Endothelium-Dependant Vasomotor Function in Spontaneously Hypertensive Rats Following Chronic Dietary Treatment with Resveratrol

by

Christopher S. Smith

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ABSTRACT

Essential hypertension is a disease involving impaired endothelium-dependant vasomotor function which is partially mediated through an increase in reactive oxygen species. Recent evidence has demonstrated that resveratrol (RSV), a polyphenol with antioxidant capabilities, alleviates this impaired endothelium-dependant vasomotor function and can provide cardiovascular health benefits. The aim of this study was to examine the effects of chronic resveratrol treatment for 28 days on the endothelium-dependant vasomotor function and hemodynamic measures of the common carotid artery (CCA) of Spontaneously Hypertensive rat (SHR) aged 20-22 weeks at a high (2.7 mg per day, equivalent to a 500mg dose in humans) and a low (0.027mg per day, equivalent to moderate red wine consumption in humans) dose. The 20-22 week old SHR (n=9) demonstrated an elevated mean arterial blood pressure compared to the normotensive control, Wistar Kyoto rats (WKY) (n=9) ($p<0.001$). The SHR also demonstrated decreased endothelium-dependant vasorelaxation and increased endothelium-dependant contraction, indicating impaired endothelium-dependant vasomotor function. Following chronic treatment with resveratrol for 28 days at a high dose maximal relaxation to acetylcholine (ACh) of phenylephrine (PE) pre-contracted CCA vessels was increased when compared to SHR CON (High 100.4 $\pm$ 5.2%, CON 53.6 $\pm$ 6%) ($p<0.001$). This difference is possibly mediated by improved nitric oxide (NO) bioavailability. This study also confirmed that SHR demonstrate increased endothelium-dependant contractions in quiescent, non pre-contracted rings of the CCA ($P<0.001$). High dose resveratrol treatment reduced SHR endothelium-dependant contraction in the CCA compared to SHR CON (High 55 $\pm$ 5.4%, CON 68 $\pm$ 5%) ($p<0.05$). This effect was likely mediated by an observed reduction in prostacyclin (PGI_2) production, when compared to the SHR CON

($p<0.05$). This result is likely to be cause by inhibition of cycloxygenase 1 (COX 1) since reversal of SHR endothelium-dependant contraction is demonstrated following inhibition of COX 1, an interpretation supported by the observation that resveratrol treatment had no effect on the sensitivity of the thromboxane-prostaglandin receptor, and no effect on the protein expression of COX 1. This study indicates that chronic treatment with resveratrol at a high dose improves CCA endothelium- dependent vasomotor function in SHR via improved NO bioavailability and a reduction in endothelium-dependant PGI2-mediated contraction. These improvements in vasomotor function produced an alteration in vascular tone to a less contracted state. If these observations extend to the resistance vasculature they could contribute to the explanation for resveratrol-dependant reduction in mean arterial blood pressure.

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-ONOO	*Peroxynitrite*
AA	*Arachidonic acid*
AICAR	*5-aninoimidazole-4-carboxamide-1-β-Diribofuranoside*
AMPK	*5' adenosine monophosphate-activated protein kinase*
AUC	*Area under the curve*
ACh	*Acetylcholine*
BCA	*bicinchoninic acid*
CCA	*Common carotid artery*
cGMP	*Cyclic guanosine monophosphate*
COX 1	*Cycloxygenase 1*
COX 2	*Cycloxygenase 2*
EDCF	*Endothelial derived contraction factor*
eNOS	*Endothelial nitric oxide synthase*
EDRF	*Endothelial derived relaxation factor*
HDL	*High density lipoprotein*
H_2O_2	*Hydrogen peroxide*
INDO	*Indomethacin*
IP	*Prostacyclin receptor*
KCl	*Potassium chloride*
L-NMMA	*L-NG-Nitroarginine methyl ester*
LDL	*Low density lipoprotein*
MR	*Maximal response*
NADPH oxidase	*Nicotinamide adenine dinucleotide phosphate-oxidase*
NO	*Nitric oxide*

NOS	*Nitric oxide synthase*
PE	*Phenylephrine*
PGI_2	*Prostacyclin*
PKG	*Protein kinase G*
PLA2	*Phospholipase A2*
ROS	*Reactive oxygen species*
RSV	*Resveratrol*
sGC	*Soluble guanylyl cyclase*
SHR	*Spontaneously hypertensive rats*
SNP	*Sodium nitroprusside*
SOD	*Superoxide dismutase*
TA	*Thoracic aorta*
TP	*Thromboxane-prostaglandin receptor*
TXA_2	*Thromboxaine A2*
VSM	*Vascular smooth muscle*
WKY	*Wistar Kyoto Rats*

Resveratrol is a chemical compound with numerous cardiovascular health benefits. Previous studies conducted by this laboratory have indicated that resveratrol may alleviate vasomotor dysfunction often associated with hypertension. This study will examine the effects of chronic dietary resveratrol treatment on aspects of endothelial dysfunction not previously examined; namely its effect on endothelium-dependant contractions. A brief introduction to hypertension and its prevalence in society will be outlined with a focus on hypertension's association with endothelial dysfunction. Two key regulators of endothelial dysfunction, nitric oxide (NO) and prostacyclin (PGI_2) will be introduced and the changes occurring to these chemicals during the progression of endothelial dysfunction will be characterized. The cardiovascular effects of resveratrol will be introduced in more detail, outlining the rationale for the positive effects seen in previous studies and identifying the novel effects that will be illuminated in the present study. This will be followed by the experimental methods used to examine changes in the vascular function of both Spontaneously Hypertensive Rats (SHR) and Wistar Kyoto Rats (WKY), the animal models used in this study. The methods will be followed by the results of the study, and finally the main findings of the study will be discussed and who they relate to the original hypothesis.

Introduction:

Hypertension is defined as chronically elevated blood pressure consisting of a systolic pressure in excess of 140 mmHg and a diastolic pressure in excess of 90 mmHg (1,2). This disorder is associated with a multitude of diseases including coronary artery disease, obesity and diabetes, all of which have an increasing prevalence in Canadian society. One in every five Canadians suffers from some form of hypertension and this number is expected to

increase in the coming years (3). For this reason, it is important to develop an understanding of the pathophysiological changes in the heart and blood vessels associated with hypertension.

The endothelium, a single layer of endothelial cells located on the luminal side of the blood vessel wall, plays an important role in control of vascular tone, which can be altered in hypertension. Under normal conditions, the endothelium can be stimulated by a large number of physical and biological factors to elicit either dilation or constriction of blood vessels. However, in diseased states, the endothelial control of vascular tone becomes compromised, resulting in endothelial dysfunction (2,4,5). This dysfunction is known to result from a number of contributing factors including; vascular injury, altered molecular signaling and an increase in reactive oxygen species (6-8).These factors result in impaired endothelium-mediated dilation and an increase in endothelium-dependant contractions, which are hallmarks of endothelial dysfunction (9-12). Endothelial dysfunction is often correlated with an increase in blood pressure seen in hypertensive patients (13). To fully understand the effects of endothelial dysfunction on vascular control, it is first necessary to comprehend the normal cellular mechanisms which become dysfunctional with hypertension.

Endothelial Cell Control of Vascular Tone:

It was initially thought that the layer of endothelial cells located on the luminal side of the blood vessel wall did not play a role in the control of vascular tone. However, this idea was changed in 1980 with the important discovery of an endothelial derived relaxation factor (EDRF) by Furchgott and Zawadzki, now known as nitric oxide (14). This discovery resulted in a change in the focus of vascular research to illuminate the role of the endothelium in the control of vascular tone through mechanisms of both relaxation and contraction.

It has since been shown that a number of vaso-active substances or stimuli known to elicit a response from the vascular smooth muscle (VSM) are produced and released by the endothelium (15). In response to a stimulus, the endothelium can release two main classes of metabolites, which affect the tonic state of VSM: endothelial derived relaxing factors, and endothelial derived contraction factors (EDCF) (16,17). The balance between EDRFs and EDCFs is a determinant of the basal tone of an artery. Two of the most notable EDRFs, NO and PGI_2, play an important role in the control of vascular tone in a normotensive state, and are main foci of endothelial dysfunction research (4). NO, normally an important signaling molecule responsible for producing dilation, decreases in bioavailability during the progression of endothelial dysfunction. The reduction in NO bioavailability is apparent in hypertensive patients who suffer from impaired NO mediated dilation (18). This result has also been reproduced in isolated sections of the common carotid artery (CCA) and thoracic aorta of hypertensive animal models (4). PGI_2, an EDRF, is normally involved in the control of vascular tone predominantly producing relaxation of the blood vessel. However during endothelial dysfunction, there are multiple alterations in the PGI_2 signaling pathway, the end result being a transition in the functional role of this molecule from an EDRF to an EDCF (1). To understand the functional alterations occurring to these EDRFs it is important to outline the normal function of NO and PGI_2 and the underlying mechanisms causing these alterations.

Nitric Oxide:

NO is a gaseous transmitter with a very short half life (19). It is produced from L-arginine by endothelial nitric oxide synthase (eNOS) in response to an increase in intracellular calcium (20,21). Once NO is released by the endothelium, it permeates the cell membrane of the VSM. The predominant mechanism is that NO stimulates soluble guanylyl cyclase (sGC),

which increases cyclic guanosine monophosphate (cGMP). cGMP then causes relaxation of the VSM through a number of pathways in the VSM (1). The basic mechanism of NO synthesis and function on the VSM, as well as the determinants of NO bioavailability, are illustrated in figure 1. (2)

NO has been shown to have a role in the maintenance of blood pressure. When Sainz and colleagues chronically treated rats with the NOS inhibitor L-NG-Nitroarginine methyl ester (L-NMMA), a significant increase in blood pressure resulted. Similarly eNOS knock-out models also display an increase in blood pressure (22). As mentioned previously, patients with essential hypertension exhibit impaired NO mediated dilation, indicating hypertensive patients suffer from dysfunctional NO signaling (18). Though this impaired NO mediated dilation has also been documented in animal models of essential hypertension, such as the spontaneously hypertensive rats (SHR). The SHR have demonstrated an increase in eNOS expression and phosphorylation in both the CCA and thoracic aorta (12). This finding indicates that the impaired NO signaling in hypertension is not necessarily due to a lack of NO production.

NO bioavailability has been shown to be heavily influenced by the state of oxidative stress of the cell. In models with increased oxidative stress, such as the SHR, there is an associated decrease in NO bioavailability. This decrease is mediated in part through reactive oxygen species such as the superoxide anion (4,5,23). NO is a highly unstable molecule which, when exposed to superoxide, reacts readily to form peroxynitrite and loses its ability to cause VSM relaxation, as seen in figure 1. Support for superoxide's role in the reduction of NO bioavailability has come from multiple sources in the literature. A number of experimental methods have conclusively proven that superoxide scavenges NO and impairs

its ability to act on the VSM. These include; treatment with exogenous superoxide, inhibition of superoxide dismutase (SOD), treatment with exogenous SOD and SOD mimetics, and inhibition of superoxide production through inhibition of NADPH oxidase (24-26). These findings support the theory that reactive oxygen species is a major determinant of decreases in bioavailability of NO leading to impaired signaling, a hallmark of endothelial dysfunction in hypertension.

Prostacyclin:

PGI_2 is an EDRF that can play a significant role in the development of endothelial dysfunction. In a normotensive state, the production of PGI_2 begins with the stimulation of receptors on the endothelial cell membrane. Through a G protein coupled mechanism, phospholipase A2 (PLA2) is activated, which mobilizes arachidonic acid, a fatty acid found within the cell membrane (27). Phospholipase A2s are members of a super family of intracellular and secreted enzymes which is responsible for the deacylation of arachidonic acid from glycerol. The phospholipase A2 super family is split into categories based on structure, intracellular location, and regulation properties (28). The most abundant and well characterized category is the cytosolic phospholipase A2, This category contains both calcium-independent and calcium-dependant isoforms (28). It is the calcium dependant isomer of cytosolic phospholipase A2 that is thought to be stimulated as a part of the PGI_2 signaling pathway. However some recent studies by Vanhoutte et al. indicate that both calcium-dependant and calcium-independent isoforms of phospholipase A2 could be contributing to the mobilization of arachidonic acid in the PGI_2 pathway (29). Once arachidonic acid had been mobilized from the cell membrane it is then converted by cycloxygenase 1, 2 (COX 1,2) into endoperoxides, which then are converted into the different

prostaglandins through their respective synthases. PGI_2 is one of the most abundant prostaglandins, and is produced by prostacyclin synthase (9,30). Once PGI_2 is produced it is able to stimulate receptors on the VSM cell membrane. PGI_2 stimulates the Prostacyclin receptor on the VSM membrane, which undergoes a conformational change and activates a stimulatory G protein resulting in dilation. (1,31). Figure 2 illustrates the production of PGI_2 and its possible effects on the VSM.

During endothelial dysfunction, $PGI_{2's}$ influence on the VSM cell is altered, losing its effects as an EDRF and producing VSM responses as an EDCF. The changed role of PGI_2 in endothelial dysfunction was first noted by Luscher and Vanhoutte in 1986 using SHR (27). These investigators noticed impaired dilation during acetylcholine (ACh) dose-responses curves, followed by a re-contraction in aortas of SHR compared to WKY controls. The dilation was attributed to the effects of NO as normally seen, but the metabolite responsible for the re-contraction was unknown. Luscher et al. further examined this re-contraction by repeating the ACh dose-response curve in quiescent (non pre-contracted) rings incubated for 30 minutes with the NOS inhibitor L-NMMA. Under these conditions, ACh stimulation results in endothelium-derived contraction that was greatly enhanced in SHR thoracic aorta compared to WKY. This contraction could be abolished with the introduction of indomethacin (INDO), a non-selective COX inhibitor (9,10,12,32). These experiments indicate that the re-contraction observed during an ACh dose-response curve can be attributed to COX-mediated mechanisms. Since this initial discovery, much has been done to elucidate the role of PGI_2 as the main contributor responsible for this re-contraction, and the cellular mechanisms of the PGI_2 pathway that become altered during endothelial dysfunction to produce contraction as

opposed to relaxation (12). Figure 3 outlines the two types of experiments used by Luscher and Vanhoutte to identify the endothelium-derived contractions.

Changes in expression of COX in the endothelium and VSM may play a large role in the increased EDCFs produced during endothelial dysfunction. Both COX 1 and COX 2 are inducible molecules with the ability to become over-expressed in response to stimuli such as increased shear stress (33). The endothelium has almost a 20 times greater expression of COX than does the VSM, and though both COX 1 and 2 are present, the endothelium preferentially expresses COX 1 (4). The SHR CCA also has a much greater expression of COX when compared to WKY normotensive controls, indicating an increased COX expression with the development of hypertension (12). Another factor of the PGI_2 pathway which may lead to an increase in EDCF production is prostacyclin synthase, which has been shown to have increased expression in SHR. These differences may explain the increase in PGI_2 production in SHR and the increased magnitude of endothelium-derived contractions exhibited by these animals compared to their normotensive controls (WKY) (9,10,32). Similarly to the difference in expression of COX seen in the CCA of SHR, the production of EDCF's between COX 1 and 2 sub groups is also not equal. By performing ACh dose-response curves in quiescent rings following incubation with selective inhibitors of both COX1 and COX2, it was determined that COX1 appears to be the dominant contributor to endothelium-derived contractions, while COX2 contributes minimally to the response (34,35).

Another important modification that occurs in endothelial dysfunction related to EDCF signaling is a change in PGI_2 receptor stimulation on the VSM cell membrane. There are multiple receptors found on the VSM cell membrane that are affected by the different prostanoids. PGI_2 in a non-dysfunctional state stimulates the prostacyclin receptor, which

causes relaxation of the VSM as stated above. During conditions of vascular endothelial dysfunction there is a loss of the dilatory effect normally observed with PGI_2 stimulation. It is not clear what causes dysfunction of the prostacyclin receptor leading to a loss of relaxation, but it has been shown to occur as early as 3 months of age in SHR (36). It is thought that this may be caused by a decrease in expression of the prostacyclin receptor. However, it is still unclear if this is the sole cause of prostacyclin receptor dysfunction (36,37).

The thromboxane-prostanoid receptor (TP) becomes the main receptor stimulated by PGI_2 in endothelial dysfunction, resulting in EDCF-mediated contractions. This was demonstrated by the blockade of ACh-induced endothelium-dependant contractions using thromboxane-prostanoid receptor antagonists in quiescent ring experiments (27). Though the thromboxaine-prostanoid receptor can be stimulated by a multitude of COX metabolites including thromboxane A2 (TXA_2), PGH_2 and others, it has been demonstrated by Vanhoutte and colleagues that the main contributor to endothelium-dependant contraction during endothelial dysfunction is PGI_2 (12,30). Figure 4 outlines the dysfunctional roles of PGI_2. (38)

Both NO and PGI_2 play a key role in vasodilation of blood vessels and are contributors to the basal tone of the vasculature in a normotensive state. In hypertension, changes in the signaling pathways of these molecules are main contributors to endothelial dysfunction (4). Reactive oxygen species have also been shown to increase EDCF production by stimulating COX and also propagating its signal in the VSM, indicating reactive oxygen species possible role in endothelial dysfunction (30,39).

Reactive Oxygen Species in Endothelial Dysfunction:

Hypertension and endothelial dysfunction are associated with an increase in oxidative stress in both endothelial and VSM cells (4). This is mediated by an increase in reactive oxygen species including superoxide, hydrogen peroxide, and peroxynitrite(2,4,40). These species are produced to some extent in normal physiological conditions, but are increased under pathophysiological conditions such as hypertension (4). Increases in reactive oxygen species are seen both in patients with essential hypertension and in different animal models used to study hypertension such as the SHR (6,41). There are many sources of reactive oxygen species in the vasculature which can account for the increased reactive oxygen species production. NADPH oxidase, xanthine oxidase, eNOS, and COX 1, and 2, all contribute, but NADPH oxidase appears to be the largest contributor of reactive oxygen species in the vasculature, producing superoxide anions (2,6). NADPH oxidase is stimulated by angiotensin II, a molecule commonly elevated in patients suffering from essential hypertension, which leads to an increase in superoxide production and an alteration in the state of oxidative stress of the cell (4,42). NADPH oxidase expression has been shown to increase in hypertensive animal models, contributing to the increase in reactive oxygen species (42).

It was determined by Kerr and colleagues that the majority of superoxide anions produced in the vasculature are endothelium-derived. This was observed in the thoracic aorta of SHR and WKY using chemiluminecent dyes, noting there was a significant decrease in superoxide production when the endothelium is removed in comparison to the endothelium being intact. SHR aorta had a much larger production than those of WKY (6,43). The increase in reactive oxygen species is a main contributor to the development of endothelial dysfunction, affecting both NO and PGI_2 signaling. As stated earlier, superoxide reacts with

NO to form peroxynitrite. Both of these molecules are highly unstable and quickly react with one another, reducing NO bioavailability, which can account for the impaired dilation apparent in endothelial dysfunction. The formation of peroxynitrite not only decreases NO bioavailability, but also has a number of destructive consequences due to the instability of this molecule. Firstly, peroxynitrite can inhibit SOD, leading to an increase in superoxide anion concentration and a decrease in the antioxidant capabilities of the cell (44). Secondly, peroxynitrite also inhibits GC which is the downstream target of NO. When sGC is inhibited there is a reduction in the vaso-dilatory effect of NO. Resveratrol's ability to scavenge superoxide may reduce peroxynitrite formation and increase NO bioavailability, improving NO mediated dilation which is impaired in endothelial dysfunction (45).

