Ischemia reperfusion injury in isolated hearts from spontaneously hypertensive rats following chronic resveratrol treatment

by

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AUTHOR'S DECLARATION

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

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ABSTRACT

Hypertension is a risk factor for myocardial ischemia via the promotion of endothelial dysfunction and atherosclerosis (Ogita et al., 2004). Hypertension not only a predisposes the heart to ischemic events, but as shown by clinical and experimental studies, exacerbates the heart's susceptibility to ischemia-reperfusion injury (Golden et al., 1994; Besík et al., 2007; Snoeckx et al., 1986) due to impairments in regulation of calcium handling, ion homeostasis, and energy metabolism (Galiñanes et al., 2004). Nutraceuticals have demonstrated beneficial and protective effects on both hypertension and ischemia reperfusion injury. Resveratrol, a naturally occurring polyphenol which is present in grapes and wine, acts as a cardioprotective agent and can be used to protect the heart against ischemia reperfusion injury.

Here we investigate the effectiveness of *chronic* dietary resveratrol intake in normotensive and hypertensive rats on protection against ischemia-reperfusion injury, assessed in an isolated perfused Langendorff heart model. Rats ingested either a High dose of 2.7mg/day-which mimicked the resveratrol content in daily supplemental intake levels, a Low dose of 0.027mg/day- which mimicked the resveratrol content in moderate red wine intake, or no resveratrol in the drinking water for 28 days, at which point hearts were excised and mounted on a Langendorff apparatus. Once stable conditions were established all hearts were subjected to 30 minutes of no flow ischemia followed by 2 hours of reperfusion.

Interestingly, SHR animals did not exhibit reduced recovery, or increased infarct size as compared to WKY. Infarct size as measured by triphenyltetrazolium chloride staining after 2 hours of reperfusion was significantly reduced in High and Low groups (combined WKY and SHR) as compared to Controls (19.9±0.9 and 19.4±0.8 vs 27.7±0.7 % of baseline, $p<0.0001$). Left ventricular developed pressure was significantly improved 2 hours post ischemia in both High and Low groups (combined SHR and WKY) compared to Controls (83.1±4.1 and 78.6±3.4 vs.67.9±3.2% of baseline, $p<0.01$). A higher rate of maximal pressure development was maintained in High and Low groups (combined SHR and WKY) compared to Controls (90.5±4.7 and 95.6±5.0 vs.73.5±4.8% of baseline, $p<.05$). Resveratrol treatment at a High and

Low dose reduced contracture of the myocardium as compared to Control (7.2±3.0 and 6.9±2.9 vs. 20.1±2.9 mmHg- LVEDP, $p<0.01$).

In conclusion resveratrol treatment at both a High and Low dose protects against a decline in cardiac function, and reduces infarct size post ischemia reperfusion. Additionally, tolerance to ischemia reperfusion injury in SHR is not reduced as compared to WKY.

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LIST OF ABBREVIATIONS

ΔBW	*Change in body weight*
+dP/dt	*Maximal rate of pressure development*
A1, A3	*Adenosine receptors 1 and 3*
AKT	*Protein kinase B*
AMPK	*AMP-activated protein kinase*
ATP	*Adenosine triphosphate*
AUC	*Area under the curve*
Bad	*Bcl-2 associated death promoter*
Bcl-2	*B cell lymphoma 2*
BW	*Body weight*
Ca^{2+}	*Calcium*
Con	*Control*
COX	*Cyclooxygenase*
CO_2	*Carbon dioxide*
CDT	*Conductance*
CPP	*Coronary perfusion pressure*
CREB	*cAMP response element binding protein*
CVR	*Coronary vascular resistance*
DNA	*Deoxyribonucleic acid*
DWC	*Daily water consumption*
eNOS	*Endothelial nitric oxide synthase*
ERK	*Extracellular signal-regulated kinase*
FC	*Food consumption*
H^+	*Hydrogen*
HW	*Heart weight*
iNOS	*Inducible nitric oxide synthase*
i.p.	*Intraperitoneal injection*
IR	*Ischemia reperfusion*
IRI	*Ischemia reperfusion injury*
K^+	*Potassium*
K_{ATP}	*ATP dependant potassium channels*

KHB	*Krebs Heinsleit buffer*
LDL	*Low density lipoprotein*
L-NAME	*N^G-nitro-L-arginine-methyl ester*
LV	*Left ventricular*
LVEDP	*Left ventricular end diastolic pressure*
LVDP	*Left ventricular developed pressure*
MAPK	*Mitogen-activated protein kinase*
MI	*Myocardial ischemia*
mmHg	*Millimetres of mercury*
mtNO	*Mitochondrial nitric oxide*
mtPTP	*Mitochondrial permeability transition pore*
n	*Number of animals per group*
Na^+	*Sodium*
NADPH	*nicotinamide adenine dinucleotide phosphate*
NO	*Nitric oxide*
NOS	*Nitric oxide synthase*
O_2	*Oxygen*
pH	*Power of Hydrogen*
ROS	*Reactive oxygen species*
SEM	*Standard error of means*
SHR	*Spontaneously hypertensive rat*
SR	*Sarcoplasmic reticulum*
TTC	*Triphenyltetrazolium chloride*
WKY	*Wistar Kyoto rat*

INTRODUCTION

The most recent Canadian statistics indicate that cardiovascular disease accounts for approximately 30% of all deaths in Canada, 54% of which are caused by ischemic heart disease (CANISM, 2010). Hypertension not only predisposes the heart to ischemic events, but as shown by clinical and experimental studies, exacerbates the heart's susceptibility to myocardial ischemia-reperfusion injury (Golden et al., 1994; Besík et al., 2007; Snoeckx et al., 1986). Standard treatment of myocardial ischemia attempts to restore blood flow. A paradoxical effect occurs upon reperfusion in that it allows for treatment of ischemia, but causes further damage and dysfunction to the myocardium. Studies have shown that treating an individual with a pharmacologic agent before ischemia occurs can provide protection against the ischemic insult. Resveratrol, a phenolic component of red wine, protects against myocardial ischemia reperfusion injury (IRI) when used as a nutraceutical in healthy and diseased models. As recently demonstrated in an isolated perfused heart model, *acute* treatment with red wine extract can elicit cardioprotective effects on spontaneously hypertensive rat (SHR) hearts (Fantinelli et al., 2007), though these effects cannot be solely attributed to resveratrol. Experimentally, resveratrol prevents the development of pathological cardiac hypertrophy and contractile dysfunction in SHR (Thandapilly et al., 2010), though at present there is a lack of evidence regarding the cardioprotective effects of *chronic* resveratrol treatment in SHR undergoing ischemia reperfusion (IR). Additionally, numerous studies have proven the cardioprotective effects of direct resveratrol administration to an isolated heart, undergoing ischemia reperfusion, via the perfusate in wild type animals (Mokni et al., 2007; Ray et al., 1999; Lin et al., 2008; Dernek et al., 2004; Hung et al., 2004). *Acute* application to a perfused heart does

not take *chronic* dietary intake and digestion into consideration, and these methods of treatment may result in different mechanistic approaches to cardioprotection. Here we attempt to investigate the effectiveness of *chronic* dietary and supplemental levels of resveratrol intake in normotensive and hypertensive rats on protection against ischemia-reperfusion injury, assessed in an isolated perfused Langendorff heart model. Using functional and biochemical data, the effects of resveratrol on cardiac function and viability post ischemia reperfusion will be studied.

The Spontaneously Hypertensive Rat as a Model of Hypertension

Hypertension, a disorder which affects 20% of Canadian adults, is a modifiable risk factor for cardiovascular disease (Wilkins et al., 2010), and can negatively affect the structure and function of vital organs including the heart and kidney (Yusuf et al., 2004). It is defined by an elevated systolic blood pressure of 140mmHg and higher, and a diastolic pressure of 90mmHg and higher (Quinn et al., 2010). Hypertension is a risk factor for myocardial ischemia (Yusuf et al., 2004; Klabunde, 2005) via the promotion of endothelial dysfunction and atherosclerosis (Ogita et al., 2004). Clinical and experimental studies on hypertensive individuals also show increased susceptibility to myocardial IRI (Golden et al., 1994; Besík et al., 2007; Snoeckx et al., 1986), and reasons for this will be discussed below.

The spontaneously hypertensive rat will be used as a model of inherited spontaneous hypertension in this study, with the Wistar Kyoto rat (WKY) as a normotensive control. Essential hypertension accounts for 95% of cases that are diagnosed in patients (Quinn et al., 2010). The SHR model is an experimental model that closely mimics the course of untreated essential hypertension. The relative changes in hemodynamic characteristics such as peripheral resistance, cardiac output, and heart rate are comparable in their course of action throughout

the development of hypertension (Yamori et al., 1973). Common to both is the development of responsive secondary cardiovascular diseases, including left ventricular (LV) hypertrophy (Yamori et al., 1973). LV hypertrophy is an important contributor to cardiovascular morbidity and mortality (Ho et al., 1993), as it can lead to the development of heart failure, and can cause altered responses of the myocardium to IRI (Ferdinandy et al., 2007).

