Effects of a Chronic Mental Stress Intervention Protocol on Vasomotor Function in Common Carotid Artery from Wistar-Kyoto and Spontaneously-Hypertensive Rats

by

Laura Victoria Bennett

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Kinesiology

Waterloo, Ontario, Canada, 2017

© Laura Victoria Bennett 2017

AUTHOR'S DECLARATION

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

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

ABSTRACT

Chronic mental stress is emerging as a cardiovascular disease (CVD) risk factor; however, the underlying physiological mechanisms to explain how chronic mental stress may be causing CVD are still under investigation. Previous literature has demonstrated that chronic mental stress can induce endothelial dysfunction, which is a well-known independent risk factor for CVD. The purpose of this study was to further investigate the underlying pathophysiological mechanisms that may be contributing to this endothelial dysfunction, in the normotensive Wistar-Kyoto (WKY) and Spontaneously Hypertensive (SHR) rat models. A chronic mental stress protocol, known as the Unpredictable Chronic Mild Stress (UCMS) protocol, was used to induce chronic mental stress in the animals in order to determine its effects on endothelial function. Animals were divided up into four groups: WKY CON, WKY UCMS, SHR CON, and SHR UCMS. To test the efficacy of the UCMS protocol, behavioural tests were performed to confirm the presence of mental stress in the animals in the UCMS groups compared to the control groups (**Coat status:** WKY: $p<0.0001$, SHR: $p<0.0006$; **Splash test (Grooming Frequency):** WKY: $p=0.5581$, SHR: $p=0.0050$). Unexpectedly, compared to WKY CON endothelium-dependent, acetylcholine (ACh)-stimulated vasocontraction (Maximum Amp (MAX): 27.25±5.95, Area Under the Curve (AUC): 54.22±10.98, EC_{50}: 2.58±0.79), UCMS was found to significantly *attenuate* endothelium-dependent, ACh-stimulated MAX (11.86±2.91; $p=0.043$) and AUC (24.87±5.43; $p=0.037$), but not EC50 (2.48±1.20; $p=0.913$). Likewise, compared to SHR CON endothelium-dependent, ACh-stimulated vasocontraction (MAX: 48.5±9.0, AUC: 84.3±15.4, EC_{50}: 2.44±0.70), UCMS was found to attenuate endothelium-dependent, ACh-stimulated MAX (33.40±6.51; $p=0.083$), AUC (65.42±12.76; $p=0.170$), and EC50 (1.62±0.43; $p=0.202$), however this attenuation did not reach significance. Furthermore,

compared to WKY CON endothelium-dependent, ACh-stimulated vasorelaxation MAX: 92.43±3.08, AUC: 300.6±17.84, EC_{50}: 95.25±32.16), UCMS was found to significantly *augment* endothelium-dependent, ACh-stimulated AUC (349.9±13.42; p=0.040), and insignificantly augment endothelium-dependent, ACh-stimulated MAX (101.0±1.35; p=0.241) and EC50 (41.0±8.44; p=0.120). Likewise, compared to SHR CON endothelium-dependent, ACh-stimulated vasorelaxation (MAX: 83.81±3.19, AUC: 282.4±12.19, EC_{50}: 69.41±25.53), UCMS was found to augment endothelium-dependent, ACh-stimulated MAX (88.65±2.63; p=0.250), AUC (287.0±11.09; p=0.781), and EC50 (87.83±32.3; p=0.669), however this augmentation did not reach significance. Dose-dependent response curves to sodium nitroprusside (SNP) and U46619 were similar across all groups, suggesting that the attenuated vasocontraction and enhanced vasorelaxation was not due to UCMS having an effect on vascular smooth muscle (VSM) sensitivity, but is more likely due to a reduction in endothelium-derived contracting factor (EDCF) production/bioavailability and/or an increase in endothelium-derived relaxing factor (EDRF) production/bioavailability.

ACKNOWLEDGEMENTS

I would first like to thank my Supervisor, **Dr. Jim Rush**, for the opportunity to complete my thesis in your lab. Thank you for your constant encouragement and for always making time to meet with me and for keeping me a priority… even on your busiest of days!

Thank you to my committee members, **Dr. Russell Tupling** and **Dr. Marina Mourtzakis**, for providing your keen insight to ensure that my project reaches its fullest potential.

I would also like to thank the former Rush-lab member, **Peter Holder**, for your unwavering patience when teaching me new lab techniques and for always making working in lab so enjoyable.

Dr. Steven Denniss, I cannot thank you enough for always having confidence in me, even when I did not! Thank you for all of the support and guidance you have given me. Your love for science, and knowledge in general, is contagious and I am so grateful for all you have taught me; these skills will undoubtedly continue to aid me in future endeavors.

I would like to thank the **Kinesiology Physiology Floor** for always offering your help and your support during my years as a Masters student. My time in the lab would not have been nearly as enjoyable without each and every one of you. I would specifically like to thank **Juan Henao** who helped me with my project several days a week. I would not have been able to do it without you!

Emily McLaughlin, I would not have been able to complete my project without all of your help. Thank you so much for always showing up and being excited to help in any way possible!

Mom and Dad, thank you for always encouraging me to challenge myself and for letting me make my own mistakes instead of doing things for me; I learnt a great deal from those mistakes. Thank you for always believing that I could do anything I set my mind to. I don't know what I would do without your endless love and support.

I would also like to thank **Tyler, Caitlin, Kim, Hailey, and "The Wolfpack"**, for always reminding me that life is a balance between work and play. Thank you for always being there for me and for all of the great times you have given me when I needed a break from everything!

Finally, I would like to thank my amazing husband **David**. Thank you for always believing in me, for always being there to celebrate both the big and small successes, and for always comforting me in any failures. I can't imagine going through life with anyone else.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

6-keto $PGF_1\propto$ - 6-keto prostaglandin $F_1\propto$ (stable metabolite of prostacyclin)

AA – Arachidonic acid

ACh – Acetylcholine (muscarinic receptor agonist)

BHR – Borderline hypertensive rat

BW – Body weight

Ca^{2+} - Calcium

CAF – Central Animal Facility

CCA – Common carotid artery

CON – Control

COX – Cyclooxygenase

CVD – Cardiovascular disease

EDCF – Endothelium-derived contracting factor

EDHF – Endothelium-derived hyperpolarizing factor

EDRF – Endothelium-derived relaxing factor

eNOS – Endothelial nitric oxide synthase

ET-1 – Endothelin 1

ET_A – Endothelin receptor type A

ET_{B1} – Endothelin receptor type B_1

Indo – Indomethacin (non-selective cyclooxygenase inhibitor)

KCl – Potassium chloride

L-name or LN – L-N^G-nitroarginine methyl ester (non-selective nitric oxide synthase inhibitor)

LV – Left ventricle

LV:BW – Left ventricle to body weight ratio

LVH – Left ventricular hypertrophy

ND – No drug (control)

NO – Nitric oxide

NOS – Nitric oxide synthase

NS-398 – Selective cyclooxygenase-2 inhibitor

PE – Phenylephrine (∝1-adrenergic receptor agonist)

PGD_2 – Prostaglandin D_2

PGE_2 – Prostaglandin E_2

$PGF_2\propto$ - Prostaglandin F_2-alpha

PGI_2 – Prostacyclin

SC-560 – Selective cyclooxygenase-1 inhibitor

SEM – Standard error of the mean

SHR – Spontaneously hypertensive rat

SNP – Sodium nitroprusside (nitric oxide donor)

SQ29548 – Selective thromboxane-prostanoid receptor antagonist

TP – Thromboxane-prostanoid

TXA_2 – Thromboxane A_2

U46619 – Selective thromboxane-prostanoid receptor agonist

UCMS – Unpredictable chronic mild stress

VSM – Vascular smooth muscle

WKY – Wistar-kyoto rat

1.0 Introduction

This thesis focused on the effect of chronic mental stress on endothelium-dependent vasomotor function in the common carotid artery (CCA) of hypertensive and normotensive rats. The following offers an introduction to the concepts required to better understand the purpose and objectives of this study.

1.1 Structure and Related Functions of Blood Vessels

Most blood vessels are comprised of three layers including the adventitia, the media, and the intima (1). Of particular importance, are the several layers of vascular smooth muscle (VSM) cells within the media and the single layer of endothelial cells that line the innermost surface of blood vessels, composing the intima (2, 3, 4).

The endothelium not only functions to produce a barrier between the lumen and outer layers of the blood vessel, it also plays a crucial role in regulating several vascular homeostatic processes, including: platelet aggregation, leukocyte adhesion, inflammatory processes, VSM proliferation, and vascular tone (Figure 1). The endothelium produces several endothelium-derived substances (see *Endothelium-Derived Relaxing and Contracting Factors*, below), which assist in the regulation of these vascular homeostatic processes (5, 6, 7, 8, 9, 10). The main function of the VSM is to contract and relax in a graded manner, thereby regulating vascular tone. Precise control of vascular tone plays a crucial role in regulating blood flow and pressure. A number of endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors (EDCFs) contribute to the regulation of VSM function and vascular tone.

1.2 Vascular Tone

Vascular tone refers to the extent of vasocontraction that is present in a blood vessel. Most blood vessels, particularly small arteries and arterioles, exhibit some level of vasocontraction under basal physiological conditions and therefore exhibit some degree of vascular tone in their basal state (11). From this point of partial contraction, the smooth muscle surrounding the vessel can either contract further or relax, resulting in graded vasoconstrictory or vasodilatory responses, respectively. These responses maintain homeostatic blood flow and blood pressure in response to disturbances. The precise control of vascular tone is regulated through a healthy balance of vasodilator and vasoconstrictor substances (2, 6, 7, 8, 9, 10, 12, 13), many of them working via paracrine and endocrine signalling mechanisms, often involving the endothelium (5, 6, 12, 13, 14).

1.3 The Endothelium

The endothelium plays a crucial role in maintaining vascular tone and overall health of the blood vessel by secreting several diffusible substances; namely, vasodilators and vasoconstrictors, known as EDRFs and EDCFs, respectively (6, 7, 15, 16). The relative balance of EDRF and EDCF production and activity significantly contributes to the dynamic regulation of vascular tone and overall health of the blood vessel (Figure 1) (7).

1.3.1 Endothelium-Derived Relaxing and Contracting Factors

A powerful EDRF is nitric oxide (NO), which has the ability to diffuse across the cell membrane into VSM cells and induce relaxation of a blood vessel (17). Other EDRFs typically include endothelium-derived hyperpolarizing factors (EDHF) and cyclooxygenase (COX)-derived prostacyclin (PGI_2) (4, 16, 17). Importantly, the extent to which NO, EDHFs, or PGI_2

contributes to endothelium-dependent vasorelaxation varies depending on the vascular bed, size of the blood vessel as well as the species and strain of animal being investigated (18).

Some major EDCFs are also COX-derived, meaning they are produced in the endothelial cell via a COX-mediated reaction from arachidonic acid (AA) (16, 19). They include thromboxane A_2 (TXA_2), prostaglandin F_2-alpha ($PGF_2\propto$), prostaglandin D_2 (PGD_2), and prostaglandin E_2 (PGE_2); all of which are collectively known as prostanoids (4, 17). In addition, major non-COX-mediated EDCFs include endothelin-1 (ET-1) and angiotensin II.

These EDRFs and EDCFs are continually and simultaneously being secreted by the endothelium, producing a delicate balance of vasodilator and vasoconstrictor substances (13, 16, 17). Subsequently, these substances variously contribute to the regulation of vascular homeostatic processes including platelet aggregation, leukocyte adhesion, thrombosis, inflammation, VSM proliferation, and/or they can diffuse directly into the VSM or bind to and stimulate receptors on the VSM cell membrane to control vascular tone (Figure 1) (15).

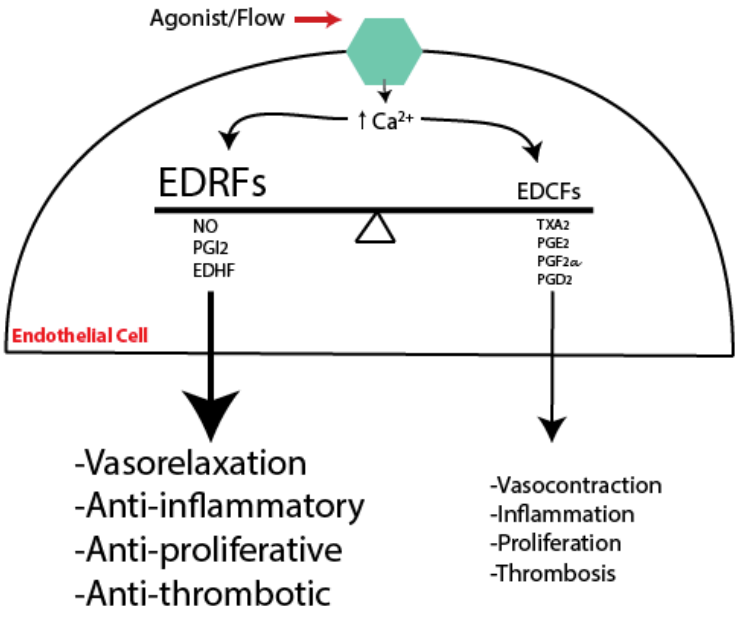

Figure 1: Healthy endothelium-dependent regulation of vascular homeostatic processes.

1.3.2 Endothelial Control of Vascular Health

In larger arteries (e.g., aorta and CCA), the primary function of EDRFs and EDCFs is in regulating the overall health of the vessel. While EDRFs are involved in preventing platelet aggregation, leukocyte adhesion, thrombosis, inflammation, and VSM proliferation within the blood vessel, EDCFs can counteract these effects by promoting platelet aggregation, leukocyte adhesion, thrombosis, inflammation, and VSM proliferation (15, 20, 21, 22). For example, PGI_2 can bind to prostacyclin IP receptors that are located on platelet cells and inhibit platelet aggregation (23, 24), whereas TXA_2 can bind to thromboxane-prostanoid (TP) receptors, also located on platelets cells, and activate platelet aggregation (25).

As long as a "healthy" balance of EDRF and EDCF production and activity exists within the blood vessel, a healthy anti-thrombotic/anti-inflammatory/anti-oxidative/anti-proliferative environment will be maintained and the overall health of the blood vessel will be preserved (Figure 1).

1.3.3 Endothelial Vasomotor Function

In smaller arteries and arterioles, the primary function of EDRFs and EDCFs is to aid in vasomotor functional responses through regulation of vascular tone. Vasodilation and/or vasoconstriction can occur in response to three types of biophysical stimuli: mechanical, electrochemical, and/or receptor-mediated (1, 17, 26, 27, 28, 29, 30). Each of these stimuli can act on the endothelium or the VSM, ultimately leading to a change in intracellular calcium (Ca^{2+}) within the endothelial and/or VSM cell triggering a cascade of events that results in either vasodilation or vasoconstriction (16, 30). Stimulation of the endothelium, for example, leads to an increase in intracellular Ca^{2+} resulting in the activation of several enzymes that ultimately produce EDRFs and EDCFs. These EDRFs and/or EDCFs can then act on the underlying VSM

cells to cause either relaxation, and resultant vasodilation, or increased contraction, and resultant vasoconstriction, which is necessary in the regulation of vascular tone (6, 7, 8, 9, 16, 30).

It is important to note, however, that a single vasoactive substance has the capacity to bind to and activate a variety of receptor types. Furthermore, these different receptor types are often connected to varied subcellular signalling mechanisms that often have opposing effects on one another. For example, while ET-1 tends to have a higher affinity for endothelin receptor type A (ET_A), which acts through contractile subcellular signalling mechanisms, it can also bind to endothelin receptor type B_1 (ET_{B1}) to elicit VSM relaxation (1). Similarly, PGI_2 most often elicits vasorelaxation because it has a higher affinity for IP receptors, but in some cases will activate the TP receptor and elicit vasocontraction (31, 32, 33) (see *Endothelial Dysfunction and Vascular Disease*). Therefore, the overall vascular tone will not only be influenced by which vasoactive substances are being produced, but also by whether these vasoactive substances act on receptors that are connected to contractile or dilatory subcellular signalling mechanisms. Moreover, the affinity for a particular receptor by a vasoactive substance may be strongly influenced by the overall state of the blood vessel (e.g., health of the endothelium) (34, 35).

1.3.4 Endothelial Dysfunction and Vascular Disease

Endothelial dysfunction can be defined as an imbalance in EDRF and EDCF production/activity impairing the regulation of vascular homeostasis (e.g., vascular tone) (15). Importantly, this imbalance can be the result of reduced EDRF activity, increased EDCF activity, or in some cases, a combination of the two (6, 16). An imbalance in these vasoactive substances predisposes blood vessels to the pro-constrictory/pro-thrombotic/pro-inflammatory/proliferative state that is associated with so many cardiovascular disease (CVD) states, including atherosclerosis and hypertension (9, 30, 36). For example, endothelial dysfunction has been

demonstrated in a frequently studied animal model of hypertension, known as the Spontaneously Hypertensive (SHR) rat, which was shown to be in part due to over-active EDCF activity (31, 32, 37, 38, 39). It was further determined that dysfunctional IP receptors on the VSM wall further contributes to the over-active EDCF activity and thereby further contributes to the resulting endothelial dysfunction seen in this model of hypertension (31, 32, 39). Like many endothelium-derived vasoactive substances, PGI_2 can bind to multiple receptor types. In healthy vasculature, PGI_2 has a higher affinity for IP receptors, which are connected to dilatory subcellular signalling mechanisms that elicit vasorelaxation when activated (31, 32, 33). In SHR rats, however, an increase in dysfunctional IP receptors instead results in activation of TP receptors by PGI_2 resulting in the activation of contractile subcellular signalling mechanisms, and vasocontraction (31, 32, 33). As a result, PGI_2 becomes a potent vasoconstrictor in SHR rats, resulting in over-active EDCF activity and further promoting a pro-constrictory state and, therefore, endothelial dysfunction (16, 31, 32, 33).

1.3.5 Measuring Endothelial Function

Since the discovery of the importance of endothelial health, many techniques have been developed over the years to assess endothelial function in humans and a variety of animal models, through both in-vivo and in-vitro methods.

While it is possible to directly measure endothelial function in human coronary arteries, this technique is highly invasive and considered very high risk for the patient (1, 15, 40). For these reasons, endothelial function in humans is most commonly measured in the peripheral circulation by analyzing the blood vessel's response to a mechanical stimulus known as shear stress, which is the result of an increase in blood flow. An increase in blood flow and resultant shear stress on the blood vessel can stimulate the endothelium to release NO; this collective

process is known as flow-mediated dilation (1, 15). Flow-mediated dilation is often measured in the brachial artery using Doppler ultrasound, allowing for a non-invasive measure of NO-mediated endothelium-dependent vasorelaxation (15). This is of importance because impairment in endothelium-dependent vasorelaxation is considered a hallmark of endothelial dysfunction, which is an independent predictor of cardiovascular events (e.g., myocardial infarction) (41, 42).

Animal models allow for the assessment of endothelial health through direct assessment of endothelium-dependent vasorelaxation and endothelium-dependent vasocontraction. This can be accomplished, in-vitro, by isolating a segment of the artery of interest followed by vascular myography (see *Vasomotor Activity* in the Methods section, below) (39). The advantage to studying endothelial function in-vitro (e.g., using vascular myography), is that the environment in which the artery segment is being investigated can be easily controlled and manipulated (43). Furthermore, physiological/mechanistic adaptations made in vivo, as a result of any intervention/perturbation, can be pharmaco-dissected in vitro (39, 43). This is a commonly used method for better understanding the physiological adaptations to any intervention/perturbation on a mechanistic level (43).

1.4 Cardiovascular Disease and Associated Risk Factors

Cardiovascular disease (CVD) is a class of chronic conditions involving the heart and/or blood vessels that develop over time as the result of the presence of and exposure to non-modifiable and modifiable risk factors (40).

1.4.1 Modifiable and Non-modifiable CVD Risk Factors

Non-modifiable risk factors, such as age and genetics (e.g., sex, race, family history), cannot be altered by an individual's choices, actions, or behaviours. Conversely, an important

distinguishing feature of modifiable (non-genetic) risk factors is that they can be changed (i.e., modified), as they are sensitive to an individual's choices, actions, and behaviours. Traditional modifiable risk factors include diet, exercise, and tobacco use (44). Much research currently exists describing the influences of these factors on vascular function. Notably, a less thought-of, yet equally modifiable risk factor to consider is chronic mental stress.

There is evidence to suggest that psychosocial factors (e.g., anxiety, depression, and chronic life stress) significantly increase the risk of CVD (45, 46, 47, 48). This may, in part, be due to the unhealthy decision-making (e.g., smoking, unhealthy diet, lack of exercise) that often accompanies negative psychosocial factors, including chronic mental stress (45, 49, 50). Importantly, however, even independent of the unhealthy decision-making, the negative psychosocial stress itself has been shown to be associated with CVD and, more specifically, endothelial dysfunction (48, 51).

1.5 Chronic Mental Stress

1.5.1 Chronic Mental Stress as a Modifiable CVD Risk Factor

Chronic mental stress has long been associated with adverse health outcomes, including CVD, but is often ignored when considering the risk factors that can be modified by an individual's behaviours (45, 46, 52, 53, 54). A surge in interest to better understand the physiological outcomes of psychological factors, such as chronic mental stress, has become apparent over the past decade (55, 56). However, much of the physiology demonstrating how chronic mental stress can affect (patho)physiological processes (e.g., endothelial function) remains to be investigated (56).

1.5.2 Unpredictable Chronic Mild Stress (UCMS) Protocol

Several animal models of chronic mental stress, including the Crowded Housing Stress model, the Chronic Behavioural Stress model, and the Unpredictable Chronic Mild Stress (UCMS) protocol, have been developed for investigating behavioural and physiological outcomes of chronic mental stress (56, 57, 58, 59, 60, 61). In recent years these chronic mental stress models have been used to better understand the underlying mechanisms involved in psychological stress-induced CVD (56, 57, 58, 59, 60, 61). To date, the UCMS protocol is the most validated model for studying the (patho)physiological outcomes of chronic mental stress (61, 62). This protocol involves intermittently exposing rats to one or more of the "mild" stressors, chosen by the investigators, in a repetitive but unpredictable manner (See *Appendix I* for the stress schedule used in this study) (56). Examples of mild stressors commonly used by investigators using the UCMS model include tilting the rats' cage, altering the rats' light/dark cycle, and removing the rats' bedding from their cage (63, 64, 65, 66, 67). While the protocol varies slightly from study-to-study, a total of approximately six stressors are generally chosen for each of the UCMS protocols. Then, from this list of stressors, approximately three stressors are randomly selected and applied to the rats each day. The time at which each stressor is applied, as well as the duration that each stressor will last, is also randomized each day. This randomization allows the investigator to apply each stressor to the rodents in an unpredictable manner and avoid adaptation by the rodents to these stressors (56).

1.6 Chronic Mental Stress and Endothelial Dysfunction

Several investigators have used the UCMS protocol to investigate a causal link between chronic mental stress and CVD (63, 64, 65, 66, 67). For example, numerous studies that have utilized the UCMS protocol have reported induction of vascular dysfunction in rodents,

suggesting that chronic mental stress is physiologically linked to the resulting vascular dysfunction (65, 66, 67, 68, 69). More specifically, investigators have been studying the *endothelial* dysfunction resulting from the chronic mental stress associated with the UCMS protocol (65, 66, 67).

1.6.1 Chronic Mental Stress and Impaired Endothelium-Dependent Vasorelaxation

To date, studies of endothelial dysfunction in UCMS have focused on reduced endothelium-dependent vasorelaxation, and the possible involvement of NO in this impairment (65, 68, 69, 70, 71). Previous literature has demonstrated that UCMS impairs endothelium-dependent vasorelaxation in aorta of Wistar rats and Balb/cJ mice (65, 66, 68, 69). A significant reduction in endothelial nitric oxide synthase (eNOS) expression as a result of the UCMS protocol has also been demonstrated in Aorta of Wistar rats (68, 69). Moreover, vasorelaxation in response to sodium nitroprusside (SNP), an external source of NO, has been shown to not be impaired after the UCMS protocol in these animals (65, 69). These data suggest that the impairment in endothelium-dependent vasorelaxation is, at least in part, caused by a significant reduction in production of NO, rather than a decreased sensitivity of the VSM to the NO.