Superoxide also plays a role in EDCF production, and endothelium-dependant contractions of the VSM. It was noted that treatment with antioxidants and antioxidant enzymes such as SOD and catalase can reduce endothelium-dependant contractions to ACh in quiescent rings of SHR. This indicated that superoxide can play an important role in the activation of COX1, leading to the production of EDCF such as PGI_2 (41,46). Superoxide's role in the production and propagation of the EDCF signal is shown in figure 4. Reducing the increase in reactive oxygen species though scavenging of superoxide could also reduce endothelium-dependant contractions, which arise during endothelial dysfunction and hypertension. The role of reactive oxygen species in the dysfunctional endothelial signaling makes reactive oxygen species a possible target for intervention using antioxidants like resveratrol.

Resveratrol

Resveratrol, or 3,5,4- trihydroxystilbene, is a naturally occurring polyphenol that can be found in a number of plant species including grapes, berries and some nuts and it has been shown to confer a number of health benefits following consumption. Resveratrol exists as both cis and trans isomers and though both isomers are associated with health benefits, the vast majority of these benefits have been attributed to the more common trans isomer (47). Trans resveratrol was most notably known for its proposed role in providing cardiovascular protection in the "French Paradox". The "French Paradox" refers to the French lifestyle which incorporates a large number of risk factors for cardiovascular disease, including a high fat diet, smoking, minimal physical activity and alcohol consumption. However, as a population, the French have one of the lowest rates of cardiovascular disease (48). The low prevalence of cardiovascular disease in French society has partially been attributed to the moderate red wine consumption of the French population. Resveratrol, which is found in red wine, has been proposed as a molecule responsible for the reduction of cardiovascular disease of the French population. Since this proposition numerous research studies have examined the effects of resveratrol on the cardiovascular system in both models of health and disease. This research has indicated that resveratrol provides a number of benefits for the cardiovascular system, some of which act through an endothelium-dependant mechanism. Though research indicates resveratrol consumption is associated with a number of benefits, the dosage of resveratrol required to provide these benefits is unclear. It is also still yet to be determined if excessive consumption can have adverse side effects possibly resulting in toxicity.

Resveratrol is readily metabolized by the body, and following oral administration it is estimated 70 % of resveratrol ingested is absorbed by the gastrointestinal tract. This is

partially accomplished through the lipophilic properties of resveratrol enabling it to cross the plasma membranes of cells. This passive diffusion through cell membranes allows for trans-epithelial diffusion of resveratrol through the intestinal walls, eventually diffusing into the vasculature of the gastrointestinal tract (49,50). Once resveratrol is orally ingested it rapidly enters the blood stream reaching its peak plasma concentration within 15 minutes. Though resveratrol is readily absorbed by the digestive tract, it has a low bioavailability in plasma due to its short half life of 8-14 minutes, its absorption by a number of tissues including the liver, kidneys, cardiac and vascular tissue, and its conversion into a number of metabolites including RSV-3-sulfate and RSV-3-O-glucuronide (47,49). The resveratrol concentration in the plasma rapidly diminishes after reaching its peak concentration. However, there is a second spike in the resveratrol plasma concentration, following the peak plasma concentration, indicating there is a small resveratrol storage capacity within the body. This storage capacity is a result of resveratrol's ability to react with hemoglobin and possibly bile, being re-released as free resveratrol as the plasma concentration diminishes (49). The peak resveratrol plasma concentration was determined in Sprague-Dawley rats by Juan et al. This study found that after oral ingestion of 2mg/kg resveratrol the peak plasma concentration reaches 586.77 ng/ml 15 minutes following ingestion (51). Similar experiments have also shown that the plasma concentrations of resveratrol increase linearly with increasing dosages of resveratrol. In addition to this linear relationship it has also been found that chronic dietary treatment with resveratrol over a period of days also leads to an increase in plasma concentration (47,49,51). Though these dosages greatly exceed the weight adjusted amount of resveratrol consumed by a moderate red wine drinker, there do not appear to be any side effects to such a large single dose. Resveratrol has also been found to be a nontoxic

compound when chronically administered in an excessive dosage of 20mg/kg for a period of 28 days to Sprague-Dawley rats. This treatment did not result in any changes in growth or appearance of the vital organs, and did not alter hematological variables, including red blood cell count, hemoglobin measures, white blood cell count and platelet measures. The animal's clinical biochemical markers were also unaltered; these markers include the HDL, LDL, triglycerides, blood glucose and a number of metabolite measures. These results indicate that resveratrol is safe for chronic dietary treatment at dosages that greatly exceeds normal dietary consumption (51).

Resveratrol treatment has been shown experimentally to have positive effects in a number of disease models associated with free radicals, which includes hypertension (52). This is accomplished through resveratrol's strong antioxidant capabilities, which allows for buffering of a number of different reactive oxygen species that can affect endothelial function. These reactive oxygen species include hydrogen peroxide; the hydroxyl ion, peroxynitrite and the superoxide ion (49). Resveratrol's antioxidant scavenging ability is associated with its hydroxyl groups which allow for the scavenging of free radicals. Specifically, it is the 4 - hydroxystilbene group which enables resveratrol to reduce reactive oxygen species effectively (53). Once the hydroxyl group has reduced the free radical, the structure of resveratrol allows for the transition of the radical through a number of resonance structures following the reaction. These resonance structures enable resveratrol to be stable when reacting with free radicals, and give resveratrol its strong antioxidant capabilities. Figure 5 shows the structure of resveratrol and highlights the 4 – hydroxystilbene group in the Para position which is a key component in its antioxidant role.

Though resveratrol has been shown to be a strong antioxidant in-vitro, scavenging superoxide, hydrogen peroxide, and the hydroxyl radical, some endogenous antioxidants such as superoxide dismutase, catalase and glutathione have been shown to be more potent than resveratrol (54). Though resveratrol treatment can play a role in quenching the increase in reactive oxygen species that occurs during a disease state like hypertension, through its primary antioxidant role, it is likely to have a small impact compared to these more potent endogenous antioxidants. Resveratrol has also been shown to be less potent than some other exogenous antioxidants such as ascorbate. However resveratrol has been shown to elicit effects which can be attributed to more than just its role as a primary antioxidant. These additional mechanisms include modulation of endogenous antioxidants, inhibition of some pro-oxidant enzymes and also alterations in certain protein expression, all of which have beneficial effects to the cardiovascular system (54).

Resveratrol has been shown to increase circulating antioxidant activity in plasma; this is accomplished by unabsorbed polyphenols acting locally in the gastrointestinal tract. These residual polyphenols are able to scavenge free radicals, and prevent lipid peroxidation which will in turn spare other antioxidants from oxidation, thus increasing plasma antioxidant activity (55). Resveratrol can also mediate benefits that are not related to its antioxidant capabilities through its effect on gene regulation of a number of different enzymes and proteins, some of which include SOD, catalase, glutathione peroxidase, NADPH oxidase, eNOS, and tumor necrosis factor α (56-58). Though the mechanisms by which resveratrol influences the expression of these different proteins and enzymes are still not completely understood, it has been shown that resveratrol stimulates a number of different transcription factors. For instance, resveratrol has been shown to stimulate the Sirtuin family of proteins

which are associated with a number of benefits which prolong cellular life span. These benefits include modulation of gene expression and mitochondrial function (49). Resveratrol is thought to most notably stimulate Sirtuin 1 which in turn stimulates the forkheaded box family of transcription factors which controls the expression of a number of proteins, some of which become altered in hypertension (49). Resveratrol stimulation of Sirtuin 1 may alleviate some of these alterations associated with hypertension.

Resveratrol also stimulates the nuclear factor-E_2-related factor-2 (Nrf2) transcription factor and indirectly, though Nrf2, the antioxidant response element pathway. This process targets numerous reactive oxygen species detoxifying and antioxidant genes. These genes include NADPH: quinone oxidoreduxtase and heme-oxygenase-1, and γ-glutamylcystine synthase, the rate limiting enzyme for glutathione synthesis (59). Though the pathways by which resveratrol regulates gene expression are still not fully characterized it has been conclusively shown that resveratrol treatment does alter the protein expression of those listed above in a number of studies(56,57,60). Resveratrol treatment has been shown to increase SOD, catalase, glutathione, heme-oxygenase-1 and eNOS expression while reducing the expression of tumor necrosis factor α and NADPH oxidase. These alterations will have a positive effect on relieving the oxidative stress seen during endothelial dysfunction (56,57,59).

Resveratrol has been shown to reduce oxidative stress through inhibition of pro-oxidant enzymes including NADPH oxidase and COX. Resveratrol's down regulation of tumor necrosis factor α leads to a decrease stimulation of NADPH oxidase, resulting in a reduction in superoxide production (56). Resveratrol has also been shown to inhibit angiotensin II stimulation of NADPH oxidase, further decreasing the production of

superoxide (49). The decreased production of reactive oxygen species, which is accomplished through resveratrol's direct or indirect inhibition of NADPH oxidase, is an important contributor to the reduction in oxidative stress seen following resveratrol treatment. The combination of resveratrol's primary antioxidant effects and its secondary effects on the modulation of exogenous antioxidants and pro-oxidant enzymes indicate chronic resveratrol treatment will improve alterations that are seen during endothelial dysfunction dependent on increases in oxidative stress. These effects of resveratrol include improvement of the EDRF signaling and reductions of the EDCF signal. Thus resveratrol treatment could lead to positive effects on vascular tone and possibly blood pressure in these cases. Non antioxidant effects of resveratrol such as alterations in expression of eNOS and COX may also play a role in the positive effects of resveratrol (49,61).

Results from previous studies conducted in our laboratory using chronic dietary resveratrol treatment demonstrated an improvement in NO-mediated dilation. This was initially hypothesized to be due to improved NO bioavailability, through an improved oxidative state and possibly by an increase in eNOS expression, which has been shown to occur in cell culture experiments (61). However resveratrol treatment did not cause an increased eNOS expression in either SHR or WKY. This indicates that an improvement in NO bioavailability could be mediated through a decrease in reactive oxygen species scavenging of NO (52), and also points to a clear possibility that there is a NO-independent component of the resveratrol effect. However, this previous chronic resveratrol study had multiple limitations. For instance it did not account for possible changes occurring to the EDCF pathway following chronic dietary resveratrol treatment (47,49,51,52,57). The study also used a high dose of resveratrol which was lower than commercially available resveratrol

supplementation. Also the study did not examine the effects of resveratrol treatment on endothelium-dependant contractions, PGI_2 production, hemodynamic measures, or resveratrol-mediated dilation. Furthermore this study also did not use normotensive control animals for all experiments.

Results from our laboratory have also shown that *acute* incubation with resveratrol in vitro reduces endothelium-dependant contractions and decreases the production of PGI_2 (Jeffery in press). These results indicate that resveratrol has an effect on COX1 function in the vasculature which may be mediated through a reduction in reactive oxygen species. Further evidence for resveratrol treatment affecting COX1 function is shown through the effect of oxidized resveratrol. Resveratrol, when oxidized, has an inhibitory effect on COX function (62). The *acute* incubation resveratrol study also demonstrated that resveratrol treatment leads to an increase in 5' adenosine monophosphate-activated protein kinase phosphorylation, a protein capable of relaxation of the VSM and possibly the inhibition of the endothelium-dependant contractions (63). Thus, previous findings from this laboratory indicate that chronic dietary resveratrol treatment may improve endothelial function through a number of mechanisms affecting NOS- and COX-mediated signaling.

Purpose:

The following study aims to investigate the effects of chronic dietary resveratrol treatment on endothelium-dependant vasomotor function of the CCA as well as on hemodynamics of SHR and WKY animals. The study will specifically focus on examining

1. Resveratrol's effects on endothelium-dependant vasorelaxations. This will focus on identifying the influence of resveratrol on NO mediated vasorelaxation including

dose-dependent effect on endothelium-dependant relaxation, NO sensitivity of the VSM and eNOS expression in the CCA.

2. Resveratrol's effects on endothelium-dependant contractions. This will focus on examining prostacyclin-mediated responses. This will include assessment of resveratrol's dose-dependent effect on endothelium-dependant contraction, PGI_2 production, sensitivity of thromboxane-prostanoid receptor on the VSM, and COX 1 expression.
3. Resveratrol's effects on hemodynamics. This will focus on assessing the dose-dependent effect of resveratrol on blood flow, conductance and blood pressure.

Other secondary experiments examined the effects of chronic dietary resveratrol treatment on acute resveratrol-mediated relaxation, 5' adenosine monophosphate-activated protein kinase-mediated relaxation, hydrogen peroxide-mediated contractions and phenylephrine-mediated contractions.

Hypotheses:

Hypothesis one: Chronic dietary treatment with resveratrol at a high dose, which mimics a resveratrol supplement dose, and at a low dose, which mimics moderate (2 glasses) red wine consumption, will improve the relaxation of the SHR CCA to ACh, but have no effect on this response in WKY CCA. (Both doses will be weight adjusted to mimic resveratrol doses in humans.)

Hypothesis two: Chronic dietary resveratrol treatment will cause a dose-dependent reduction in endothelium-dependant contractions of the CCA VSM in the SHR, but not in the WKY. This improvement will be due to a reduction in COX production of EDCFs.

Hypothesis three: Chronic dietary resveratrol treatment will decrease PGI_2 production in the CCA in a dose-dependent manner in both SHR and WKY.

Hypothesis four: Chronic dietary resveratrol treatment at a high dose will decrease blood pressure and alter blood flow of the SHR but have no effect on the hemodynamics of WKY. Treatment at low dose of resveratrol will have no effect on either SHR or WKY

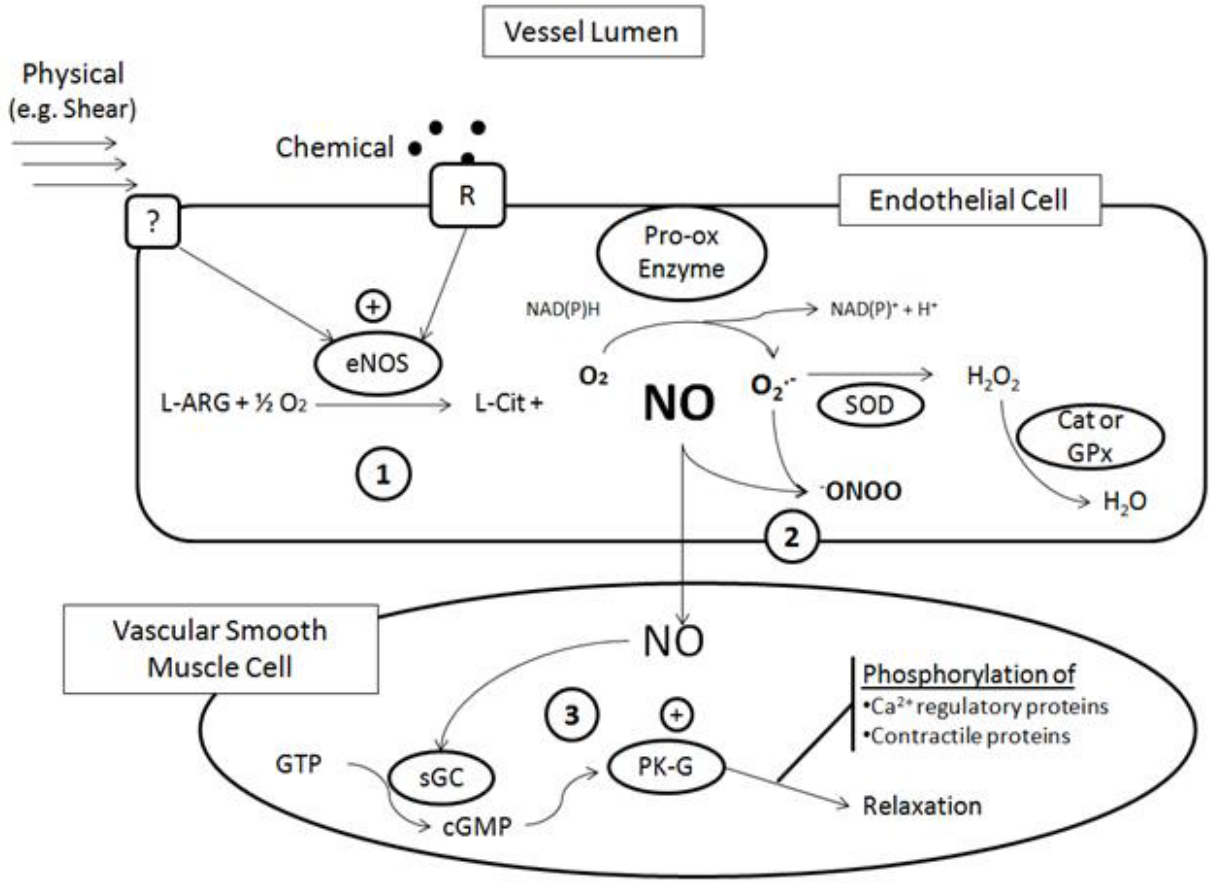

Figure1: Both physical and chemical stimuli can activate eNOS acutely through various post transcriptional mechanisms, determining the rate of NO production (1). NO that reacts with ROS will be reduced to -ONOO and become inactivated (2). Chemicals such as resveratrol and antioxidant enzyme systems like SOD,,GPx and Catalase can quench ROS leading to increased NO bioavailability. NO initiates relaxation through a sGC-PKG cascade (3). Adapted from (5)

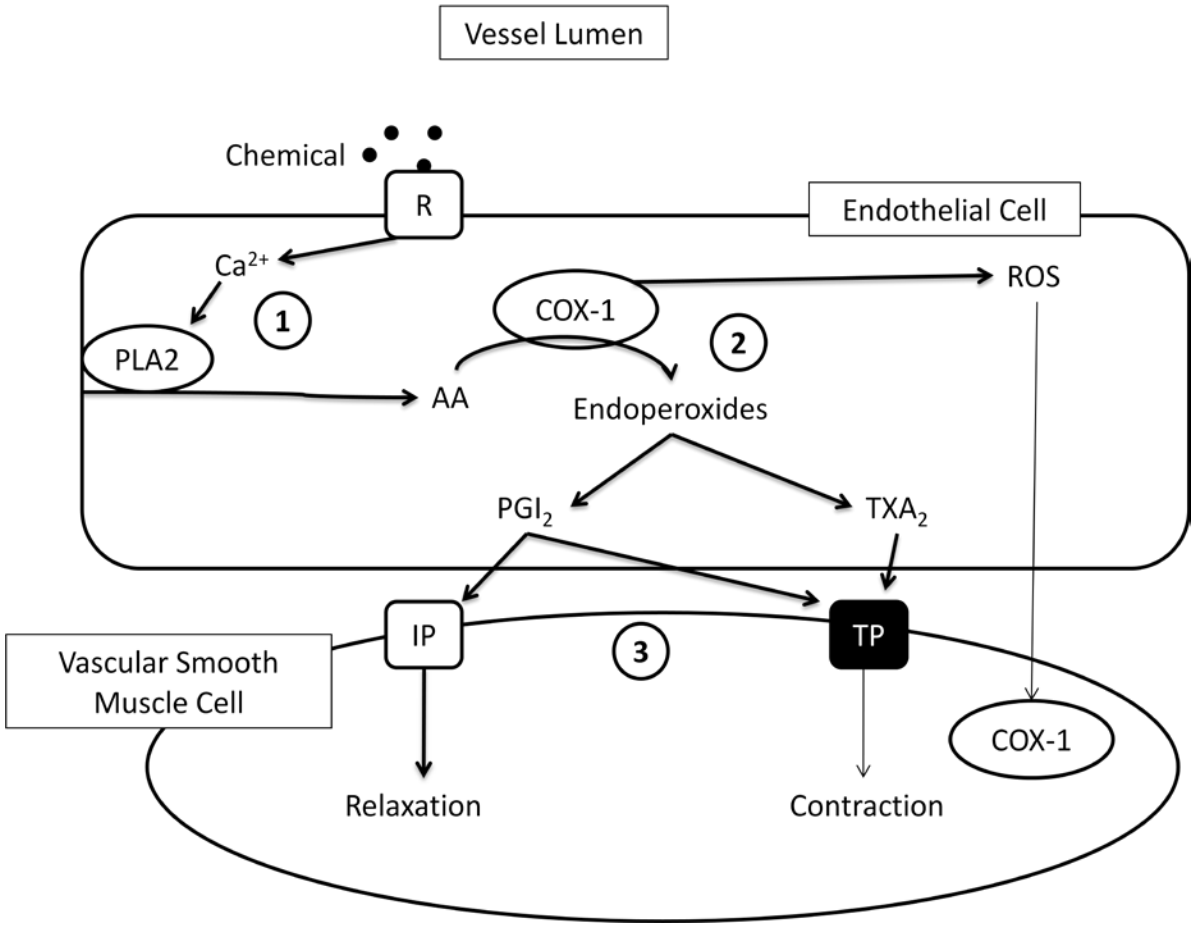

Figure 2: Chemical stimulation of the endothelial cell causes an increase in intracellular calcium (1.)This activates PLA2 which mobilizes AA from the cell membrane. AA is converted by COX-1 in to endoperoxides (2.) The endoperoxides are then converted into PGI_2 and TXA_2 which then act through the IP or TP receptor on the VSM to alter vascular tone (3).