Myocardial Ischemia-Reperfusion Injury

Partial or complete occlusion in one or more areas of the coronary tree renders an ischemic myocardial environment. In a clinical setting of spontaneous myocardial ischemia (MI), the occlusion is typically a result of atherosclerotic plaque (Ogita et al., 2004). An infarction occurs when prolonged ischemia and/or reperfusion results in irreversible cell death (Cokkinos et al., 2006). Infarcted tissue is unable to contribute to the generation of contractile activity, leaving non infracted areas to compensate. Over time the increased demand on the non infracted tissue may lead to detrimental functional changes and heart failure (Bolli et al., 1999); therefore it is of utmost importance to prevent or limit the amount of infracted tissue.

Initial treatment of myocardial ischemia (MI) requires removal of the blockage in order to salvage remaining viable tissue, resulting in reintroduction of oxygenated blood to the previously ischemic myocardium. Reperfusion can be obtained clinically via angioplasty, thrombolytic therapy, and bypass surgery (Cokkinos et al., 2006). In combination with irreversible cell death, ischemia causes reversible damage that can be eliminated by reperfusion (Bolli et al., 1999). Paradoxically, reperfusion is fundamental for the treatment of ischemia but also generates an environment which can cause further damage or death to the myocardium with mechanisms distinct from those of ischemic injury (Yellon et al., 2007). Both ischemia and reperfusion are responsible for myocardial injury and infarction, as the abrupt metabolic and

biochemical changes of reperfusion compound the damage by ischemia (Yellon et al., 2007) and these mechanisms will be discussed below. Most importantly, ischemic and reperfusion damage are not homogeneous and numerous factors combine to produce the overall result of injury and infarction. Common to both pathways of injury are calcium overload and oxidative stress, both of which can interact with numerous biological substrates, and are the main pathways of IRI (Piper et al., 2003).

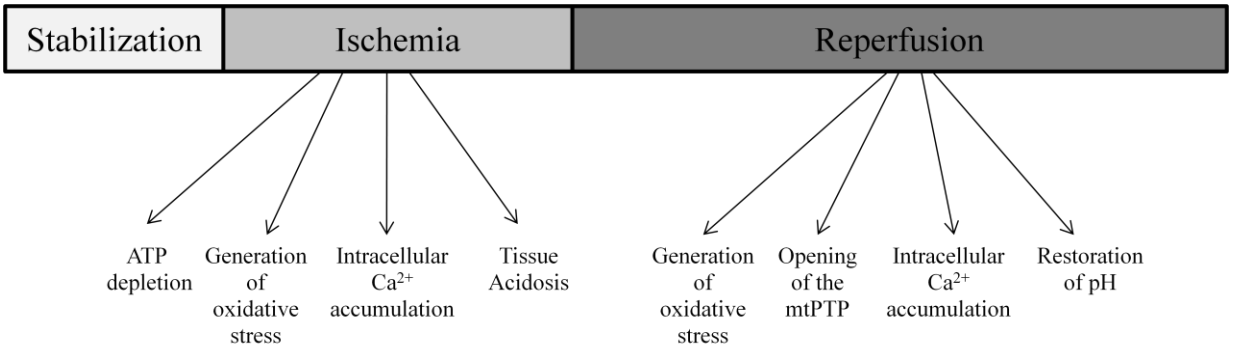

Figure 1: Ischemia reperfusion schematic of a Langendorff Heart, and respective mechanisms of ischemia reperfusion injury. Abbreviations: ATP- adenosine triphosphate; Ca^{2+}- calcium; mtPTP- mitochondrial permeability transition pore; pH- power of Hydrogen.

Mechanisms of Ischemic Injury

Myocardial ischemia results in a reduction of the oxygen and nutrients required by cardiomyocytes for normal performance, and ultimately survival. Cellular homeostasis relies on regulation of energetic ATP supply; therefore under ischemic conditions aerobic production of ATP is severely reduced, and the cell relies heavily on anaerobic ATP production despite its inefficiency to produce ATP in comparison to aerobic metabolism (Hoffman et al., 2004). The inefficiency of glycolysis is paired with a high output of inorganic phosphate, lactate, and H^+ (Cokkinos et al., 2006). Under long durations of ischemia, anaerobic glycolysis is unable to provide sufficient energy, and combined with the resultant acidic conditions can cause damage

and death to cardiomyocytes (Cokkinos et al., 2006). The time course of events during ischemia can be found in Table 1.

Table 1: Time course of events during myocardial ischemia in an isolated rat heart

TIME	EVENT
1 - 2min	ATP levels fall; cessation of contractility
10 min	~50% depletion of ATP; cellular edema, decreased membrane potential and susceptibility to arrhythmias
20 - 24 min	Irreversible cell injury

During ischemia, the impaired ATP synthesis rate causes imbalances in ionic state across cellular membranes, largely due to the inability of ATP-dependant pumps to function. Major contributors to the imbalance are the intracellular accumulation of sodium (Na^+) and depletion of potassium (K^+), which can cause osmotic swelling of the cell and damage to the sarcolemma (Klabunde, 2005; Cokkinos et al., 2006). This imbalance is an effect of the impaired NA^+/K^+ ATP dependant pump, and the activation of the sodium-hydrogen exchanger upon intracellular acidosis (Cokkinos et al., 2006). The overall ion imbalance results in influx of calcium (Ca^{2+}) through activation of voltage sensitive Ca^{2+} channels, as well as the reversal of the Na^+/Ca^{2+} exchanger (Cokkinos et al., 2006). The loss of high energy phosphates also leads to impaired sarcoplasmic reticulum (SR) Ca^{2+} uptake, allowing for even greater accumulation of cytosolic Ca^{2+} (Cokkinos et al., 2006).

The rise in cytosolic Ca^{2+} results in cellular damage by activation of Ca^{2+} dependent phospholipases, proteases, and calpains, causing membrane swelling, rupture, in addition to ischemic contracture (Cokkinos et al., 2006; Ferdinandy et al., 2007). Ischemic contracture is a consequence of the presence of Ca^{2+} and lack of ATP within a cell, which results in non-

cycling, attached actin-myosin crossbridges (Piper et al., 2003), and causes a distinct rise in left ventricular end diastolic pressure (LVEDP). Contracture can results in cytoskeletal defects that render cardiomyocytes more fragile and susceptible to mechanical damage (Piper et al., 2003).

In addition to disruptions in ionic homeostasis, cellular systems are overwhelmed by an increase in ROS production during ischemia. Oxidative stress can cause damage to all components of a cell including lipids, proteins, and DNA, which exacerbates the dysfunction at the myocardium and causes both irreversible and reversible damage (Cokkinos et al., 2006). The major sources of ROS production during ischemia include the mitochondrial electron transport chain, xanthine oxidase, and the NADPH oxidase system (Cokkinos et al., 2006). ROS directly affect membrane enzymes, pumps and protein channels that are involved in the regulation of ion transport and lipid peroxidation, and can cause a change in membrane permeability (Buja, 2005). Additionally, ROS can result in defective Ca^{2+} handling (Saini et al., 2005), and therefore add to the negative effects of ATP depletion on ion transport. More specifically free radicals can impair Ca^{2+} stimulated SR and sarcolemmal Ca^{2+}ATPase activity, thereby reducing calcium transport into the SR or out of the cell, and exacerbating Ca^{2+} overload within the ischemic cell (Netticadan et al., 1999; Bolli et al., 1999).

Evidence reveals that SHR have increased susceptibility to ischemia which may be due to impairments in regulation of calcium handling, ion homeostasis, and energy metabolism (Galiñanes et al., 2004). SHR with left ventricular hypertrophy exhibit faster onset and greater extent of ischemic contracture (Peyton et al., 1982; Anderson et al., 1987). Energy metabolism is altered in SHR, with lesser ATP and creatine phosphate content during ischemia as compared to normotensive controls (Snoeckx et al., 1986; Anderson et al., 1990). Reduced antioxidant potential (Batist et al., 1989) and increased intracellular Na^{+} accumulation during ischemia (Golden et al., 1994) may also contribute to their increased susceptibility to ischemia.

Mechanisms of Reperfusion Injury

Reperfusion of the ischemic environment is necessary in order to reoxygenate and salvage remaining viable tissue. Restoration of blood flow to the previously ischemic myocardium results in mitochondrial reenergization, rapid restoration of pH, an increase in oxidative stress, inflammatory signaling, and intracellular Ca^{2+} overload, together which can lead to opening of the mitochondrial permeability transition pore (mtPTP), and hypercontracture (Yellon et al., 2007). Although the pathogenesis of reperfusion injury is continually being investigated, the signaling pathways are complex and not completely understood.

During reperfusion, mitochondria respond to the re-established delivery of oxygen and replenish energy stores that were lost during ischemia. By doing so the mitochondria produce high amounts of ROS, and leakage of ROS at the electron transport chain (Ferdinandy et al., 2007; Ruuge et al., 1991). The quick increase of oxygen in the acidic myocardium also favours the reaction of a hydroperoxyl radical, and this stress results in the decreased bioavailability of nitric oxide (Yellon et al., 2007). High levels of ROS induce cellular damage to lipids, proteins, and deoxyribonucleic acid (DNA), can cause apoptosis (Becker, 2004), and can damage the SR, leading to Ca^{2+} leak (Yellon et al., 2007). Additionally ROS attract neutrophils, and signal other inflammatory mediators, which can mediate cardiomyocyte death in the later stages of reperfusion (Yellon et al., 2007).