Impairment in a blood vessel's ability to relax, however, may not only be due to under-active EDRFs, but may also be due to over-active signalling of COX-mediated EDCFs, or some combination of both (6, 16). It is therefore necessary to consider not only the bioavailability and VSM-sensitivity of NO and other EDRFs (i.e., prostaglandins, EDHFs) but also the potential changes in EDCFs when investigating endothelial responses in any stress adaptation model, including UCMS.

1.6.2 Chronic Mental Stress and Augmented Endothelium-Dependent Vasocontraction

No literature currently exists whereby the effects of UCMS on endothelium-dependent vasocontraction and the contributing EDCF activity have been specifically investigated. A study by Fuchs et al., however, could suggest that EDCF activity becomes augmented, which may contribute to an enhancement in endothelium-dependent vasocontraction (59). Fuchs et al. used "chronic behavioural stress" to induced impairment in endothelium-dependent vasorelaxation in Borderline Hypertensive Rats (BHR), a first-generation cross between SHR and wistar-kyoto (WKY) rats (59). Rather than using the UCMS protocol, the investigators used air-jets to blow compressed air on young (3 mo) and old (18 mo) BHR for 2 hours/day and the protocol lasted for a total of ten days. In this experiment, the "chronic behavioural stress" was sufficient to significantly impair acetylcholine (ACh)-induced, endothelium-dependent vasorelaxation in coronary arteries of the old stressed rats compared to the old control rats. Interestingly, preincubation with the nitric oxide synthase (NOS) inhibitor L-N^G-nitroarginine methyl ester (L-name) did not eliminate the impairment in relaxation; however, preincubation with the COX inhibitor indomethacin (Indo) did (59). While Fuchs et al. suggest that the impairment in ACh-induced endothelium-dependent vasorelaxation in the old stressed rats may in part be due to an increase in endothelium-dependent vasoconstrictor prostaglandins, which may counteract the endothelium-dependent vasodilators, the study did not specifically investigate COX-mediated EDCF activity. It can therefore be hypothesized that if endothelium-dependent vasocontractile activity would have been specifically investigated in this study, the investigators may have found an augmentation in endothelium-dependent, COX-mediated vasocontraction in the old stressed rats compared to the old control rats.

1.7 Animal Model

In this study, the effects of UCMS were measured and compared within and between normotensive Wistar-Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats.

In 1951, Wistar rats were sent from the Wistar Laboratory in the United States to Kyoto University in Japan where the first generation of WKY rats was bred (72). The SHR rat strain was later developed by breeding several generations of spontaneously hypertensive WKY rats (72). Today, the SHR rat is the most commonly studied animal model of essential hypertension, and WKY rats are often used as the normotensive control strain to which findings in SHR rats are compared against (73).

Endothelial dysfunction, as characterized by reduced endothelial-dependent vasorelaxation and/or enhanced endothelium-dependent vasocontraction, has been demonstrated in arteries from SHR rats compared to WKY (39). These data suggest that the underlying cause of hypertension in SHR may also be associated with the endothelial dysfunction that is present in this strain of rat.

Early studies investigating the cause of endothelial dysfunction in SHR rats suggested that the endothelium could not produce and/or release a sufficient amount of EDRFs, most notably NO, to sustain healthy vascular tone (7). However, more recent literature investigating impaired endothelium-dependent vasorelaxation in SHR CCA has shown that preincubation with the COX-inhibitor indomethacin can reverse the impairment in endothelial-dependent vasorelaxation (39). These data suggest that the reduced endothelium-dependent vasorelaxation in CCA of SHR rats is, at least in part, COX-mediated. Furthermore, this reduced endothelium-dependent vasorelaxation has also been shown to be associated with a significant increase in endothelial-dependent vasocontraction (39).

Importantly, the majority of studies investigating endothelial dysfunction focuses on the impairment in endothelium-dependent vasorelaxation and do not specifically investigate whether an enhancement in endothelium-vasocontraction is present (39, 74, 75, 76). Since it is now known that endothelial dysfunction can be also be caused by an increase in EDCF activity/bioavailability, and not only a reduction in EDRF activity/bioavailability, it is important that investigators specifically investigate EDCF activity by studying endothelium-dependent vasocontraction (28, 77, 78, 79). Therefore, in the current study, SHR animals were used because both a reduction in EDRF activity as well as an increase in EDCF activity have been shown to contribute to the endothelial dysfunction present in this animal model (7, 31, 32, 37, 38, 39). Furthermore, the mechanisms underlying this endothelial dysfunction have been well defined. Additionally, WKY animals were used in the current study since they are often the control strain to which findings in SHR animals are compared against (73).

2.0 Purpose

The purpose of this thesis was to further investigate the underlying pathophysiological mechanisms contributing to endothelial dysfunction associated with chronic mental stress in a rat model. Whether the endothelial dysfunction is a result of a reduction in EDRF activity, an augmentation of EDCF activity, or some combination of the two was further analyzed in this thesis. More specifically, we examined:

1. The effect of UCMS on endothelium-dependent vasorelaxation in the CCA of WKY and SHR rats.
2. The effect of UCMS on endothelium-dependent, COX-mediated vasocontraction in the CCA of WKY and SHR rats.
3. Whether the impairments of endothelium-dependent vasorelaxation and/or endothelium-dependent vasocontraction were greater or less in the SHR UCMS group compared to the WKY UCMS group, compared to their respective strain control (CON) group.

3.0 Hypotheses

3.1 Hypothesis 1

Hypothesis 1: Compared to their respective controls, UCMS will impair endothelium-dependent vasorelaxation in both WKY and SHR

The majority of previous studies that have investigated the endothelial dysfunction associated with chronic mental stress have demonstrated that endothelium-dependent vasorelaxation is significantly impaired in the stress groups compared to their respective control groups (65, 66, 68, 69, 70, 71). Furthermore, some studies have demonstrated that the UCMS protocol, specifically, impaired endothelium-dependent vasorelaxation in aorta of Wistar rats and Balb/cJ mice (65, 66, 68, 69).

3.2 Hypothesis 2

Hypothesis 2: Compared to their respective controls, UCMS will augment endothelium-dependent, COX-mediated vasocontraction in both WKY and SHR

As mentioned previously, Fuchs et al. demonstrated that chronic behavioural stress impaired endothelium-dependent vasorelaxation that was reversed by indomethacin but not NO, suggesting that the impairment was, at least partly, due to increased EDCF activity (59). Furthermore, a study by Stanley et al. found that the vascular production of COX-mediated vasoconstrictor, TXA_2, was significantly greater in Balb/cJ mice after UCMS compared to control mice (65). Together, these data suggest that an increase in COX-mediated EDCF activity may contribute to the impairment in vasorelaxation associated with the chronic stress. Furthermore, it suggests that, if specifically investigated, increased EDCF activity may also cause enhanced endothelium-dependent vasocontraction.

Based on these data, it is hypothesized that the UCMS protocol will significantly enhance endothelium-dependent vasocontraction in both WKY and SHR rats. Furthermore, it is hypothesized that this augmentation will be the result of an increase in COX-mediated EDCF production/activity, rather than an increased sensitivity of the VSM (i.e., TP receptor sensitivity) to the EDCFs.

3.3 Hypothesis 3

Hypothesis 3: The UCMS-induced impairment in endothelium-dependent vasorelaxation (Figure 2A) and the UCMS-induced augmentation in endothelium-dependent vasocontraction (Figure 2B) will be greater in WKY compared to SHR.

Previous literature has established that endothelium-dependent vasorelaxation is significantly attenuated in arteries from SHR compared to WKY and, furthermore, that endothelium-dependent vasocontraction is significantly augmented in arteries from SHR compared to WKY (37, 38, 39).

Based on these findings, it is hypothesized that the UCMS protocol will exaggerate the, already existing, impairments in vasomotor function in the SHR rats overall resulting in significantly reduced endothelium-dependent vasorelaxation and significantly greater endothelium-dependent vasocontraction in SHR compared to WKY (i.e., significantly greater absolute impairment in vasomotor function in the SHR UCMS group compared to the WKY UCMS group).

In contrast, because little impairment in vasomotor function exists in arteries of control WKY rats, unlike in SHR, it is reasonable to assume that a larger capacity for impairment in vasomotor activity exists in the WKY rats compared to the SHR rats. Therefore, it is hypothesized that a greater relative/overall change in impairment in vasomotor function (i.e.,

reduced endothelium-dependent vasorelaxation and augmented endothelium-dependent vasocontraction) will be observed between the WKY UCMS and control groups compared to the relative impairment between the SHR UCMS and control groups. These data would suggest that chronic mental stress has a greater *relative* effect on endothelial function in normotensive WKY rats compared to hypertensive SHR rats.

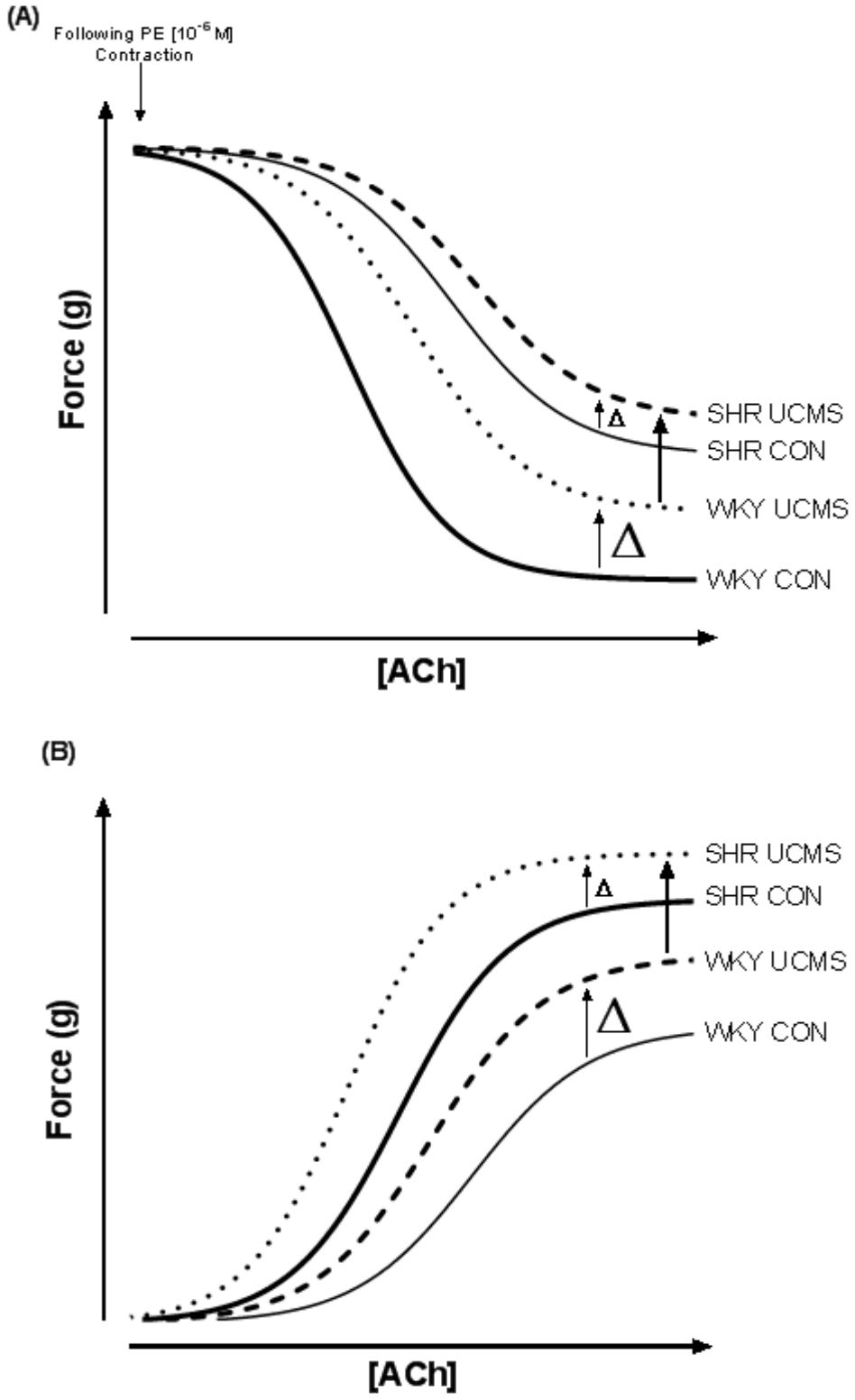

Figure 2: Hypothesized UCMS-induced impairment in endothelium-dependent vasorelaxation and vasocontraction. The UCMS-induced impairment in endothelium-dependent vasorelaxation (A) and the UCMS-induced augmentation in endothelium-dependent vasocontraction (B) will be greater in WKY compared to SHR.

4.0 Materials and Methods

4.1 Animal Model

Male WKY (n=24) and SHR (n=24) aged 30-40 weeks were obtained from the University of Waterloo breeding colony. Animals were housed two per cage in the Central Animal Facility (CAF) at the University of Waterloo, and had access to standard chow (Harlan Laboratories) and tap water ad libitum. Food and water intake of all animals was monitored throughout the study.

Animals were divided into four groups including two control (CON) groups (n=12 WKY; n=12 SHR) and two chronic mental stress (UCMS) groups (n=12 WKY; n=12 SHR). All control animals were housed in a separate quiet room from the UCMS group. After six weeks under either condition, rats were anesthetized via an intraperitoneal injection using Sodium Pentobarbital at a dose of 45 mg/kg of body weight (BW). Once deemed adequately anesthetized (i.e., no pain response with toe pinch test), the rat was sacrificed via exsanguination for vasomotor function testing (see *4.4 Vasomotor Activity*) and biochemical analyses (see *4.5 Biochemical Analyses*).

4.2 Unpredictable Chronic Mild Stress

Animals were stressed 3 times/day for a total of six weeks and were stressed, on average, 58% of the time in a 24-hour period. The same stressor was not used more than once per day or in succession (i.e., the last stressor one day was never the first stressor the following day) to avoid any adaptation by the animals to the stressor.

The stressors used in this experiment included (1) *removal of bedding:* bedding was removed from the cage (65, 66, 67, 70, 71), (2) *damp bedding:* Enough water was added to the cage to cause damp but not fully saturated bedding (i.e., no pools of water were present; ~75 mL

water per 1 cup of bedding) (65, 66, 67, 68, 69, 70, 71, 80, 81), (3) *altered light/dark cycle:* the light/dark cycle was interrupted by turning the lights on and off every 30 minutes (65, 67), (4) *cage tilting:* the cage was tilted 45^{o} (65, 66, 67, 70, 71), (5) *predator odour*: coyote urine was placed near, but outside, the cages, and (6) *predator sounds:* a recording of cat growling/hissing was placed in the room (65, 67). For ethical reasons, the stressors did not include food and/or water deprivation.

4.3 Efficacy Measures of UCMS Protocol

As described in previous literature using the UCMS protocol, behavioural tests were completed regularly during the UCMS protocol to test the efficacy of the protocol itself (65, 66, 67, 70, 71, 82, 83). The behavioural tests included the splash test and monitoring coat status, as described in detail below. Furthermore, the same behavioural tests were also regularly completed in the control groups to allow for comparison of the chronic stress in the rats in the UCMS group compared to the rats in the control group.

4.3.1 Coat Status

Consistent with past studies, coat status was evaluated weekly throughout the duration of the protocol (65, 66, 67, 71). Eight different body parts (i.e., head, neck, dorsal coat, ventral coat, tail, forelimb, hind limb, and genital region) were individually assessed and assigned a score of either 0 or 1 depending on whether the coat was clean or dirty, as described in previous literature, and a total cumulative score was obtained for each rat by calculating the sum of these eight scores (65, 66, 67, 71). The Animal Care Technician provided guidelines for what was considered as a “clean” or “dirty” coat.

Due to the subjective nature of the test, one person determined the coat status each week for the duration of the protocol, to ensure consistency each week. Furthermore, a second observer rated the coats independently but at the same time as the primary rater 25% of the time to determine the degree of agreement between the two raters.

4.3.2 Splash Test

The dorsal coat of the rats were sprayed, or "splashed", twice with a 10% sucrose solution, as described in previous literature (65, 66, 67, 70). The viscosity of the sucrose solution was sufficient to dirty the coat and initiate grooming. A lack of grooming is considered a sign of depression in rodents. Therefore, reduced grooming activity was indicative of depressive behaviour in the rat (65, 66, 67, 70).

The amount of time spent grooming, defined as licking and/or scratching, during the first five minutes after being sprayed was recorded. More specifically, the number of times the rat engaged in grooming (i.e., frequency) and the idle time between the spray and the initiation of grooming (i.e., latency time) was observed, recorded, and compared between animals (65, 66, 67, 70).

4.4 Vasomotor Activity

Both the left and right CCAs were extracted from anaesthetized animals for the measurement of vasomotor activity in these arteries. Vasomotor activity was measured, as previously published by our laboratory (39, 74). Briefly, immediately following extraction, cleaned CCAs were placed in 4^{o}C Kreb's 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). Next, the arteries were cut into 2mm sections and mounted onto a force transducer that is part of

a wire myography unit. Once mounted, the arteries were placed in individual water-jacketed tissue baths each containing 5mL of 37^{o}C Kreb's Bicarbonate Buffer that were constantly aerated with 95% O_2 and 5% CO_2 (39, 74).

The vessel segments were then equilibrated by washing with fresh buffer, followed by applying an optimal passive tension of 2.85g (as determined by Denniss and Rush, 2009); this process was performed a total of 3 times in 10 minute intervals. The segments were then tested for viability by maximally contracting the vessels with 10^{-4} M potassium chloride (KCl). This maximal contraction was left to plateau for 30 minutes before washing the vessels with fresh buffer in three 5-minute intervals. This 30-minute KCl contraction was then repeated for a second time before the segment went on to be used for measurements of vasomotor activity (e.g., endothelium-dependent vasorelaxation or endothelium-dependent vasocontraction), as described in detail below (39, 74). The maximum amplitude produced by each ring as the result of the second dose of KCl was utilized as each ring's reference contraction.

4.4.1 Endothelium-dependent vasorelaxation

Following the equilibration period (described in 4.4), arteries were first pre-incubated for 30 minutes in buffer containing either no drug (ND); L-name (10^{-4} M in bath) to inhibit NOS; indomethacin (10^{-5} M in bath) to non-selectively inhibit both COX-1 and COX-2; L-name and indomethacin to inhibit NOS and COX-1/-2; SC-560 (10^{-6} M in bath) to selectively inhibit COX-1; or L-name and SC-560 to inhibit both NOS and COX-1. Next, all segments were precontracted with phenylephrine (PE; 10^{-6} M in bath). Once a plateau had been sustained for a minimum of 15 minutes, the endothelium-dependent vasorelaxation was measured in a dose-dependent manner, in response to ACh (10^{-10} -10^{-4} M in bath) (39, 74).

Following the Ach dose-response measurements, the vessel segments were re-equilibrated with 4 washes in 15-minute intervals. The same drug conditions, as used for the endothelium-dependent vasorelaxation measurements, were maintained during each of these washes. Following the final wash, all segments were again precontracted with PE (10^{-6} M in bath). Once a plateau had been sustained for a minimum of 15 minutes, vasorelaxation in response to the NO donor sodium nitroprusside (SNP; 10^{-12} – 10^{-4} M in bath) was measured in a dose-dependent manner to examine the sensitivity of the VSM to an external source of NO (i.e., independent of the production/bioavailability of NO by the endothelium).

4.4.2 Endothelium-dependent vasocontraction

Following the equilibration period (described in 4.4), arteries were first preincubated for 30 minutes with L-name (10^{-4} M in bath) to inhibit NOS; L-name and indomethacin to inhibit both NOS and COX-1/-2; L-name and SC-560 to inhibit both NOS and COX-1; L-name and NS-398 to inhibit both NOS and COX-2; or L-name and SQ29548 to inhibit both NOS and the TP receptor. Endothelium-dependent vasocontraction was then measured in a dose-dependent manner, in response to ACh (10^{-10} -10^{-4} M in bath) (39, 74).

The buffer from these endothelium-dependent vasocontraction experiments was collected when the vessel segment reached its peak contraction (i.e., 1-2 minutes after the final dose of ACh was added to the tissue bath). These samples were flash frozen at -80^{o}C for biochemical analyses that were performed at a later date (described in *4.5 Biochemical Analyses*) (74).

Following the ACh dose-response measurements, the vessel segments were re-equilibrated with 4 washes in 15-minute intervals. The same drug conditions, as used for the endothelium-dependent vasocontraction measurements, were maintained during each of these washes. Following the final wash, vasocontraction in response to the TP receptor agonist

U46619 (10^{-9} – $10^{-5.5}$ M in bath) was measured in a dose-dependent manner to examine the sensitivity of the TP receptors to elicit vasocontraction.

4.5 Biochemical Analyses

Once maximal ACh-stimulated vasocontraction had been achieved (i.e., 1-2 minutes after the final dose of ACh had been added) during the endothelium-dependent vasocontraction protocol, the buffer from these tissue baths was collected and frozen at -80^{o}C. This tissue buffer was later used to measure PGI_2 level by measuring its stable metabolite, 6-keto-prostaglandin F1∝, as described by Cayman's Chemical EIA Assay Kit, as we have done previously (74, 84).

4.6 Data Analysis and Statistics

All data are expressed as the average ± standard error of the mean (SEM). Curve fitting and statistical comparisons were performed using GraphPad Prism. Ring cumulative tension responses to a vasomotor stimulus were individually best fit using a non-linear regression model to a sigmoidal dose-response curve. Maximum amplitude (MAX), dose resulting in 50% of MAX (EC_{50}), and area under the curve (AUC) were generated then averaged within their respective group. One-way or two-way ANOVA with Tukey post-hoc tests, when necessary, were used for multiple within and/or between group comparisons. Independent groups were compared using two-tailed, unpaired Student's *t*-tests. Statistical significance was considered if $p<0.05$.

5.0 Results

5.1 Physical Characteristics of Animal Models

Table 1 presents the data describing the physical characteristics of the animals used for this study. All animals gained weight over the 6-week treatment period. The final body weights of WKY were ~5-10% greater than SHR (Table 1). Furthermore, two-way ANOVA analyses demonstrate both a strain and stress effect on weight gain, wherein WKY gained more weight compared to SHR and UCMS animals gained more weight compared to control animals throughout the 6-week protocol (Table 1). Food and water monitoring throughout the treatment period determined that SHR consumed ~10-25% more food and ~15-25% more water than WKY (Table 1), a finding that has been previously documented (84, 85). However, it is unknown whether these increases are a result of increased consumption by SHR or increased spillage (84, 85). Stress had no significant effect on food and/or water consumption in WKY (Table 1). Stress had no significant effect on water consumption in SHR, however SHR in the UCMS group consumed ~10% more food than SHR in the control group (Table 1).

Measuring the left ventricle to body weight ratio (LV:BW) is a commonly used surrogate in place of measuring blood pressure to determine the presence of hypertension in an animal. The left ventricle to body weight ratio is significantly greater in animals with hypertension due to systemic pressure-induced left ventricular hypertrophy (LVH), compared to normotensive animals (39). As has been observed previously in our lab (39), the left ventricle to body weight ratio was significantly greater (~15-30%) in SHR compared to WKY (Table 1), demonstrating the presence of hypertension in the SHR animals used in this study. No significant stress effect on left ventricle to body weight ratio was observed in either strain (Table 1).

5.2 Behavioural Characteristics of Animal Models

5.2.1 Splash Test

Using the Fisher's Exact Test it was determined that significantly fewer WKY groomed at all during the splash test, compared to SHR ($p=0.005$). Of all of the animals that underwent the splash test, 13 of the 24 WKY and 3 of the 24 SHR did not groom at all when sprayed with the 10% sucrose solution (Figure 3A). Of the 13 WKY that did not groom, 7 were from the control group and 6 were from the UCMS group. Of the 3 SHR that did not groom, 1 was from the control group and 2 were from the UCMS group.

Of those that did groom, the latency of grooming was greatest in the SHR UCMS group and, unexpectedly, was lowest in the WKY UCMS group (Figure 3B). No significant stress effect on latency of grooming was seen in SHR ($p=0.3024$), however, there was a significant stress effect in WKY ($p=0.0036$). No significant strain effect was seen between the control groups ($p=0.9221$), however, grooming latency was significantly greater in SHR UCMS compared to WKY UCMS ($p=0.0082$).