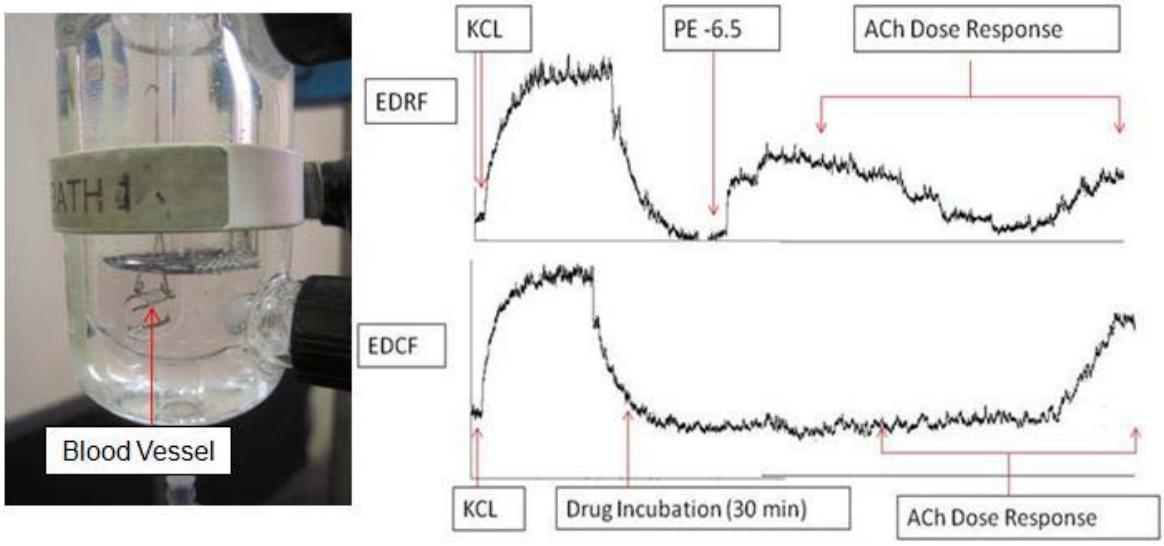

Figure 3: Blood vessels (TA, CCA etc) are dissected and cut to a uniform length and loaded on a wire myograph. Vessels equilibrate and are then subjected to one of the following protocols shown. EDRF: viability of the vessel is tested using potassium chloride (KCL) which is washed out of the tissue bath repeatedly. Once a basal amount of tension is reached the vessel is pre-contracted with phenylehprine (PE). Once the force created by the vessel plateaus, an ACh dose response curve follows (protocol examines NO mediated dilation). EDCF: viability is tested with KCL and washed, once basal tension is reached incubation with L-NMMA follows to inhibit production of NO. Following a 30 min incubation period an ACh dose response curve is done (protocol examines endothelium derived contractions)

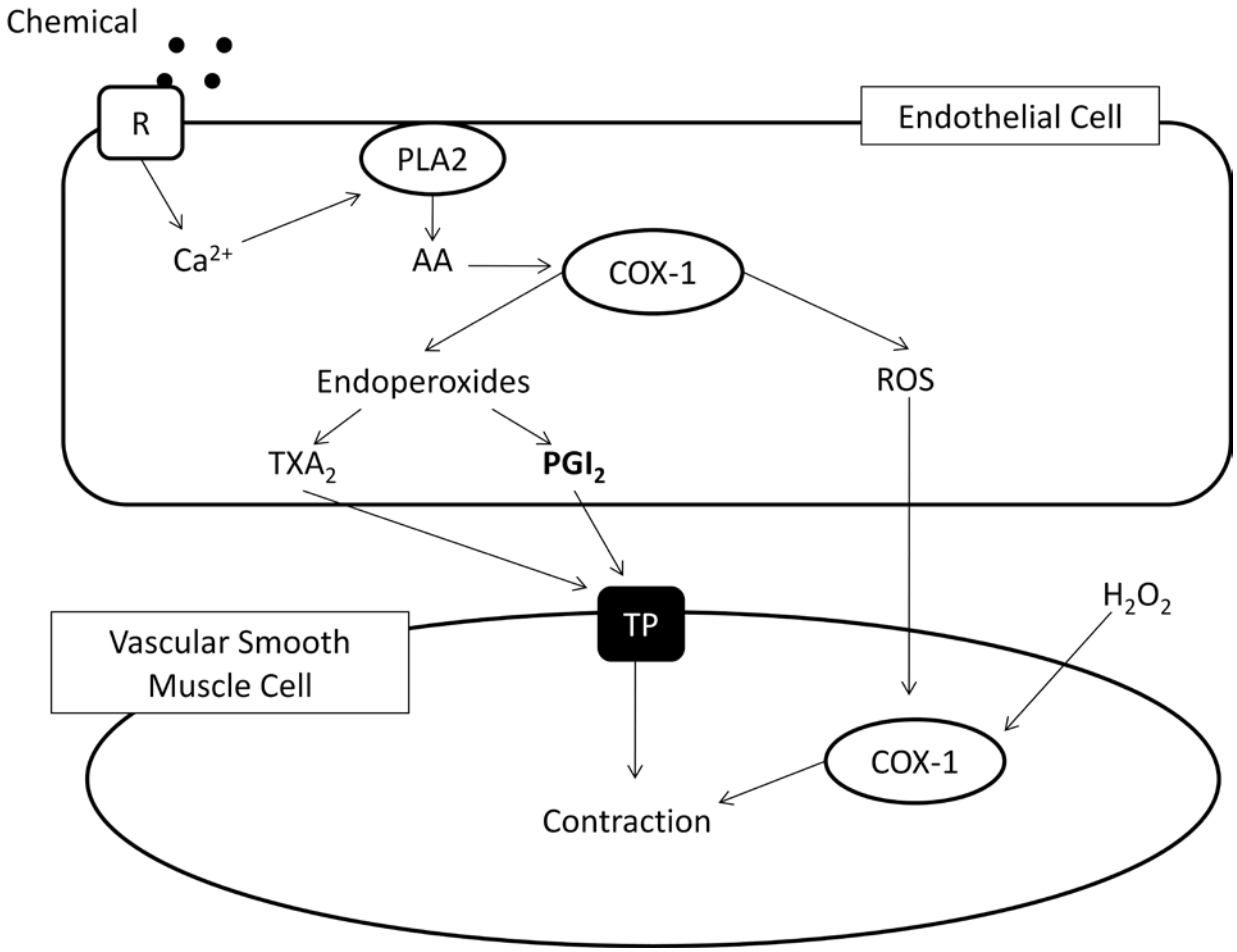

Figure 4: The mechanisms involved in endothelium derived contractions seen during endothelial dysfunction. A stimulus causes an increased intracellular calcium concentration, which activates PLA_2, releasing arachidonic acid. This is then metabolized by COX1 into endoperoxides and then converted in to predominantly PGI_2 and, to a lesser extent, TXA_2. PGI_2 then causes contraction through stimulation of the TP receptor. ROS are also generated in the endothelium and are able to stimulate COX in the VSM, propagating the TP receptor mediated contraction. Adapted From (30)

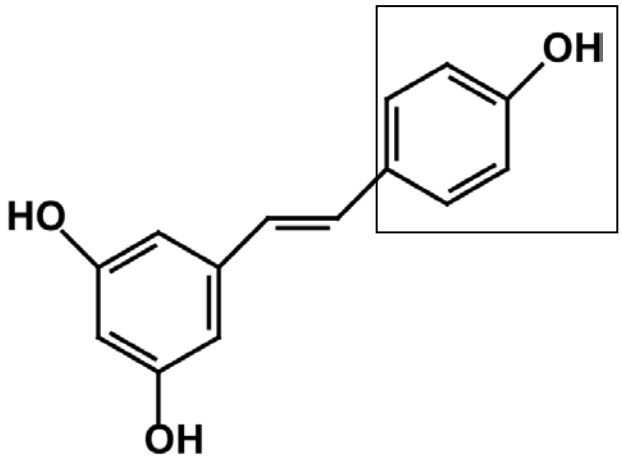

Figure 5: 3,5,4- trihydroxystilbene chemical structure, 4 – hydroxystilbene group in the Para position. The hydroxyl groups of resveratrol provide its antioxidant activity.

Methods:

Animals:

Male Spontaneously Hypertensive Rats (n=35) and male Wistar Kyoto Rats (n=35) were purchased from Harlan (Indianapolis,IN) at 13 weeks of age. Rats were group housed in a temperature and humidity controlled room and acclimatized to the 12 hour reverse light cycle. Rats had free access to standard lab chow (Harlan) and tap water until the age of 16 to 20 weeks. At this age, the animals were weighed and individually housed in a temperature and humidity controlled room with free access to standard lab chow and treated water depending on group. The water was changed daily and consumption was monitored. At 20-24 weeks age, following 4 weeks of treatment, animals were weighed and then injected with sodium pentobarbital (50-65 mg/kg i.p.; Bimeda-MYC, Cambridge, ON). Level of sedation was monitored by measuring the withdrawal from a toe pinch, to ensure proper sedation for hemodynamic measures. Following hemodynamic measurements the animals were sacrificed by exanguination.

Resveratrol Treatment:

Resveratrol treatment was designed to mimic moderate red wine consumption of 500 ml/day, and pharmalogical supplementation with resveratrol in a 70 kg human. Moderate red wine consumption of 500 ml/day was estimated to be 4.68 mg (64,65). Pharamalogical supplementation with resveratrol was estimated to be approximately 500 mg (66). The resveratrol consumption for 500ml/day of red wine was adjusted to a value per kg which was 0.0668 mg/kg. The per kg value was adjusted to a dosage for a rat, assuming an average rat weight of 0.333 kg, which resulted in each rat needing to consume 0.022263 mg/day of

resveratrol. Water consumption per rat was estimated to be 35 ml/day(52), which meant the resveratrol concentration must be 0.0006361 mg/ml for the low resveratrol group which represents moderate red wine consumption (52). The pharmalogical supplementation group was determined to be 100 times the dosage of the low group receiveing 0.06361 mg/ml of resveratrol.

All animal groups were fed standard laboratory chow and tap water while being group-housed. Upon isolation food consumption was recorded over the 4 week period, and the drinking water was customized to each experimental group. The control group received tap water with 1g/100ml low viscosity carboxymethyl cellulose (MP Biomedicals, Solon OH). The low dose resveratrol groups received tap water with 1g/100ml low viscosity carboxymethyl cellulose and 0.6361mg/L trans Resveratrol (Toronto Research Chemicals, Toronto On). The High dose resveratrol groups received tap water with 1g/100ml low viscosity carboxymethyl cellulose and 63.61mg/L trans Resveratrol. Water bottles were changed daily and the amount of fluid consumed by each animal was recorded.

Hemodynamic Measures:

Rats were injected with sodium pentobarbital and placed supine on a heating pad heated to 38 °C (Gaymar TP-500, Orchard Park, New York and Temp-Pad; Seabrook Medical Systems)**.** The left common carotid artery (CCA) was exposed through a small incision in the neck and connective tissue was removed. Blood flow through the left CCA was measured using a TS240 flowmeter module and a MA1PRB transit-time perivascular flow probe (ultrasound frequency, 7.2 MHz; bidirectional flow scale setting, 1V=20ml/min, Transonic systems Inv., Ithaca, NY). The flow probe was gently placed around the exposed CCA with 1

ml of surgical lubricant, and then the CCA was placed back in to a normal anatomical position. A stable blood flow was measured for 5 min after which the probe was removed.

Immediately following the measure of blood flow, the CCA was cleaned using a saline solution. Blunt ended forceps were used to disrupt flow; once flow has been disrupted a small incision was made at the cephalic end of the CCA. A Mikro-Tip pressure catheter (Model SPR-320, 2F Mikro-Tip Pressure Transducer Catheter, Millar Instruments, INC., Houston, TX) was inserted into the CCA and secured using a removable tie (4-0 surgical suture). Flow was restored to the artery and systolic blood pressure, diastolic blood pressure, mean arterial blood pressure, and heart rate was measured on a Powerlab console (ADInstuments, Colorado Springs, CO) using Chart 5 software (ADInstuments, Colorado Springs, CO, v5.5.4).)

Vasomotor Assessment:

Following the hemodynamic measures the animals were sacrificed via exsanguination. The Right CCA was excised, placed in a Krebs-Henseleit buffer (131.5 NaCl, 13.5 NaHCO, 11.2 glucose, 5.0 KCl, 2.5 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, and 0.023 EDTA (all in mM)) at 4 °C, and cleaned using blunt ended forceps**.** The CCA was sliced into 2 mm rings using a surgical blade under a microscope (Zeiss; VWR, Mississauga, ON) and mounted on a wire myograph system (vascular myography unit, Radnoti Glass Technology Inc., Monrovia, CA). Two adjacent wires were carefully threaded through the vessel lumen. One wire was attached to a force transducer the other was attached to a glass foot. The suspended vessels were placed in tissue baths containing 5 ml Krebs buffer at 37°C**.** Force transduction was measured by means of an isometric transducer (Model MLT0201/D, ADInstruments) and software (8SP PowerLab).

The rings were slowly adjusted to a basal tension of 2.85 g (previously determined to be optimal resting tension for generation of active responses), washing with Krebs buffer every 10 minutes. Once allowed to equilibrate at 2.85 g, tension viability was tested using 60mM of KCl to elicit contraction. After a period of 30 minutes, the rings were washed with Krebs buffer 3 times with a spacing of 5 minutes, restoring them to the basal tension in preparation for vasomotor function testing.

Vasorelaxation studies:

Rings were pre-contracted with Phenylephrine (PE) ($10^{-6.5}$ M). Once the contraction had reached its plateau, a cumulative dose-response curve was performed using a variety of protocols to examine dilatory responses of the CCA to different agonists. The chemicals used include ACh, Sodium Nitroprusside (SNP), resveratrol, and 5-aninoimidazole-4-carboxamide-1-β-Diribofuranoside (AICAR), and indomethacin all of which use different concentrations for their respective protocols determined from previous work in the lab. The ACh protocol exposed the pre-contracted vessels to increasing concentrations of ACh (10^{-9} to 10^{-4}M) in a cumulative manner. Following the plateau of each concentration of Ach, subsequent concentrations were added. The SNP protocol exposed pre-contracted vessels to increasing concentrations of SNP (10^{-10} to 10^{-4}M) in a cumulative manner. Following the plateau of each concentration of SNP, subsequent concentrations were added. For the assessment of NO bioavailability the ACh protocol was used following 30 minute incubation with indomethacin, this incubation removed the endothelium-dependant contractions and allowed for the assessment of NO-mediated relaxation. The resveratrol protocol exposed pre-contracted vessels to increasing concentrations of resveratrol (10^{-6} to 10^{-4}M) in a cumulative manner. Each concentration of resveratrol was administered in 25minute intervals. The AICAR

protocol exposed pre-contracted vessels to three concentration of AICAR (10^{-7}, 10^{-4}, 10^{-2}M) in a cumulative manner. Each concentration of AICAR was administered in 30 minute intervals.

Vasocontraction studies:

Quiescent rings were incubated with L-NMMA (10^{-4} M) for 30 minutes before addition of any chemicals. Following incubation, an EDCF curve was examined using multiple concentrations for each chemical. The chemicals used in the EDCF protocols were ACh and H_2O_2. The ACh protocol exposed the quiescent vessels to increasing concentrations of ACh (10^{-9} to 10^{-4}M) in a cumulative manner. Following the plateau of each concentration of Ach, subsequent concentrations were added until maximal contraction was achieved. The H_2O_2 protocol exposed the quiescent vessels to increasing concentrations of H_2O_2 (10^{-6} to 10^{-3}M) in a cumulative manner. Following the plateau of each concentration of H_2O_2, subsequent concentrations were added until maximal contraction was achieved. A cumulative dose-response curve to phenylephrine was performed in quiescent rings that have not been incubated with L-NMMA. The rings were exposed to increasing concentration of PE (10^{-9} to 10^{-4}).

Biochemical Analysis:

Prostacyclin Release:

The buffer from the tissue bath was collected and frozen in liquid nitrogen, and stored at -80 °C immediately following peak contraction during the ACh dose-response in quiescent rings incubated with L-NMMA. PGI_2 production was measured by assessing the concentration

of 6-keto-PGF1 in the buffer (stable metabolite of PGI_2) using a competitive EIA kit according to the manufacturer's instructions (Cayman Chemical). These PGI_2 values were expressed in reference to the length of the CCA ring.

Vascular Protein Expression:

Frozen CCA were hand homogenized in 150µl of ice cold extraction buffer (10mM NaH_2PO_4, 1% SDS, 6M urea at pH 7.4). The homogenates were then incubated at 60 °C for 3 hours being intermittently vortexed, and then centrifuged (12min, 12000rpm). Following this, the Supernatant was removed and frozen at -80°C. The Supernatant was then thawed and a bicinchoninic acid protein assay was performed by combining the samples with the BCA working reagent (50 parts bicnchoninic acid + 1 part copper II suphate) and compared to a bovine serum albumin protein standard. The protein concentration was read on a spectrometer (OD 527nm, SpectraMax plus 384, Molecular Devices, Sunnyvale, CA). Following this procedure, these samples were prepared for Western blotting by diluting them to 1µg/µl.

