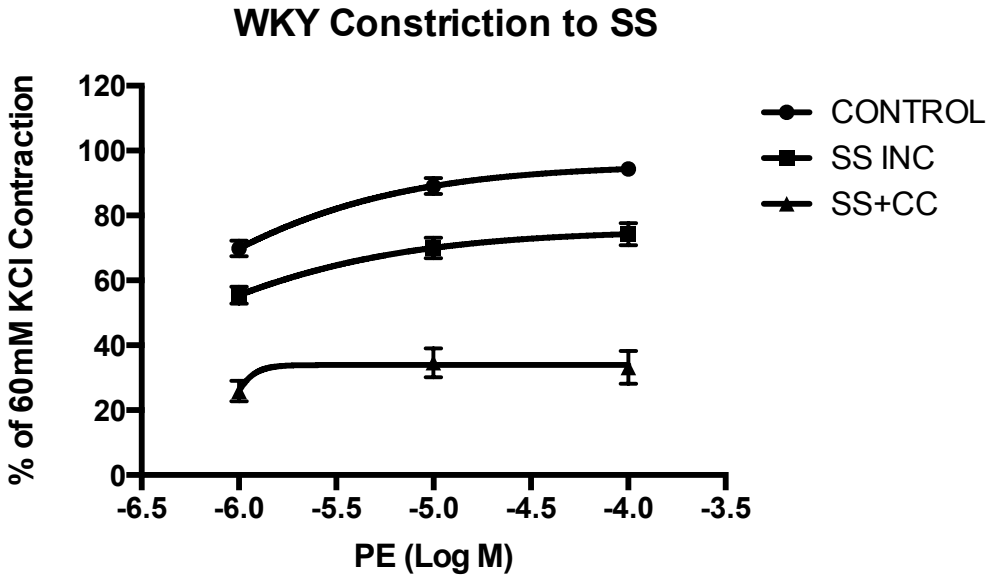
Supplement 1: Pilot work examining the effect of sodium salicylate (SS) and AMPK inhibition on relaxation in isolated WKY CCA segments (E^+) stimulated with PE ($10^{-6.0}$ M).



Supplement 2: Pilot work examining the effect of sodium salicylate (SS) and AMPK inhibition on relaxation in isolated SHR CCA segments (E^+) stimulated with PE ($10^{-6.0}$ M).



Supplement 3: Force readout confirming the lack of endothelium (E^-) in CCA preparation. After the addition of PE ($10^{-6.0}$ M), ACh was administered in a dose-dependent fashion ($10^{-8.0}$ – $10^{-4.0}$ M) and no contraction was elicited, indicating the segment was denuded.



Supplement 4: Pilot work examining the effect of sodium salicylate incubation and AMPK inhibition on PE-induced contraction. Vessels were incubated with sodium salicylate (SS; $10^{-2.5}$ M) and/or the AMPK inhibitor Compound C (CC; 20 μM).

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