Additionally, there was a significant strain effect ($p<0.0001$) on grooming frequency, wherein WKY groomed significantly less frequently in both the Control and UCMS groups, compared to SHR (Figure 3C). A significant stress effect was present in the SHR animals ($p=0.0050$), wherein SHR in the UCMS group groomed significantly less frequently than SHR in the control group. Unexpectedly, stress had no significant effect on grooming frequency in WKY ($p=0.5581$).

5.2.2 Coat Status

The intraclass correlation coefficient to assess the inter-rater reliability (i.e., the degree of agreement among the two raters) ranged between 0.69-1.0 across the eight different body

regions. The neck had the lowest degree of agreement (0.69) and the forelimb had the greatest degree of agreement (1.0).

Coat status significantly increased (i.e., coats became significantly dirtier) over time ($p<0.0001$) in both WKY and SHR (Figure 3D). A significant strain effect was also present wherein the coats of the WKY were significantly dirtier than those of the SHR in both the control ($p=0.0009$) and the UCMS groups ($p=0.0062$) (Figure 3D).

A significant difference in the coat status between UCMS and control groups was also detected in both WKY ($p<0.0001$) and SHR ($p=0.0006$), wherein the coats of animals in the UCMS groups were significantly dirtier than those in the control groups. Coats were found to be significantly dirtier after only one week of UCMS in SHR ($p=0.0027$) and remained significantly dirtier for each week that followed. Likewise coats were found to be significantly "dirtier" after only one week of UCMS in WKY ($p=0.0114$), however, this significant difference was not observed at weeks 4 ($p=0.0620$), or 5 ($p=0.0548$) (Figure 3D).

5.3 Vascular Myography Data

5.3.1 Absolute and Relative KCl and PE Contractile Activity

KCl-stimulated contractions were greater in UCMS compared to their respective control group in WKY ($p<0.0001$; Figure 4A) and SHR ($p<0.0001$; Figure 4A). Likewise, KCl-stimulated contractions were greater in SHR compared to WKY in the control ($p<0.0001$; Figure 4A) and UCMS ($p<0.0001$; Figure 4A) groups. Contractile responses to the first dose of PE (10^{-6}M) were ~25% lower in SHR compared to WKY (Figure 4B) across all drug conditions, as has been observed previously in our lab (39, 86). No significant differences in developed tension in response to the first dose of PE (PE1; 10^{-6}M) were found between Control and UCMS groups in WKY ($p=0.9027$; Figure 4B) or SHR ($p=0.0668$ Figure 4B). Similar results were obtained when

developed tension in response to PE1 (10^{-6}M) was analyzed relative to each ring's maximal contraction to KCl (Figure 4C). No significant differences in developed tension to the second dose of PE (PE2; 10^{-6}M) were found between strains (p=0.6117) or between control and UCMS groups (p=9135) (Figure 4D).

Previous research in our lab (39) has observed that the ratio of developed tension in response to PE (10^{-6}M) in drug conditions containing L-name compared to those that do not contain L-name are greater in WKY compared to SHR. These data suggest that L-name has a greater effect in CCA from WKY compared to SHR. This is most likely due to production and/or bioavailability of NO already being lower in SHR (39, 87). Similar results were also observed in the current study. In SHR CON (Figure 5A) and SHR UCMS (Figure 5B), the developed tension in response to PE1 (10^{-6}M) was ~15% greater in the L-name, L-name+Indo, and L-name+SC-560 drug conditions compared to the ND, Indo, and SC-560 drug conditions, respectively. However, the developed tension is response to PE1 (10^{-6}M) was ~30% greater in the L-name, L-name+Indo, and L-name+SC-560 drug conditions compared to ND, Indo, and SC-560 drug conditions, respectively, in WKY CON (Figure 5C) and WKY UCMS (Figure 5D).

5.3.2 Agonist-stimulated Vasocontractile Responses

Three different figure formats are provided to demonstrate differences between strains (Fig. 6-7), drug conditions (Fig. 8, 11), and stress and control groups (Fig. 9-10).

5.3.2.1 ACh-stimulated vasocontraction is attenuated in quiescent SHR and WKY CCA that underwent UCMS compared to their respective control groups

As has been shown previously in our lab (39), ACh-stimulated vasocontraction in quiescent CCA preincubated with L-name was augmented in SHR compared to WKY (Table 2).

This result was consistent across the control (Table 2; Figure 6A) and UCMS (Table 2; Figure 7A) groups.

The effects of COX- and TP receptor-inhibitors were investigated in the presence of L-name to optimize the ACh-stimulated contractions (39, 29, 88, 89). Non-selectively inhibiting COX through preincubation with indomethacin impeded endothelium-dependent vasocontraction in all four groups (i.e., WKY control and UCMS and SHR control and UCMS) (Table 2; Figure 8). Similarly, blocking the TP receptor through preincubation with SQ29548 also impeded the contractile response to ACh in all four groups (Table 2; Figure 8). Furthermore, selective inhibition of COX-1 or COX-2 through preincubation with SC-560 or NS-398, respectively, also blunted endothelium-dependent vasocontraction in all four groups (Table 2; Figure 8).

Unexpectedly, UCMS attenuated endothelium-dependent vasocontraction in response to increasing doses of ACh, in both WKY (Table 2; Figure 9A) and SHR (Table 2; Figure 10A). Furthermore, this attenuation was greater in WKY (~55%) compared to SHR (~40%).

5.3.2.2 U46619-stimulated vasocontraction elicited similar vasocontractile responses across all groups

Increasing doses of U46619 elicited similar sigmoidal dose-response curves in the presence of L-name, the COX-1 inhibitor SC-560, the COX-2 inhibitor NS-398, and the non-selective COX-inhibitor indomethacin, across all four groups (Table 3; Figure 11).

In all four groups the lower doses of U46619 (10^{-9} – $10^{-6.5}$M) were insufficient to elicit any vasocontraction in the presence of the TP-receptor antagonist, SQ29548. Higher concentrations of U46619 (10^{-6} and $10^{-5.5}$M), however, elicited similar vasocontractile responses in all four groups (Table 3; Figure 11).

5.3.3 Agonist-stimulated Vasodilatory Responses

Three different figure formats are provided to demonstrate differences between drug conditions (Fig. 12,17), strains (Fig. 13-14), and stress and control groups (Fig. 15-16).

5.3.3.1 ACh-stimulated, endothelium-dependent vasorelaxation is augmented in precontracted SHR and WKY CCA that underwent UCMS compared to their respective control groups

CCA precontracted with PE (10^{-6}M) from both WKY and SHR exhibited ACh-stimulated, endothelium-dependent vasorelaxation in both the control and UCMS groups (Table 4; Figure 12). Preincubation with L-name to inhibit NOS impeded this vasorelaxation in all four groups (Figure 12), suggesting that this ACh-stimulated vasodilatory response is NOS-mediated. Additionally, when CCA was preincubated with L-name in addition to the COX-inhibitors, indomethacin and SC-560, endothelium-dependent vasodilatory responses to ACh were also impeded, due to the blocking of the NOS-system (Figure 12).

Non-selective COX-inhibition, with indomethacin, slightly augmented endothelium-dependent vasodilatory responses to ACh in SHR control (Table 4; Figure 12B) and UCMS (Table 4; Figure 12D) groups compared to the ND condition. Non-selective COX-inhibition, with indomethacin, also augmented the endothelium-dependent vasodilatory response in the WKY control group compared to the no drug condition (Table 4; Figure 12A), but had no effect in the WKY UCMS group (Table 4; Figure 12C).

As has been previously demonstrated in our lab (39, 74), SHR exhibited attenuated ACh-stimulated, endothelium-dependent vasorelaxation compared to WKY in both control (Table 4; Figure 13A) and UCMS (Table 4; Figure 14A) groups.

Unexpectedly, UCMS augmented endothelium-dependent vasorelaxation in response to increasing doses of ACh, in both WKY (Table 4; Figure 15A) and SHR (Table 4; Figure 16A). Furthermore, this augmentation was slightly greater in WKY (~10%) compared to SHR (~7%).

5.3.3.2 SNP-stimulated vasorelaxation elicited similar vasodilatory responses across all groups

Increasing doses of SNP elicited similar sigmoidal dose-response curves across all drug conditions in all four groups, wherein no significant differences were seen between strains or between the UCMS and Control groups (Table 5; Figure 17). Furthermore, no significant differences were seen between drug conditions, within each of the four groups (Table 5; Figure 17).

5.4 Biochemical PGI_2 Assessment

PGI_2 levels were measured through measuring levels of the PGI_2 stable metabolite, 6-keto $PGF_1\propto$, in the buffer collected from the tissue baths following Ach dose-response curves (i.e., the same rings as displayed in Figure 8). As has been observed previously in our lab (84), PGI_2 levels were significantly greater in SHR compared to WKY in the LN condition ($p=0.0087$; Figure 18A). However, no significant differences in PGI_2 levels were seen between WKY and SHR in the LN+Indo ($p=0.1144$; Figure 18B) or LN+SQ29548 ($p=0.5462$; Figure 18C) drug conditions. Furthermore, COX-inhibition with indomethacin reduced PGI_2 levels compared to the L-name condition, as has been demonstrated previously in our lab (84). This decrease reached significance in the WKY CON ($p=0.0440$; Figure 19A), SHR CON ($p=0.0124$; Figure 19B), and SHR UCMS ($p=0.0074$; Figure 19B) groups, but not in the WKY UCMS group ($p=0.0520$; Figure 19A). Stress had no significant effect on PGI_2 levels in WKY or SHR (Figure 18).

Table 1: Physical Characteristics of WKY and SHR in Control and UCMS groups

	WKY		SHR		p value		
	CON	UCMS	CON	UCMS	Stress Effect	Strain Effect	Stress x Strain
Age (weeks)	34.2±0.59	33.9±0.26	33.5±0.72	32.8±0.17	0.3090	0.0717	0.7673
Initial body weight (g)	380±4.0	377±4.6	350±8.4[b]	365±8.6	0.3711	0.0034	0.1842
Final body weight (g)	413±6.5	415±5.2	365±9.2[b]	390±9.1	0.0938	<0.0001	0.1307
Change in body weight (g)	33.9±4.3	38.2±3.1	15.7±1.9[b]	25.6±2.2[b]	0.0221	<0.0001	0.3578
Food consumption (g/day)	18.6±0.38	18.1±0.27	20.2±0.43[b]	22.2±0.25[a,b]	0.0378	<0.0001	0.0010
Water consumption (mL/day)	36.1±0.69	35.2±1.01	42.3±1.77[b]	43.0±1.76[b]	0.8769	<0.0001	0.6095
Left ventricle (mg) : body weight (g)	2.12±0.08	2.22±0.14	2.78±0.07[b]	2.65±0.07[b]	0.8763	<0.0001	0.2421

Data are represented as the average mean ± SEM. P<0.05 was considered statistically significant. From post-hoc analyses: [a] p<0.05 vs. CON within WKY or SHR; [b] p<0.05 vs. WKY within corresponding CON or UCMS group.

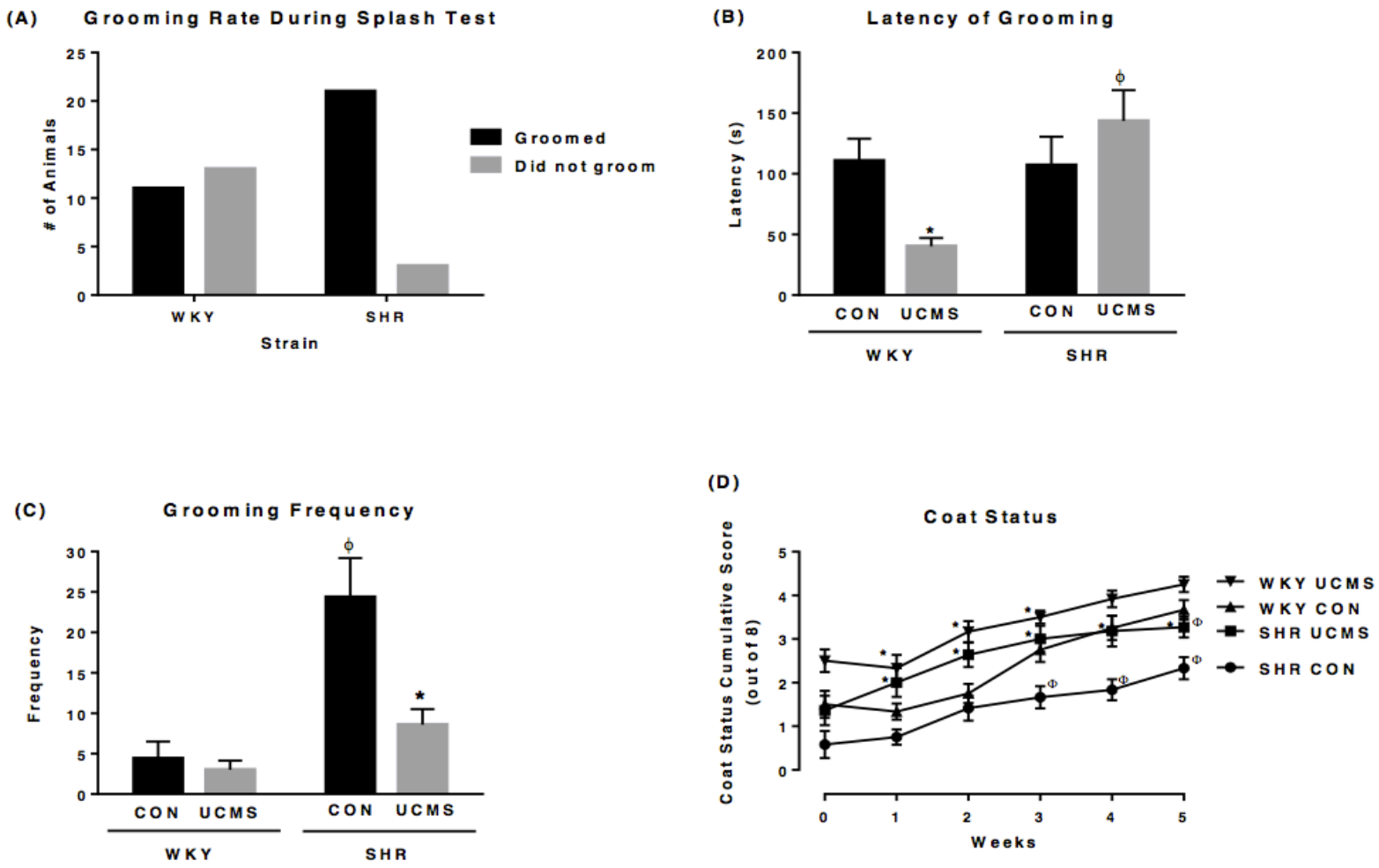

Figure 3: Behavioural tests (splash test and coat status) used to examine the efficacy of the UCMS protocol. Grooming Rate (A), Latency (B) and frequency (C) of facial grooming following a 10% sucrose solution spray. Grooming rate was analyzed using the Fisher's Exact Test and any difference where $p<0.05$ was considered statistically significant. Data for grooming latency and frequency are represented as the average mean ± SEM. $p<0.05$ was considered statistically significant. From post-hoc analyses: * $p<0.05$ vs. CON within WKY or SHR; Φ $p<0.05$ vs. WKY within corresponding CON or UCMS group. (D) Cumulative weekly coat status score represented as the average mean ± SEM; analyzed using 2-way ANOVA where $p<0.05$ was considered statistically significant.

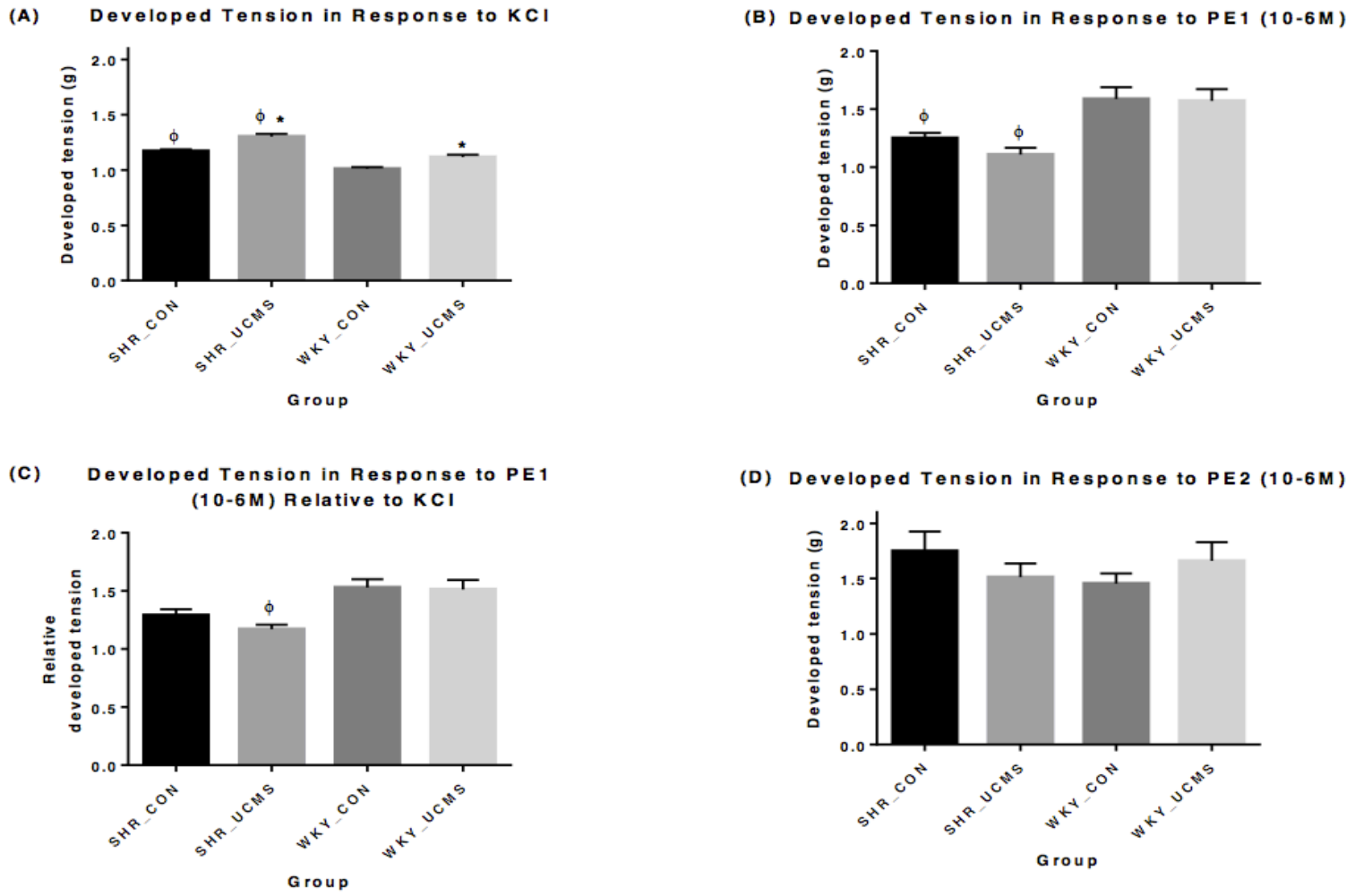

FIGURE 4: Absolute developed tension in response to 4M KCl, and developed tension to PE (10^{-6}M) in both absolute terms and relative to KCl contractions. Viability of arterial rings was assessed through contractile response to 4M KCl (A). Then, rings used to investigate endothelium-dependent vasorelaxation were precontracted with a first dose of PE (PE1; 10^{-6}M); this data is represented in both absolute terms (B), and relative to the KCl contractions (C). These same rings were precontracted a second time, following the Ach dose-response curves, with a second dose of PE (PE2; 10^{-6}M) (D) before investigating VSM sensitivity with increasing doses of SNP. Data are represented as the average mean ± SEM. $p<0.05$ was considered statistically significant. From post-hoc analyses: * $p<0.05$ vs. CON within WKY or SHR; Φ $p<0.05$ vs. WKY within corresponding CON or UCMS group.

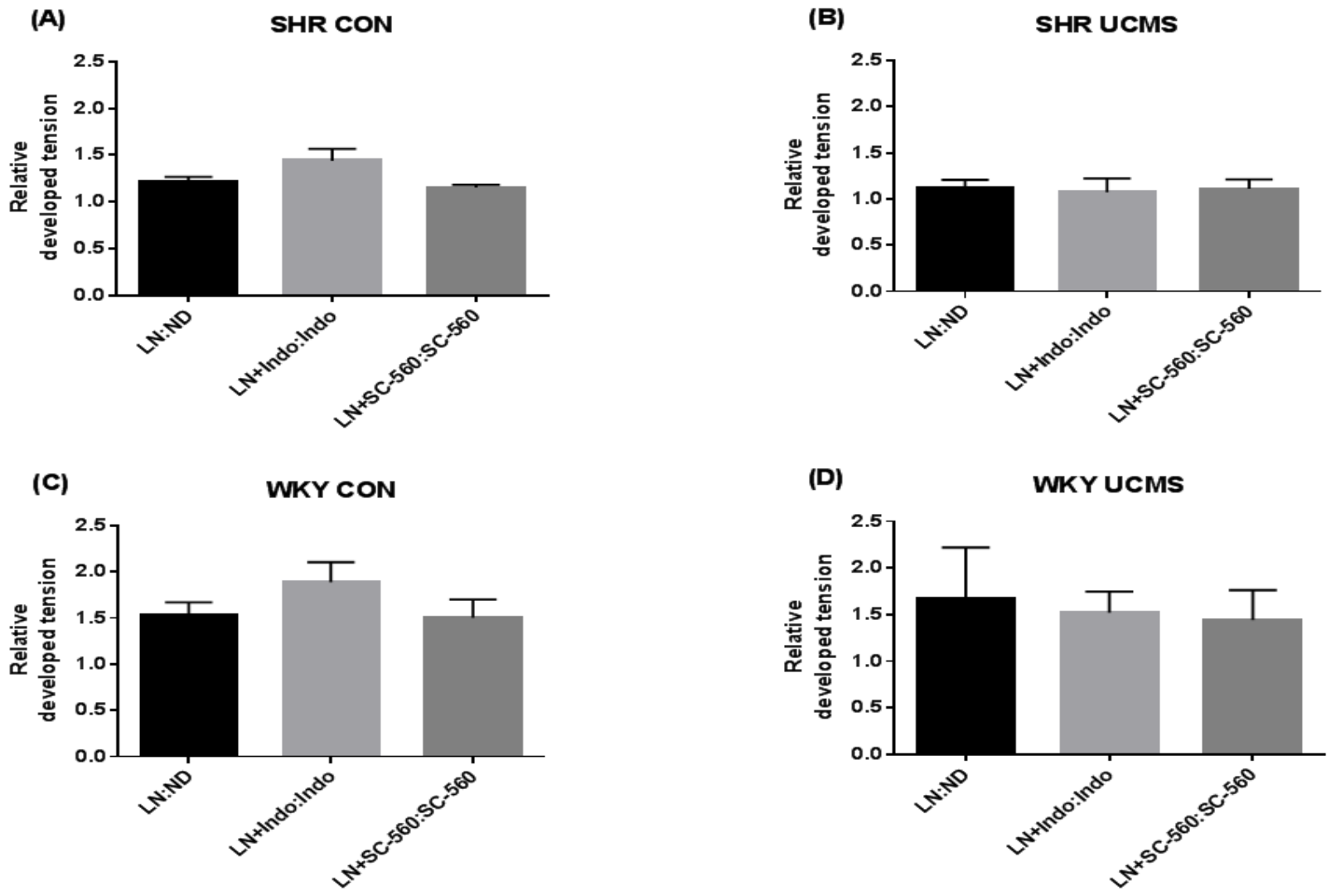

Figure 5: The ratio of developed tension in response to PE1 in drug conditions with L-name compared to drug conditions without L-name. The relative developed tension in reponse to the first dose of PE (PE1; 10^{-6}M) in the LN, LN+Indo, and LN+SC-560 drug conditions relative to the ND, Indo, and SC-560 drug conditions, respectively. Data are represented as the average mean $\pm$ SEM. $p<0.05$ was considered statistically significant.