Prior to electrophoresis (120v for 60-80min) samples were denatured at 95°C for 5 min and then loaded into the lanes (30µg/lane) of the sodium dodecyl sulphate-polyacrylamide gels (7.5% SDS-PAGE). The proteins were then transferred onto microporous PVDF membranes (25V for 40 min). Immunodetection was performed by first blocking the membranes for 1 hour in 5% bovine serum albumin (BSA), then incubating for a specific amount of time depending on the primary anti-body (eNOS (1:750):24H, COX1 (1:200):1H). This was followed by 1 hour incubation with either horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies. Detection was performed using enhanced

horseradish peroxidase/luminol chemiluminescence reagents (Amersham, Little Chalfront, UK) and a syngene system (syngene, Cambridge, UK).

Statistical Analysis:

For contraction curves, the tension values were expressed as a percentage of the KCl pre-contractions. For relaxation curves, relaxations were expressed as a percentage of relaxation from phenylephrine pre-contraction.

The pre-contractions and parameters of the dose-response curves were compared using 2 way ANOVA. The animal characteristics, and hemodynamic measurements, prostacyclin production and Western blot results were also compared using a 2 way ANOVA. (α=0.05)

Analysis was done using the statistical software SAS for two way and one way ANOVAs', while curve fitting data including Area under the Curve (AUC) and maximal response was assessed using GraphPad Prisim v4.03 software (San Diego, CA).

Results:

Animal Characteristics:

Food consumption was significantly different between the SHR and WKY strains however, there were no differences found in food consumption between the three treatment groups within each strain (Table 1). As observed in the previous chronic dietary resveratrol treatment study there was a difference in water consumption between the two strains ($p<0.0001$). However, there were no differences found in water consumption between the treated groups of each strain (Table 1). Resveratrol consumption of the low and high groups successfully reached a level of resveratrol ingestion which represented both moderate red wine consumption (SHR=0.0296 mg/day, WKY=0.0216 mg/day) and pharmacological supplementation levels (SHR=2.78mg/day, WKY=2.1mg/day) (Table 1). These values, once weight adjusted to represent a 70 kg human, represent moderate red wine consumption (6.2 mg) and pharmacological supplementation (583.8 mg) levels of resveratrol.

Similar to previous studies there was a difference in final body weight between the two strains. There were no differences observed in the final body weight of the three treatment groups of each strain (Table 1). There were also no statistical differences seen in the body weight change during the 4 week treatment period with resveratrol in all of the groups. (Table 1)

Hemodynamic Measures:

The SHR animals had a significantly reduced *mean* CCA blood flow in all treatment groups when compared to respective WKY groups (Table2). The SHR CON had a *mean*

blood flow of (3.8 ± 0.36) ml/min whereas the WKY CON had a *mean* blood flow of (5.0 ± 0.36) ml/min (p=.0254). This finding is consistent with a previous study conducted in our laboratory which found SHR blood flow to be reduced (12). In terms of *maximal* blood flow, the SHR CON, LOW, and HIGH groups were significantly reduced compared to their WKY counterparts with the exception of the WKY LOW. All groups had similar *minimal* blood flow. Resveratrol treatment had no effect on the blood flow of either strain, with the treated groups showing no differences in *mean, maximal* and *minimal* blood flow when compared to control groups (Table 2).

As anticipated, the mean arterial pressure, systolic blood pressure, diastolic blood pressure and heart rate of the SHR animals were all significantly greater when compared to the WKY animals ($P<0.05$). Within the SHR strain there was a treatment effect in the SHR HIGH group when compared to the SHR CON group with a reduction in mean arterial pressure ($P<0.0001$), systolic blood pressure ($P<0.05$), and diastolic blood pressure ($P<0.0001$) . These differences in blood pressure parameters were seen without a significant reduction in heart rate of the animals (Table 2). A graphical representation of the changes in the mean arterial pressure can be found in figure 6.

The mean vascular conductance of the SHR CON was significantly different when compared to the WKY CON group (p=0.0001) (Table 2). This finding was similar to previous results conducted in this laboratory (12)

Vasomotor Function:

For clarity, the vasomotor function assessment will be reported in two sections. The first being a comparison between SHR and WKY control groups, examining differences

between the strains in the absence of resveratrol treatment. The second section will report the results of the comparisons within each strain, examining the differences between the control and resveratrol treated groups.

KCl Contraction

Comparison between SHR and WKY control group responses

There were no differences between the strains in the response to KCl stimulation. The SHR CON produced a contraction of (0.99 ± 0.027) grams of tension compared to the WKY CON which produced a contraction of (0.91 ± 0.048) grams of tension.

Comparison within SHR and WKY treatment groups

There were no significant differences in the KCl contraction between the treatment groups of each strain.

Phenylephrine Pre-Contraction

Comparison between SHR and WKY control group responses

The phenylephrine pre-contractions were significantly different between SHR CON and WKY CON. The SHR CON had a reduction in the amount of tension produced with its pre-contractions reaching (0.514 ± .033) grams of tension after administration of -6.5 Log M of phenylephrine. Whereas the WKY CON pre-contractions reached (0.65 ± .037) grams of tensions (p=.05) This finding is similar to previous reports from this laboratory (12).

Comparison within SHR and WKY treatment groups

There were no significant differences in phenylephrine pre-contraction observed between the three treatment groups within the WKY strain. However treatment at a high dose of resveratrol appears to increase the contractile response to phenylephrine with the SHR HIGH group reaching pre-contractions of (0.6 $\pm$ .026) grams of tension compared to the SHR CON, which reached (0.514 $\pm$ .033) grams of tension (p=.044).

***Vasorelaxations**:*

Endothelium-Dependant Vasorelaxations - ACh Dose-Response Curves

Comparison between SHR and WKY control group responses

The SHR CON had a greater maximal relaxation compared to WKY CON (P<.0001) in response to ACh at a concentration of 10-4 Log M (Table 3A). This is consistent with results previously seen in the literature and indicates the SHR have impaired maximal endothelium-dependant vasorelaxations (12).

Though this difference in maximal vasorelaxation following a dose of 10^{-4} Log M ACh was seen, there was no difference found between strains following assessment of the area under the curve (AUC). (Table 3A)

Point-by-point analysis of the ACh dose-response curve reveals that this is because the SHR exhibit greater vasorelaxation to smaller concentrations of ACh compared to the WKY (Figure 7). To accompany this, the SHR CON group showed impaired vasorelaxations compared to the WKY CON group at higher concentrations of ACh (Figure 7). This result

indicates that the SHR CON animals exhibit altered endothelium-dependant vasorelaxations with impairment of the maximal response to ACh.

Comparison within SHR and WKY treatment groups

As hypothesized there was an increase in endothelium-dependant vasorelaxation observed within the treatment groups of the SHR animals. The SHR HIGH showed a greatly improved endothelium-dependant vasorelaxation to a maximal dose of ACh compared to SHR CON ($p<.0001$) indicating a treatment effect of resveratrol (Table 3A) (Figure 8). This result differs from a previous study performed by our laboratory, which indicated improvement at both low and high doses of resveratrol. However differences in the blood vessel used for vasomotor assessment may explain these differing results in the vessels` response to ACh.

The WKY HIGH, LOW and CON groups exhibited no statistical differences in vasorelaxation when compared with eachother with respect to maximal response, point by point analysis, and AUC analysis. (Table 3A) (Figure 8)

<u>Endothelium-Independent Vasorelaxations - Sodium Nitroprusside Dose-Response Curves</u>

Comparison between SHR and WKY control group responses

There were no differences in maximal endothelium-independent vasorelaxations to Sodium Nitroprusside between the SHR and WKY control groups. With the SHR CON and WKY CON groups demonstrating over 100% endothelium-independent vasorelaxation to a maximal dose of Sodium Nitroprusside (Table 3A). Likewise there were no differences between SHR CON and WKY CON groups with respect to AUC and point by point

comparisons of each SNP concentration. These data indicate that there were no differences in NO sensitivity or response to NO between the two strains. (Figure 9)

Comparison within SHR and WKY treatment groups

There were no differences in maximal vasorelaxations to Sodium Nitroprusside between treatment groups within either SHR or WKY groups. Likewise there were no differences found between treatment groups within either SHR or WKY groups with respect to AUC and point by point comparisons. Indicating there were no differences in NO sensitivity following chronic dietary resveratrol treatment. (Table 3A) (Figure 9)

Endothelium-Dependant Vasorelaxation With COX 1 and 2 Inhibition - ACh Dose-Response Curve Following INDO Incubation

Comparison between SHR and WKY control group responses

Unlike the large difference present in the absence of INDO, there were no differences in maximal vasorelaxations to ACh between the SHR and WKY control groups following incubation with INDO (Figure 10). Likewise there were no differences observed with respect to AUC (Table 3A). Though there was no difference seen between the control groups of each strain there was a difference between the entire strains following comparison of AUC. Point-by-point analysis of control groups illustrated differences between the stains. The SHR CON group showed greater relaxations to the smaller concentrations of ACh (Figure 10).

Comparison within SHR and WKY treatment groups

There were no significant differences observed in the maximal responses to ACh in the presence of INDO between the three treatment groups of the SHR (Figure 9). Likewise there were no differences with respect to AUC analysis between the SHR groups. Point by point analysis illustrated differences between both SHR HIGH and LOW groups compared to the SHR CON. The SHR HIGH group illustrates greater vasorelaxation to multiple concentrations of ACh (Figure 10). The SHR LOW group illustrates a reduction in vasorelaxations from at a number of concentrations of ACh. The WKY treatment groups illustrated no differences in terms of maximal responses, AUC or point by point analysis. (Figure 10)

AICAR Mediated Vasorelaxation

Comparison between SHR and WKY control group responses

The WKY CON group had a greater maximal relaxation to AICAR compared to the SHR CON group, but this difference was not statistically significant (Table 3A). Point by point analysis indicated a difference between the WKY and SHR responses to AICAR. Treatment with 10^{-4} Log M AICAR the SHR CON group elicited less of a contraction than the WKY CON ($p=0.0061$). (Figure 11)

Comparison within SHR and WKY treatment groups

SHR HIGH and SHR LOW groups have an increased maximal vasorelaxation to AICAR compared to the SHR CON group, with the difference between SHR HIGH and CON groups reaching statistical significance. Likewise both SHR HIGH and SHR LOW show a

significant difference in response to 10^{-4} Log M AICAR, with both groups displaying vasorelaxation compared to the contraction demonstrated by the SHR CON group. (Figure 11)

There were no differences were observed within the WKY treated groups, indicating resveratrol has no effect on AICAR mediated dilation in the WKY stain.

Resveratrol Mediated Vasorelaxation

Comparison between SHR and WKY control group responses

No differences were observed between SHR CON and WKY CON groups with respect to vasorelaxation responses to acute resveratrol in vitro (Table 3A) (Figure 12).

Comparison within SHR and WKY treatment groups

The SHR HIGH group displayed greater vasorelaxation throughout the resveratrol dose-response curve compared to the SHR CON. This only reached significance at a concentration of 10^{-5} ($p=.0199$).

The WKY HIGH and WKY LOW groups reached a maximal relaxation that was much greater than that of the WKY CON group ($p<.0001$). The WKY HIGH group illustrated significantly greater vasorelaxation than the CON group at all concentrations of resveratrol, whereas the WKY LOW group had greater relaxations than the CON group only at the high concentrations of resveratrol. (Figure 12)

Vasocontractions:

Endothelium-Dependant Vasocontractions – ACh Dose Response Curve

Comparison between SHR and WKY control group responses

As anticipated there were differences in endothelium-dependant contraction between the SHR CON and WKY CON groups. The SHR CON had markedly larger maximal contractions when compared to WKY CON (Table 3B). This result confirms the SHR dependant increase in endothelium-dependant contractions that has been illustrated in the literature (12). To accompany the increase in maximal contraction the SHR CON endothelium-dependant contractions are initiated at a lower concentration of ACh when compared to the WKY CON (Figure 13). As expected, the SHR CON group displayed a greater AUC when compared to the normotensive controls ($p=0.0021$). These results indicate that the SHR have a larger endothelium-dependant contraction response.

Comparison within SHR and WKY treatment groups

High resveratrol treatment reduced the maximal endothelium-dependant contraction of the SHR compared to SHR CON (Table 3B). This was accompanied by a significant decrease in endothelium-dependant contractions in response to ACh from $10^{-4.5}$ to 10^{-6} Log M in the SHR HIGH compare to the SHR CON (Figure 14). This indicates that High resveratrol treatment affects endothelium-dependant contractions stimulated by varying concentrations of ACh and not just contractions stimulated by a maximal dose of ACh. Though there were differences seen in both maximal contractions and point by point analysis, the area under the curve was not significantly different in the SHR HIGH group compared to the SHR CON.

Though improvements were seen in the SHR HIGH group, the SHR LOW group did not have similar reductions in endothelium-dependant contractions as had been hypothesized. There were also no differences seen in the response of the treated WKY groups compared to the WKY CON.

Thromboxane-Prostanoid Receptor Sensitivity – U46619 Dose-Response Curve

Comparison between SHR and WKY control group responses

There were no differences found between SHR CON and WKY CON groups in terms of maximal contraction, area under the curve and point by point analysis of the U46619 dose-response curve as shown in table 3B. This indicates that the sensitivity of thromboxane-prostanoid receptor is unaltered between SHR and WKY animals.

Comparison within SHR and WKY treatment groups

There were no differences found between the resveratrol treatment groups of each strain in terms of maximal contraction, area under the curve and point by point analysis of the U46619 dose-response curve. These results indicate that chronic dietary resveratrol treatment has no effect on sensitivity of the thromboxane-prostanoid receptor. (Figure 15)

Hydrogen Peroxide

Comparison between SHR and WKY control group responses

There were no differences found between SHR CON and WKY CON in terms of maximal contraction, area under the curve and point by point analysis of the H_2O_2 dose-

response curve (Table 3B). This indicates that H_2O_2 has a similar effect on both SHR and WKY.

Comparison within SHR and WKY treatment groups

There were no differences seen within the SHR groups. This seems to be due to the large variability in the responses to hydrogen peroxide as can be seen in figure 14. There were differences seen in the WKY groups with the WKY HIGH having greater contractions at a number of concentrations of H_2O_2 compare to WKY CON (Figure16). Unlike the WKY HIGH group, there were no differences between the WKY LOW and the WKY CON groups.

Phenylephrine Dose-Response Curve

Comparison between SHR and WKY control group responses

The maximal contraction to PE is reduced in the SHR CON compared to the WKY CON ($p=0.0238$) (Table 3B), confirming previous results from this laboratory (12). The reduced contractions to PE stimulation were observed at multiple concentrations throughout the dose-response (Figure 17). Though there were multiple differences seen in the point by point analysis there was no difference in the AUC between the two strains.

Comparison within SHR and WKY treatment groups

Chronic dietary resveratrol treatment at a high dose increased the maximal response to PE in the SHR. The SHR HIGH groups had an increased maximal contraction to PE compared to the SHR CON ($p<0.0001$). This indicated that the maximal response to PE was altered following resveratrol treatment at a high dose. Following point by point analysis the

SHR HIGH group had increased contraction to PE at a number of different concentrations (Figure 17). Though there were differences seen at a number of different concentrations of PE in the SHR the AUC data was not significantly different.

There were no differences between the WKY groups in terms of maximal response, point by point analysis, and area under the curve analysis. These results indicate that resveratrol treatment does not affect the response of the WKY animals to PE.

Biochemical analysis:

6 keto PGF 1α Competitive EIA Assay – Prostacyclin Production

As expected there was an increase in the prostacyclin production in the SHR CON group compared to the WKY CON group. This increase in production in the SHRs CCA coincides with results from previously shown in this laboratory in both the CCA and the thoracic aorta (Jeffery in press) (67).

Treatment with resveratrol at a high dose blunted the SHR-dependant increase in prostacyclin production; however the resveratrol treatment at a lower dose had no effect. The SHR HIGH group prostacyclin production was 189 $\pm$ 30 pg/ml compared to the SHR CON production of 519 $\pm$ 93 pg/ml. This reduction in SHR HIGH prostacyclin production resulted in a similar production as the WKY CON and corresponded with a reduction in endothelium-dependant contraction (Figure 18).

Vascular protein expression:

Western Blotting - COX 1 Protein Expression

As previously characterized in the literature (38,68) and shown by this laboratory in previous studies (12) the SHR CON had a greater COX 1 protein content when compared to WKY CON, which expressed 1.9 ± 0.26 and 1 ± 0.10 (arbitrary normalized units) (p=0.834) respectively. Though this difference is not a significant difference, there is an SHR dependant increase in COX 1 protein content. There were no differences seen between the resveratrol treated groups of either strain (Figure 19).

Western Blotting - eNOS Expression

The eNOS protein content of the SHR CON was elevated compared to WKY CON as had been shown in the CCA previously (12). The SHR CON expressed eNOS at a level 3.11 ± 0.6 compared to the WKY CON 1 ± 0.3 (arbitrary normalized units) (p=.05). There were no differences seen in eNOS protein expression in response to treatment with resveratrol in either of the SHR and WKY strains (Figure 20).

Table 1: Animal and Consumption Parameters

Strain		**WKY**			**SHR**	
Resveratrol Group	CON	LOW	HIGH	CON	LOW	HIGH
Water Consumption (ml)$^{\delta}$	37±1†	35±0.85†	34±0.85†	45±1.8†	48±2.2†	45±1.9†
Resveratrol Consumption (mg)	0	0.021	2.1	0	0.030	2.78
Food Consumption (g) $^{\delta}$	487.4±11†	491.6±8	500.6±10†	542.95±12†	519.7±6.3	550.2±11†
Final Body Weight (g) $^{\delta}$	312.8±10	313.6±5.2	321.44±4.5	345.5±5	329.4±2.8	343.2±11
Body Weight Change (g)	22.7±7	14.7±5.5	17.9±4.2	19.2±4.6	20.8±2	12±5.2

Table 1: Physical and consumption parameters: δ represents differences between strains, † represents differences within treatment group.. Values are mean ± s.e.m.: significance levels p<0.05.

Table 2: Hemodynamic Parameters

Strain	**WKY**			**SHR**		
Resveratrol Group	CON	LOW	HIGH	CON	LOW	HIGH
Heart rate[#]	264±13[†]	257±15[†]	260±11[†]	382± 13[†]	375± 14[†]	359±14[†]
CCA Blood Flow, ml/min						
Maximum[δ]	19.0±1.7	15.5±1.9	22.0±1.7[†]	11.6±1.7	9.8±1.7	11.4±1.7[†]
Minimum	1.90±0.58	1.58±0.63	1.88±0.58	0.62±0.58	0.87±0.58	-0.26±0.58
Mean[δ]	5.0±0.36[†]	4.4 ±0.365	5.5±0.36[†]	3.8±0.36[†]	3.4±0.36	2.9±0.36[†]
CCA Blood Pressure, mmHg						
Systolic[δ]	89±4[†]	93±4[†]	91±3[†]	215±4[†]	207±4[†]	173±4[†θ]
Diastolic[δ]	67±4[†]	72±5[†]	69±4[†]	162±4[†]	157±5[†]	131±5[†θ]
Mean[δ]	78±7[†]	82±8[†]	80±6[†]	176±6[†]	171±7[†]	159±6[†θ]
CCA Vascular Conductance, mmHg (µl/min)						
Mean[δ]	64.8±6[†]	59.8±4[†]	62.9±9[†]	23.2±4[†]	21.3±0.26[†]	23.5±2.8[†]

Table 2: Hemodynamic parameters: δ represents differences between strains, † represents differences within treatment group, and θ represents differences within strain compared to control group. Values are mean ± s.e.m.: significance levels p<0.05.