Calcium plays an important role in the pathogenesis of reperfusion injury. At reperfusion the high level of cytosolic Ca^{2+} from ischemia is compounded by the abrupt influx of Ca^{2+} through reenergized membrane transporters, and potentially by release of Ca^{2+} from damaged SR (Yellon et al., 2007), which provokes an uncontrolled myofibrillar activation (Piper et al., 2004). The SR also begins to sequester excess Ca^{2+} upon reperfusion and when filled can

result in a cycle of Ca^{2+} uptake and release which requires excessive ATP usage without achieving calcium ion homeostasis (Hoffman et al., 2004). In response to reperfusion, SHR exhibit a faster onset of Ca^{2+} overload during reperfusion than normotensive rats (Friehs et al., 2003). The sustained high cytosolic Ca^{2+} levels can cause hypercontracture; a state of even greater LVEDP than during ischemia, and can induce cardiomyocyte death (Piper et al., 2004). Additionally, contracture can result in compression of microvessels within the myocardium, resulting in reduced perfusion (Piper et al., 2003).

The mitochondria themselves are susceptible to reperfusion injury and can signal cell death via necrosis or apoptosis (Ferdinandy et al., 2007). Apoptosis is an energy dependant event and prolonged ischemia results in energy depletion (Cokkinos et al., 2006). Cell death occurs mainly by necrosis with some apoptosis in the early stages of reperfusion, and once energy stores are replenished, can transition to a larger proportion of apoptosis (Ferdinandy et al., 2007). The mtPTP is a nonspecific pore located between the mitochondrial membranes (Ferdinandy et al., 2007). The mtPTP is signaled to open when there is an overload of Ca^{2+} in the mitochondrial matrix (Ferdinandy et al., 2007), and allows for release of apoptotic signalers (Yellon et al., 2007). Additionally, opening of the mtPTP causes swelling of the mitochondria due to influx of electrolytes and water, and subsequent loss of membrane potential (Ferdinandy et al., 2007). Other metabolic changes that occur during reperfusion, including the restoration of pH due to the washout of H+ and the buildup of ROS, favour opening of the mtPTP (Yellon et al., 2007). Cell death caused by opening of the mtPTP is a result of either apoptosis, proving sufficient energy for caspase activation, or necrosis (Machado et al., 2009).

Pharmacologic Cardioprotection

Protection of the myocardium against IRI is imperative as loss of myocardial function is the key factor responsible for cardiovascular morbidity and mortality. With increasing emphasis on preventing and treating chronic disease through nutritional modification, further exploration has resulted in the use of nutraceuticals to improve tolerance to IRI. By producing a state of cellular protection, nutraceuticals reduce the injury from ischemia, and manipulate the response to reperfusion.

One of the recently popular cardioprotective agents being studied is resveratrol. The first reports on resveratrol as a cardioprotective agent showed its potential to directly protect isolated hearts from IRI (Ray et al., 1999). Recent studies using *acutely* treated hearts establish that the mechanisms of cardioprotection provided by resveratrol mimic certain pathways common to ischemic preconditioning (Ray et al., 1999; Hattori et al., 2002; Das et al., 2005; Bradamante et al., 2004). These include pathways relating to inflammation, K^+ dependant ATP channels, oxidative stress, and apoptosis, and will be discussed below.

Resveratrol as a Cardioprotective Agent

Resveratrol, 3,5,4'-trihydroxystilbene, is a plant-derived polyphenol found in dietary sources such as grape skin, peanuts, and red wine (Wenzel et al., 2005). It is thought to play a significant role in the French Paradox, an observation that there is a low incidence of cardiovascular disease in the French population despite having a diet high in saturated fats (Kopp, 1998). Purified resveratrol is currently being sold in supplemental form as a nutraceutical, in addition to being available in dietary sources.

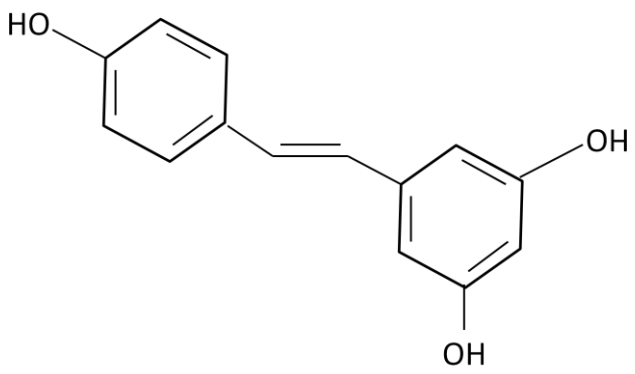

Figure 2: Structure of Resveratrol

Resveratrol is well absorbed and distributed to organs in both rodents and humans. It is metabolized primarily to glucuronide and sulfate conjugates, though the biological activity of these metabolites remains unknown (Wenzel et al., 2005). Additionally resveratrol is capable of binding to human serum albumin and hemoglobin (Lu et al.,). Resveratrol is detectable in plasma 15 minutes after oral administration, whether it is ingested from red wine or as a supplement, and peak plasma concentrations are reached around 30-60 minutes post ingestion (Wenzel et al., 2005). A secondary peak occurs 6 hours post ingestion, suggesting release from hemoglobin, or reabsorption of conjugated metabolites from the intestine (Wenzel et al., 2005).

Cardioprotective agents attempt to reduce irreversible cell death, and promote a safe transition to normal contractile function following ischemia. Numerous studies using normotensive isolated rat hearts elucidate the cardioprotective abilities of resveratrol against IRI with both *chronic* and *acute* treatment. *Acute* resveratrol treatment refers to the *in vitro* direct application of resveratrol to an isolated heart through perfusate, and *chronic* treatment refers to the *in vivo* treatment of an animal for several days before the heart is removed. Difficulty arises when attempting to apply the results of *acute*ly treated isolated hearts undergoing IR to a clinical setting as one is unable to predict an ischemic occurrence. *Chronic*

ingestion of resveratrol in wild type rats affords greater functional recovery to isolated hearts undergoing IR as compared to *acute* treatment (Dernek et al., 2004; Bradamante et al., 2003), though currently the dosages used greatly exceed even the supplemental dose available to humans. It is therefore of importance to understand the cardioprotective effects of *chronic* resveratrol treatment using doses that would be attainable to humans.

At varying doses and delivery methods, resveratrol reduces IRI as evidenced by reduction of infarct size, reduction in apoptotic cardiomyocytes, and greater recovery of left ventricular contractility (Das et al., 2006). Resveratrol acts to attenuate myocardial ischemia-reperfusion injury (Das et al., 2006; Goh et al., 2007) by affecting multiple pathways including inflammation, oxidative stress, potassium channels, and apoptosis. These pathways are targets for protection against IRI (Turer et al., 2010), and furthermore these pathways are disrupted in the SHR (Yuhki et al., 2010; Lassègue et al., 2004; Cameron et al., 1988; Peng et al., 1999). Additionally, *chronic* resveratrol treatment for protection against IRI in hypertensive rats has not yet been studied. Altered characteristics of these pathways in SHR animals, and lack of research relating resveratrol to IRI in SHR, provide basis for *chronic* treatment with resveratrol in SHR. The mechanisms of cardioprotection provided by resveratrol in normotensive animals will be discussed below, though the examination of pharmacologic cardioprotection by resveratrol in SHR is lacking.