Table 2: Ach-stimulated vasocontractile curve-fit parameter analyses

	WKY		SHR		p value		
	CON	UCMS	CON	UCMS	Stress Effect	Strain Effect	Stress x Strain
LN (L-name)							
EC_{50} (uM)	2.58±0.79	2.48±1.20	2.44±0.70	1.62±0.43	0.5568	0.4011	0.5309
MAX (%)	27.25±5.95	11.86±2.91[a]	48.5±9.0	33.40±6.51	0.0337	0.0037	0.9854
AUC	54.22±10.98	24.87±5.43[a]	84.3±15.4	65.42±12.76	0.0560	0.0081	0.7046
LN+Indo							
EC_{50} (nM)	---	---	---	---	---	---	---
MAX (%)	0.05±0.95	0.85±0.32	1.58±1.12	-0.58±0.88	0.4508	0.9144	0.0996
AUC	2.83±1.48	4.21±0.85	7.42±1.47	5.02±1.18	0.6924	0.0498	0.1560
LN+SC-560							
EC_{50} (uM)	---	---	---	---	---	---	---
MAX (%)	0.92±0.90	1.06±0.59	2.69±0.59	1.28±0.81	0.3860	0.1767	0.2932
AUC	9.82±1.91	8.26±1.39	8.33±1.15	7.14±1.03	0.3310	0.3552	0.8935
LN+NS-398							
EC_{50} (nM)	---	---	---	---	---	---	---
MAX (%)	3.07±1.33	1.12±0.94	-0.48±1.49	2.40±1.45	0.7184	0.3817	0.0723
AUC	11.96±4.40	10.35±2.53	9.42±1.45	6.13±1.20	0.3752	0.2257	0.7588
LN+SQ-29,548							
EC_{50} (nM)	---	---	---	---	---	---	---
MAX (%)	1.65±0.91	1.45±0.81	5.51±1.16	3.15±1.41	0.2355	0.0124	0.3127
AUC	9.62±2.06	8.67±2.15	14.48±2.65	9.56±1.60	0.1846	0.1934	0.3671

Curve-fit parameter analyses corresponding to data depicted in Figure 8. EC_{50}, dose resulting in 50% of maximum curve amplitude; MAX, maximum curve amplitude; AUC, area under the curve. From post-hoc analysis: [a] $p<0.05$ vs. CON within WKY or SHR; [b] $p<0.05$ vs. WKY from corresponding CON or UCMS condition. Data represented as the average ± SEM and are expressed relative to a maximal 60 mM KCl dose.

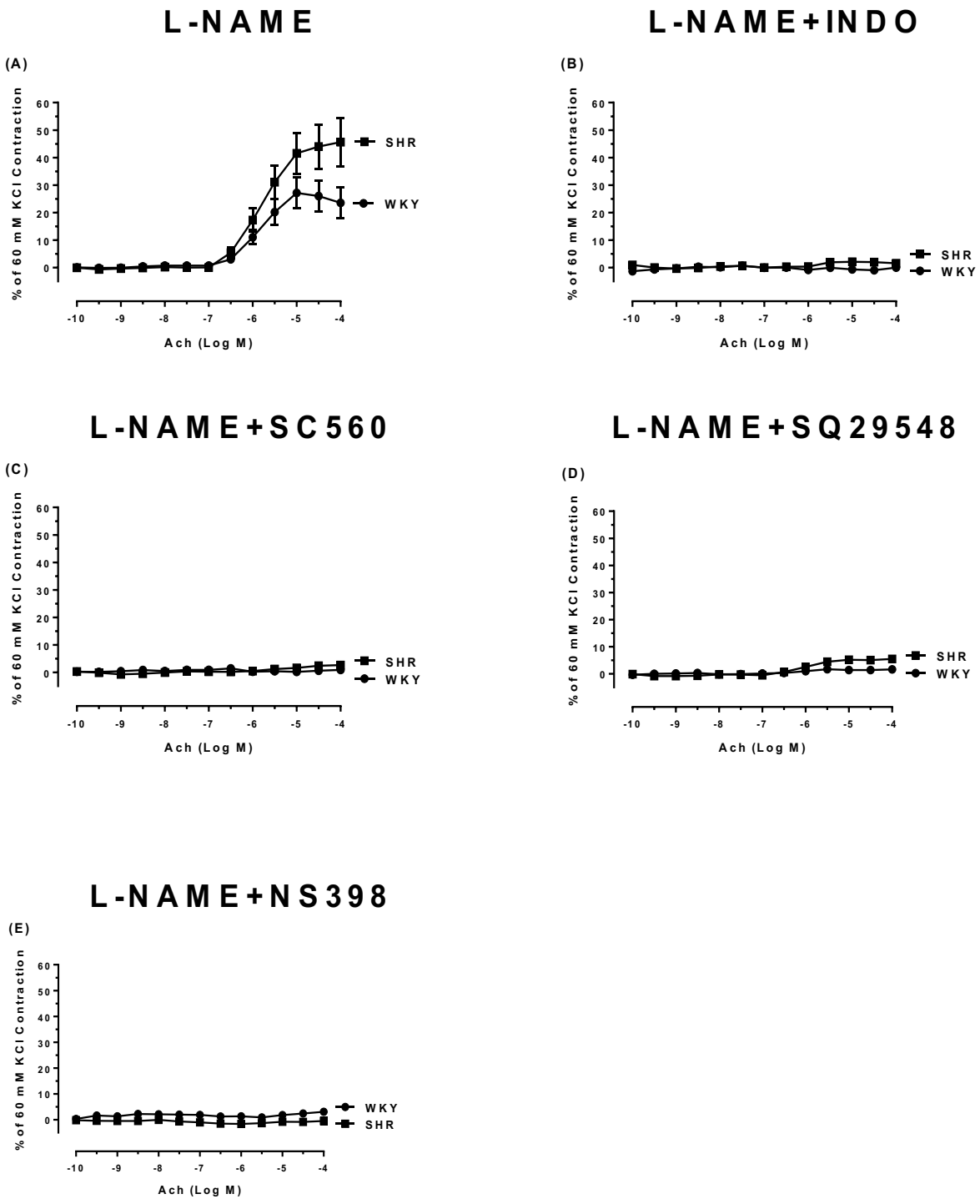

Figure 6: ACh-stimulated vasocontraction in quiescent CCA of Control animals, comparing WKY versus SHR. Arterial rings were preincubated with L-name, L-name+Indo, L-name+SC-560, L-name+SQ29548, or L-name+NS-398 before exposure to cumulative doses of ACh. Data represents average ± SEM and are expressed relative to each ring's maximal contraction to KCl; $p<0.05$ was considered statistically significant.

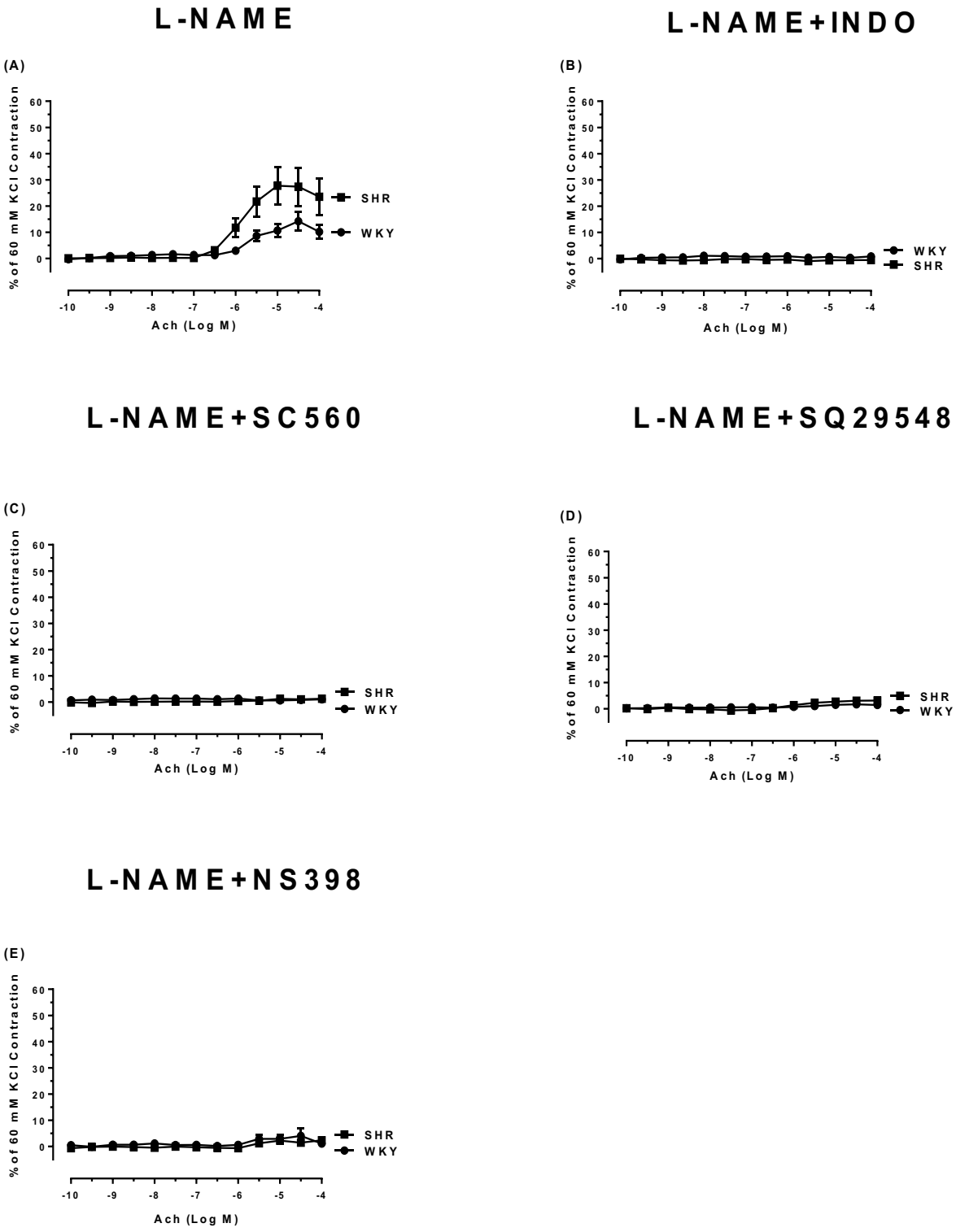

Figure 7: ACh-stimulated vasocontraction in quiescent CCA of UCMS animals, comparing WKY versus SHR. Arterial rings were preincubated with L-name, L-name+Indo, L-name+SC-560, L-name+SQ29548, or L-name+NS-398 before exposure to cumulative doses of ACh. Data represents average ± SEM and are expressed relative to each ring's maximal contraction to KCl; $p<0.05$ was considered statistically significant.

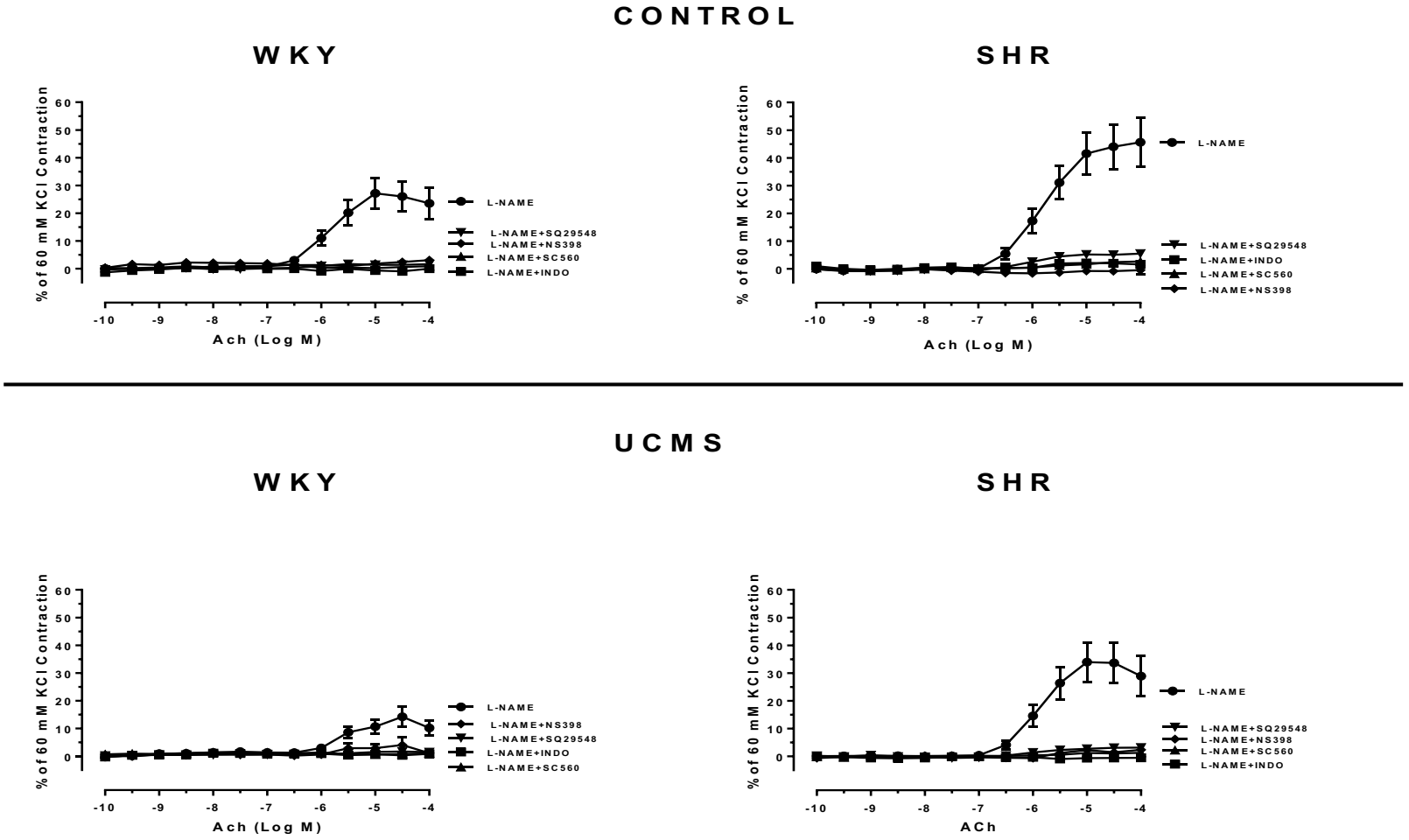

Figure 8: ACh-stimulated vasocontraction in quiescent CCA of Control (*top*) and UCMS (*bottom*) animals across various drug conditions. Arterial rings were preincubated with L-name, L-name+Indo, L-name+SC-560, L-name+SQ29548, or L-name+NS-398 before exposure to cumulative doses of ACh. Data represents average ± SEM and are expressed relative to each ring's maximal contraction to KCl; $p<0.05$ was considered statistically significant.

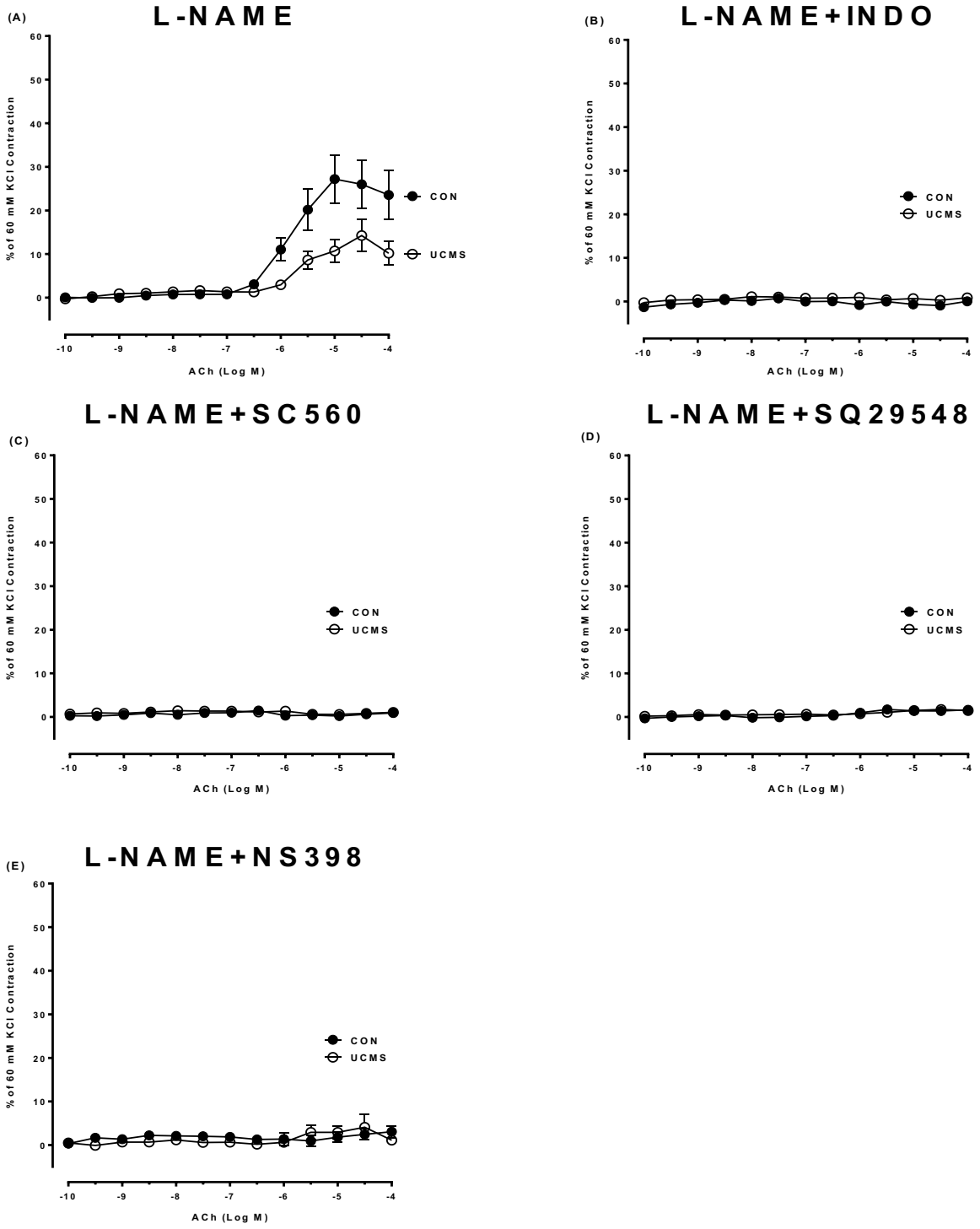

Figure 9: ACh-stimulated vasocontraction in quiescent CCA of WKY animals, comparing CON versus UCMS. Arterial rings were preincubated with L-name, L-name+Indo, L-name+SC-560, L-name+SQ29548, or L-name+NS-398 before exposure to cumulative doses of ACh. Data represents average ± SEM and are expressed relative to each ring's maximal contraction to KCl; $p<0.05$ was considered statistically significant.

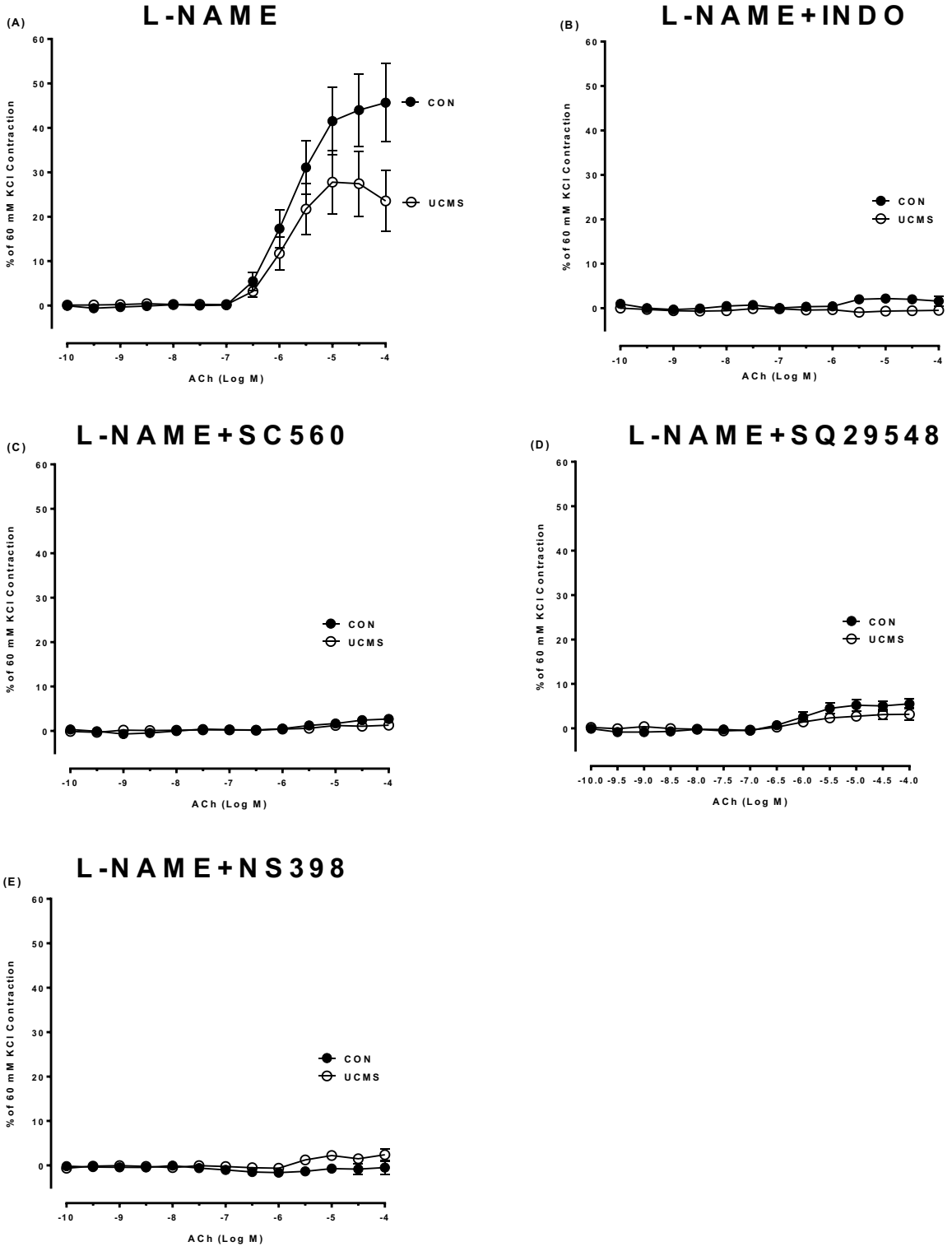

Figure 10: ACh-stimulated vasocontraction in quiescent CCA of SHR animals, comparing CON versus UCMS. Arterial rings were preincubated with L-name, L-name+Indo, L-name+SC-560, L-name+SQ29548, or L-name+NS-398 before exposure to cumulative doses of ACh. Data represents average ± SEM and are expressed relative to each ring's maximal contraction to KCl; $p<0.05$ was considered statistically significant.

Table 3: U46619-stimulated vasocontractile curve-fit parameter analyses.

	WKY		SHR		p value		
	CON	UCMS	CON	UCMS	Stress Effect	Strain Effect	Stress x Strain
LN (L-name)							
EC_{50} (uM)	12.86±1.46	11.48±1.19	17.74±1.98	14.65±3.11	0.2918	0.0616	0.6847
MAX (%)	158.7±6.45	155.4±10.44	146.1±3.75	132.6±4.50	0.2281	0.0128	0.4610
AUC	352.7±13.25	338.7±21.64	311.9±10.86	291.6±11.27	0.2593	0.0054	0.8360
LN+Indo							
EC_{50} (nM)	32.57±9.09	17.13±3.40	26.35±2.46	23.62±3.06	0.0714	0.9780	0.1979
MAX (%)	144.8±16.29	144.0±4.77	147.1±4.06	126.6±5.11	0.2091	0.3674	0.2406
AUC	274.9±16.53	310.8±13.82	295.3±8.32	257.4±10.51[b]	0.9330	0.1984	0.0076
LN+SC-560							
EC_{50} (uM)	18.61±1.04	13.18±0.85[a]	21.15±1.74	14.01±0.98[a]	<0.001	0.1369	0.4431
MAX (%)	163.1±9.34	146.2±5.19	154.7±2.71	135.8±4.93	0.0075	0.1477	0.8752
AUC	341.0±19.26	321.7±10.81	321.4±7.05	295.9±8.23	0.0884	0.0836	0.8089
LN+NS-398							
EC_{50} (nM)	15.59±1.80	17.01±5.06	20.13±3.31	9.07±1.08	0.1367	0.5897	0.0590
MAX (%)	162.4±7.75	159.2±6.17	137.1±5.11[b]	139.5±4.61	0.9487	0.0012	0.6374
AUC	350.9±11.45	328.5±10.38	282.5±18.18[b]	309.2±14.59	0.8861	0.0078	0.1106
LN+SQ-29,548							
EC_{50} (nM)	1.73±0.27	1.50±0.10	1.97±0.27	1.49±0.14	0.0876	0.5758	0.5353
MAX (%)	140.2±22.28	117.5±7.90	98.40±9.71	92.49±8.07	0.2974	0.0178	0.5396
AUC	53.52±5.99	56.86±4.24	37.80±2.87	42.18±4.23	0.3994	0.0017	0.9082

Curve-fit parameter analyses corresponding to data depicted in Figure 11. EC_{50}, dose resulting in 50% of maximum curve amplitude; MAX, maximum curve amplitude; AUC, area under the curve. From post-hoc analysis: [a] $p<0.05$ vs. CON within WKY or SHR; [b] $p<0.05$ vs. WKY from corresponding CON or UCMS condition. Data represented as the average ± SEM and are expressed relative to a maximal 60 mM KCl dose.