Table 3A: Vasomotor Assessment Parameters

Drug Condition		WKY			SHR		
		CON	LOW	HIGH	CON	LOW	HIGH
					Vasorelaxations		
Endothelium dependant vasorelaxations (ACh)	MR (%)	86.2±6%†	92.8±5%†	90.5±4%	53.6±6%†	50.2±9%†	99.4±5%$^{\theta}$
	AUC	224±18	195±17	266±12	208±21	218±14	235±15
Endothelium independent vasorelaxations (SNP)	MR (%)	108±3%	NA	109 ±3%	108±5%	NA	109±2%
	AUC	454±31	NA	463±18†	460±27	NA	320±3$^{\dagger\theta}$
Endothelium dependant vasorelaxation (INDO, ACh)	MR (%)	92.5±2%	91.3±4%	92.4±6%†	94.7±13%	92.9±4%	105.1±10%†
	AUC$^{\delta}$	210±13	226±16	235±22	293±30	213±15	325±33
Resveratrol relaxation	MR (%)	90.5±9%	118.7±6%$^{\dagger\theta}$	113.4±9%$^{\theta}$	94.3±3%	101.2±5%†	101.6±7%
	AUC	54±7	84±10	105±24	65±7	70±7	79±17
AICAR relaxation	MR (%)	112.1±8%	114.9±6%	118.4±7%	94.5±6%	112.6±2%$^{\theta}$	116.3±5%$^{\theta}$
	AUC	141±14	154±13	134±9	124±23	167±16	190±16

Table 3A: Vasomotor assessment parameters: Response to a maximal dose (MR) expressed as a percent relaxation from pre-contraction and area under the curve (AUC) of vasorelaxation assessment. δ represents differences between strains, † represents differences within treatment group, and θ represents differences with strain compared to control group. Values are mean ± s.e.m.: significance levels p<0.05

Table 3B: Vasomotor assessment parameters

Drug Condition		WKY			SHR		
		CON	LOW	HIGH	CON	LOW	HIGH
		Vasocontractions					
Endothelium dependant contractions (ACh)	MR$^{\delta}$ (%)	30±6%†	27±7%†	25±6%†	68±5%†	66±5%†	55±5%$^{\dagger\theta}$
	AUC$^{\delta}$	38±8†	34±9†	33±7	98±14†	95±14†	62±10
TP receptor sensitivity (U46619)	MR (%)	145±18%	NA	140±4%	138±10%	NA	147±4%
	AUC	327±49	NA	313±15	297±14	NA	320±3
Phenylephrine	MR$^{\delta}$ (%)	79±15%†	NA	86±3%	60±6%†	NA	91±11%$^{\theta}$
	AUC	313±69	NA	322±15	192±20	NA	304±47
Hydrogen Peroxide	MR (%)	34±12%	18±6%†	52±13%	33±6%	49±13%†	43±12%
	AUC	85±6	56±17	94±19	65±11	86±10	79±18

Table 3B: Vasomotor assessment parameters: Response to a maximal dose (MR) expressed as a percent of KCl contraction and area under the curve (AUC) of vasocontraction assessments. δ represents differences between strains, † represents differences within treatment group, and θ represents differences with strain compared to control group. Values are mean ± s.e.m.: significance levels $p<0.05$

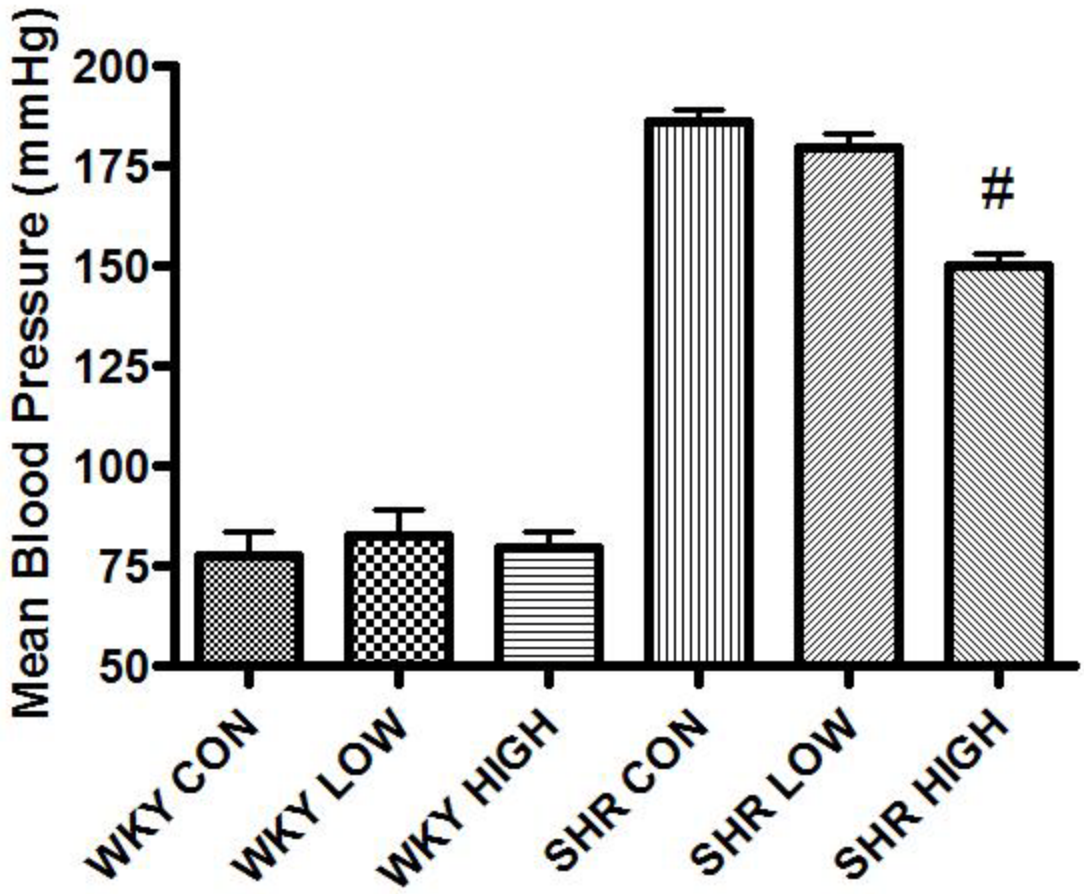

Figure 6: mean arterial blood pressure: Significant differences were seen between the CON of SHR and WKY. Differences were also seen between SHR HIGH and SHR CON. Values are mean ± s.e.m.: significance levels α= p<0.05, #=p<0.001.

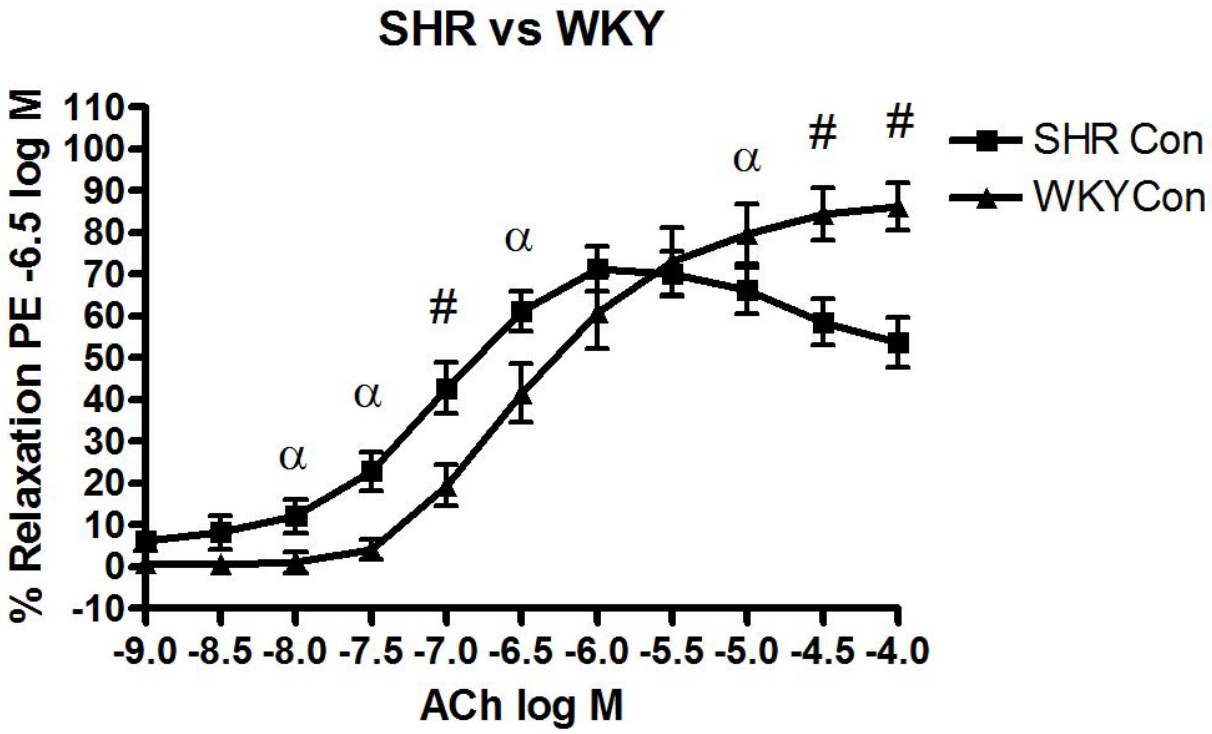

Figure 7: Endothelium-dependant vasorelaxation to ACh expressed as a percent relaxation from PE-precontraction. SHR Con compared to WKY Con, N=8 per group , significant differences seen between SHR High and Con groups. Values are means ± s.e.m.: significance levels α= p<0.05, #=p<0.001.

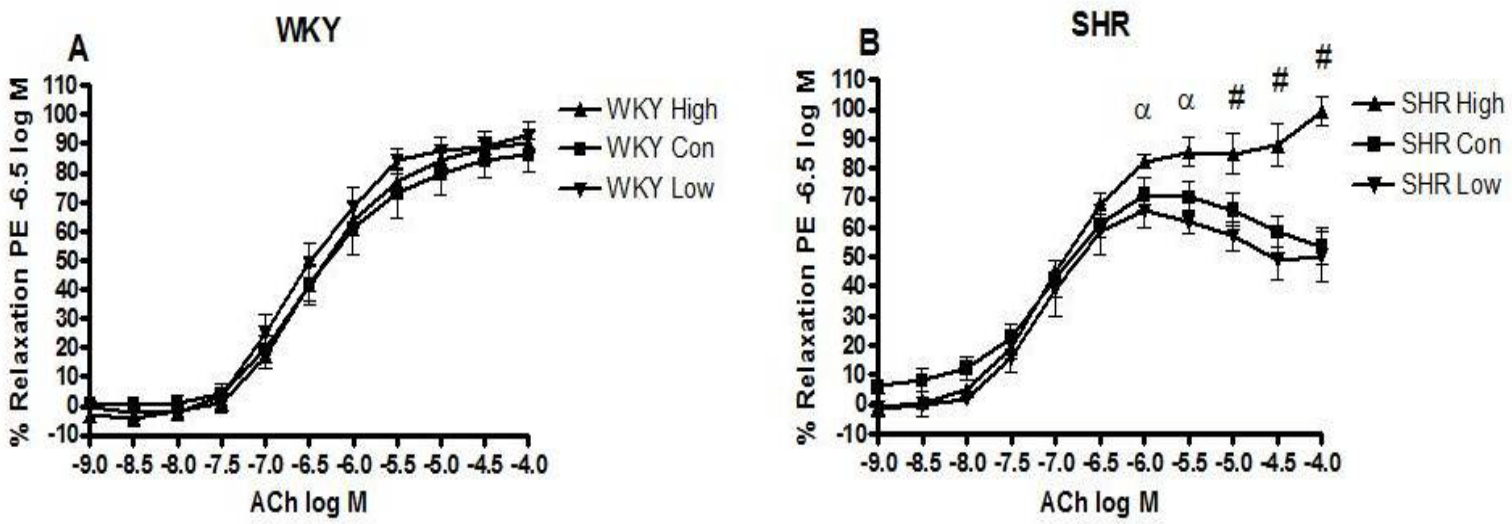

Figure 8: Endothelium-dependant vasorelaxation to ACh expressed as a percent relaxation from PE-precontraction. WKY (A) and SHR (B), N=8 per group , significant differences seen between SHR High and Con groups. Values are means ± s.e.m.: significance levels α= p<0.05, #=p<0.001.

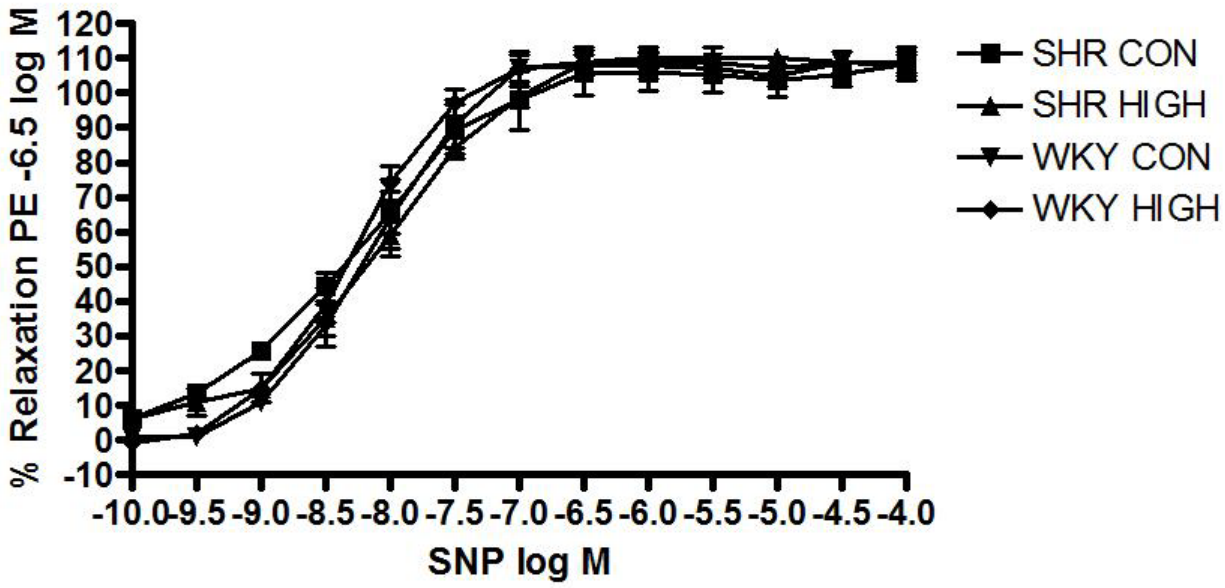

Figure 9: Endothelium-independent vasorelaxation to Sodium Nitroprusside expressed as a percent relaxation from PE precontraction. NO differences seen between groups. Values are mean ± s.e.m.: significance levels α= p<0.05, #=p<0.001.

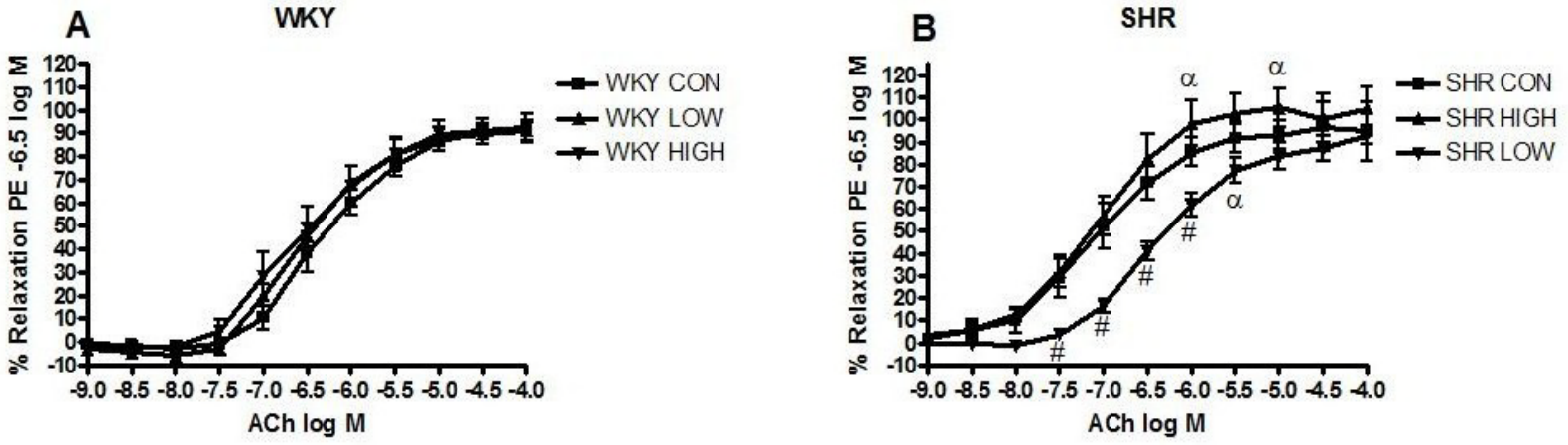

Figure 10: Endothelium-dependant vasorelaxation to ACh following the inhibition of Cycloxygenase 1 and 2 expressed as a percent relaxation from PE-precontraction. WKY(A) and SHR(B), N=5, Differences seen between SHR CON and SHR HIGH groups. Values are means ± s.e.m.: significance levels α= p<0.05, #=p<0.001.

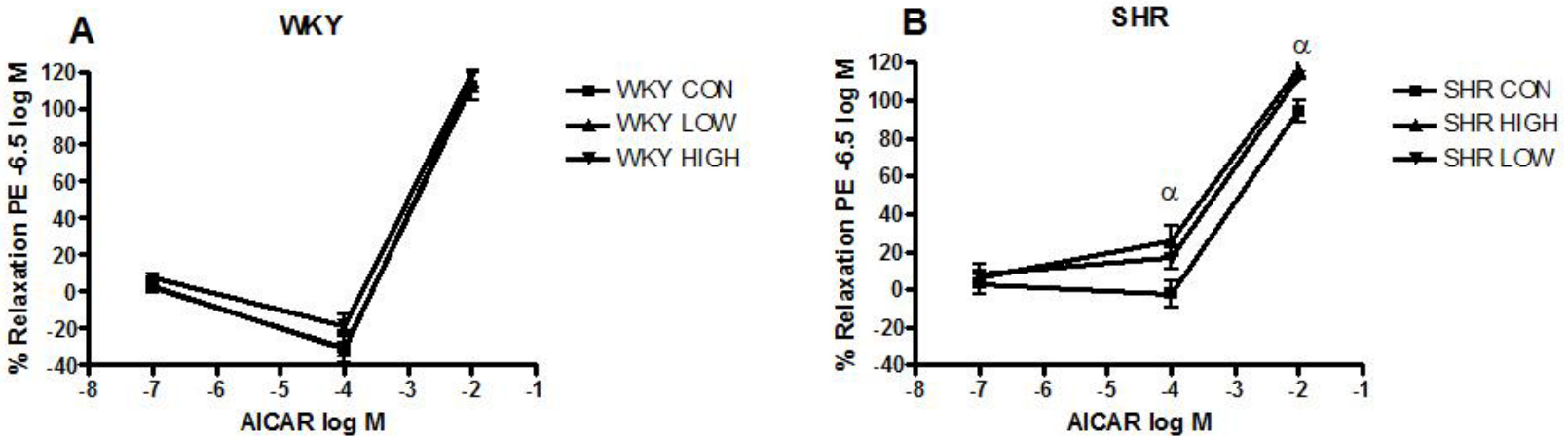

Figure 11: Vasorelaxations to AICAR expressed as a percent relaxation from PE-precontraction. WKY (A) and SHR (B), N=5, Differences seen between the SHR CON and both LOW and HIGH groups. Values are means ± s.e.m.: significance levels α= p<0.05, #=p<0.001.