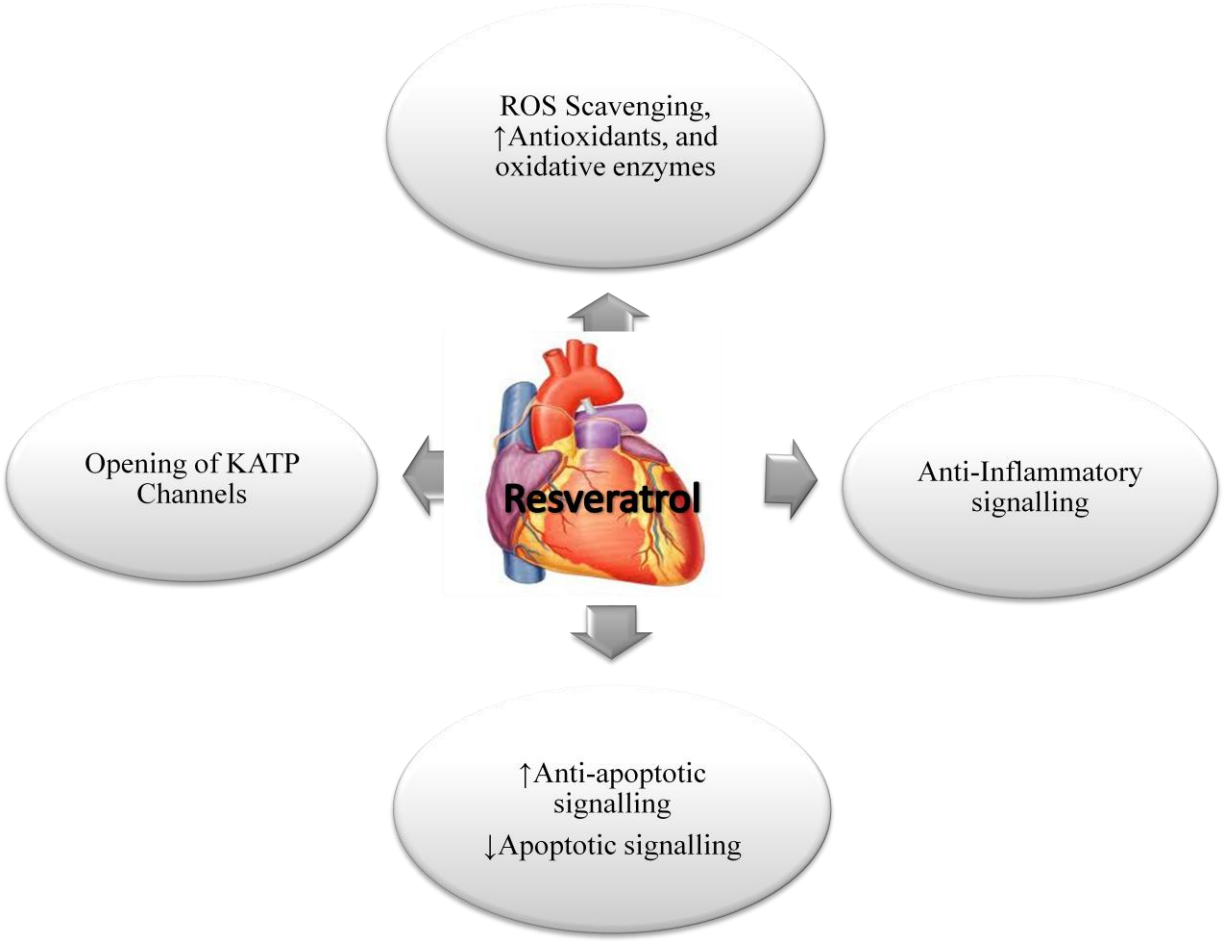

Figure 3: Main areas of cardioprotection provided by resveratrol against ischemia reperfusion injury

Inflammation

Inflammation is a property of atherosclerosis, is exacerbated by hypertension, and can cause MI (Ogita et al., 2004). Resveratrol possesses anti-inflammatory properties linked to its ability to inhibit the synthesis and release of pro-inflammatory mediators (Das et al., 2007), inhibit platelet aggregation and proadhesive molecules, and prevent endothelial disruption during IR (Shigematsu et al., 2003; Das et al., 2006). Additionally, resveratrol inhibits pro-inflammatory mediator-related enzymes such as cyclooxygenase in arteries (Das et al., 2007).

Resveratrol therefore provides cardioprotection by reducing tissue injury by inflammatory mediated pathways of the vasculature.

The use of a COX inhibitor, such as Aspirin, is often recommended at the onset of myocardial ischemia (Trialists' Collaboration, 1994), but the effects of COX inhibition in cardiomyocytes during IRI is not well understood. Recent data suggest that although there are benefits to blocking COX in the arteries to prevent inflammatory signaling, there may be a cardioprotective role played by activated COX in cardiomyocytes during IRI (Camitta et al., 2001). This protection exists when COX-2 is stimulated by NO under ischemia reperfusion conditions (Bolli et al., 2002), and when COX-2 is constitutively expressed (Inserte et al., 2009). Additionally, COX-1 and COX-2 null mice demonstrate reduced tolerance to IRI (Camitta et al., 2001; Yuhki et al., 2011). Ventricular tissue from animals treated for 14 days with 2.5mg resveratrol/Kg of body weight/day demonstrate no change in COX-1 expression, but a large increase in COX-2 expression (Dudley et al., 2008). The differences between SHR and WKY myocardial COX expression is not yet known, though the association of prostanoids with hypertension and cardiac hypertrophy would suggest there may be differences (Yuhki et al., 2010).

Antioxidant Activity

Antioxidants and free radical scavengers have favorable effects on both hypertension and IRI. Oxidants are able to elicit an inflammatory response, calcium overload, and apoptotic signaling (Zhao, 2004). Resveratrol works as a powerful antioxidant in vivo by direct and indirect mechanisms including scavenging of free radicals, and enhancement of oxidative enzyme expression (Das et al., 2006). Resveratrol directly scavenges peroxyl, hydroxyl, and superoxide radicals (Ray et al., 1999), and reduces malonaldehyde formation in the

postischemic reperfused myocardium (Das et al., 2006). Additionally, oxidation of low density lipoprotein (LDL), which is strongly associated with coronary heart disease and MI (Holvoet, 2004), is inhibited by resveratrol treatment in humans (Frankel et al., 1993). Reduction of LDL oxidation by resveratrol may act as a preventative measure against atherosclerotic plaque formation, and subsequent MI.

A large component of resveratrol's antioxidant nature arises from the ability to increase nitric oxide synthesis at rest and during ischemia reperfusion in the isolated heart (Hattori et al., 2002). In the myocardium, release of NO results in inotropic and chronotropic events, including enhancement of ventricular relaxation, and decreased myocardial O_2 consumption, and the upregulation of iNOS and eNOS is a factor involved in preparing the heart's defenses against IR (Bolli, 2001). *Chronic* resveratrol treatment at 0.5-2.5mg/Kg/day-i.p. upregulates eNOS and iNOS expression, in addition to increasing NO bioavailability by directly scavenging free radicals, and stimulating NO production (Hung et al., 2004; Bradamante et al., 2004; Das et al., 2005). The use of *acute*ly treated isolated hearts with 10uM resveratrol from iNOS knockout mice (Imamura et al., 2002), and use of the NO antagonist N^G-nitro-L-arginine methyl ester (L-NAME) in both an *acute* (10uM) and *chronic* (25mg/L for 15 days) setting (Bradamante et al., 2003) during IR abolishes the cardioprotective effects of resveratrol. This confirms the role of NOS in resveratrol induced cardiac protection.

Myocardial mitochondria also play an important role during IR as dysfunction and production of mitochondrial ROS contribute to cardiac failure and cell death. Recently resveratrol has been shown to increase mitochondrial NO (mtNO), and reduce mitochondrial lipid peroxidation which may act to decrease the ROS leak from the mitochondria (Gutiérrez-Pérez et al., 2011).

SHR possess elevated reactive oxygen species and markers of oxidation (Lassègue et al., 2004), and at 18 weeks of age have reduced eNOS expression in the left ventricle (Piech et al., 2003). The mitochondrial of SHR are susceptible to the elevation in oxidative stress, resulting increased mitochondrial damage and dysfunction (Ballinger, 2005). Additionally, lipid peroxidation in the SHR myocardium is elevated (Torii et al., 1992). SHR receiving 2.5mg/Kg/day of resveratrol by oral gavage for 10 weeks have reduced oxidative stress and recovered levels of antioxidants in the myocardium (Thandapilly et al., 2010). The above characteristics of SHR, and the antioxidant effects of resveratrol provide basis for studying resveratrol treatment in SHR against IRI.

ATP Dependant Potassium Channels

Activation of ATP dependent potassium channels (K_{ATP}) is an adaptive mechanism that provides protection from IRI (Grover et al., 2000), and is common to the ischemic preconditioning pathway (Cole et al., 1991). Initially it was found that as ATP levels within a cell decline, the K_{ATP} open, though it is now known that other endogenous factors including pH and NO, as well as pharmacologic agonists, are able to stimulate opening the K_{ATP} (Grover et al., 2000). The use of various K_{ATP} openers during IR reduces necrosis and improves functional recovery of an isolated heart (Grover et al., 2000). Opening of K_{ATP} during IR results in reduced action potential duration, and therefore reduced Ca^{2+} entry via voltage-dependant Ca^{2+} channels (Grover et al., 2000).

Resveratrol is able to enhance K_{ATP} activation in isolated cardiomyocytes receiving 0.1-100umol/L of resveratrol (Chen et al., 2008; Goh et al., 2007), though research in this area of study is lacking with respect to stressed conditions such as IR. K_{ATP} are involved in the protection against IRI by red wine extract, likely through PKC activation (Mosca et al., 2002). Additionally, it is well established that in SHR cardiomyocytes, K_{ATP} sensitivity to ATP is

reduced (Cameron et al., 1988) and together with the above suggests the relevance of K_{ATP} in resveratrol induced cardioprotection on hypertensive animals.

Apoptosis

Resveratrol provides cardioprotection against IRI by attenuating the number of apoptotic myocytes. Apoptosis can occur during the ischemic phase, be triggered at reperfusion, or accelerated by reperfusion (Cokkinos et al., 2006; Zhao, 2004). Apoptosis is upregulated in SHR cardiomyocytes (Peng et al., 1999), and SHR may therefore benefit from treatment with resveratrol. Resveratrol enhances pro-survival signaling. Activation of adenosine receptors A1 and A3, by *acute* administration of 10uM resveratrol to isolated hearts, leads to survival signaling by Bcl-2, and decreased apoptotic signaling by Bad via an Akt pathway and cAMP response-element binding protein (CREB) (Das et al., 2005). This pathway can also lead to stimulation of NO production by Akt phosphorylation (Das et al., 2005). Further to this, in studies using both *acute* (10uM) administration to isolated hearts and *chronic* gavage (2.5mg/Kg/day for 7 days), resveratrol activates a mitogen-activated protein kinase (MAPK) pathway to activate extracellular signal-regulated kinase (ERK) 1 and 2, and p38α MAPK (Das et al., 2006; Das et al., 2006). This activation acts as a part of the signaling pathway via CREB, resulting in survival signaling via Bcl-2 (Das et al., 2006).