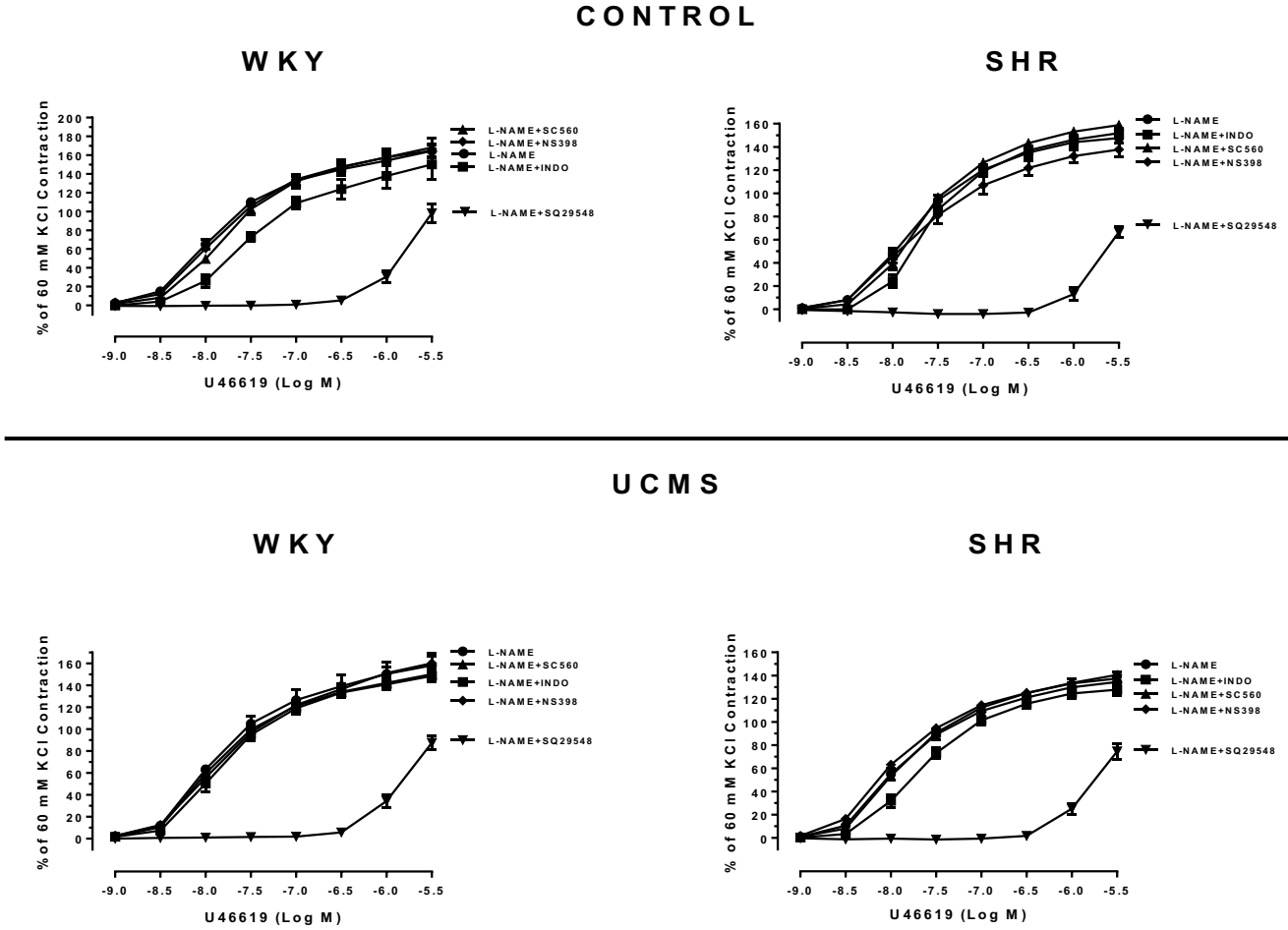

Figure 11: U46619-stimulated vasocontraction in quiescent CCA of Control (*top*) and UCMS (*bottom*) animals across various drug conditions. Arterial rings were preincubated with L-name, L-name+Indo, L-name+SC-560, L-name+SQ29548, or L-name+NS-398 before exposure to cumulative doses of U46619. Data represents average ± SEM and are expressed relative to each ring's maximal contraction to KCl; $p<0.05$ was considered statistically significant.

Table 4: ACh-stimulated vasodilatory curve-fit parameter analyses

	WKY		SHR		p value		
	CON	UCMS	CON	UCMS	Stress Effect	Strain Effect	Stress x Strain
No Drug (ND)							
EC_{50} (uM)	95.25±32.16	41.0±8.44	69.41±25.53	87.83±32.30	0.5162	0.7034	0.1916
MAX (%)	92.43±3.08	101.0±1.35	83.81±3.19	88.65±2.63[b]	0.0190	0.0004	0.5257
AUC	300.6±17.84	349.9±13.42	282.4±12.19	287.0±11.09[b]	0.0562	0.0053	0.1113
LN (L-name)							
EC_{50} (uM)	---	---	---	---	---	---	---
MAX (%)	-1.28±1.23	1.23±0.43	-0.16±1.42	-3.02±1.15[b]	0.8683	0.1529	0.0166
AUC	15.54±2.25	10.27±1.14	11.26±1.53	21.13±4.22[b]	0.3844	0.2152	0.0061
Indo							
EC_{50} (uM)	83.58±14.10	98.57±48.68	82.13±17.17	98.92±10.06	0.6602	0.9878	0.9800
MAX (%)	96.84±2.01	97.67±0.90	92.05±1.38	99.60±41.36[a]	0.0094	0.3306	0.0315
AUC	304.7±8.24	324.1±27.44	286.03±15.12	322.0±17.10	0.1455	0.5750	0.6548
LN+Indo							
EC_{50} (uM)	---	---	---	---	---	---	---
MAX (%)	-0.10±0.79	0.70±1.70	-1.96±2.80	-0.49±0.59	0.4968	0.3643	0.8393
AUC	12.05±1.33	14.39±0.95	24.81±8.47	13.03±1.78	0.2714	0.1931	0.1114
SC-560							
EC_{50} (uM)	15.40±4.23	90.07±26.21	55.41±14.31	147.9±35.00	0.0043	0.0692	0.7269
MAX (%)	102.5±0.67	101.4±0.92	97.60±1.61	98.98±2.32	0.9219	0.0324	0.4425
AUC	392.0±12.27	319.9±18.05[a]	321.3±13.25[b]	282.4±10.99	0.0019	0.0023	0.2845
LN+SC-560							
EC_{50} (uM)	---	---	---	---	---	---	---
MAX (%)	3.36±1.18	2.22±1.09	0.05±1.55	2.62±1.56	0.6081	0.2998	0.1909
AUC	13.12±2.77	9.33±1.59	8.89±1.69	9.30±1.99	0.5050	0.2763	0.3052

Curve-fit parameter analyses corresponding to data depicted in Figure 12. EC_{50}, dose resulting in 50% of maximum curve amplitude; MAX, maximum curve amplitude; AUC, area under the curve. From post-hoc analysis: [a] $p<0.05$ vs. CON within WKY or SHR; [b] $p<0.05$ vs. WKY from corresponding CON or UCMS condition. Data represented as the average ± SEM and are expressed relative to a maximal dose of PE (10^{-6}M).

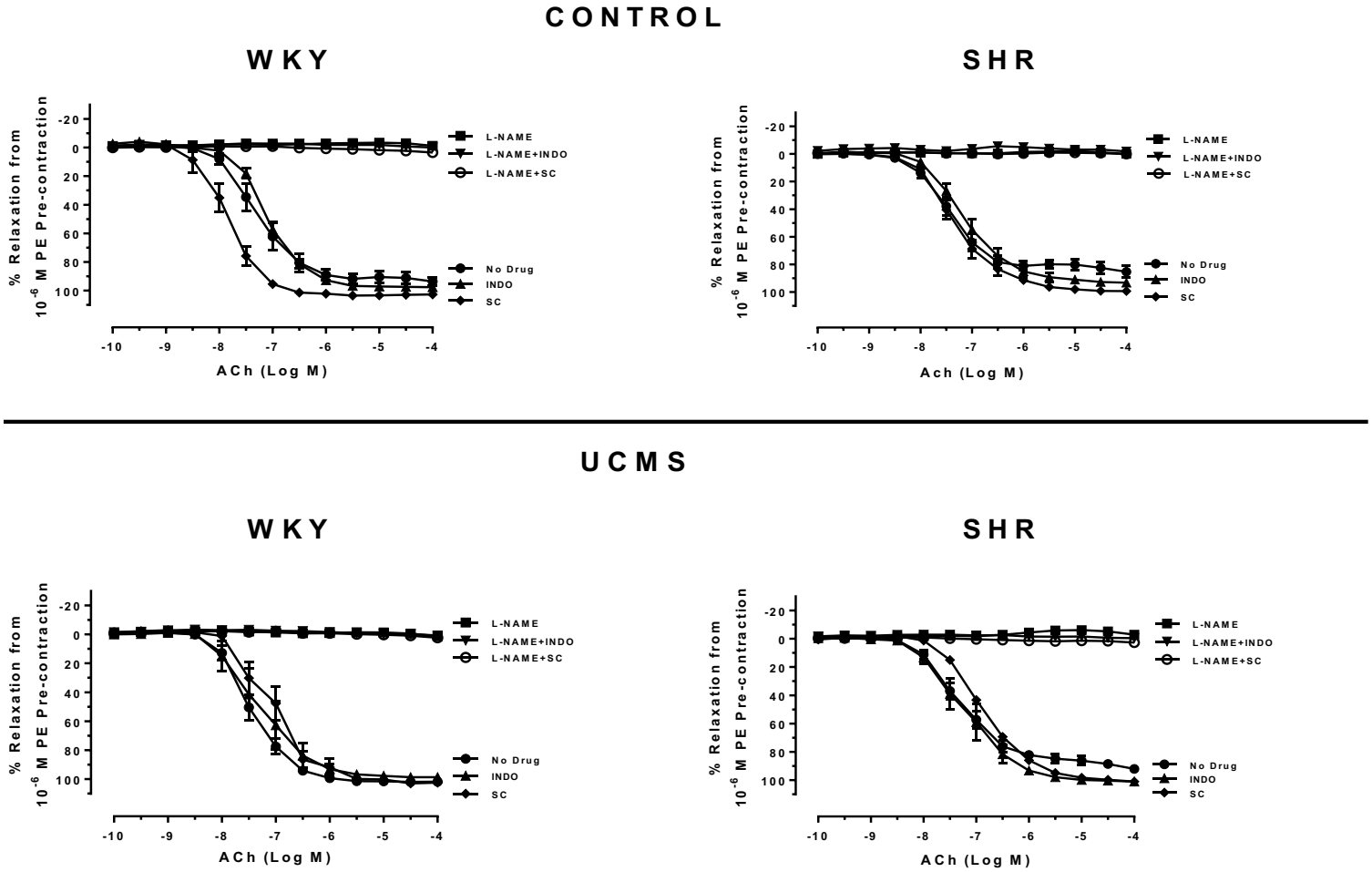

Figure 12: ACh-stimulated vasorelaxation in precontracted (PE 10^{-6}M) CCA of Control (*top*) and UCMS (*bottom*) animals across various drug conditions. Arterial rings were preincubated with ND, L-name, Indo, L-name+Indo, SC-560, or L-name+SC-560 before exposure to cumulative doses of ACh. Data represents average ± SEM and are expressed relative to each ring's maximal contraction to PE (10^{-6}M); $p<0.05$ was considered statistically significant.

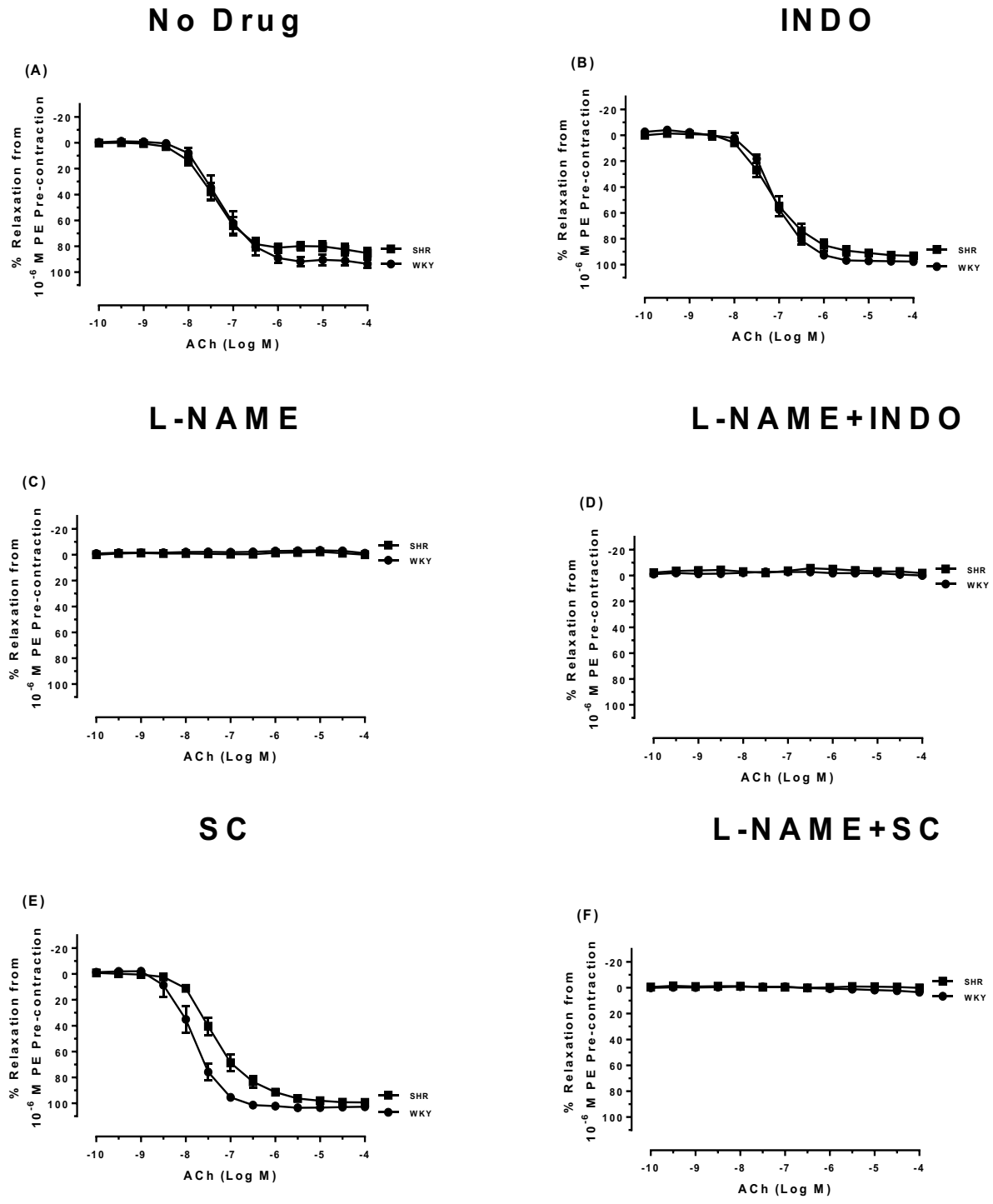

Figure 13: ACh-stimulated vasorelaxation in precontracted (PE 10^{-6}M) CCA of Control animals, comparing WKY versus SHR. Arterial rings were preincubated with ND, L-name, Indo, L-name+Indo, SC-560, or L-name+SC-560 before exposure to cumulative doses of ACh. Data represents average ± SEM and are expressed relative to each ring's maximal contraction to PE (10^{-6}M); $p<0.05$ was considered statistically significant.

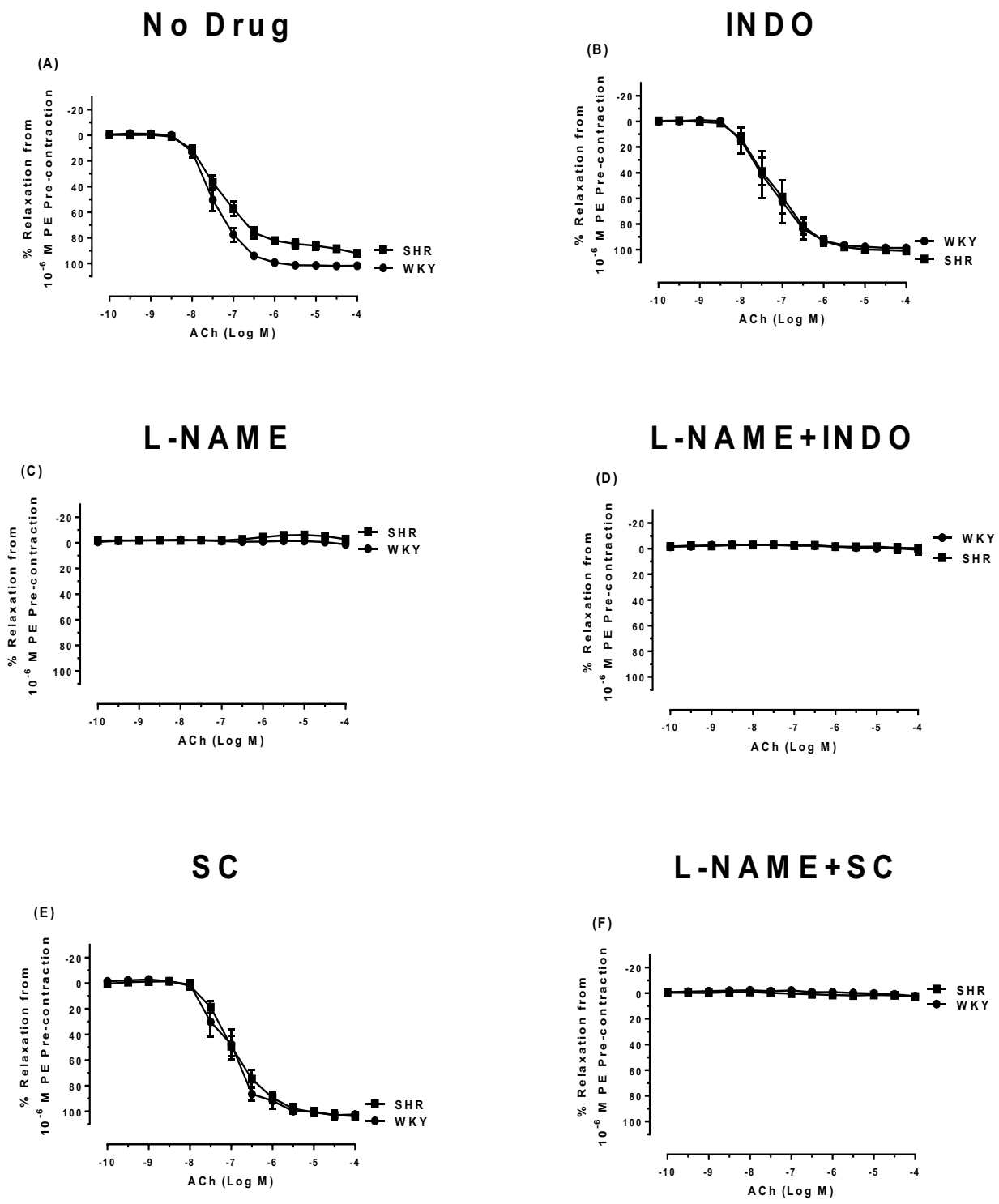

Figure 14: ACh-stimulated vasorelaxation in precontracted (PE 10^{-6}M) CCA of UCMS animals, comparing WKY versus SHR. Arterial rings were preincubated with ND, L-name, Indo, L-name+Indo, SC-560, or L-name+SC-560 before exposure to cumulative doses of ACh. Data represents average ± SEM and are expressed relative to each ring's maximal contraction to PE (10^{-6}M); $p<0.05$ was considered statistically significant.

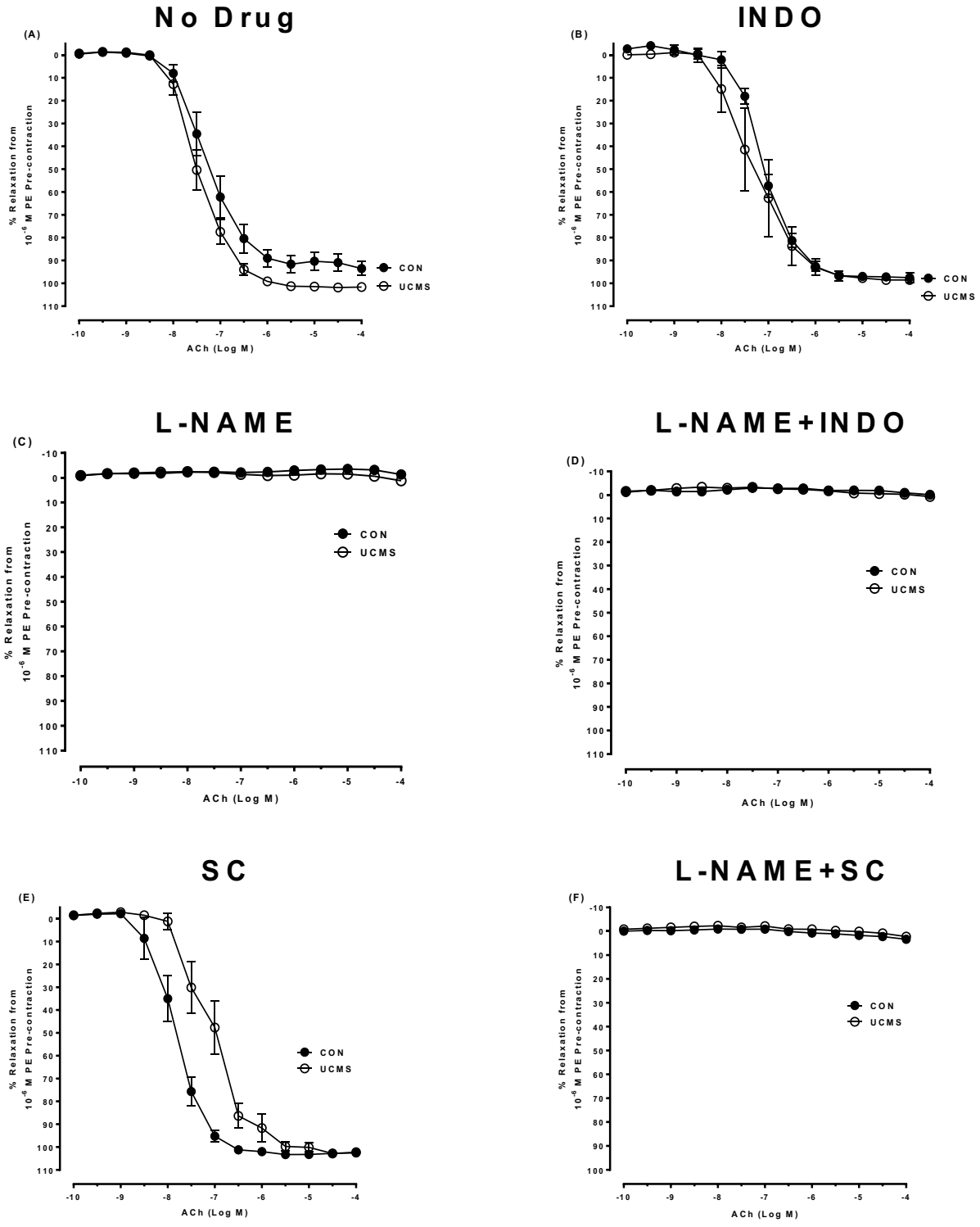

Figure 15: ACh-stimulated vasorelaxation in precontracted (PE 10^{-6}M) CCA of WKY animals, comparing CON versus UCMS. Arterial rings were preincubated with ND, L-name, Indo, L-name+Indo, SC-560, or L-name+SC-560 before exposure to cumulative doses of ACh. Data represents average ± SEM and are expressed relative to each ring's maximal contraction to PE (10^{-6}M); $p<0.05$ was considered statistically significant.