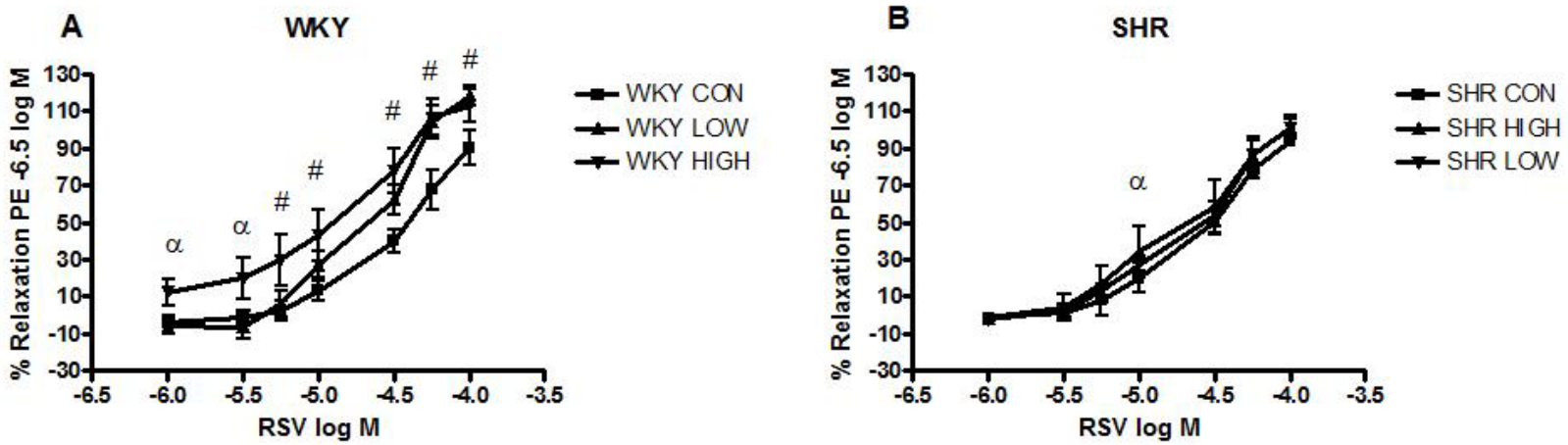

Figure 12: Vasorelaxation to resveratrol expressed as a percent relaxation from PE-precontraction. WKY(A) and SHR(B), N=5, significant differences were seen between WKY CON and both HIGH and LOW groups. Differences were also seen between SHR CON and HIGH groups. Values are means ± s.e.m.: significance levels α= p<0.05, #=p<0.001.

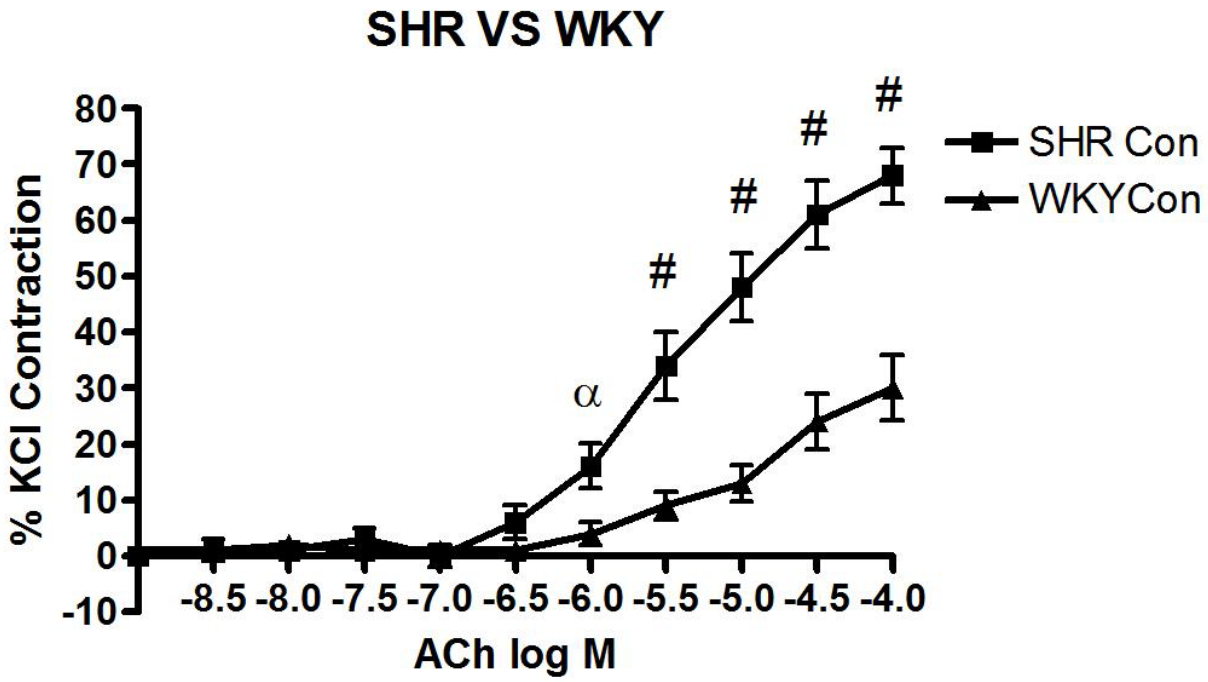

Figure 13: Endothelium-dependant contactions to ACh in quiescent rings of the CCA following L NAME incubation expressed as a percent KCl contraction. SHR Con compared to WKY Con, N=8, Significant differences were seen between the SHR CON and HIGH groups. Values are means ± s.e.m.: significance levels α= p<0.05, #=p<0.001.

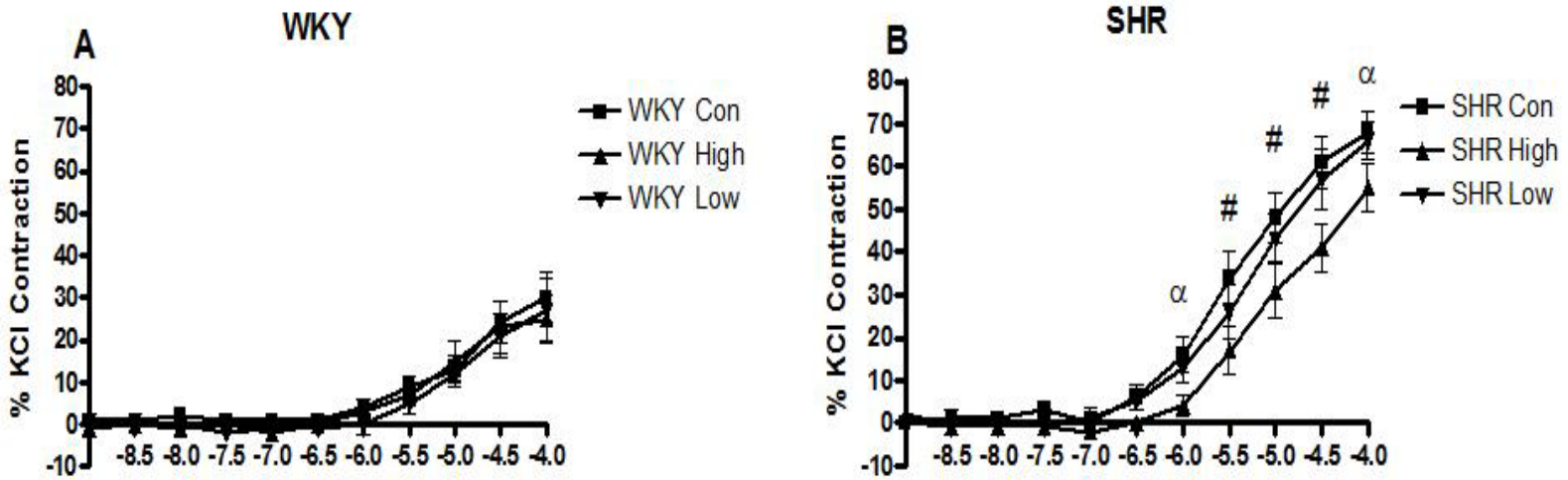

Figure 14: Endothelium-dependant contactions to ACh in quiescent rings of the CCA following L NAME incubation expressed as a percent KCl contraction. WKY(A) and SHR(B), N=8, Significant differences were seen between the SHR CON and HIGH groups. Values are means + s.e.m.: significance levels α= p<0.05, #=p<0.001.

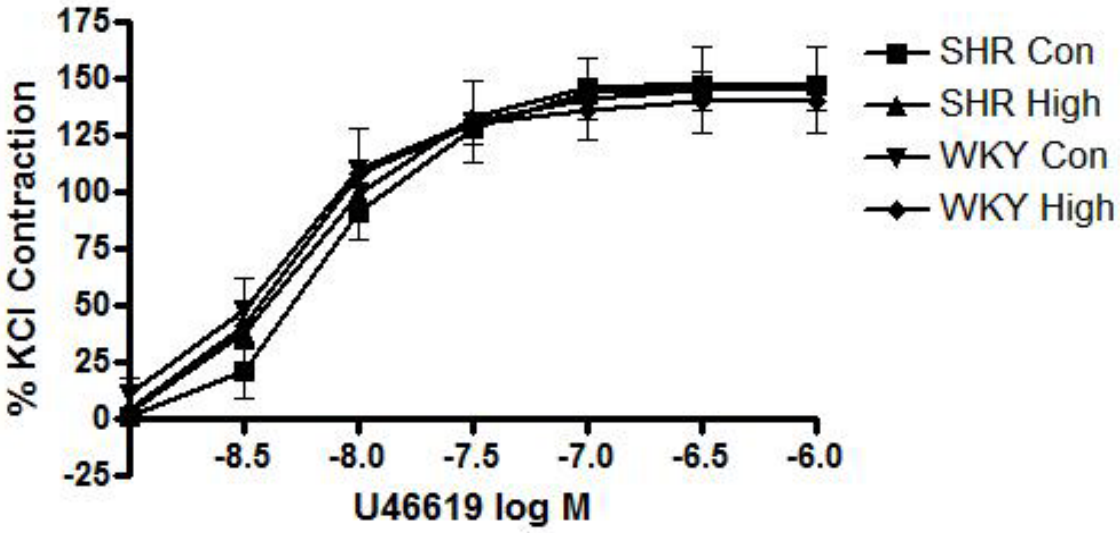

Figure 15: Endothelium-independent contractions to TP receptor agonist U46619 in quiescent rings of the CCA expressed as a percent KCl contraction. N =5. Values are means ± s.e.m.: significance levels α= p<0.05, #=p<0.001.

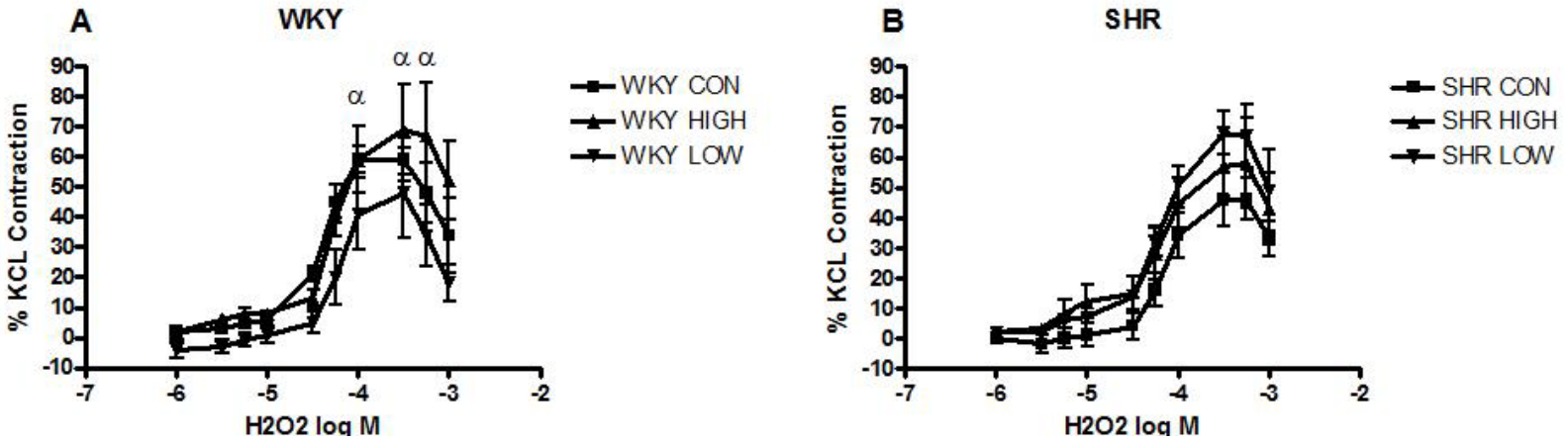

Figure 16: Contractions to hydrogen peroxide in quiescent ring of the CCA following incubation with L NAME expressed as a percent of KCl contraction. WKY(A) and SHR(B), N=5. Significant differences seen between the WKY CON and HIGH groups. Values are means ± s.e.m.: significance levels α= p<0.05, #=p<0.001.

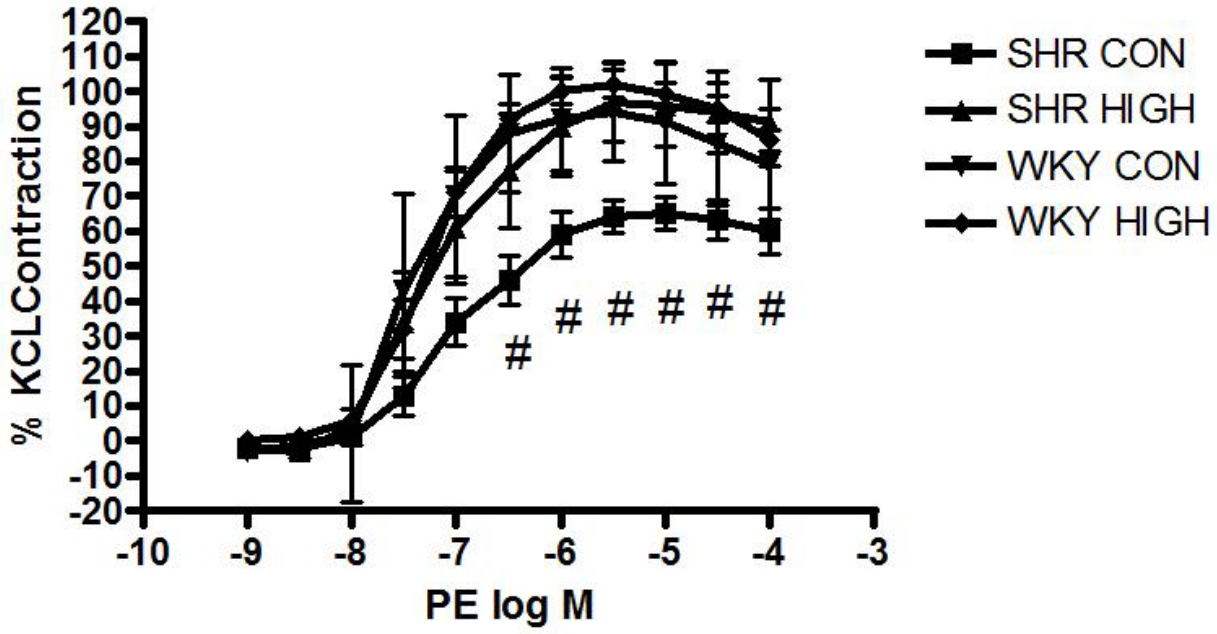

Figure 17: Contraction to Phenylephrine in quiescent rings of the CCA expressed as a percent of KCl contraction. N=5.Significant differences seen in the SHR CON contractions compared to SHR HIGH. Values are means ± s.e.m.: significance levels α= p<0.05, #=p<0.001.