As previously mentioned, opening of the mtPTP can occur during reperfusion, causing release of apoptotic stimuli and swelling of the mitochondria. *Chronic* resveratrol treatment (2.5mg/Kg/day for 14 days by gavage) reduces mitochondrial swelling during reperfusion, which is presumed to lead to the resultant reduction in cell death (Dudley et al., 2008).

Resveratrol and Hypertension

In SHR animals, resveratrol treatment has primarily been used to study the treatment of hypertension and LV hypertrophy. Previous research from this lab, using *chronic* dietary resveratrol treatment at physiologically relevant doses, showed improved vasorelaxation in aorta of SHR animals (Rush et al., 2007). Additionally, *chronic* resveratrol treatment for 10 weeks, at 2.5mg/Kg/day by oral gavage, prevents the development of pathological cardiac hypertrophy and contractile dysfunction in SHR (Thandapilly et al., 2010), though at present there is a lack of evidence regarding the effects of pharmacologic cardioprotection with resveratrol on SHR. Because of alterations in SHR myocardial pathways that are related to the pathways cardioprotection provided by resveratrol in normotensive animals, it is of interest to investigate whether resveratrol will provide protection in SHR.

PURPOSE OF STUDY

The above provides background regarding IRI and the potential for resveratrol to act as a powerful cardioprotective agent in both normotension and hypertension. This, taken together with the lack of research on *chronic* resveratrol treatment and IRI in hypertensive individuals, provides rationale for the current study. Also described above are the mechanisms by which resveratrol counteracts the detrimental effects of hypertension on various systems of the heart. The interaction of resveratrol on hypertension-induced changes to the myocardium may have a beneficial effect on IRI. Of interest to this study is whether a dietary or supplemental amount of resveratrol is required to evoke cardioprotection. We will treat animals by providing resveratrol in the drinking water at two different doses. The first dose will mimic the resveratrol content one could ingest through moderate red wine consumption (Low), and the second dose will mimic the resveratrol amount that one could ingest through daily supplemental consumption (High).

The purpose of this study is to investigate the cardioprotective effects of *chronic* resveratrol treatment against myocardial infarction and post ischemic dysfunction, in hypertensive and normotensive animals, using functional and biochemical parameters. Secondary to this we will assess baseline protein expression in SHR and WKY after resveratrol treatment at both doses.

The expression of many different proteins is negatively susceptible to hypertension, and the expression of these same proteins also can be positively altered by resveratrol treatment. In addition these proteins are known to play a role in IRI. We will therefore assess the effect of strain and resveratrol treatment on select protein expression.

HYPOTHESES

Primary Hypotheses

1. SHR animals will show greater functional susceptibility to IRI, and infarct size than WKY
2. *Chronic* resveratrol treatment will protect against post ischemic dysfunction in both SHR and WKY animals
3. *Chronic* resveratrol treatment will decrease myocardial infarct size in both SHR and WKY
4. Resveratrol treatment at a high dose will provide greater cardioprotection than at a low dose in both SHR and WKY animals

Secondary Hypotheses

Hypertension will negatively affect protein expression, and resveratrol treatment will beneficially affect the expression of the same proteins.

METHODS

Animals

Experiments were performed using a total of 78 male WKY (n= 39) and SHR (n=39) rats, obtained from Harlan (Indianapolis, IN) at the age of 13 weeks. Animals were group housed at a constant air temperature (20-21°C), and humidity (~50%) in a 12h:12h reverse light-dark cycle. Animals had access to standard 22/5 (W) Rodent Diet lab chow and tap water until 16 weeks of age at which point they began the resveratrol (trans-3,4',5-trihydroxystilbene) treatment stage and were housed individually.

Treatment

At 16 weeks of age animals began the 4 week treatment protocol ingesting tap water containing 1g/100ml low viscosity carboxymethylcellulose and one of the following; 6.361mg/100mL Resveratrol (High), 0.06361mg/100mL Resveratrol (Low), or no Resveratrol (Control) (Toronto Research Chemicals, Ontario). Consumption was measured on a 24 hour basis, and drink was prepared and replaced daily under dim light conditions. Bottles were wrapped in tin foil to create a light-negative container environment, which prevented resveratrol decomposition.

Isolated Heart Preparation

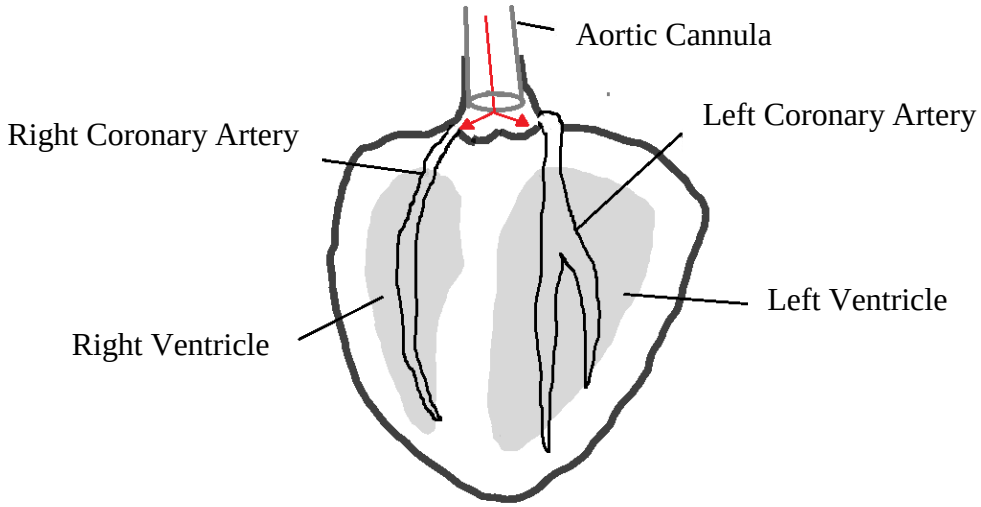

Figure 4: Retrograde flow through an isolated heart. Perfusate (represented by the arrows) begins at the coronary ostia, flows through the coronary tree into the myocardium, and exits through the coronary sinus and right atrium.

The Langedorff technique uses an isolated heart to maintain cardiac activity and permits for the measurement of left ventricular functional parameters and coronary vasomotor function. The heart is perfused in a retrograde fashion, causing the aortic valve to remain closed, forcing perfusate through the coronary system, and out through the coronary sinus and right atrium (Figure 3). Hearts remain stable without an appreciable decline in function for up to 4 hours (Figure 7).

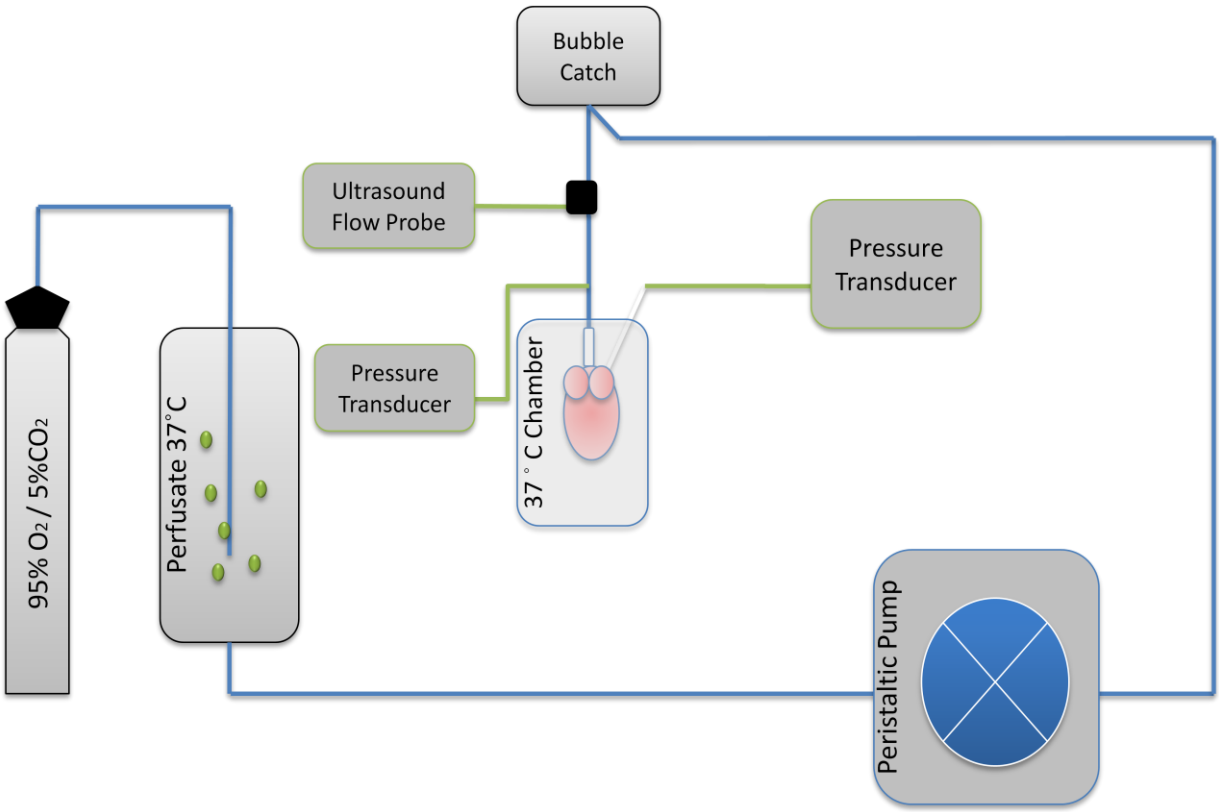

Figure 5: Schematic of the Langendorff Isolated Perfused Heart Set Up.