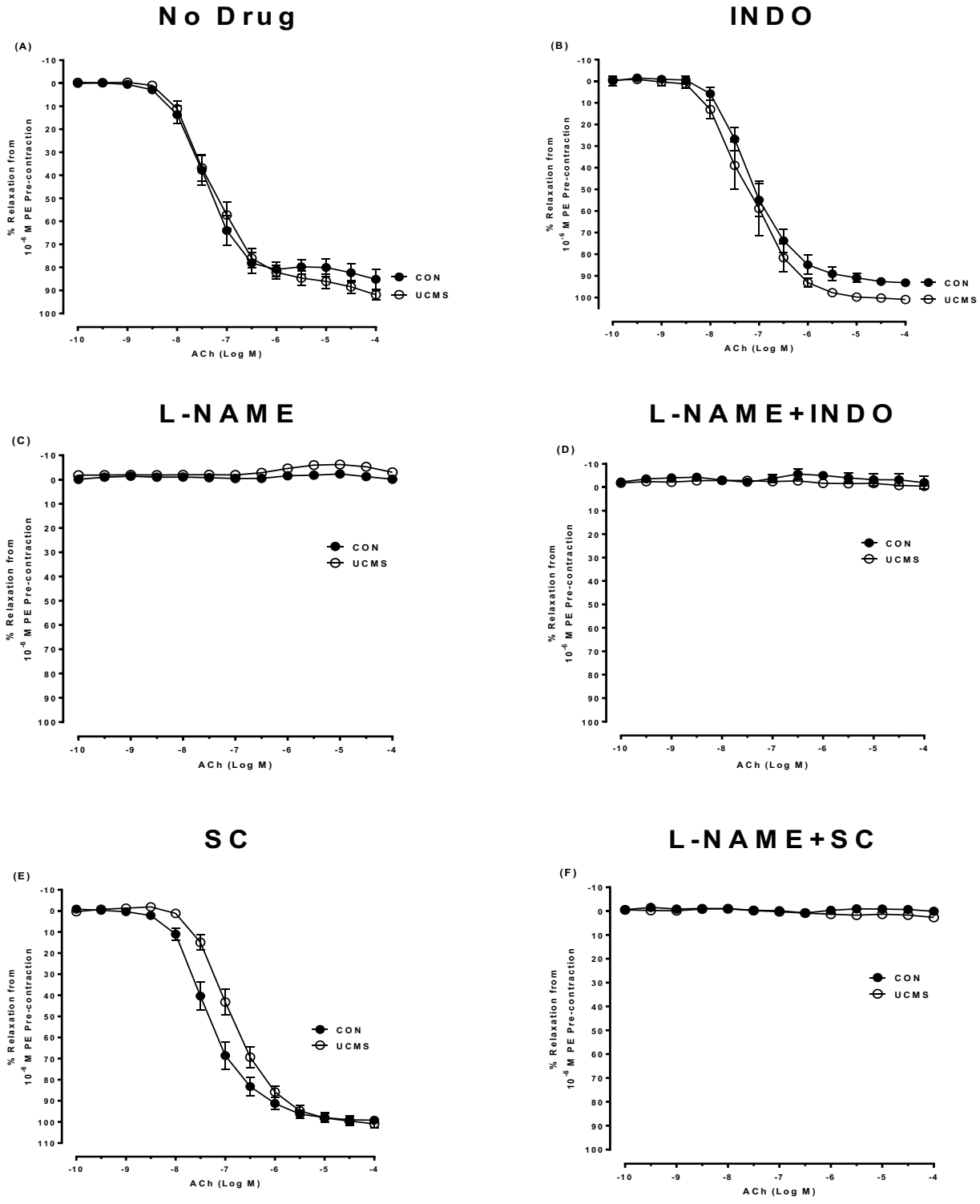

Figure 16: ACh-stimulated vasorelaxation in precontracted (PE 10^{-6}M) CCA of SHR animals, comparing CON versus UCMS. Arterial rings were preincubated with ND, L-name, Indo, L-name+Indo, SC-560, or L-name+SC-560 before exposure to cumulative doses of ACh. Data represents average ± SEM and are expressed relative to each ring's maximal contraction to PE (10^{-6}M); $p<0.05$ was considered statistically significant.

Table 5: SNP-stimulated vasodilatory curve-fit parameter analyses

	WKY		SHR		p value		
	CON	UCMS	CON	UCMS	Stress Effect	Strain Effect	Stress x Strain
No Drug (ND)							
EC_{50} (uM)	6.32±0.74	6.62±0.68	8.97±1.70	15.76±9.98[a,b]	0.0494	0.0016	0.0708
MAX (%)	104.5±1.51	101.1±0.62	103.0±1.71	101.4±2.84	0.1709	0.7331	0.6364
AUC	444.0±8.87	427.3±3.89	427.8±13.37	399.4±19.09	0.0719	0.0781	0.6324
LN (L-name)							
EC_{50} (uM)	5.59±0.59	5.54±0.58	7.65±0.91	7.88±0.85	0.9072	0.0063	0.8537
MAX (%)	105.2±0.95	102.8±1.11	101.9±0.79	102.2±1.85	0.4170	0.1357	0.3103
AUC	449.8±6.24	437.2±4.19	422.5±6.34	411.3±14.20	0.1927	0.0049	0.9407
Indo							
EC_{50} (uM)	4.74±1.29	6.42±2.65	6.53±1.85	11.33±2.73	0.2049	0.1908	0.5338
MAX (%)	108.8±2.13	102.3±1.41	111.6±4.46	99.31±1.86[a]	0.0100	0.9747	0.3754
AUC	474.4±13.56	442.8±22.70	481.3±26.08	400.0±16.13	0.0246	0.4339	0.2828
LN+Indo							
EC_{50} (uM)	4.95±0.39	5.69±0.82	4.88±0.82	10.76±3.57	0.0948	0.1999	0.1891
MAX (%)	105.0±1.76	101.8±1.73	104.1±2.45	100.7±0.76	0.0815	0.5872	0.9616
AUC	453.6±6.64	432.5±4.58	451.1±14.47	409.4±13.90	0.0088	0.2492	0.3506
SC-560							
EC_{50} (uM)	3.17±0.47	4.39±0.66	7.36±2.14	8.68±3.01	0.2394	0.0286	0.8945
MAX (%)	103.8±1.00	101.8±1.06	102.8±1.69	103.7±4.01	0.6377	0.8948	0.4940
AUC	470.3±4.89	445.4±8.61	435.4±19.03	442.3±34.34	0.2986	0.2156	0.2778
LN+SC-560							
EC_{50} (uM)	3.17±0.47	4.82±0.60	7.36±2.14	9.12±2.16	0.2868	0.0135	0.9706
MAX (%)	103.8±1.00	103.4±1.66	102.8±1.69	101.0±2.51	0.5419	0.3395	0.7077
AUC	470.3±4.89	446.6±9.71	435.4±19.03	395.1±26.12[a]	0.0751	0.6317	0.0197

Curve-fit parameter analyses corresponding to data depicted in Figure 17. EC_{50}, dose resulting in 50% of maximum curve amplitude; MAX, maximum curve amplitude; AUC, area under the curve. From post-hoc analysis: [a] $p<0.05$ vs. CON within WKY or SHR; [b] $p<0.05$ vs. WKY from corresponding CON or UCMS condition. Data represented as the average ± SEM and are expressed relative to a maximal dose of PE (10^{-6}M).

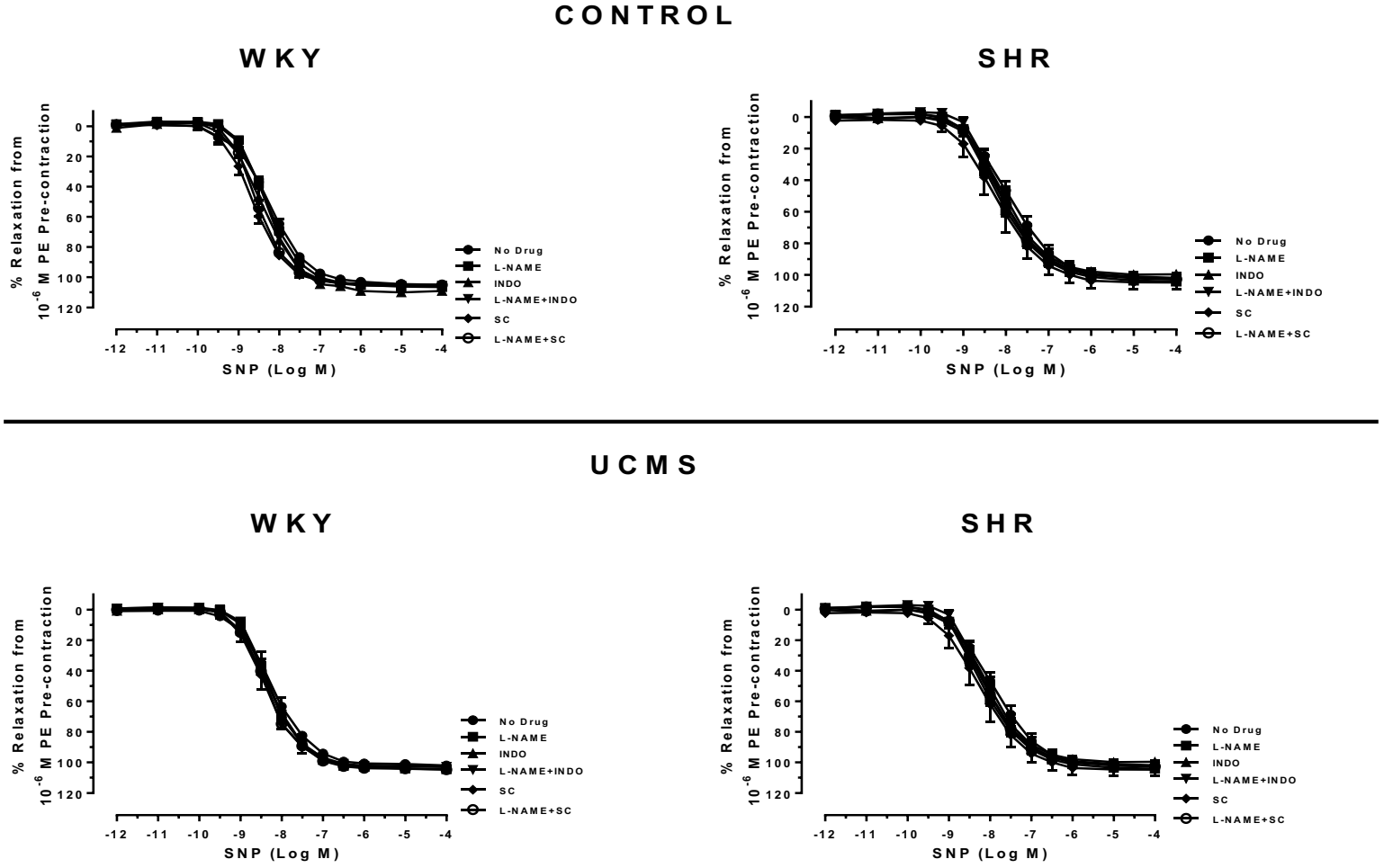

Figure 17: SNP-stimulated vasorelaxation in precontracted (PE 10^{-6}M) CCA of Control (*top*) and UCMS (*bottom*) animals across various drug conditions. Arterial rings were preincubated with ND, L-name, Indo, L-name+Indo, SC-560, or L-name+SC-560 before exposure to cumulative doses of SNP. Data represents average ± SEM and are expressed relative to each ring's maximal contraction to PE (10^{-6}M); $p<0.05$ was considered statistically significant.

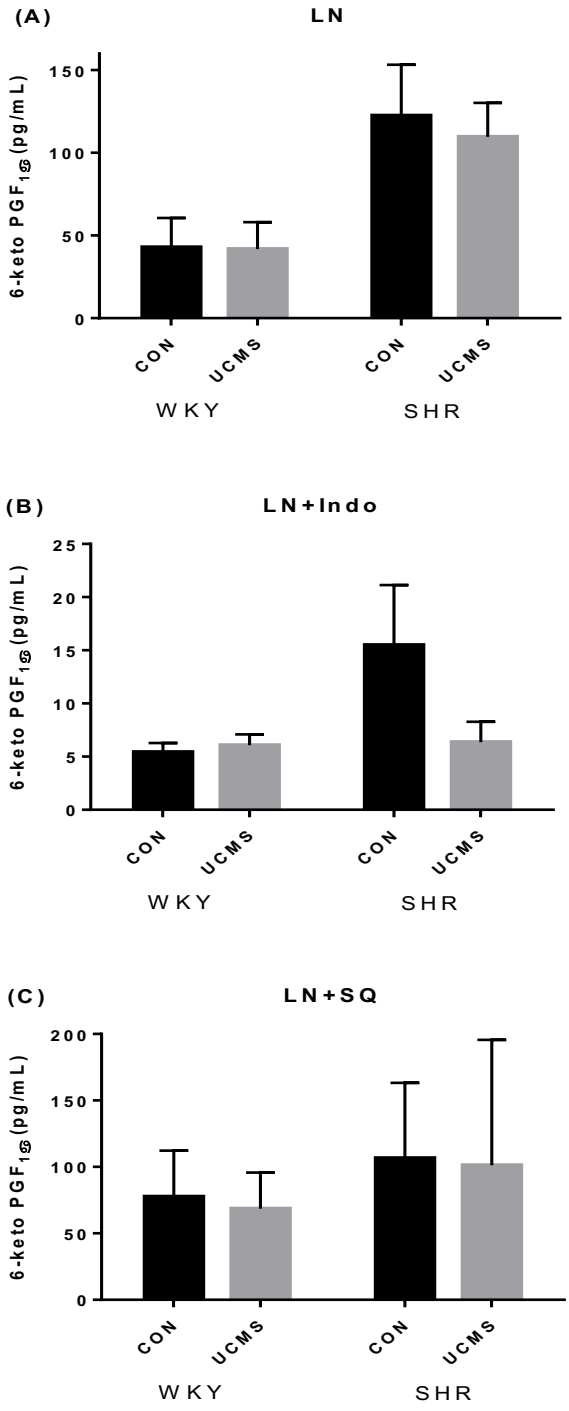

Figure 18: PGI_2 levels in the tissue buffer surrounding CCA segments from WKY and SHR comparing the Control and UCMS groups. The concentration of the stable PGI_2 metabolite, 6-keto prostaglandin F1∝ (PGF1∝), in the tissue buffer surrounding CCA segments from WKY and SHR in the Control versus UCMS groups incubated with LN (A), LN+Indo (B), or LN+SQ29548 (C) following the maximal dose of ACh (10^{-4}M) in the vasocontractile dose-response curves (Figure 8). Data are represented as the average ± SEM. $p<0.05$ was considered statistically significant. From post-hoc analyses: Φ $p<0.05$ vs. WKY within corresponding CON or UCMS group.

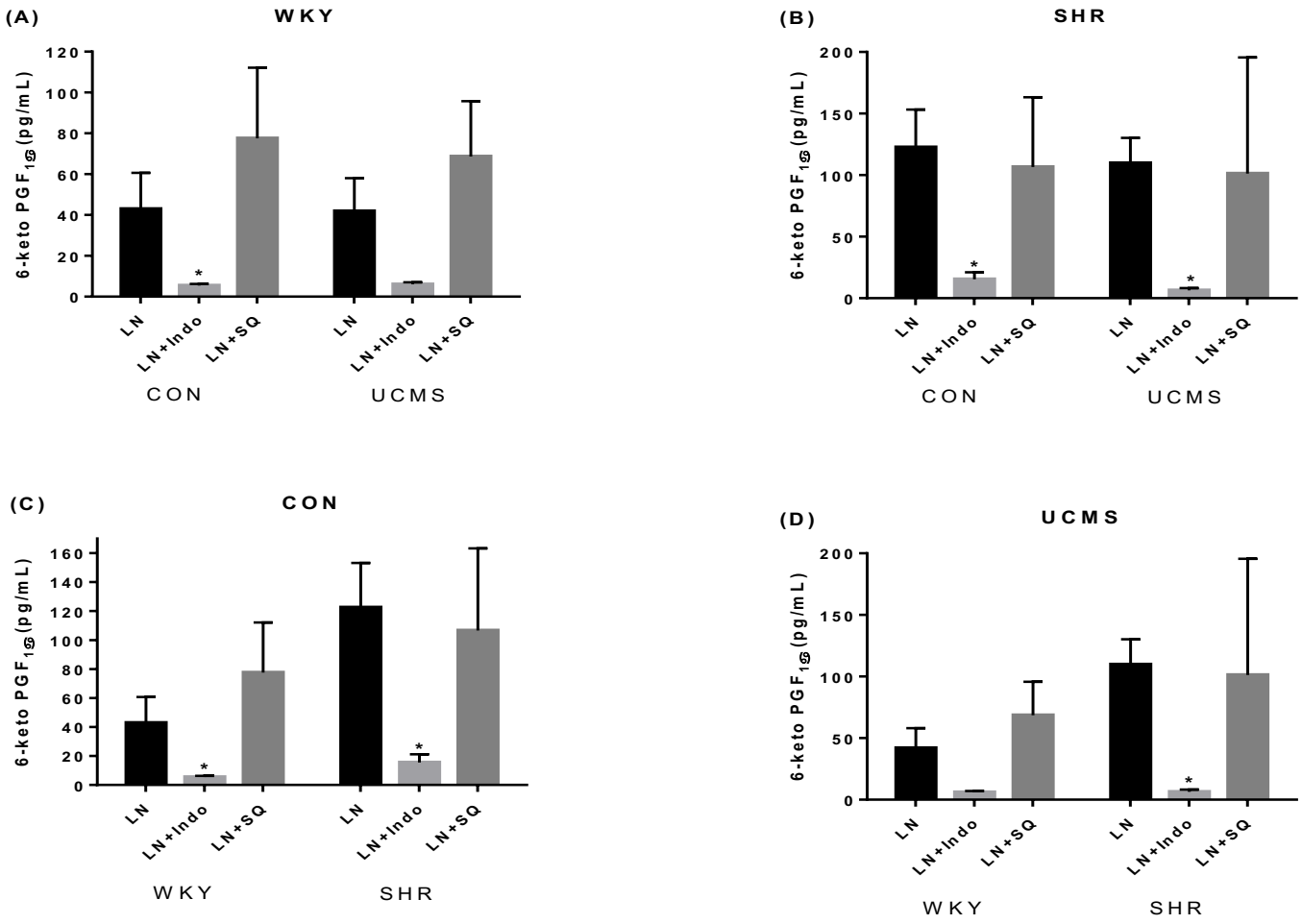

Figure 19: PGI_2 levels in the tissue buffer surrounding CCA segments from WKY and SHR comparing across drug conditions. The concentration of the stable PGI_2 metabolite, 6-keto prostaglandin F1∝ (PGF1∝), in the tissue buffer surrounding CCA segments from WKY (A) and SHR (B), comparing the Control and UCMS groups, and from Control (C) and UCMS (D), comparing WKY and SHR, following the maximal dose of ACh (10^{-4}M) in the vasocontractile dose-response curves (Figure 8). Data are represented as the average ± SEM. $p<0.05$ was considered statistically significant. From post-hoc analyses: * $p<0.05$ vs. LN+Indo within corresponding strain or stress group.

6.0 Discussion

Chronic mental stress has been shown to be associated with several CVD risk factors and has therefore been emerging as an independent risk factor for CVD (45, 46, 52, 53, 54, 90, 91, 92). However, a significant amount of investigation remains to identify how chronic mental stress can lead to CVD, on a mechanistic level (56). This thesis aimed to investigate the effects of UCMS on endothelial function in WKY and SHR rats. While some of the hypotheses were accepted, others were rejected. Overall, the main findings from this project were:

1. Splash test and coat status results suggest that the UCMS protocol was sufficient to induce mental stress.
2. Animal strain, but not stress, significantly affected left ventricle to body weight ratio.
3. Compared to their respective controls, UCMS augmented endothelium-dependent vasorelaxation.
4. Compared to their respective controls, UCMS attenuated endothelium-dependent vasocontraction.
5. UCMS-induced augmentation of endothelium-dependent vasorelaxation and attenuation of endothelium-dependent vasocontraction were not due an effect of UCMS on VSM sensitivity, an increase in NO bioavailability, or a reduction in PGI_2 levels

6.0.1 Splash test and coat status results suggest that the UCMS protocol was sufficient to induce mental stress

Behavioural analyses, such as monitoring coat status and performing splash tests (as described in *4.3 Efficacy Measures of UCMS Protocol*), often accompany the physiological and biochemical analyses when using a psychological intervention/protocol, such as the UCMS protocol (65, 66, 67, 70, 71). This is of importance because the physiological and biochemical data are not relevant if the intervention did achieve the desired outcome. Performing behavioural analyses, such as the splash test and coat status, to ensure that the UCMS was inducing stress in the UCMS animals was particularly important in the current study, since it is the first time that the UCMS protocol has been used in our lab.

The splash test and coat status data from the SHR animals in our study is comparable to what has been found in previous studies (65, 67, 70) and suggests that the UCMS protocol was sufficient to induce depressive symptoms in the animals in the SHR UCMS group compared to the SHR control group. However, the splash test data and coat status data in WKY was not consistent with previous literature (65, 67, 70). Grooming frequency during the splash test was significantly lower (i.e., to a similar degree as SHR UCMS animals) in both the WKY UCMS group and the WKY control group. Additionally, the coat status scores of WKY were only significantly greater (i.e., dirtier) in the UCMS group compared to the control group at weeks 1 and 2. Moreover, the coat status scores were higher (i.e., dirtier) in WKY compared to SHR, in both the CON and UCMS groups.

With this study being the first to investigate the effects of the UCMS protocol in WKY and SHR rats it is possible that these unexpected results may be due the strain differences in the animals we used compared to animals used in previous studies. For instance, WKY rats are

classified as an animal model of depression (93, 94, 95), therefore substantiating the possibility that the strain of rat chosen for this study could have contributed to these results. Our splash test and coat status data build on the already existing literature that have demonstrated that WKY are significantly more depressed than several other rat strains (e.g., SHR, Wistar, Sprague-Dawley, etc.) (93, 94, 95). This was determined by the various behaviours exhibited by each of these rat strains in a series of behavioural tests. For example, social avoidance was demonstrated by WKY in the social interactions test (93). Learned helplessness has also been demonstrated in WKY, as determined by the WKY's lack of motivation to escape the forced swim test (93) and the shuttlebox escape test (93). Moreover, these investigators established that these behaviours were not present in Wistar, Sprague-Dawley, Fisher-344, or SHR rats, further demonstrating the increased presence of depressive behaviours in WKY compared to other rat strains (93).

Additionally, while no significant strain effect in the latency of grooming was detected during the splash test, statistical analysis could only be performed for the animals that groomed at all. Since significantly fewer WKY (13 of 24) groomed at all during the splash test compared to SHR (21 of 24), together with the fact that a reduction in grooming is considered a sign of depressive behaviour, this data would further suggest that WKY rats are an animal model of depression.

Overall, the lack of grooming by WKY during the splash test, and the dirtier coats in WKY compared to SHR further demonstrates the presence of depression in the WKY rat strain and should be considered when choosing the animal model for any experiment involving a behavioural component.