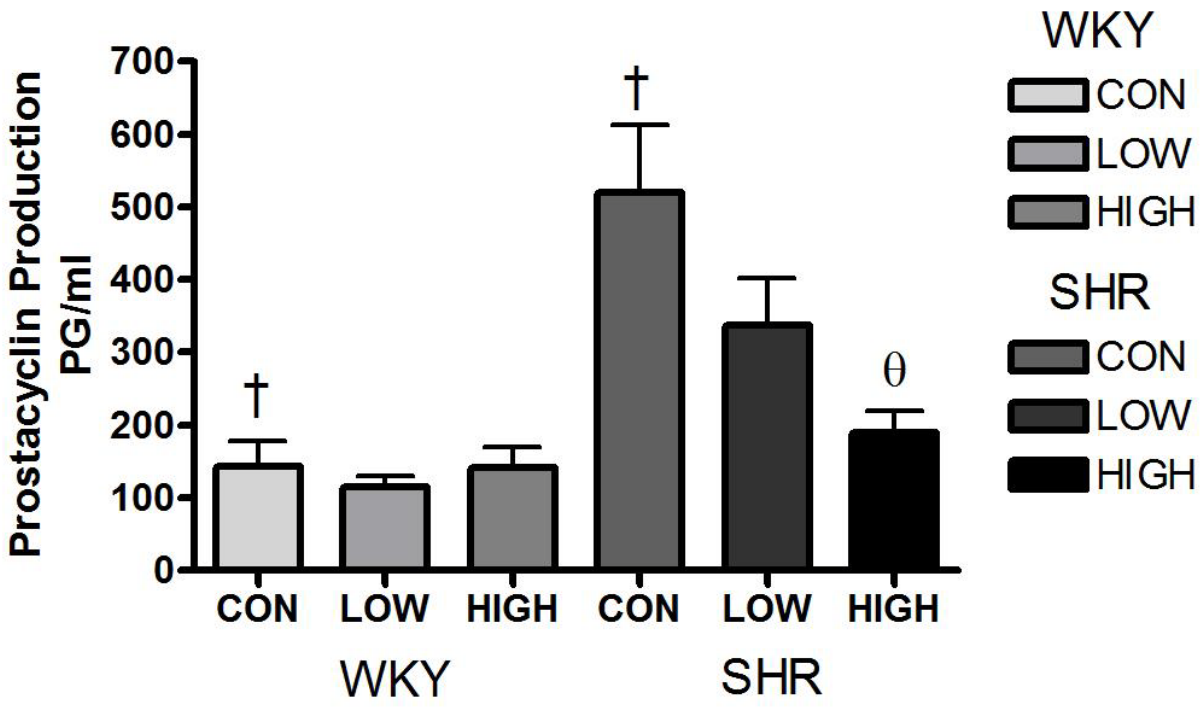

Figure 18: Prostacyclin production in CCA rings stimulated with ACh. N=7. † represents differences within treatment group, and θ represents differences within strain compared to control group. Values are means ± s.e.m.: significance levels $p<0.05$

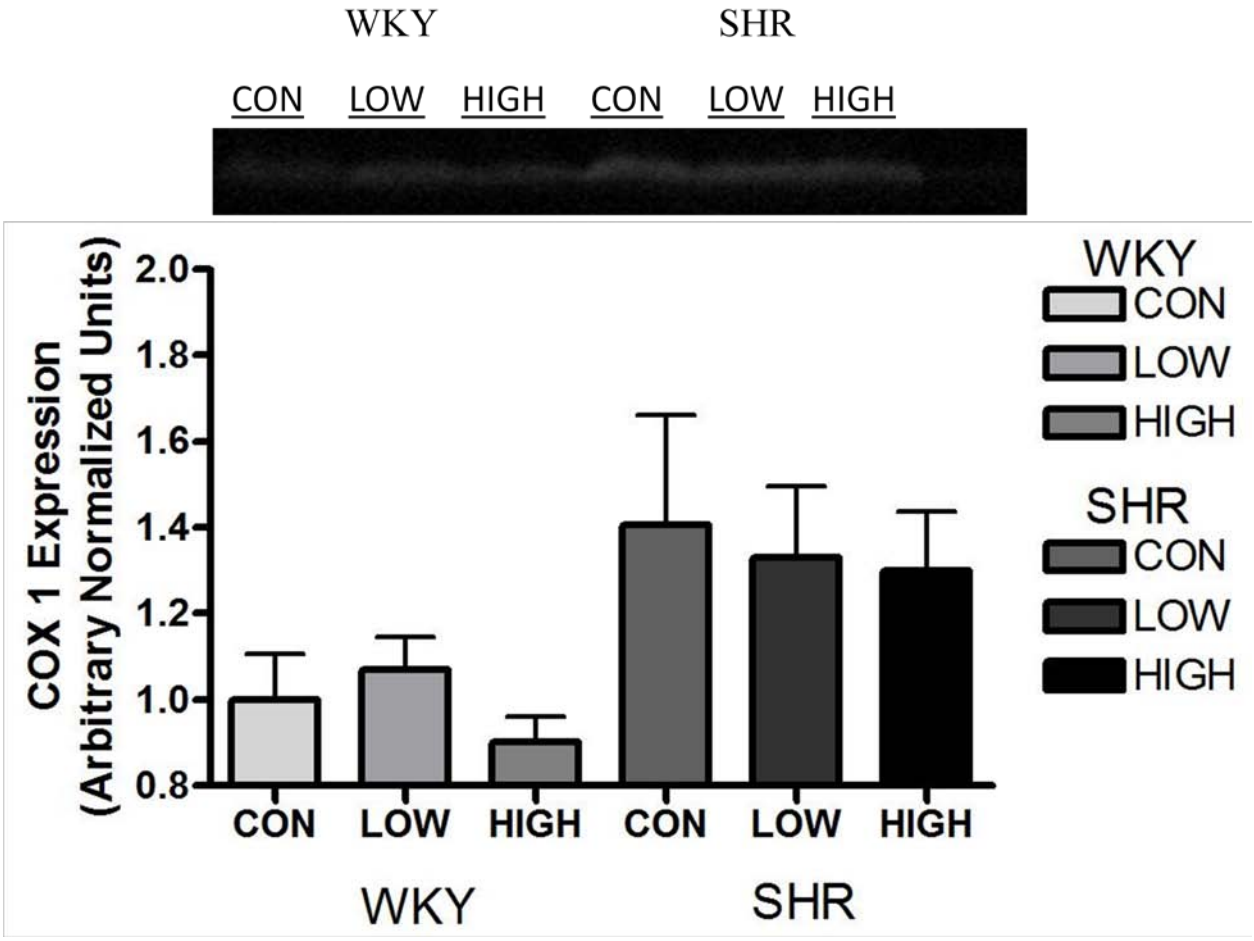

Figure 19: Protein values of COX 1 normalized to WKY CON group. N= 4 per group. Values are means ± s.e.m.:

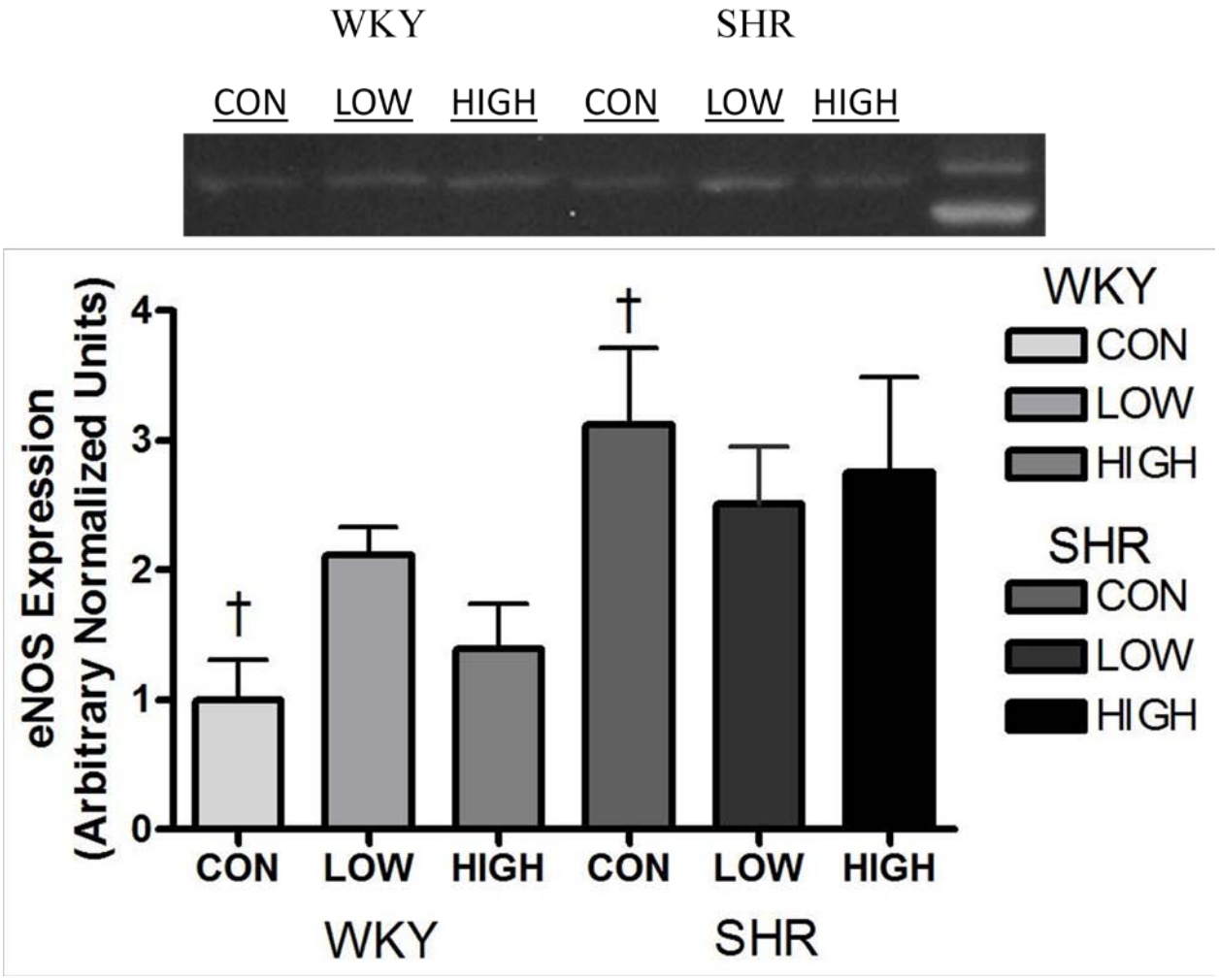

Figure 20: Protein values of eNOS normalized to WKY CON group, N= 4 per group. † represents differences within treatment grou.p Values are means ± s.e.m.: significance levels $p<0.05$.

Discussion:

The purpose of this study was to investigate the effects of chronic dietary resveratrol treatment on endothelium-dependant control of vasomotor function in the CCA, and to elucidate the differences in hypertensive and normotensive animal models. The main findings of this study are:

1. Resveratrol treatment at a high dose improves endothelium-dependant vasorelaxation to ACh in the SHR; however a low dose of resveratrol does not have the same effect.
2. Resveratrol treatment at a high dose reduces the endothelium-dependant contraction to ACh in the SHR; however a low dose of resveratrol does not have the same effect.
3. Resveratrol treatment at a high dose reduces PGI_2 production of the CCA of the SHR. Treatment at a low dose causes only a small reduction in the SHR PGI_2 production.
4. Resveratrol treatment had no effect on the sensitivity of the thromboxane-prostanoid receptor in the SHR or WKY.
5. SHR exhibit markedly increased blood pressure and an altered blood flow profile compared to WKY. Resveratrol treatment at a high dose reduces the blood pressure of the SHR, but does not affect the blood flow profile.
6. Resveratrol treatment also had minor effects on AICAR mediated relaxation, resveratrol mediated relaxation and phenylephrine contraction in both WKY and SHR.

Animal Characteristics:

The SHR control group had a greater average final body weight of 33 grams when compared to the WKY ($p=0.0556$) however, this difference was not significant. The lack of significance between these two control groups could possibly be due to the large age range at which the animals were sacrificed ranging from 21 weeks and 3 days to 24 weeks of age. However, there was a difference in final body weight following comparison of the entire stains, this coincides with previous studies conducted by this laboratory which have conclusively shown an increase in body weight in the SHR when compared to the WKY(12,32).

Resveratrol treatment had no effect on the final body weight of either strain which is supported by the previous chronic dietary resveratrol treatment study conducted by Rush et al. (52). Table 1 illustrates that both the SHR and WKY groups follow a similar trend, indicating that resveratrol treatment had no effect on the final body weight of the animals.

There was a significant difference between strains in the total amount of food consumed throughout the 4 week treatment period (Table 1). There were no differences seen in the amount of food consumption between the control groups and the resveratrol treated groups of the respective strains indicating that resveratrol did not have an effect on the appetite of the animals.

As had been demonstrated in previous chronic dietary resveratrol studies, there were significant differences in the daily water consumption of the animals with the SHR consuming considerably more water in comparison to the WKY (Table 1). The resveratrol treated groups

consumed similar amounts of treated water as the control groups indicating that the resveratrol treatment did not affect the amount of water consumed by the animals.

Consistent water consumption in the resveratrol treated groups in comparison to control groups allowed for resveratrol treatment to achieve levels that were calculated in the methods section (Table 1). These dosages of resveratrol were then weight adjusted to a weight of 70 kg. Assuming 0.333 kg for the average rat, the low group would have received 6.2 mg following weight adjustment to 70 kg, which is a level of resveratrol consumption in the realm of moderate red wine consumption (64,65). The high group would have received 583.8 mg of resveratrol following weight adjustment to 70 kg. This 583.8 mg dosage is equivalent to pharmacological resveratrol supplementation currently available (66).

Due to the difference in water consumption between the two strains, SHR and WKY animals will have received slightly different levels of resveratrol during the treatment (Table 1).

Endothelium-Dependant Vasorelaxation:

SHR display impaired endothelium-mediated vasorelaxation to ACh in phenylephrine pre-contracted vessels (4). Vessels from the SHR have a robust relaxation to small concentrations of ACh but this relaxation is abolished by a re-contraction with the administration of higher concentrations. The re-contraction of the CCA has been demonstrated to occur due to alterations in both NO and PGI_2 signaling from the endothelium in the SHR but not the WKY (2,4,5,12). The impaired endothelium dependant vasorelaxation in the CCA has been confirmed by the current study with the SHR control group demonstrating a reduction in relaxation from phenylephrine pre-contraction (Figure 7).

Resveratrol treatment at both a low and high dosage was hypothesized to reverse the reduction in endothelium-dependant vasorelaxations through actions on the NO pathway. However, unlike the previous chronic dietary resveratrol treatment study conducted by Rush et al., treatment at a low dose of resveratrol did not provide improvements in the response to ACh (52), (Table 3A) (Figure 8). This result indicates that treatment with a low dose of resveratrol does not affect the impaired relaxation seen in the CCA. The discrepancy between the previous chronic dietary resveratrol study conducted by Rush et al. and the present study may be due to the use of a different blood vessel. Though both are conduit arteries, the thoracic aorta used by Rush et al. may have been more susceptible to resveratrol treatment than the CCA used in the present study (52). The Rush et al. study also used fewer concentrations of ACh during the endothelium-dependant relaxation experiments and was unable to reproduce the re-contraction in SHR at higher dosages of ACh. This re-contraction during ACh dose-response curve in SHR has been established in the literature and was able to be reproduced in the current study (4).

Resveratrol treatment at a high dose illustrated improvements in the ACh mediated relaxations with the SHR high group, reaching a relaxation of almost 100% following a maximal dose of ACh (Table 3A). The resveratrol treatment provided the SHR high group with a similar relaxation to the normotensive WKY (Figure 8). This improvement in endothelium-dependant relaxation may have been partially caused by a number of alterations throughout the NO signaling pathway, including improved VSM's sensitivity to NO, increased NO bioavailability through decreased NO scavenging, and an increase in NO production mediated through improved eNOS activity or increased eNOS expression.

Results from the current study using Sodium Nitroprusside, a NO donor, illustrated that there were no differences in the VSM sensitivity to NO between SHR and WKY following resveratrol treatment (Figure 9). This finding is supported by the previous work by Rush et al. indicating that the SHR had no alteration in the relaxation to Sodium Nitroprusside following chronic dietary resveratrol treatment (52). These results indicate that treatment with resveratrol has no effect on the NO sensitivity of the VSM which means that the improvements in endothelium-dependant relaxations, which occurred following resveratrol treatment, cannot be attributed to changes in the VSM sensitivity to NO and must have resulted from other alterations in the NO pathway.

ACh mediated relaxation following COX inhibition with indomethacin was used to functionally assess the alteration in NO bioavailability. The SHR high group has increased relaxations throughout the ACh dose-response curve; however this only reached significance at two concentrations. Low resveratrol treatment appeared to have the opposite effect on NO bioavailability compared to the high resveratrol treatment, resulting in reduced relaxations throughout the ACh dose-response curve (Figure 10). This result points to low resveratrol treatment reducing NO bioavailability or having a complex interaction with the PGI_2 pathway in an ACH-dose-dependent manner. This is perplexing considering the previous findings which illustrate low resveratrol treatment to have a positive effect on endothelium-mediated relaxation (52). A low bioavailability of resveratrol at the CCA could explain the lack of improvements in endothelium-dependant relaxation observed in the SHR low group when compared to the SHR high group. Though the current and previous resveratrol study conducted by the Rush laboratory are the first to our knowledge to use multiple dosages of resveratrol in an in vivo resveratrol administration study examining endothelium-dependant

vasomotor response, a number of in vitro studies have illustrated a concentration dependency on resveratrol effects (61,69). The dose dependency of resveratrol's effects may explain why the SHR low group does not show an improvement in the functional measure of NO production. The current study's improvements in NO bioavailability in the SHR high group could be attributed to alterations in NO production and NO scavenging.

As anticipated, the SHR exhibit an increased eNOS expression in comparison to the WKY (Figure 20). This increased eNOS expression in the SHR has been shown in numerous studies (2,12). However, this increased expression is not necessarily associated with an increase in NO production as was shown by Bhatt et al., who have shown that eNOS uncoupling can result in a decrease production of NO (70). Resveratrol treatment had no effect on the expression of eNOS in either SHR or WKY. This result was surprising due to the fact that a number of cell culture experiments have linked resveratrol treatment to increased eNOS expression (61). Though the previous chronic dietary resveratrol study also did not show increase in eNOS expression, the alteration in the resveratrol dosage used for the present study was hypothesized to have an effect on eNOS expression. Recent studies from other laboratories have shown increases in eNOS expression using resveratrol dosages of 5mg/kg/day and above (70-72), however, studies using red wine polyphenols did not show this increase in eNOS expression (73). Despite the recent evidence that resveratrol can increase eNOS expression in vivo this study was unable to replicate these results. Alterations in NO signaling pathway, which have provided improvements in the endothelium-dependant relaxations, must be attributed to other alterations and not an increase in eNOS expression. These alterations could include increased eNOS activity and reduced scavenging of NO.

The improvements in endothelium-dependant relaxation can be partially attributed to alterations in NO signaling. Recent studies have shown that resveratrol treatment has a number of effects on the NO pathway which could lead to the increased NO bioavailability demonstrated by the current study. Both Rush et al. and Bhatt et al. have shown that resveratrol treatment alters the oxidative state of the endothelial cells in the vasculature showing a decrease in reactive oxygen species production (52,70). This reduction in reactive oxygen species leads to a decreased production of peroxynitrite, which in turn leads to an increased NO bioavailability. This is supported by Bhatt et al. who showed an increased production of NO in the SHR following resveratrol treatment. Resveratrol's improvements in NO-mediated relaxation appear to be partially mediated by a reduction in NO scavenging by reactive oxygen species.

Bhatt et al. have demonstrated that NO production in SHR is actually smaller than WKY, even though there is a greater expression of eNOS in the SHR animals (70). This decreased NO production is attributed to eNOS uncoupling, which decreases eNOS's ability to produce NO and instead leads to the production of superoxide. This is caused by a decrease in the necessary co-factors needed for eNOS to convert L-Arginine to NO. One of these co-factors is tetrahydrobiopterin which is essential for stabilization of the eNOS dimer and greatly affects its affinity for L-Arginine. Tetrahydrobiopterin is sensitive to the increase in reactive oxygen species that is seen during hypertension and this leads to a decrease in tetrahydrobiopterin. Reduction in tetrahydrobiopterin in turn results in an increase in eNOS uncoupling leading to a decreased production of NO and an increased production of superoxide (74). Chronic dietary resveratrol treatment has recently been shown by Bhatt et al. to prevent eNOS uncoupling. The decrease in eNOS uncoupling resulted in a reduction in

superoxide produced by eNOS and increased NO production (70). An increase in NO production via this mechanism could be the key contributor to improved NO-mediated relaxation to ACh observed in SHR of the current study following chronic dietary resveratrol treatment.

Alterations in endothelium-dependant contractions can also contribute to the improvements observed in endothelium-dependant relaxations. Figure 10 shows that following COX inhibition there is a remarkable improvement in the endothelium-dependant relaxation to ACh when compared to the result without COX inhibition in figure 7.

Endothelium-Dependant Vasocontraction:

As expected, the response to ACh in quiescent CCA rings following incubation with L-NMMA were much greater in the SHR controls when compared to the WKY controls (Figure 13). This increase in endothelium-dependent contractions is a hallmark of endothelial dysfunction caused by an alteration in the PGI_2 signaling pathway, which has been documented in the literature to be the main contributor to endothelium-dependant contractions (4,9,10,68,75,76) (12,32). The difference in endothelium-dependant contractions between SHR and WKY has been replicated by multiple studies and confirms that the SHR used in the present study do suffer from endothelial dysfunction (4,12,27,32,38,68,75).

As opposed to the hypothesis that resveratrol would reduce endothelium-dependant contraction at both dosages, only the SHR high resveratrol group exhibited a reduction in the endothelium-dependant contractions. The SHR low group produced similar contractions as the SHR control group (Table 3B), this result is consistent with the lack of improvement in the endothelium-dependant relaxations of the SHR low resveratrol group (Figure 8). The SHR

high group displayed a reduction in the endothelium-dependant contractions (Figure 14), indicating that resveratrol treatment has an effect on the PGI_2 pathway.