At 28 days of treatment body mass was recorded and a thoroacotamy was performed under anesthesia by pentobarbital sodium injection (40-50mg/Kg intraperitoneally [i.p.]). Rats first underwent invasive carotid artery blood pressure and flow measurement prior to thoroacotamy to confirm a hypertensive or normotensive state, though the procedure is not part of this study. Hearts were excised, arrested in 4°C modified Krebs Heinsleit buffer (KHB) (118mM NaCl, 4.7mM KCl, 1.25mM $CaCl_2$*$2H_2O$, 1.2mM $MgSO_4$*$7H_2O$, 1.1mM KH_2PO_4, 24mM $NaHCO_3$, 10mM Glucose), and cannulated to a Langendorff constant flow apparatus at the aorta. Hearts were perfused in a retrograde fashion using oxygenated ($95\%O_2/5\%CO_2$), normothermic (37°C) KHB. Using an ultrasound probe, flow rate was set to mimic an in vivo flow rate by using an equation which estimates coronary flow (CF) from heart weight (Döring, 1990) using the equation $CF = 7.43*HW^{0.56}$, and heart weight from body weight using the equation

HW=(BW*0.0027)+0.6. A fluid filled balloon connected to a pressure transducer was inserted into the left ventricle through an incision in the left atria and inflated to an end diastolic pressure of 7-10mmHg to monitor and record left ventricular function. A second pressure transducer was connected to the perfusate flow line just above the heart to record coronary perfusion pressure. An ultrasonic probe measured flow of perfusate into the heart. Coronary perfusion pressure (CPP), heart rate (HR), coronary flow rate, left ventricular systolic and end diastolic pressure (LVSP, LVEDP), and rate of pressure development (+dP/dt) were recorded. Langendorff hearts completed one of two procedures; an ischemia-reperfusion protocol, or a 20 minute stabilization protocol as outlined below. During stabilization hearts were inspected for exclusion criteria as depicted in Table 2.

Parameter	Criteria
Time to perfusion from excision (min)	>2 min
Heart Rate	<100bpm, >400bpm
Left ventricular developed pressure	<70mmHg

Table 2: Exclusion criteria for the Langendorff perfused rat hearts. Any criteria residing outside of these values suggests damage to an isolated heart during excision and cannulation.

Langendorff Protocol

All hearts underwent a 20 minute stabilization period. Prior to this hearts were cleared of fat and the left ventricular balloon was allowed to equilibrate to the temperature of the ventricle. After stabilization, 10 hearts from each group underwent 30 minutes of no flow ischemia, followed by 2 hours of reperfusion at the original flow rate. These hearts were then removed from the Langendorff, weighed, and frozen for infarct size measurement. A subset of 3 hearts from each group completed only the 20 minute stabilization protocol to flush blood from the system. These hearts were removed as described below, and used for biochemical analysis.

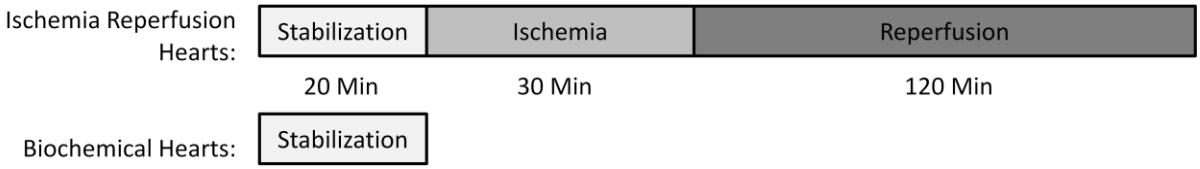

Figure 6: Langendorff isolated perfused heart protocols

The reperfusion period of 2 hours was chosen in order for effective washout of dehydrogenases, and therefore accurate infarct size staining (Schwarz et al., 2000), as well as to capture emergence from stunning, should stunning be apparent in our study. In order to establish viability of the preparation, we perfused isolated hearts from WKY and SHR animals for 3 hours (the maximal duration of our Langendorff protocol). As shown in Figure 6 there is a small, if any, decline in contractile function after 3 hours of isolation and perfusion. Two hearts from each group, and only one from WKY High were excluded from the study as they presented one or more exclusion factor as in Table 1, or they presented sustained fibrillation at reperfusion.

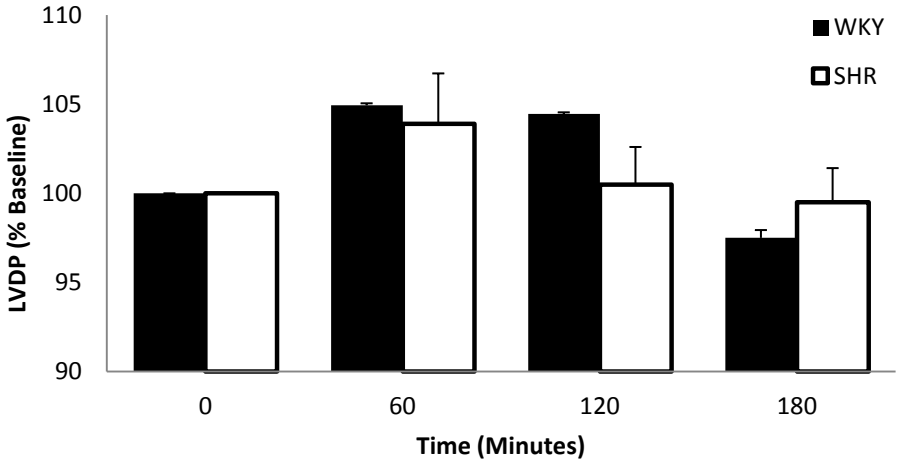

Figure 7: Contractility of Isolated Perfused Hearts over 3 Hours of baseline perfusion. Each group represents the average of 2 hearts. Abbreviations: SHR-spontaneously hypertensive rat, WKY-wistar Kyoto rat, LVDP- left ventricular developed pressure.

Infarct Size Measurement

At the end of reperfusion the hearts were excised, trimmed of remaining aorta and atrial appendage, and stored at -70°C sealed in plastic wrap and tin foil for future processing. Frozen hearts were sliced transversely from base to apex in 1mm slices using a razor blade. The slices were thawed at room temperature, blotted, and incubated in 1% wt/vol triphenyltetrazolium chloride (TTC) in a phosphate buffer solution at 37°C for 20 min. The slices were flipped after 10 min of incubation, and the containers were agitated at 5 min intervals. Slices were then blotted, fixed between 2 cover slides spaced at 1mm, and imaged using a dissection microscope and a digital camera. Infarct size was quantified using NIH Image 5.1 by dividing each image into ischemic but viable (TTC stained) and infarcted (TTC negative) zones. The total area (Figure 7A) and total infarct area (Figure 7B) of each slice was calculated and converted to millimeters squared. Volume was then calculated using the known thickness of 1mm. Total heart volume was quantified by adding slice volumes together, and total infarct volume of the heart was found by the sum of the slice infarct volumes. Infarct size was then quantified as a ratio of total heart infracted volume (Figure 7B) to total heart volume (Figure 7A).

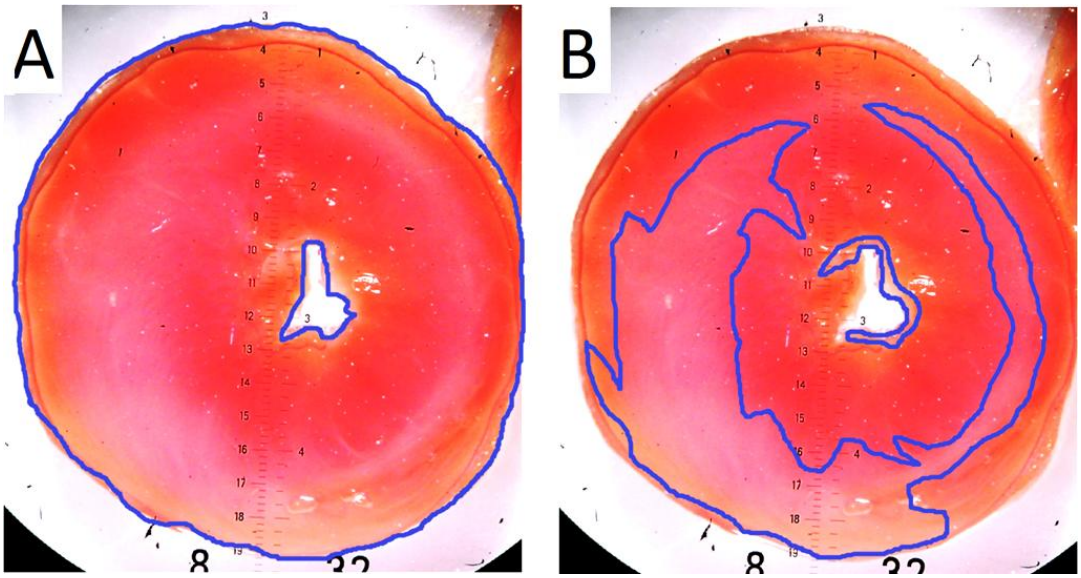

Figure 8: TTC Staining of a Heart Slice. Images do not represent slices from this study. A. Total heart area is outlined. B. Total infarct area is outlined. Infarct size is quantified as a ratio of infarcted area (B) to total slice area (A).