6.0.2 Animal strain, but not stress, significantly affected left ventricle to body weight ratio

An increase in left ventricle to body weight ratio is present in animals with hypertension as a result of the systemic pressure-induced left ventricular hypertrophy (LVH) (39, 96, 97). The left ventricle to body weight ratio was significantly greater in SHR compared to WKY in both the control and UCMS groups, suggesting the presence of LVH in these animals. These results were expected and have been observed previously in our lab (39) and by others (96, 97, 98, 99). However, since chronic mental stress has been shown to be associated with several CVD risk factors, including hypertension (90, 91, 92), it may have been hypothesized that the UCMS would have caused LVH in WKY and exaggerated the already existing LVH in SHR. However, while LVH can develop over the course of only a few weeks, it usually takes months to develop (98). It is therefore likely like that the 6-week UCMS protocol was not long enough for the structural changes associated with LVH to occur. A study published by Puzserova et al. demonstrated that 8 and 12 weeks of chronic stress, induced by crowded housing, was insufficient to induce left ventricular hypertrophy in WKY (58), further suggesting that a longer protocol may need to be used for LVH to develop.

6.0.3 Compared to their respective controls, UCMS augmented endothelium-dependent vasorelaxation

As expected, endothelium-dependent, ACh-stimulated vasorelaxation in precontracted CCA was attenuated in SHR compared to WKY. Additionally, inhibiting NOS with L-name inhibited endothelium-dependent vasorelaxation, suggesting that endothelium-dependent vasorelaxation in WKY and SHR is NO-mediated. Furthermore, non-selectively inhibiting COX with indomethacin reversed the impairment in endothelium-dependent vasorelaxation in both

SHR groups, suggesting that this impairment is, at least in part, due to over-active EDCF activity. These results were expected, as they have been observed previously in our lab (39, 74) and by others in aorta of WKY and SHR (75, 76).

Unexpectedly, UCMS augmented endothelium-dependent vasorelaxation in both WKY and SHR. However, previous studies that have investigated the effects of the UCMS protocol on endothelial function have done so using mostly mice and Wistar rats (65, 66, 68, 69). The current study is the first to examine the effects of the UCMS protocol on endothelial function in WKY and SHR CCA. A previous study by Puzserova et al. investigated endothelium-dependent, ACh-stimulated vasorelaxation in femoral arteries from WKY following 8 or 12 weeks of chronic stress, induced by crowded housing (i.e., 5 rats/cage instead of 4 rats/cage) (58). Eight weeks of crowded housing resulted in augmented endothelium-dependent vasorelaxation compared to the control group, similar to what was observed in the current study. Interestingly, 12 weeks of crowded housing resulted in *attenuated* endothelium-dependent vasorelaxation compared to the control group (58). It is, therefore, possible that the duration of the UCMS protocol used in the current study was not sufficiently long enough to induce endothelial dysfunction. If the protocol had been longer in duration, the UCMS may have led to attenuated vasorelaxation and/or augmented vasocontraction, as originally hypothesized, and as observed in the Puzserova et al. study.

6.0.4 Compared to their respective controls, UCMS attenuated endothelium-dependent vasocontraction

Endothelium-dependent, ACh-stimulated vasocontraction in quiescent CCA was augmented in SHR compared to WKY, due to overactive EDCF activity, as has been

demonstrated previously in our lab (39, 74) and by others in aorta of WKY and SHR (31, 75). Non-selectively inhibiting COX with indomethacin blocked endothelium-dependent vasocontraction in WKY and SHR, in both the control and UCMS groups. Moreover, blocking the TP receptor with the TP receptor-agonist SQ-29548 also blocked endothelium-dependent vasocontraction. These results are consistent with previous work in our lab (39, 74), and demonstrate that the endothelium-dependent vasocontraction in the WKY and SHR, in both the control and UCMS groups, is both COX- and TP receptor-mediated.

Unexpectedly, however, UCMS attenuated endothelium-dependent vasocontraction in both WKY and SHR. To date, no studies have specifically investigated the effects of UCMS on endothelium-dependent vasocontraction. However, Fuchs et al. (59) demonstrated that chronic behavioural stress, induced by air-jets blowing compressed air on the animals, impaired endothelium-dependent vasorelaxation in old (18 mo.) BHR, and suggested that this impairment may have been due overactive EDCF activity (see *1.6.2 Chronic Mental Stress and Augmented Endothelium-Dependent Vasocontraction*). However, this same study demonstrated that the chronic behavioural stress enhanced endothelium-dependent vasorelaxation in young (3 mo.) BHR (59). It is therefore possible that the age of the animals in the current study (i.e., ~ 8 mo.) could have contributed to the unexpected UCMS-induced attenuation of endothelium-dependent vasocontraction and/or augmentation of endothelium-dependent vasorelaxation.

6.0.5 UCMS-induced augmentation of endothelium-dependent vasorelaxation and attenuation of endothelium-dependent vasocontraction were not due an effect of UCMS on VSM sensitivity, an increase in NO bioavailability, or a reduction in PGI_2 levels

Impairment in endothelium-dependent vasorelaxation and/or augmentation in endothelium-dependent vasocontraction can be the result of either a reduction in EDRF activity/bioavailability, an increase in EDCF activity/bioavailability, and/or a change in VSM sensitivity to these endothelium-derived factors (6, 15, 16). Likewise, an augmentation in endothelium-dependent vasorelaxation and/or attenuation in endothelium-dependent vasocontraction can be the result of an increase in EDRF activity/bioavailability, a reduction in EDCF activity/bioavailability, and/or a change in VSM sensitivity to these endothelium-derived factors.

In the current study, increasing doses of SNP, an exogenous NO donor, elicited similar vasodilatory response curves across all groups. Likewise, increasing doses of U46619, a TP receptor agonist, elicited similar vasocontractile response curves across all groups. Together, these data suggest that neither strain nor chronic mental stress had any effect on VSM sensitivity to either NO or TP receptor stimulation.

Additionally, UCMS did not significantly affect the ratio of PE contraction in the L-name drug condition compared to the ND condition, a putative indicator of NO effect on basal tone (39, 86), suggesting that UCMS did not increase NO levels. These data would suggest that the UCMS-induced augmentation in endothelium-dependent vasorelaxation and attenuation in endothelium-dependent vasocontraction might be due to a reduction in EDCF levels. As expected, PGI_2 levels were greater in SHR compared to WKY in the L-name condition. This increase in PGI_2 levels has been shown to contribute to the attenuation of endothelium-dependent

vasorelaxation in SHR compared to WKY (39, 84). Since PGI_2 has been shown to be one of the most active prostaglandins in the WKY and SHR rat models (31, 32, 33, 37, 38, 39), it was originally hypothesized that PGI_2 would be greater in UCMS groups compared to their respective controls. However, the unexpected UCMS-induced augmentation in endothelium-dependent vasorelaxation and attenuation in endothelium-dependent vasocontraction would have suggested that PGI_2 would instead be reduced in the UCMS groups compared to their respective controls. Unexpectedly, the current study demonstrated that UCMS had no effect on PGI_2 levels in WKY and only slightly reduced PGI_2 levels in SHR. It is possible that the number of animals in each group did not provide enough power for this reduction to reach significance. Alternatively, Stanley et al. found that the vascular production of COX-mediated vasoconstrictor, TXA_2, was significantly greater in Balb/cJ mice after UCMS compared to control mice (65). Therefore, it is possible that UCMS has a greater effect on TXA_2 levels than on PGI_2 levels and that, if measured in the current study, a reduction in TXA_2 levels may have been detected in the UCMS groups compared to control groups.

6.1 Limitations

This thesis aimed to investigate the effects of UCMS on endothelial function in both hypertensive and normotensive animal models. The WKY and SHR rat strains were desirable to use for the current study because SHR have become the preferred animal model for studying hypertension (100) and WKY rats are often used as the normotensive control strain to which findings in SHR rats are compared against (73). As such, a significant amount of data already exists regarding the differences in endothelial function between the strains, and the related mechanisms through which these differences evolve (31, 39, 74, 75, 76). Furthermore, the effects of UCMS in SHR and WKY had not yet been investigated, which added to the desire to study

these strains in the current study. Therefore, even though it was known that WKY have been used in previous literature as an animal model of depression and have been shown to be hyper-reactive to stress compared to SHR (93, 94, 95), SHR and WKY were the animal models chosen for this study.

Another potential limitation to this study is how unstandardized the UCMS protocol remains, and as a result how much the protocol varies between investigators. Four important criteria should be considered when investigating the effects of chronic mental stress on physiological outcomes, as each one could potentially affect the physiological response. These criteria include (1) the duration of the intervention, (2) the time in the subject's life when the intervention is being introduced (i.e., the age of the subject), (3) the intensity of each individual stressor, as defined not only by how powerful the stressor is but also by how long the stressor lasts and how frequently the stressor occurs, and (4) the mode (i.e., type) of stressor. For example, a study by Puzserova et al. demonstrated that endothelium-dependent vasorelaxation in femoral arteries from WKY was enhanced following 8 weeks of chronic stress induced by crowded housing (i.e., 5 animals/cage instead of 4 animals/cage) (58). However, endothelium-dependent vasorelaxation was impaired following 12 weeks of crowded housing (58). These data suggest that the length (i.e., duration) of the chronic mental stress intervention can affect the physiological outcome. Additionally, a study by Fuchs et al. demonstrated that, following chronic behavioural stress, endothelium-dependent vasorelaxation was enhanced in coronary arteries from young (3 mo.) BHR, but was impaired in coronary arteries from old (18 mo.) BHR (59). These data suggest that the time during the life course at which the intervention is introduced (i.e., the age of the animals) can affect the physiological response(s) to the chronic stress. Furthermore, while no studies currently exist whereby the physiological responses to

varying intensities of stressors have been systematically investigated, it is logical to assume that the degree of physiological response will increase as the intensity of the stressor increases. Likewise, no studies currently exist whereby the mode (i.e., type) of stressors have been systematically investigated to ensure that the protocol induces the outcome of interest. For example, Zhu et al. demonstrated that while both the UCMS protocol and chronic restraint stress were sufficient to induce anxiety-like behaviours in C57BL/6 mice, only the UCMS protocol was sufficient to induce depressive-like behaviours, suggesting that choosing a specific mode of stressor is likely important in achieving a certain desired outcome (101).

The UCMS protocol is considered to be the most validated model for studying the (patho)physiological outcomes of chronic mental stress (61, 62). However, the duration of the protocol, the optimal time to introduce the protocol, and the intensity of the stressors within the protocol have not yet been standardized. Previous literature has demonstrated that these criteria can affect the physiological outcome in response to the chronic mental stress and therefore the lack of standardization of the UCMS protocol was likely a limitation within this study.

6.2 Future Directions

Chronic mental stress has been shown to be associated with CVD, and is now emerging as a CVD risk factor (45, 46, 52, 53, 54, 90, 91, 92). Currently, researchers are investigating the underlying mechanisms to explain a cause and effect relationship between chronic mental stress and CVD risk factors (65, 66, 67). However, since the UCMS protocol shows the most promise to be the most validated model for studying the effects of chronic mental stress, a logical next step is to establish a standardized protocol so that all investigators are using the same protocol and so that reliable results can be obtained and compared between studies. In order to standardize

the protocol, a study needs to be done wherein the physiological and behavioural responses to varying intensities of stressors are investigated. The intensity of the stressors within the protocol should aim to closely represent the type of stress that is trying to be imitated. For example, the mild stressors most commonly used in the UCMS protocol try to imitate the level of stress that is experienced in everyday life (61, 62).

Once the individual stressors within the UCMS protocol have been standardized, the effects of the duration of the UCMS protocol and the age of the animals can then be further investigated. This will allow investigators to develop their research questions based on the knowledge of how each of these factors can affect the results. Puzserova et al. demonstrated that chronic mental stress might improve endothelial function before leading to endothelial dysfunction (58). This was demonstrated by the fact that 8 weeks of chronic stress enhanced endothelium-dependent vasorelaxation where as 12 weeks of chronic stress impaired endothelium-dependent vasorelaxation (58). However, because measures were only taken at 8 and 12 weeks, it is unknown at what time-point maximal endothelial function (i.e., maximally enhanced endothelium-dependent vasorelaxation) and maximal endothelial dysfunction (i.e., maximally impaired endothelium-dependent vasorelaxation) may have been achieved. Therefore, a study should be performed wherein measures are taken weekly to determine the time-course of the physiological effects of chronic mental stress. It would be important for this study to include animals of various ages since Fuchs et al. demonstrated that the physiological effects of chronic mental stress may be vary depending on the age of the animals being studied (59). Therefore, it might be hypothesized that the time-course of the physiological effects of chronic mental stress may also be different in animals of different ages. For example, younger animals may adapt to the chronic mental stress earlier than older animals and may be able to compensate for longer

before endothelial dysfunction develops. Unfortunately, no studies, to date, have specifically investigated this theory.

Ideally, this research will reveal how chronic mental stress can cause endothelial dysfunction, on a mechanistic level. Additionally, since chronic mental stress is considered one of the modifiable CVD risk factors people could potentially reduce their risk for CVD by modifying their behaviour in such a way that reduces their stress levels (45, 46, 52, 53, 54). Therefore, once the underlying mechanisms explaining how chronic mental stress increases an individual's risk of CVD, biomarkers for monitoring one's stress levels may be identified and the effect of various interventions can be tested. Similarly to how monitoring one's blood glucose levels might encourage diabetics to improve their diets, as demonstrated by Ong et al., monitoring one's stress levels may also encourage chronically stressed individuals to partake in stress-relieving activities (102). Ultimately, the objective is for researchers to use a standardized chronic mental stress protocol to better understand how chronic mental stress causes endothelial dysfunction on a mechanistic level, so that prevention and treatment strategies for chronic mental stress can be developed.

6.3 Conclusion:

The data in the current study demonstrated that 6 weeks of UCMS (as defined in *4.2 Unpredictable Chronic Mild Stress*) improved endothelial function, as determined by an attenuation of endothelium-dependent vasocontraction and an augmentation of endothelium-dependent vasorelaxation, in CCA from 30-40 week old WKY and SHR. In contrast, a large amount of data from previous studies has demonstrated that chronic mental stress impairs endothelium-dependent vasorelaxation. However, a wide variety of factors have been shown to

contribute to the physiological response to chronic mental stress, including the duration, intensity, and mode of the stress as well as the age and strain of the animal model being investigated. As such, a great deal of investigation remains to standardize a chronic mental stress protocol in order to identify the underlying mechanisms by which chronic mental stress can lead to CVD.

References

1. Sandoo, A., Zanten, J., Metsios, G., Carroll, D., & Kitas, G. (2010). The Endothelium and Its Role in Regulating Vascular Tone. *The Open Cardiovascular Medicine Journal TOCMJ, 4*, 302-312.
2. Dora, K., Doyle, M., & Duling, B. (1997). Elevation of intracellular calcium in smooth muscle causes endothelial cell generation of NO in arterioles. *Proceedings of the National Academy of Sciences, 94*(12), 6529-6534.
3. Hasan, A., Paul, A., Memic, A., & Khademhosseini, A. (2015). A multilayered microfluidic blood vessel-like structure. *Biomedical Microdevices Biomed Microdevices, 17*(5).
4. Favero, G., Paganelli, C., Buffoli, B., Rodella, L., & Rezzani, R. (2014). Endothelium and Its Alterations in Cardiovascular Diseases: Life Style Intervention. *BioMed Research International, 801896*, 1-28.
5. Galley, H., & Webster, N. (2004). Physiology of the endothelium. *British Journal of Anaesthesia, 93*(1), 105-113.
6. Vanhoutte, P. (2003). Endothelial Control of Vasomotor Function. *Circulation Journal Circ J, 67*, 572-575.
7. Félétou, M., Köhler, R., & Vanhoutte, P. (2012). Nitric oxide: Orchestrator of endothelium-dependent responses. *Annals of Medicine Ann Med, 44*, 694-716.
8. Wong, M., & Vanhoutte, P. (2010). COX-mediated endothelium-dependent contractions: From the past to recent discoveries. *Acta Pharmacologica Sinica Acta Pharmacol Sin, 31*(9), 1095-1102.
9. Golbidi, S., & Laher, I. (2013). Exercise and the Aging Endothelium. *Journal of Diabetes Research, 2013*, 1-12.
10. Vanhoutte, P. (2010). Regeneration of the Endothelium in Vascular Injury. *Cardiovasc Drugs Ther Cardiovascular Drugs and Therapy, 24*(4), 299-303.
11. Bevan, J., & Laher, I. (1991). Pressure and Flow-Dependent Vascular Tone. *FASEB Journal, 5*(9), 2267-73.
12. Kerage, D., Brindley, D., & Hemmings, D. (2013). Review: Novel insights into the regulation of vascular tone by sphingosine 1-phosphate. *Placenta, 28*.
13. Félétou, M. (2011). The Endothelium, Part I: Multiple Functions of the Endothelial Cells -- Focus on Endothelium-Derived Vasoactive Mediators. *Colloquium Series on Integrated Systems Physiology: From Molecule to Function,* 1-306.
14. Doshi, S., Lewis, M., & Goodfellow, J. (2001). Improving endothelial vasomotor function. *Bmj, 323*(7309), 352-353.
15. Poredos, P., & Jezovnik, M. (2013). Testing Endothelial Function and its Clinical Relevance. *JAT Journal of Atherosclerosis and Thrombosis, 20*(1), 1-8.
16. Matsumoto, T., Goulopoulou, S., Taguchi, K., Tostes, R., & Kobayashi, T. (2015). Constrictor prostanoids and uridine adenosine tetraphosphate: Vascular mediators and therapeutic targets in hypertension and diabetes. *British Journal of Pharmacology Br J Pharmacol, 172*(16), 3980-4001.
17. Tang, E., & Vanhoutte, P. (2010). Endothelial dysfunction: A strategic target in the treatment of hypertension? *Pflügers Archiv - European Journal of Physiology Pflugers Arch - Eur J Physiol, 459*, 995-1004.

18. Feletou, M., & Vanhoutte, P. (2006). Endothelial dysfunction: A multifaceted disorder (The Wiggers Award Lecture). *AJP: Heart and Circulatory Physiology, 291*(3), H985-1002. doi:10.1152/ajpheart.00292.2006
19. Furchgott, R., & Zawadzki, J. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature, 288*(5789), 373-376.
20. Kubes, P., Suzuki, M., & Granger, D. (1991). Nitric oxide: An endogenous modulator of leukocyte adhesion. *Proceedings of the National Academy of Sciences, 88*, 4651-4655.
21. Nong, Z., Hoylaerts, M., Pelt, N., Collen, D., & Janssens, S. (1997). Nitric Oxide Inhalation Inhibits Platelet Aggregation and Platelet-Mediated Pulmonary Thrombosis in Rats. *Circulation Research, 81*, 865-869.
22. Peiro, C., Redondo, J., Rodriguez-Martinez, M., Angulo, J., Marin, J., & Sanchez-Ferrer, C. (1995). Influence of Endothelium on Cultured Vascular Smooth Muscle Cell Proliferation. *Hypertension, 25*(4 Pt 2), 748-751.
23. Corsini, A., Folco, G., Fumagalli, R., Nicosia, S., Noe, M., & Oliva, D. (1987). (5Z)-carbacyclin discriminates between prostacyclin-receptors coupled to adenylate cyclase in vascular smooth muscle and platelets. *British Journal of Pharmacology, 90*(1), 255-261.
24. Chow, K., Jones, R., & Wise, H. (2003). Protein kinase A-dependent coupling of mouse prostacyclin receptors to Gi is cell-type dependent. *European Journal of Pharmacology, 474*, 7-13.
25. Fitzgerald, G. (1991). Mechanisms of platelet activation: Thromboxane A2 as an amplifying signal for other agonists. *The American Journal of Cardiology, 68*, 11B-5B.
26. Davies PF. (1995) Flow-mediated endothelial mechanotransduction. *Physiol Rev, 75,* 519-60.
27. Miller VM, Vanhoutte PM (1985). Endothelium-dependent contractions to arachidonic acid is mediated by products of cyclooxygenase. Am J Physiol 248: H432–H437.
28. Katusic ZS, Shepherd JT, Vanhoutte PM (1987). Endothelium-dependent contraction to stretch in canine basilar arteries. Am J Physiol 252: H671–H673.
29. Yang D, Gluais P, Zhang JN, Vanhoutte PM, Félétou M (2004). Endothelium-dependent contractions to acetylcholine, ATP and the calcium ionophore A 23187 in aortas from spontaneously hypertensive and normotensive rats. Fundam Clin Pharmacol 18: 321–326.
30. Félétou, M., Huang, Y., & Vanhoutte, P. (2011). Endothelium-mediated control of vascular tone: COX-1 and COX-2 products. *British Journal of Pharmacology, 164*, 894-912.
31. Gluais, P., Lonchampt, M., Morrow, J., Vanhoutte, P., & Feletou, M. (2005). Acetylcholine-induced endothelium-dependent contractions in the SHR aorta: The Janus face of prostacyclin. *British Journal of Pharmacology, 146*(6), 834-845.
32. Gomez, E., Schwendemann, C., Roger, S., Simonet, S., Paysant, J., Courchay, C., . . . Félétou, M. (2008). Aging and prostacyclin responses in aorta and platelets from WKY and SHR rats. *American Journal of Physiology - Heart and Circulatory Physiology Am J Physiol Heart Circ Physiol, 295*(5), H2198-211.
33. Félétou, M., Verbeuren, T., & Vanhoutte, P. (2009). Endothelium-dependent contractions in SHR: A tale of prostanoid TP and IP receptors. *British Journal of Pharmacology, 156*(4), 563-574.
34. Dagassan, P., Breu, V., Clozel, M., Künzli, A., Vogt, P., Turina, M., . . . Clozel, J. (1996). Up-Regulation of Endothelin-B Receptors in Atherosclerotic Human Coronary Arteries. *Journal of Cardiovascular Pharmacology, 27*, 147-153.

35. Bacon, C., Cary, N., & Davenport, A. (1995). Distribution of Endothelin Receptors in Atherosclerotic Human Coronary Arteries. *Journal of Cardiovascular Pharmacology, 26*(Suppl 3), S439-S441.
36. Drexler, H., & Hornig, B. (1999). Endothelial Dysfunction in Human Disease. *Journal of Molecular and Cellular Cardiology, 31*(1), 51-60.
37. Cohen, M., & Berkowitz, B. (1976). Decreased Vascular Relaxation in Hypertension. *The Journal of Pharmacology and Experimental Therapeutics, 196*(2), 396-406.
38. Hongo, K., Nakagomi, T., Kassell, N., Sasaki, T., Lehman, M., Vollmer, D., . . . Torner, J. (1988). Effects of aging and hypertension on endothelium-dependent vascular relaxation in rat carotid artery. *Stroke, 19*(7), 892-897.
39. Denniss, S., & Rush, J. (2009). Impaired hemodynamics and endothelial vasomotor function via endoperoxide-mediated vasoconstriction in the carotid artery of spontaneously hypertensive rats. *AJP: Heart and Circulatory Physiology, 296*, H1038-H1047.
40. Cox, D., Vita, J., Treasure, C., Fish, R., Alexander, R., Ganz, P., & Selwyn, A. (1989). Atherosclerosis impairs flow-mediated dilation of coronary arteries in humans. *Circulation, 80*(3), 458-465.
41. Versari, D., Daghini, E., Virdis, A., Ghiadoni, L., & Taddei, S. (2009). The ageing endothelium, cardiovascular risk and disease in man. *Experimental Physiology, 94*(3), 317-321. doi:10.1113/expphysiol.2008.043356
42. Lerman, A., & Zeiher, A. (2005). Endothelial Function: Cardiac Events. *Circulation, 111*(3), 363-368. doi:10.1161/01.cir.0000153339.27064.14
43. Spiers, A., & Padmanabhan, N. (2005). A Guide to Wire Myography. *Hypertension, 108*, 091-104. doi:10.1385/1-59259-850-1:091
44. History of the Framingham Heart Study. (2015). Retrieved September 11, 2015, from https://www.framinghamheartstudy.org/about-fhs/history.php
45. Rozanski, A., Blumenthal, J., & Kaplan, J. (1999). Impact of Psychological Factors on the Pathogenesis of Cardiovascular Disease and Implications for Therapy. *Circulation, 99*(16), 2192-2217.
46. Lombard, J. (2010). Depression, psychological stress, vascular dysfunction, and cardiovascular disease: Thinking outside the barrel. *Journal of Applied Physiology, 108*, 1025-1026.
47. Sivasankaran, S., Pollard-Quintner, S., Sachdeva, R., Pugeda, J., Hoq, S., & Zarich, S. (2006). The effect of a six-week program of yoga and meditation on brachial artery reactivity: Do psychosocial interventions affect vascular tone? *Clin Cardiol Clinical Cardiology, 29*(9), 393-398.
48. Ghiadoni, L., Donald, A., Cropley, M., Mullen, M., Oakley, G., Taylor, M., . . . Deanfield, J. (2000). Mental Stress Induces Transient Endothelial Dysfunction in Humans. *Circulation, 102*(20), 2473-2478.
49. Pecoraro, N., Reyes, F., Gomez, F., Bhargava, A., & Dallman, M. (2004). Chronic Stress Promotes Palatable Feeding, which Reduces Signs of Stress: Feedforward and Feedback Effects of Chronic Stress. *Endocrinology, 145*(8), 3754-3762.
50. Zellner, D., Loaiza, S., Gonzalez, Z., Pita, J., Morales, J., Pecora, D., & Wolf, A. (2006). Food selection changes under stress. *Physiology & Behavior, 87*, 789-793.