The results from the current study expand upon previous, unpublished findings from an *acute* incubation resveratrol treatment study conducted in this laboratory, which demonstrated diminished endothelium-dependant contraction in the SHR (Jeffery in press), and now demonstrated similar findings in a chronic dietary treatment model. Previous research by Kane et al. has illustrated that chronic dietary treatment using wine polyphenols at 150mg/kg/day produced a reduction in endothelium-dependant contractions in an Angiotensin II rodent model of hypertension, which supports the current findings (77).

While the reduction in endothelium-dependant contractions is able to partially explain the improvements seen in the endothelium-dependant relaxations it is evident that the reduction cannot fully explain the improved endothelium-dependant relaxations, indicating that the NO component discussed earlier had a partial role in improved relaxation (Figure 8,14). The reduction in the endothelium-dependant contraction could be caused by a number of alterations in the PGI_2 pathway which include an alteration in VSM PGI_2 sensitivity, a decrease in PGI_2 production which could be caused by an alteration in arachidonic acid production, COX activity, COX expression or prostacyclin synthase expression all of which will be discussed.

The thromboxane-prostanoid receptor is the receptor stimulated by PGI_2 during the signaling cascade of endothelium-dependant contractions (68). This receptor produces contraction of the VSM and when selectively inhibited, abolishes the endothelium-dependant contractions. The sensitivity of the thromboxane-prostanoid receptor to the agonist U46619

was not different between the SHR and WKY CCA following treatment with resveratrol (Figure 15). This result indicates that the reduced endothelium-dependant contractions following resveratrol treatment are not mediated through an alteration in the thromboxane-prostanoid receptor sensitivity (Figure 15) (Table 3 B). Interestingly, the thromboxane-prostanoid receptor sensitivity to U46619 and other agonists has been reported to be different between WKY and SHR, the SHR exhibiting hypersensitivity to thromboxane-prostanoid receptor agonists. However, these results were seen in the thoracic aorta and not in the CCA, which may help to explain the discrepancy (38).

As expected, PGI_2 production was significantly increased in the SHR control group compared to the WKY (Figure 18). This SHR dependant increase in PGI_2 production has been documented throughout the literature and is thought to be a key contributor to the increase in endothelium-dependant contractions and progression of endothelial dysfunction (68). Resveratrol treatment produced a reduction in the SHR dependant increase in PGI_2 production. The SHR high group illustrated the greatest reduction in the PGI_2 production as can be seen in figure 18 with production levels reaching those similar to the WKY control group. The SHR low group demonstrated a reduction in PGI_2 production but this was not significantly different compared to the SHR control group, and did not result in a decrease in endothelium-dependant contractions (Figure 14). The reduction in PGI_2 production could have been mediated through a reduction in arachidonic acid, an inhibition of COX 1, a reduction in the COX 1 expression or possibly a reduction in prostacyclin synthase expression. Resveratrol has been shown to inhibit PGI_2 production as was shown in the previous *acute* incubation resveratrol study (Jeffery in press) and documented in the literature (49). This reduction in PGI_2 production is a key contributor to the positive effects on

endothelium-dependant vasomotor function that are seen following chronic dietary resveratrol treatment.

As expected there was an increase in COX 1 expression in the SHR control group when compared to the WKY control group. Though this increase was not significant it is supported by results from previous studies showing an SHR dependant increase in COX 1 expression (11,12,75). This increase in COX 1 expression is likely to contribute to the SHR increase in PGI_2 production and the increase in endothelium-dependant contraction seen in the progression of hypertension. Following resveratrol treatment there is a slight reduction in SHR COX 1 expression but this change is not significant (Figure 19). The reduction in COX 1 that was demonstrated in the current study may contribute to the reduced PGI_2 production; however this reduced PGI_2 production also appears to be mediated to a greater extent by the inhibition of COX 1by resveratrol or possibly through a decrease in arachidonic acid production resulting in a reduction in PGI_2 production.

The reduction in endothelium-dependant contraction produced by chronic dietary resveratrol treatment plays a key role in the improvement of the endothelium's control of vascular tone. Resveratrol's affect on endothelium-dependant contractions appears to be mediated through alterations in the production and signaling pathway of PGI_2. Inhibition of PGI_2 production through COX inhibition eliminates endothelium-dependant contractions similar to *acute* incubation resveratrol treatment and to a degree, chronic dietary resveratrol treatment (9,12) (Jeffery in press). Evidence that the reduction in endothelium-dependant contractions is mediated through a reduction in PGI_2 is provided by the lack of effect of resveratrol on the sensitivity of the thromboxane-prostanoid receptor (Figure 15) (68).

A reduction in the PGI_2 signal is the most likely alteration to provide the reduction in endothelium-dependant contractions in the current chronic resveratrol study. Support for the reduction in PGI_2 production being the key contributor to the blunted contractions seen in the current study comes from multiple sources in the literature. Kane et al. studied the effect of wine polyphenol treatment in the angiotensin II model of hypertension; these researchers demonstrated a reduction in blood pressure and also a reduction in the endothelium-dependant contractions angiotensin II model (77). Though Kane et al. did not measure PGI_2 production directly, it was reported that following treatment with wine polyphenols there was a significant reduction in COX 1 and COX 2 expression which was attributed to an improved oxidative state provided by the red wine polyphenols. COX expression had been demonstrated to be unregulated by an increase in reactive oxygen species, a reduction in reactive oxygen species production or increase in scavenging could reduce COX expression (78). The reduction in COX 1 and 2 expressions demonstrated by Kane et al. was associated with a reduction in endothelium-dependant contractions and was likely caused by a reduction in PGI_2 production (77).

Though the current study did not demonstrate a significant reduction in COX 1 expression (Figure 19) a reduction in PGI_2 production was still noted. Resveratrol could have been able to produce reduced PGI_2 production through inhibition of COX 1, a reduction in arachidonic acid production, mediated through an alteration in phospholipase A2 activity, or a reduction in prostacyclin synthase expression. Resveratrol has been shown to inhibit COX 1 activity in a dose-dependent manner in vitro (79). Resveratrol's ability to reduce oxidant content in the cell can reduce the activity of COX 1 (49,62,80). The reduced activity is thought to be mediated through inhibition of the production of PGH_2 the precursor to PGI_2,

The inhibition is a result of the lack of oxidation of an iron catalytic subunit in the COX molecule which results in a lack of production of PGH_2 (49,81). Resveratrol's effect on phospholipase A2 and prostacyclin synthase has yet to be detailed in the literature and could be partially responsible for the reduction in PGI_2 production exhibited in the present study.

The reduction in PGI_2 production is the key contributor to the reduced endothelium-dependant contractions, and this has been mediated through either resveratrol's inhibitory effect on COX 1 or also possibly through a reduction in arachidonic acid through actions possibly effecting phospholipase A2. The inhibitory effect on COX 1 is due to resveratrol's antioxidant capabilities within the cell decreasing the presence of reactive oxygen species, (49,52,56,57,59,60,70,82,83).

Hemodynamic Measures:

The mean, maximum and minimum CCA blood flow values in the SHR were all reduced compared to the normotensive WKY, indicating impairment in the hemodynamic measures of the SHR hypertensive model (Table 2). This finding was similar to previous results from Denniss et al. which found both decreased blood flow and conductance in to CCA of the SHR when compared to WKY (12). The altered hemodynamics measure in the SHR is also supported by the reduction in CCA blood flow in human patients with essential hypertension (84). The current study further established the impairment in CCA blood flow seen in models of essential hypertension and examined the effects of chronic dietary resveratrol treatment on this measure. Following chronic dietary treatment with resveratrol there were no alterations in the CCA blood flow in either strain (Table 2), indicating that resveratrol treatment does not improve the impaired blood flow seen in the SHR. To our

knowledge this is the first study to assess the CCA blood flow of a hypertensive model following resveratrol treatment so no literature is available to support the finding that resveratrol treatment does not have an effect on CCA blood flow.

The MAP of the SHR control group was 176 $\pm$ 6 mmHg compared to the WKY control groups MAP of 78 $\pm$ 7 mmHg indicating that the SHR had a marked increase in blood pressure which is congruent with results from the literature (12,52). This result indicates that the SHR do suffer from hypertension and endothelial dysfunction that is associated with this increase in blood pressure.

The current study found that, following 4 weeks of treatment with resveratrol at a high dose of 2.7 mg/kg/day, the blood pressure was reduced in comparison to age matched SHR (Table 2). This reduction in blood pressure is likely due to improvements of the endothelium-dependant vasomotor function altering the vascular tone of the hypertensive animals. A recent study has shown similar effects of resveratrol treatment of a much greater dose of 10mg/kg/day for 8 weeks in obese Zucker rats causing a reduction in blood pressure (71). A study by Diebolt et al. illustrated a reduction in blood pressure following treatment with wine polyphenols and attributed the reduced blood pressure to the polyphenols effects on NO signaling (45). Kane et al. demonstrated a reduction in blood pressure in the angiotensin II model following treatment with red wine polyphenols, the reduction in blood pressure illustrated by this study was attributed to a reduction in endothelium-dependent contractions that was deomostrated during vascular assessment (77). The current study attributes resveratrol's reduction in the SHR blood pressure to both improvements in NO signaling and a reduction in endothelium-dependant contractions combining to produce improved vascular tone.

A number of studies have been conducted using chronic resveratrol application which have shown improvements in endothelial function, however no alterations in blood pressure were found in these studies (52,85). However, these studies used varying dosages and treatment periods, whereas the studies listed above and a recent study conducted by Bhatt et al. used a resveratrol or wine polyphenol treatment in excess of 5 mg/kg/day. The Bhatt et al. study which used a similar resveratrol dosage to the current study found a similar reduction in blood pressure in the SHR (70). Bhatt et al. attributed this reduction to improvements in endothelial function similar to the results of the current study (70).

AICAR and Resveratrol-Mediated Relaxation:

SHR and WKY control group responses to AICAR differed throughout the dose-response curve. Following administration of AICAR at a -4 Log M the WKY control group showed a slight contraction which was 30% greater than the phenylephrine pre-contraction. Whereas the SHR control group showed almost no contraction following this dose of AICAR (Figure11).

Resveratrol treatment at both a low and high dose resulted in greater relaxations throughout the AICAR dose-response curve in the SHR (Figure 11). This increase in relaxation could possibly be caused by an increase in 5' adenosine monophosphate-activated protein kinase phosporylation. In a previous *acute* incubation resveratrol study it was found that resveratrol increases 5' adenosine monophosphate-activated protein kinase phosphorylation (Jeffery in press). The increase 5' adenosine monophosphate-activated protein kinase phosphorylation can lead to an increased eNOS stimulation and production of NO leading to improved relaxations (63). In a number of models that display endothelial

dysfunction expression and activation of 5' adenosine monophosphate-activated protein kinase are reduced, which may contribute to impaired NO-mediated signaling (63,86). Resveratrol treatment has been shown to salvage the 5' adenosine monophosphate-activated protein kinase expression and activation in some models of endothelial dysfunction (86). It is possible that the resveratrol treatment has had a similar effect in the SHR of the present study leading to an increase in NO production and in turn an increase in relaxation.

Resveratrol, along with its numerous antioxidant and gene expression effects, has been shown to cause relaxation of pre-contracted vessels in both endothelium-dependant and independent manners (Jeffery in press) (87,88). This process has been shown to be partially mediated through the NO signaling pathway, demonstrated by the partial inhibition of resveratrol mediated relaxation following incubation with L-NMMA (87,89). Resveratrol has had an effect on the NO mediated component of the endothelium-dependant vasorelaxation of the SHR, however the resveratrol mediated relaxation in the SHR appears to be unaffected by the resveratrol treatment. All three of the SHR groups reached similar levels of maximal relaxation and no differences were seen in the area under the curve analysis (Table 3A). The only difference occurred at a concentration of -5 Log M with the high resveratrol group showing slightly increased relaxation. Interestingly the WKY animals resveratrol mediated relaxation was greatly affected by resveratrol treatment. The resveratrol treatment led to an increase in maximal relaxation and an alteration in the resveratrol dose-response curve that can be seen in (figure 12). This novel finding merits further investigation into the mechanisms behind the increase in resveratrol mediated relaxations to determine if this alteration is endothelium-dependant or independent, working through the NO signaling pathway or through a number of different ion channels.

Phenylephrine-Mediated Contractions:

Phenylephrine contractions have been shown to be reduced in the SHR when compared to WKY (12). This alteration is thought to be caused by an alteration in the α-1-adrenoreceptor subtypes in the SHR, which have been shown to be altered as early as 12 weeks of age (90).In this study the SHR control group demonstrated a reduction in the response to phenylephrine stimulation in comparison to the WKY control group, which is characteristic of the alterations seen in the α-1- adrenoreceptor. The reduced response to phenylephrine is illustrated in figure 17 and table 3B. Resveratrol treatment restores the response to phenylephrine, producing similar contraction to the WKY control group (Table 3 B) (Figure 17). This is possibly accomplished by altering the expression of the α-1-adrenoreceptor subtypes to one that is similar to the WKY (90). Chronic dietary resveratrol treatment had no effect on the response of the WKY to phenylephrine. Further research into the possible alterations that are occurring to the α-1- adrenoreceptor subtypes during resveratrol treatment should be conducted to elucidate the mechanisms involved.

Limitations:

Though resveratrol supplementation in the drinking water of the animals is a common method used in the research field, this method has its limitations (52,70,77). Firstly resveratrol treatment through drinking water supplementation is not as precise as other methods such as an intraperitoneal injection with resveratrol. Resveratrol treatment using water supplementation assumes that the water that is removed from the bottle is consumed by the animal which does not take into account residual dripping and other factors such as manipulation by the animal. Finally, resveratrol treatment using water supplementation is

done ad libitum, which can cause a discrepancy in the amount of resveratrol removed from the water bottles either through consumption or manipulation by the animals. This discrepancy was demonstrated in the present study by the different amounts of resveratrol received by the SHR and WKY groups. Given the alternative of daily intraperitoneal injections which has its own limitations including stressing the animal we chose to use water supplementation. Though there were limitations to the resveratrol treatment of this study it is apparent from the results that the treatment did have an effect, indicating that the treatment was successful.

This study was limited in its ability to assess the tissues uptake of resveratrol. The inability to monitor the free resveratrol concentration in the vasculature limits the capability of this study to attribute the results to either acute effects of resveratrol following recent ingestion or chronic effects of resveratrol treatment. However the resveratrol concentration that is found in the blood plasma following dietary treatment at a similar dose to the present studies high dose is minimal, reaching 600 ng/ml of plasma (51). The small plasma concentration of resveratrol reduces the chance that the results seen in this study were due to acute ingestion effects and more likely to be a result of the chronic effects of dietary resveratrol treatment. To further support that the results from the present study can be attributed to the chronic effects of resveratrol, the time elapsed from when the animals are sedated for hemodynamic measurement to the actual assessment of vasomotor function is much greater than the half life of resveratrol in the body. This supports the conclusion the results from this study are due to the chronic effects of resveratrol and not acute effects of recent resveratrol ingestion.

This study was limited by the method in which hemodynamic measures were taken. Blood pressure and blood flow measurements were preformed while under anesthesia. Taking hemodynamic measures under anesthesia has been criticized due to the fact that under anesthesia heart rate and blood pressure values can be reduced. However, the hemodynamic results from the present study match trends in anesthetized animals from previous studies and these results were similar to those recorded in conscious SHR using noninvasive methods such as the tail cuff method (12,91).

This study was also limited in its ability to directly assess NO production, with methods previously used by this laboratory lacking the sensitivity to measure differences in NO production. Functional assessments were used in an attempt to measure differences in the NO production; however this method is not a direct measure of NO production. Firstly, the differences in relaxation may not fully represent differences in NO production. Secondly, inhibiting COX 1 and 2 may decrease the production of reactive oxygen species altering the effects on NO bioavailability. The relaxation occurring may not be able to be attributed to just NO. Other endothelium-derived relaxation factors may also play a role such as hydrogen sulfide and hydrogen peroxide, though these appear to have a greater role in the resistance arteries rather than conduit arteries (92,93). Though this is not a direct measure of NO production it provides a good functional assessment of NO contribution to endothelium-dependant relaxation.

Finally, biochemical analysis was limited by the amount of viable tissue available. Tissue used in vasomotor assessment could not be used due to stimulation from chemical agonists and tissue used in hemodynamic measures could not be used due to the possible

removal of the endothelium. Not being able to use tissue from vasomotor or hemodynamic assessment limited the amount of viable tissue available for western blotting analysis.

Conclusion:

This study demonstrated that chronic dietary resveratrol treatment improves the endothelial function of SHR in the CCA, though not as originally hypothesized. Treatment at a low dose of resveratrol which mimics red wine consumption did not have as marked an effect as previous studies, providing no improvements in endothelium-dependant relaxation and endothelium-dependant contractions (52). Treatment at a high dose of resveratrol, which mimicked pharmacological supplementation, demonstrated a number of changes in endothelial function. This treatment alleviated dysfunction seen in endothelium-dependant relaxations, reduced endothelium-dependant contractions, which lead to a reduction in blood pressure of SHR. This was achieved through a number a possible effects of resveratrol treatment. Resveratrol's effect on NO signaling is possibly mediated through a reduction in reactive oxygen species reducing NO scavenging by superoxide. This reduction was shown in previous studies (52,70). Resveratrol's ability to increase in SOD activity may have contributed to this as was shown by Bhatt et al. (52,65,70). A reduction in eNOS uncoupling also could have improved NO bioavailability.

Reduced endothelium-dependant contractions that were observed following high resveratrol treatment were mediated by a reduction in PGI_2 production, which was accomplished by the inhibition of COX 1. This is supported by the lack of change in the sensitivity of the thromboxaine prostanoid receptor, and the reduction in PGI_2 production without a significant change in COX 1 expression.

Although this study was limited in identifying the exact mechanisms by which resveratrol altered both NO and PGI_2 signaling, it is evident that resveratrol treatment acting through these pathways was able to improve endothelium-dependant control of vascular smooth muscle in a model of hypertension associated with endothelial dysfunction. This makes resveratrol treatment an interesting option for further research as a possible treatment in humans.

Future directions:

This study has provided significant evidence for resveratrol's effect on endothelium-dependant control of vasomotor function. Experiments which could further develop the understanding of resveratrol's effect on endothelium-dependant vasomotor function in the present study include western blot analysis of phospholipase A2 expression in the CCA, as well as measurement of arachidonic acid production. This analysis would further elucidate resveratrol's effect on PGI_2 production and thus endothelium-dependant contractions. Future studies should focus on the biochemical changes that are occurring during chronic dietary resveratrol treatment which allow for these improvements. These include assessment of reactive oxygen species production, pro oxidant and anti oxidant enzymes, and other proteins integral in the endothelial control of vascular tone. Assessment of the effect of resveratrol on the contractile proteins in the VSM is another area which needs to be elucidated. Assessment in the alterations of α-1- adrenoreceptor subtypes which were illustrated by the current study must also be assessed in future studies to fully understand resveratrol's effect on these receptors. Finally, the results that have been shown in models of essential hypertension like the SHR must be replicated in humans to solidify resveratrol's treatment effects on endothelial dysfunction and hypertension.

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