Western Blot Analysis

As described above, a separate set of hearts from each group (n=3 per group) were removed from the Langendorff apparatus following a 20 min stabilization period to assess protein expression. Hearts were cut into left and right ventricles, weighed, and the free wall of the left ventricle was excised and snap frozen in liquid nitrogen. Left ventricular tissue was hand homogenized at 0°C using Urea buffer containing phosphatase and protease inhibitors. Upon centrifugation, aliquot of homogenate, and heating of homogenate at 60°C for 3 hours, samples were denatured for 5 min at 95°C. 30µg of protein was loaded per well alongside 14µL biotin and 14µL rainbow biotin for molecular weight identification, and was resolved by SDS-PAGE (120V) and transferred to a polyvinylidene fluoride membrane (50V, 25min). The membrane was blocked in 5% Bovine Serum Albumin in 10mM Tris-HCL, 0.5M NaCl, 0.5% Tween 20, pH 7.4, for 1 hour at room temperature. The primary buffers were applied and

incubated for 12 hours at 4°C, or 1 hour at room temperature. After a washing cycle using 10mM Tris-HCL, 0.5M NaCl, 0.5% Tween 20, the membranes were incubated in the original block solution with the addition of secondary antibodies for 1 hour at room temperature. The membranes then underwent a second washing cycle and were imaged using enhanced horse-radish peroxidase chemiluminescence reagent on a SynGene (ChemiGene^2).

Statistical analysis

Values in this document are presented as mean±SEM, with *n* referring to the number of animals per group. Area under the curve was analyzed using a trapezoid method, and infarct size analysis using Image J (public domain NIH software). All results underwent two way ANOVA using SAS and Statistica 8.0 statistical software, and statistically relevant comparisons were tested using Tukey-Kramer HSD post hoc, and expected to reach a significance level of $p<0.05$.

RESULTS

Animal Characteristics

On average, across all treatment groups WKY animals are leaner than age-matched SHR (318.3±4.0 vs 344.1±3.9 g, $p<0.001$), which corresponds with lesser food (491.5±4.8 vs 532.6±4.9 g, $p<0.001$) and drink consumption (35.0±0.7 vs 47.2±0.7 mL/day, $p<0.001$) over a 4 week period. Treatment with resveratrol did not affect body weight or consumption levels (Table 3). The change in BW over 4 weeks did not differ among strain or treatment group (Table 3). Due to sacrifice of 1 animal per day, ages at the time of sacrifice ranged between 20-22 weeks.

Increased drink consumption corresponds to an increased resveratrol intake by SHR animals as compared to WKY in both treatment groups (Table 3). Consumption of resveratrol in the Low groups (WKY- 0.022mg/day, SHR- 0.030mg/day) was ~100 times less than in the High groups (WKY-2.02mg/day, SHR-2.79mg/day). As planned, when weight adjusted to the size of a 70Kg human, consumption in the Low group mimicked the resveratrol content in daily moderate red wine intake (~5.5mg/day), and in the High group mimicked the resveratrol content of supplemental intake (~508mg/day).

As expected, across strain, SHR animals show greater indices of left ventricular hypertrophy than WKY which is evidenced by an increased heart weight to body weight ratio (4.44±0.09 vs 3.89±0.10 mg/g, $p<0.001$), and increased left ventricular weight to body weight ratio (3.21±0.09 vs 2.75±0.09 mg/g, $p<0.01$). On average across treatment groups, Low resveratrol treatment significantly reduced heart weight as compared to Control (1.30±0.05 vs 1.46±0.04g, $p<0.05$); however, treatment with resveratrol did not affect other indices of hypertrophy including HW:BW and LVW:BW of either strain (Table 4).

Baseline Myocardial Contractility Data

In isolated hearts, all groups stabilized to a similar level of HR, left ventricular end diastolic pressure (LVEDP), left ventricular developed pressure (LVDP), and maximal rate of pressure development (+dP/dt) (Table 5). No strain, drug, or interaction effects exist.

Baseline Coronary Parameters

All coronary parameters were first analyzed unnormalized, and then normalized to BW to account for the appropriate setting of flow based on BW. Subsequently all coronary parameters were normalized to HW to allow for analysis per gram of heart tissue. Baseline coronary flow rates were higher in SHR animals, due to the setting of flow based on body weight (Table 6). When normalized to BW and to HW, coronary flow did not differ by treatment group or strain (Table 6).

Coronary perfusion pressure is a measure of the pressure gradient between the aorta and the right atrium. CPP at baseline was higher in SHR animals compared to WKY across all treatment groups, and this effect is abolished when normalized to HW (Table 6).

Strain and treatment did not affect other baseline parameters including coronary vascular resistance (CVR) and coronary vascular conductance (CDT), though differences emerged when normalized to HW. Across strain groups, when normalized to HW, CVR and CDT were affected by strain (Table 6). SHR animals had a higher level of CVR than WKY (9.9±0.46 vs 7.9±0.44 mmHg.min/mL/g $p<0.01$), and CDT was lower in SHR animals compared to WKY (10.9±0.5 vs 13.3±0.5 mL.min/mmHg/100g $p<0.01$). Interestingly, CDT was significantly lower SHR Control and Low hearts as compared to WKY Low, when normalized to HW (9.7±0.9 and 10.1±1.1 vs15.6± 0.9 mL.min/mmHg/100g $p<0.01$). In contrast, High resveratrol treatment increases conductance per gram of HW in SHR, to levels similar to those found in all WKY groups (Table 6).

Infarct Size

Across treatment groups, Figure 9 depicts a main effect of treatment, as evidenced by reduction in infarct size in both High and Low treated hearts as compared to Control hearts (19.9±0.9 and 19.4±0.8 vs 27.7±0.9 % of infarcted volume/total volume $p<0.001$). No strain or interaction effects exist.

Ischemia Reperfusion Myocardial Functional Parameters

AUC analysis was completed in attempt to tease out strain differences in cardiac functional parameters during reperfusion and ischemia. It should be stated that significant differences between groups for AUC analysis were less evident as AUC analysis takes into account variability among subjects for multiple time points, making the variability of AUC analysis between groups large. For LVDP and +dP/dt, AUC has been calculated by subtracting the area of 2 hours of baseline activity from the 2 hour reperfusion area to calculate change in area under the curve from baseline (ΔAUC).

LVEDP, a measure of contracture of the left ventricle, is maintained around baseline values during reperfusion by both High and Low resveratrol treatment, when analyzed on an individual time point basis (Figure 10). No strain differences or interaction effects exist. Interestingly resveratrol treatment at both a High and Low dose reduces the variability among subjects as compared to the Control group, and this holds true for both strains (Figure 10). During reperfusion, AUC for LVEDP is reduced in hearts treated with Low resveratrol as compared to the Control group, indicating less contracture with Low resveratrol treatment (Figure 11b).

Left ventricular developed pressure is normalized to baseline values in order to represent the percent change from baseline of an individual heart. During the early stages of reperfusion, a drug effect was not apparent (Figure 12). However, during the late stages of reperfusion, both the High and Low resveratrol treated hearts demonstrated significantly improved contractility as compared to Control hearts (Figure 12). This represents a stronger,

more protected functional response to ischemia reperfusion injury in the treated groups as compared to the Control. No strain differences or interaction effects exist. During reperfusion, the ΔAUC for LVDP is lesser in the High group as compared to the Control group (Figure 13). This represents less impaired left ventricular contractility during reperfusion with High resveratrol treatment.

Maximal rate of pressure development is normalized to baseline values in order to represent the percent change from baseline during reperfusion. During the early stages of reperfusion only the High resveratrol treatment group significantly protected against a decline in rate of pressure development (Figure 14). At the late stages of reperfusion both the High and Low resveratrol treatment significantly maintained +dP/dt as compared to no treatment (Figure 14). This represents a greater functional tolerance to ischemia reperfusion injury in the resveratrol treated hearts as compared to the Control hearts. No strain differences or interaction effects were found. ΔAUC for +dP/dt during reperfusion showed no strain differences. Both High and Low resveratrol treatment maintained ΔAUC around baseline +dP/dt, as compared to the Control group which had a large negative ΔAUC from baseline (Figure 15). This represents a reduction in +dP/dt in the Control group, and improvement of +dP/dt by resveratrol treatment during reperfusion.