51. Sarabi, M., & Lind, L. (2001). Mental stress opposes endothelium-dependent vasodilation in young healthy individuals. *Vascular Medicine, 6*(1), 3-7. doi:10.1177/1358836x0100600102
52. Black, P. (2006). The inflammatory consequences of psychologic stress: Relationship to insulin resistance, obesity, atherosclerosis and diabetes mellitus, type II. *Medical Hypotheses, 67*, 879-891.
53. Yarnell, J. (2008). Stress at work--an independent risk factor for coronary heart disease? *European Heart Journal, 29*, 579-580.
54. Divincenzo, L., Reber, M., Perera, V., & Chilian, W. (2014). Connecting the dots--Establishing causality between chronic stress, depression, and cardiovascular disease. *Journal of Applied Physiology, 117*, 957-958.
55. Smith, C. (2012). Using Rodent Models to Simulate Stress of Physiologically Relevant Severity: When, Why and How. *Glucocorticoids - New Recognition of Our Familiar Friend,* 211-230.
56. Golbidi, S., Frisbee, J., & Laher, I. (2015). Chronic stress impacts the cardiovascular system: Animal models and clinical outcomes. *Am J Physiol Heart Circ Physiol American Journal of Physiology - Heart and Circulatory Physiology, 308*(12), H1476-98.
57. Bernatova, I., & Csizmadiova, Z. (2006). Effect of chronic social stress on nitric oxide synthesis and vascular function in rats with family history of hypertension. Life Sciences, 78, 1726-1732.
58. U., Puzserova, A., Slezak, P., Balis, P., & Bernatova, I. (2013). Long-term social stress induces nitric oxide-independent endothelial dysfnction in normotensive rats. *Stress, 16*(3), 331-339.
59. Fuchs, L., Landas, S., & Johnson, A. (1997). Behavioural stress alters coronary vascular reactivity in borderline hypertensive rats. Journal of Hypertension, 301-307.
60. Giulumian, A., Clark, S., & Fuchs, L. (1999). Effect of behavioral stress on coronary artery relaxation altered with aging in BHR. *Am J Physiol Heart Circ Physiol American Journal of Physiology - Heart and Circulatory Physiology, 276*, R435-40.
61. Willner, P., Muscat, R., & Papp, M. (1992). Chronic mild stress-induced anhedonia: A realistic animal model of depression. *Neuroscience & Biobehavioral Reviews, 16*, 525-534.
62. Willner, P. (1997). Validity, reliability and utility of the chronic mild stress model of depression: A 10-year review and evaluation. *Psychopharmacology, 134*, 319-329.
63. Grippo AJ, Beltz TG, Weiss RM, Johnson AK. The effects of chronic fluoxetine treatment on chronic mild stress-induced cardiovascular changes and anhedonia. Biol Psychiatry 2006; 59: 309-16.
64. Grippo AJ, Moffitt JA, Johnson AK. Cardiovascular alterations and autonomic imbalance in an experimental model of depression. Am J Physiol Regul Integr Comp Physiol 2002; 282: R1333-41.
65. Stanley SC, Brooks SD, Butcher JT, d'Audiffret AC, Frisbee SJ, Frisbee JC. Protective effect of sex on chronic stress and depressive behavior-induced vascular dysfunction in BALB/cJ mice. J Appl Physiol (1985) 2014; pii: jap.00537.2014.
66. Isingrini, E., Belzung, C., D'Audiffret, A., & Camus, V. (2011). Early and late-onset effect of chronic stress on vascular function in mice: A possible model of the Impact of depression on vascular disease in aging. American Journal of Geriatric Psychiatry, 19(4).
67. d'Audiffret, A., Frisbee, S., Stapleton, P., Goodwill, A., Isingrini, E., & Frisbee, J. (2010).

Depressive behavior and vascular dysfunction: A link between clinical depression and vascular disease? Journal of Applied Physiology, 108, 1041-1051.
68. Bayramgurler, D., Karson, A., Yazir, Y., Celikyurt, I., Kurnaz, S., & Utkan, T. (2013). The effect of etanercept on aortic nitric oxide-dependent vasorelaxation in an unpredictable chronic, mild stress model of depression in rats. European Journal of Pharmacology, 710, 67-72.
69. Demirtaş T, Utkan T, Karson A, Yazır Y, Bayramgürler D, Gacar N. The link between unpredictable chronic mild stress model for depression and vascular inflammation? Inflammation 2014; 37: 1432-8. doi: 10.1007/s10753-014-9867-4.
70. Isingrini E, Surget A, Belzung C, Freslon JL, Frisbee J, O'Donnell J, Camus V, d'Audiffret A. Altered aortic vascular reactivity in the unpredictable chronic mild stress model of depression in mice: UCMS causes relaxation impairment to ACh. Physiol Behav 2011; 103: 540-6. doi: 10.1016/j.physbeh.2011.04.002.
71. Isingrini E, Belzung C, Freslon JL, Machet MC, Camus V. Fluoxetine effect on aortic nitric oxide- dependent vasorelaxation in the unpredictable chronic mild stress model of depression in mice. Psychosom Med 2012; 74: 63-72.Doi; 10.1097/ PSY.0b013 e31823 a43e0.
72. Okamoto, K., & Aoki, K. (1963). Development of a Strain of Spontaneously Hypertensive Rats. *Jpn Circ J Japanese Circulation Journal, 27*, 282-293.
73. Louis, W., & Howes, L. (1990). Genealogy of the Spontaneously Hypertensive Rat and Wistar-Kyoto Rat Strains. *Journal of Cardiovascular Pharmacology, 16*, S1-5.
74. Denniss, S., Jeffery, A., & Rush, J. (2010). RhoA-Rho kinase signaling mediates endothelium- and endoperoxide-dependent contractile activities characteristic of hypertensive vascular dysfunction. *AJP: Heart and Circulatory Physiology, 298*, H1391-H1405.
75. Luscher, T. F., & Vanhoutte, P. M. (1986). Endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. *Hypertension, 8*(4), 344-348. doi:10.1161/01.hyp.8.4.344
76. Imaoka, Y., Osanai, T., Kamada, T., Mio, Y., Satoh, K., & Okumura, K. (1999). Nitric Oxide-Dependent Vasodilator Mechanism is Not Impaired by Hypertension But is Diminished with Aging in the Rat Aorta. *Journal of Cardiovascular Pharmacology, 33*(5), 756-761. doi:10.1097/00005344-199905000-00012
77. Mey, J. G., & Vanhoutte, P. M. (1982). Heterogeneous behavior of the canine arterial and venous wall. Importance of the endothelium. *Circulation Research, 51*(4), 439-447. doi:10.1161/01.res.51.4.439
78. Katusic, Z. S., Shepherd, J. T., & Vanhoutte, P. M. (1988). Endothelium-dependent contractions to calcium ionophore A23187, arachidonic acid, and acetylcholine in canine basilar arteries. *Stroke, 19*(4), 476-479. doi:10.1161/01.str.19.4.476
79. Yang, D., Feletou, M., Levens, N., Zhang, J. N., & Vanhoutte, P. M. (2002). A Diffusible Substance(s) Mediates Endothelium-Dependent Contractions in the Aorta of SHR. *Hypertension, 41*(1), 143-148. doi:10.1161/01.hyp.0000047651.45322.16
80. Marcondes, M., Zhukov, V., Bradlow, H., Sanchez-Alavez, M., Gonzalez, A., Curtiss, L., & Conti, B. (2011). Effects of chronic mental stress and atherogenic diet on the immune inflammatory environment in mouse aorta. *Brain, Behavior, and Immunity, 25*, 1649-1657.
81. Barr, A., & Phillips, A. (1998). Chronic mild stress has no effect on responding by rats

for sucrose under a progressive ratio schedule. *Physiology & Behavior, 64*(5), 591-597.
82. Willner, P., Towell, A., Sampson, D., Sophokleous, S., & Muscat, R. (1987). Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. *Psychopharmacology, 93*(3), 358-64.
83. Griebel, G., Simiand, J., Steinberg, R., Jung, M., Gully, D., Roger, P., . . . Soubrie, P. (2002). 4-(2-Chloro-4-methoxy-5-methylphenyl)-N-[(1S)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl)ethyl]5-methyl-N-(2-propynyl)-1, 3-thiazol-2-amine Hydrochloride (SSR125543A), a Potent and Selective Corticotrophin-Releasing Factor1 Receptor Antagonist. II. Characte. *Journal of Pharmacology and Experimental Therapeutics, 301*(1), 333-345.
84. Denniss, S. G., Ford, R. J., Smith, C. S., Jeffery, A. J., & Rush, J. W. (2016). Chronic in vivo or acute in vitro resveratrol attenuates endothelium-dependent cyclooxygenase-mediated contractile signaling in hypertensive rat carotid artery. *Journal of Applied Physiology J Appl Physiol, 120*(10), 1141-1150. doi:10.1152/japplphysiol.00675.2015
85. Kraly, F., Coogan, L., Specht, S., Trattner, M., Zayfert, C., Cohen, A., & Goldstein, J. (1985, April). Disordered drinking in developing spontaneously hypertensive rats. *American Journal of Physiology, 248*, R464-70.
86. Graham, D. A., & Rush, J. W. (2004). Exercise training improves aortic endothelium-dependent vasorelaxation and determinants of nitric oxide bioavailability in spontaneously hypertensive rats. *Journal of Applied Physiology, 96*(6), 2088-2096. doi:10.1152/japplphysiol.01252.2003
87. Dowell FJ, Martin W, Dominiczak AF, Hamilton CA. Decreased basal despite enhanced agonist-stimulated effects of nitric oxide in 12-week-old stroke-prone spontaneously hypertensive rat. *Eur J Pharmacol* 379: 175– 182, 1999.
88. Tang, E. H., Feletou, M., Huang, Y., Man, R., & Vanhoutte, P. (2005). Acetylcholine and sodium nitroprusside cause long-term inhibition of EDCF-mediated contractions. *AJP: Heart and Circulatory Physiology, 289*(6), H2434-H2440. doi:10.1152/ajpheart.00568.2005
89. Auch-Schwelk, W., Katusic, Z. S., & Vanhoutte, P. M. (1992). Nitric oxide inactivates endothelium-derived contracting factor in the rat aorta. *Hypertension, 19*(5), 442-445. doi:10.1161/01.hyp.19.5.442
90. Carroll, D., Phillips, A. C., Gale, C. R., & Batty, G. D. (2010). Generalized Anxiety and Major Depressive Disorders, Their Comorbidity and Hypertension in Middle-Aged Men. *Psychosomatic Medicine, 72*(1), 16-19. doi:10.1097/psy.0b013e3181c4fca1
91. Patten, S. B., Williams, J. V., Lavorato, D. H., Campbell, N. R., Eliasziw, M., & Campbell, T. S. (2009). Major Depression as a Risk Factor for High Blood Pressure: Epidemiologic Evidence From a National Longitudinal Study. *Psychosomatic Medicine, 71*(3), 273-279. doi:10.1097/psy.0b013e3181988e5f
92. Ojike, N., Sowers, J. R., Seixas, A., Ravenell, J., Rodriguez-Figueroa, G., Awadallah, M., . . . Mcfarlane, S. I. (2016). Psychological Distress and Hypertension: Results from the National Health Interview Survey for 2004-2013. *Cardiorenal Medicine, 6*(3), 198-208. doi:10.1159/000443933
93. Nam, H., Clinton, S. M., Jackson, N. L., & Kerman, I. A. (2014). Learned helplessness and social avoidance in the Wistar-Kyoto rat. *Front. Behav. Neurosci. Frontiers in Behavioral Neuroscience, 8*(109), 1-18. doi:10.3389/fnbeh.2014.00109

94. Will, C. C., Aird, F., & Redei, E. E. (2003). Selectively bred Wistar–Kyoto rats: An animal model of depression and hyper-responsiveness to antidepressants. *Molecular Psychiatry Mol Psychiatry, 8*(11), 925-932. doi:10.1038/sj.mp.4001345
95. Paré, W. P. (1989). Stress ulcer susceptibility and depression in Wistar Kyoto (WKY) rats. *Physiology & Behavior, 46*(6), 993-998. doi:10.1016/0031-9384(89)90203-5
96. Pfeffer, M., & Frohlich, E. (1973, May). Hemodynamic and myocardial function in young and old normotensive and spontaneously hypertensive rats. *Circulation Research, 32*, S28-38
97. Pfeffer, M., & Frohlich, E. (1973, May). Hemodynamics of spontaneously hypertensive rats. I. Effects of pressure elevation. *American Journal of Physiology, 224*(5), 1066-1071.
98. Owusu, I. (2007). Electrocardiographic Left Ventricular Hypertrophy In Patients Seen With Hypertensive Heart Failure. *The Internet Journal of Third World Medicine, 6*(1). doi:10.5580/28b3
99. Cutilletta, A. F., Erinoff, L., Heller, A., Low, J., & Oparil, S. (1977, April). Development of left ventricular hypertrophy in young spontaneously hypertensive rats after peripheral sympathectomy. *Circulation Research, 40*(4), 428-434.
100. Trippodo, N. C., & Frohlich, E. D. (1981). Similarities of genetic (spontaneous) hypertension. Man and rat. *Circulation Research, 48*(3), 309-319. doi:10.1161/01.res.48.3.309
101. Zhu, S., Shi, R., Wang, J., Wang, J., & Li, X. (2014). Unpredictable chronic mild stress not chronic restraint stress induces depressive behaviours in mice. *NeuroReport, 25*(14), 1151-1155. doi:10.1097/wnr.0000000000000243
102. Chua, S., Ong, W. M., & Ng, C. J. (2014). Barriers and facilitators to self-monitoring of blood glucose in people with type 2 diabetes using insulin: A qualitative study. *Patient Preference and Adherence, 8*, 237-246. doi:10.2147/ppa.s57567

Appendix I: UCMS Protocol Schedule

Protocol Day	Time	Stressor						Animals	
		Damp bedding	Altered light/dark cycle	Cage tilt	Predator odour	Predator sounds	Removal of bedding	SHR	WKY
1	7AM-10AM						✓	1,2	
	10AM-2PM								
	2PM-5PM								
	5PM-9PM		✓						
	9PM-7AM			✓					
2	7AM-10AM				✓			1,2	1,2
	10AM-2PM						✓		
	2PM-5PM								
	5PM-9PM	✓							
	9PM-7AM								
3	7AM-10AM							1,2,3,4	1,2
	10AM-2PM				✓				
	2PM-5PM						✓		
	5PM-9PM	✓							
	9PM-7AM								
4	7AM-10AM							1,2,3,4	1,2
	10AM-2PM		✓						
	2PM-5PM								
	5PM-9PM	✓							
	9PM-7AM						✓		
5	7AM-10AM					✓		1,2,3,4	1,2
	10AM-2PM		✓						
	2PM-5PM								
	5PM-9PM								
	9PM-7AM				✓				
6	7AM-10AM						✓	1,2,3,4	1,2,3,4
	10AM-2PM		✓						
	2PM-5PM								
	5PM-9PM				✓				
	9PM-7AM								
7	7AM-10AM					✓		1,2,3,4,5,6	1,2,3,4
	10AM-2PM								
	2PM-5PM								
	5PM-9PM		✓						
	9PM-7AM	✓							
8	7AM-10AM							1,2,3,4,5,6	1,2,3,4,5,6
	10AM-2PM								
	2PM-5PM		✓						
	5PM-9PM					✓			
	9PM-7AM			✓					
9	7AM-10AM							1,2,3,4,5,6,	1,2,3,4,5,6

	10AM-2PM					✓		7,8	
	2PM-5PM	✓							
	5PM-9PM			✓					
	9PM-7AM								
10	7AM-10AM							1,2,3,4,5,6, 7,8	1,2,3,4,5,6, 7,8
	10AM-2PM						✓		
	2PM-5PM			✓					
	5PM-9PM					✓			
	9PM-7AM								
11	7AM-10AM				✓			1,2,3,4,5,6, 7,8	1,2,3,4,5,6, 7,8
	10AM-2PM					✓			
	2PM-5PM		✓						
	5PM-9PM								
	9PM-7AM								
12	7AM-10AM				✓			1,2,3,4,5,6, 7,8	1,2,3,4,5,6, 7,8
	10AM-2PM					✓			
	2PM-5PM								
	5PM-9PM								
	9PM-7AM		✓						
13	7AM-10AM	✓						1,2,3,4,5,6, 7,8,9,10	1,2,3,4,5,6, 7,8
	10AM-2PM								
	2PM-5PM					✓			
	5PM-9PM								
	9PM-7AM				✓				
14	7AM-10AM							1,2,3,4,5,6, 7,8,9,10	1,2,3,4,5,6, 7,8,9,10
	10AM-2PM					✓			
	2PM-5PM				✓				
	5PM-9PM								
	9PM-7AM						✓		
15	7AM-10AM		✓					1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10
	10AM-2PM						✓		
	2PM-5PM								
	5PM-9PM	✓							
	9PM-7AM								
16	7AM-10AM		✓					1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM								
	2PM-5PM								
	5PM-9PM			✓					
	9PM-7AM	✓							
17	7AM-10AM							1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM					✓			
	2PM-5PM		✓						
	5PM-9PM								
	9PM-7AM						✓		
18	7AM-10AM							1,2,3,4,5,6,	1,2,3,4,5,6,

	10AM-2PM					✓		7,8,9,10, 11,12	7,8,9,10, 11,12
	2PM-5PM			✓					
	5PM-9PM								
	9PM-7AM						✓		
19	7AM-10AM	✓						1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM						✓		
	2PM-5PM								
	5PM-9PM		✓						
	9PM-7AM								
20	7AM-10AM							1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM						✓		
	2PM-5PM		✓						
	5PM-9PM								
	9PM-7AM				✓				
21	7AM-10AM							1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM					✓			
	2PM-5PM								
	5PM-9PM						✓		
	9PM-7AM			✓					
22	7AM-10AM					✓		1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM			✓					
	2PM-5PM								
	5PM-9PM								
	9PM-7AM						✓		
23	7AM-10AM			✓				1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM					✓			
	2PM-5PM								
	5PM-9PM	✓							
	9PM-7AM								
24	7AM-10AM							1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM			✓					
	2PM-5PM	✓							
	5PM-9PM								
	9PM-7AM				✓				
25	7AM-10AM							1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM			✓					
	2PM-5PM				✓				
	5PM-9PM								
	9PM-7AM					✓			
26	7AM-10AM							1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM				✓				
	2PM-5PM								
	5PM-9PM	✓							
	9PM-7AM		✓						
27	7AM-10AM							1,2,3,4,5,6,	1,2,3,4,5,6,

	10AM-2PM			✓				7,8,9,10, 11,12	7,8,9,10, 11,12
	2PM-5PM				✓				
	5PM-9PM								
	9PM-7AM					✓			
28	7AM-10AM			✓				1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM					✓			
	2PM-5PM								
	5PM-9PM				✓				
	9PM-7AM								
29	7AM-10AM		✓					1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM								
	2PM-5PM				✓				
	5PM-9PM	✓							
	9PM-7AM								
30	7AM-10AM				✓			1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM								
	2PM-5PM			✓					
	5PM-9PM	✓							
	9PM-7AM								
31	7AM-10AM			✓				1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM						✓		
	2PM-5PM								
	5PM-9PM								
	9PM-7AM				✓				
32	7AM-10AM	✓						1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM		✓						
	2PM-5PM								
	5PM-9PM								
	9PM-7AM						✓		
33	7AM-10AM					✓		1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM								
	2PM-5PM	✓							
	5PM-9PM								
	9PM-7AM				✓				
34	7AM-10AM							1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM			✓					
	2PM-5PM					✓			
	5PM-9PM								
	9PM-7AM				✓				
35	7AM-10AM							1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM	✓							
	2PM-5PM								
	5PM-9PM					✓			
	9PM-7AM			✓					
36	7AM-10AM		✓					1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM			✓					

	2PM-5PM								
	5PM-9PM								
	9PM-7AM	✓							
37	7AM-10AM							1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM			✓					
	2PM-5PM					✓			
	5PM-9PM						✓		
	9PM-7AM								
38	7AM-10AM				✓			1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM						✓		
	2PM-5PM								
	5PM-9PM	✓							
	9PM-7AM								
39	7AM-10AM		✓					1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM				✓				
	2PM-5PM								
	5PM-9PM								
	9PM-7AM			✓					
40	7AM-10AM				✓			1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM								
	2PM-5PM		✓						
	5PM-9PM								
	9PM-7AM			✓					
41	**7AM-10AM**						✓	**1,2,3,4,5,6, 7,8,9,10, 11,12**	**1,2,3,4,5,6, 7,8,9,10, 11,12**
	10AM-2PM								
	2PM-5PM				✓				
	5PM-9PM								
	9PM-7AM		✓						
42	7AM-10AM						✓	1,2,3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM			✓					
	2PM-5PM								
	5PM-9PM								
	9PM-7AM					✓			
43	7AM-10AM						✓	3,4,5,6, 7,8,9,10, 11,12	1,2,3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM	✓							
	2PM-5PM			✓					
	5PM-9PM								
	9PM-7AM								
44	7AM-10AM							3,4,5,6, 7,8,9,10, 11,12	3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM					✓			
	2PM-5PM	✓							
	5PM-9PM		✓						
	9PM-7AM								
45	7AM-10AM							5,6, 7,8,9,10, 11,12	3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM	✓							
	2PM-5PM				✓				

	5PM-9PM								
	9PM-7AM					✓			
46	7AM-10AM							5,6, 7,8,9,10, 11,12	3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM			✓					
	2PM-5PM						✓		
	5PM-9PM				✓				
	9PM-7AM								
47	7AM-10AM							5,6, 7,8,9,10, 11,12	3,4,5,6, 7,8,9,10, 11,12
	10AM-2PM								
	2PM-5PM						✓		
	5PM-9PM	✓							
	9PM-7AM					✓			
48	7AM-10AM	✓						5,6, 7,8,9,10, 11,12	5,6, 7,8,9,10, 11,12
	10AM-2PM				✓				
	2PM-5PM								
	5PM-9PM		✓						
	9PM-7AM								
49	7AM-10AM							7,8,9,10, 11,12	5,6, 7,8,9,10, 11,12
	10AM-2PM				✓				
	2PM-5PM						✓		
	5PM-9PM		✓						
	9PM-7AM								
50	7AM-10AM				✓			7,8,9,10, 11,12	7,8,9,10, 11,12
	10AM-2PM					✓			
	2PM-5PM		✓						
	5PM-9PM								
	9PM-7AM								
51	7AM-10AM					✓		9,10,11,12	7,8,9,10, 11,12
	10AM-2PM		✓						
	2PM-5PM	✓							
	5PM-9PM								
	9PM-7AM								
52	7AM-10AM				✓			9,10,11,12	9,10,11,12
	10AM-2PM								
	2PM-5PM								
	5PM-9PM			✓					
	9PM-7AM						✓		
53	7AM-10AM			✓				9,10,11,12	9,10,11,12
	10AM-2PM						✓		
	2PM-5PM								
	5PM-9PM		✓						
	9PM-7AM								
54	7AM-10AM			✓				9,10,11,12	9,10,11,12
	10AM-2PM								
	2PM-5PM		✓						
	5PM-9PM						✓		

	9PM-7AM								
55	7AM-10AM		✓					11,12	9,10,11,12
	10AM-2PM								
	2PM-5PM								
	5PM-9PM				✓				
	9PM-7AM					✓			
56	7AM-10AM							11,12	11,12
	10AM-2PM								
	2PM-5PM				✓				
	5PM-9PM					✓			
	9PM-7AM	✓							
57	7AM-10AM					✓			11,12
	10AM-2PM						✓		
	2PM-5PM			✓					
	5PM-9PM								
	9PM-7AM								