Coronary Parameters During Reperfusion

Resveratrol treatment did not affect any coronary parameters during reperfusion, though strain differences exist.

Coronary flow during reperfusion was matched to basal flow rate and did not differ among groups when normalized to BW or HW. Coronary perfusion pressure did not show any effects of treatment or strain at 30 and 2 hours of reperfusion. This remained true when normalized to BW and HW.

Across strain groups, after 30 minutes of reperfusion CVR was lower in WKY animals as compared to SHR when normalized to HW (9.7±1.0 vs 12.8±0.9 mL.min/mmHg/100g $p<0.05$). No effects of drug or strain were apparent at 2 hours of reperfusion.

At 30 minutes of reperfusion CDT was higher in the WKY animals as compared to SHR when normalized to HW (10.8±0.4 vs 8.9±0.5 mL.min/mmHg/100g $p<0.01$) which mimicked the strain effect at baseline. By two hours of reperfusion the strain effect was abolished, and drug effects were not apparent (Table 7).

Western Analysis

Cyclooxygenase -1

As depicted in Figure 16 COX-1 protein expression is reduced by resveratrol treatment. Low resveratrol treatment significantly reduced COX-1 protein expression in both WKY and SHR as compared to Control. High resveratrol treatment depicts a reduced, but not significantly lower level of COX-1 protein as compared to Control. No strain differences exist.

Cyclooxygenase-2

Western blot analysis revealed a reduced level of COX-2 protein expression in SHR Con compared to WKY Con (0.76±0.06 vs 1.0±0.01 normalized arbitrary units, $p<0.001$) at baseline. In WKY, Low resveratrol treatment significantly reduced COX-2 expression, though High resveratrol treatment did not affect protein expression (Figure 17). In SHR, Low resveratrol treatment did not affect COX-2 protein, but High resveratrol significantly reduced COX-2 protein as compared to Control (Figure 17).

AMP Activated Protein Kinase

Western blot analysis for AMPK protein expression revealed no strain differences. AMPK expression in WKY High is significantly higher than all groups except WKY Low (Figure 18).

Phosphorylated AMPK

Western blot analysis for phosphorylated-AMPK (pAMPK) protein expression revealed no strain differences or interactions (Figure 19). Across treatment groups, Low resveratrol treatment resulted in significantly higher pAMPK protein expression compared to the High treatment group (1.13±0.09 vs 0.92±0.10 arbitrary normalized units $p<0.01$).

Ratio of Phosphorylated AMPK to AMPK

No main or interaction effects were found for the ratio of p-AMPK to AMPK, as demonstrated in Figure 20.

Endothelial Nitric Oxide Synthase

Western blot analysis of eNOS protein at baseline revealed lowered protein expression in SHR Con compared to WKY Con (0.67±0.007 vs 1.0±0.01 arbitrary normalized units $p<0.0001$). Interestingly resveratrol treatment did not affect eNOS protein levels in WKY animals, but in the SHR, High resveratrol treatment increased protein expression of eNOS, which was significantly higher than all other groups (Figure 21).

Phosphorylated Endothelial Nitric Oxide Synthase

Across treatment groups, as depicted in Figure 22, High resveratrol treatment significantly increases phosphorylated eNOS protein as compared to both Control and Low. No strain differences exist.

Ratio of Phosphorylated eNOS to eNOS

An interaction between strain and treatment exists for the ratio of activated to unactivated eNOS. WKY High hearts have an increased proportion of activated to unactivated eNOS as compared to WKY Con, and WKY Low, whereas SHR High animals show reduction in the ratio as compared to SHR Con and SHR Low (Figure 23). Additionally, SHR Con and Low animals have a larger ratio of activated to unactivated eNOS as compared to WKY Con (Figure 22).

Cytochrome C

Western blot analysis of cytochrome C protein expression revealed no differences between strain, or treatment groups (Figure 24).

Table 3: Consumption and Animal Characteristics.

	WKY			SHR		
	Con	**Low**	**High**	**Con**	**Low**	**High**
N	13	12	13	13	13	13
BW (g) †	313.23 ± 8.0	316.9 ± 4.7	324.8 ± 6.5	346.8 ± 4.1	336.5 ± 5.1	349.1 ± 9.3
ΔBW (g)	21.5 ± 6.4	18.9 ± 4.9	19.1 ± 3.5	19.5 ± 3.5	22.9 ± 1.9	12.9 ± 4.2
DDC (mL/day) †	35.6 ± 1.0	35.7 ± 0.9	33.7 ± 0.7	48.2 ± 1.5	48.4 ± 1.7	45.0 ± 1.5
Food Consumption (g) †	484.5 ± 8	492.9 ± 3	497.0 ± 9	533.1 ± 10	522.8 ± 5	541.9 ± 10
Resveratrol Consumption (mg/day)†	0	0.022 ± 0.006	2.02 ± 0.4 X*	0	0.030 ± 0.001	2.79 ± 0.1 X*

Values are from all animals being used for Langendorff analysis and western blot analysis. Values represent means ± S.E.M. Food consumption is the total consumption over 28 days. †$p<0.0001$ strain effect; $^{X}p<0.0001$ High vs Con; *$p<0.0001$ High vs Low. Abbreviations: n-number of animals; BW-body weight; ΔBW-change in body weight; DDC-average daily drink consumption.

Table 4: Animal Heart Characteristics

	WKY			SHR		
	Con	**Low**	**High**	**Con**	**Low**	**High**
n	10	9	12	10	10	12
HW (g) †	1.27 ± 0.05	1.19 ± 0.02[X]	1.26 ± 0.05	1.65 ± 0.09	1.41 ± 0.05 [X]	1.52 ± 0.07
HW:BW(mg/g) †	4.00 ± 0.4	3.78 ± 0.3	3.89 ± 0.4	4.75 ± 0.9	4.20 ± 0.5	4.37 ± 0.6
LVW:BW (mg/g) (n=3/group) †	2.97 ± 0.2	2.57 ± 0.1	2.71 ± 0.04	3.47 ±0.3	3.03 ± 0.1	3.15 ± 0.2

Values are means ± S.E.M. HW was measured at the end of the Langendorff ischemia reperfusion protocol. LVW was measured after a 20 minute stabilization period on the Langendorff. †$p<0.01$ strain effect; [X]$p<0.05$ drug effect Low vs Con;.Abbreviations: n-number of animals; BW-body weight; HW-heart weight; LVW- left ventricular weight.

Table 5 Baseline myocardial contractile parameters of the isolated perfused hearts

	WKY			SHR		
	Con	**Low**	**High**	**Con**	**Low**	**High**
n	8	8	9	8	8	8
LVEDP (mmHg)	9.84 ± 0.75	7.77 ± 0.79	8.49 ± 0.83	9.72 ± 0.98	8.3 ± 1.24	8.86 ± 0.941
LVDP (mmHg)	141.4 ± 3.4	135.1 ± 3.73	145.6 ± 4.4	143.9 ± 10.1	149.9 ± 6.3	144.1 ± 6.6
+dP/dt$_{max}$ (mmHg/s)	3852.1 ±131.1	3509.4 ± 184.9	3719.9 ± 155.6	3994.3 ± 270.9	3803.1 ± 187.6	3872.6 ± 322.2
HR (bpm)	204±8	201±9	212±7	216±5	185±11	206±11

Values are from all hearts undergoing IR. Values are means ± S.E.M. Abbreviations: n- number of animals; LVEDP-left ventricular end diastolic pressure; LVDP- left ventricular developed pressure; +dP/dt$_{max}$- peak rate of pressure development; HR- heart rate; bpm- beats per minute.

Table 6: Baseline coronary parameters of the isolated perfused hearts undergoing IR.

	WKY			SHR		
	Con	**Low**	**High**	**Con**	**Low**	**High**
n	8	8	9	8	8	8
CF (ml/min) †	9.21 ± 0.14	9.41 ± 0.13	9.29 ± 0.09	9.54 ± 0.06	9.44 ± 0.08	9.48 ± 0.07
CF:HW (ml/min/100g)	7.02 ± 0.23	7.60 ± 0.22	7.21 ± 0.36	5.90 ± 0.37	6.41 ± 0.19	6.93 ± 0.25
CPP (mmHg)	59.1 ± 3.2	47.2 ± 5.2	59.4 ± 4.2	62.5 ± 3.1	63.7 ± 4.3	56.8 ± 4.9
CVR (mmHg.min/ml)	6.42 ± 0.35	5.06 ± 0.51	6.49 ± 0.52	6.58 ± 0.36	7.30 ± 0.49	5.99 ± 0.59
CVR:HW † (mmHg min/ml/100g)	8.57±0.78	6.45±0.83	8.39±0.74	11.0±0.74	10.20±0.79	8.34±0.78
CDT:HW† (mL/min mmHg/100g)	12.2±0.9	15.2±1.0	12.3±0.8	9.7±0.8*	10.1±0.9*	12.9±0.9

Values are from all hearts undergoing IR. Values are means ± S.E.M. *p<0.05 vs WKY low; †p<0.05 strain effect.
Abbreviations: n- number of animals; CF- coronary flow; CPP- coronary perfusion pressure; CVR- coronary vascular resistance; CDT-conductance; HW-heart weight

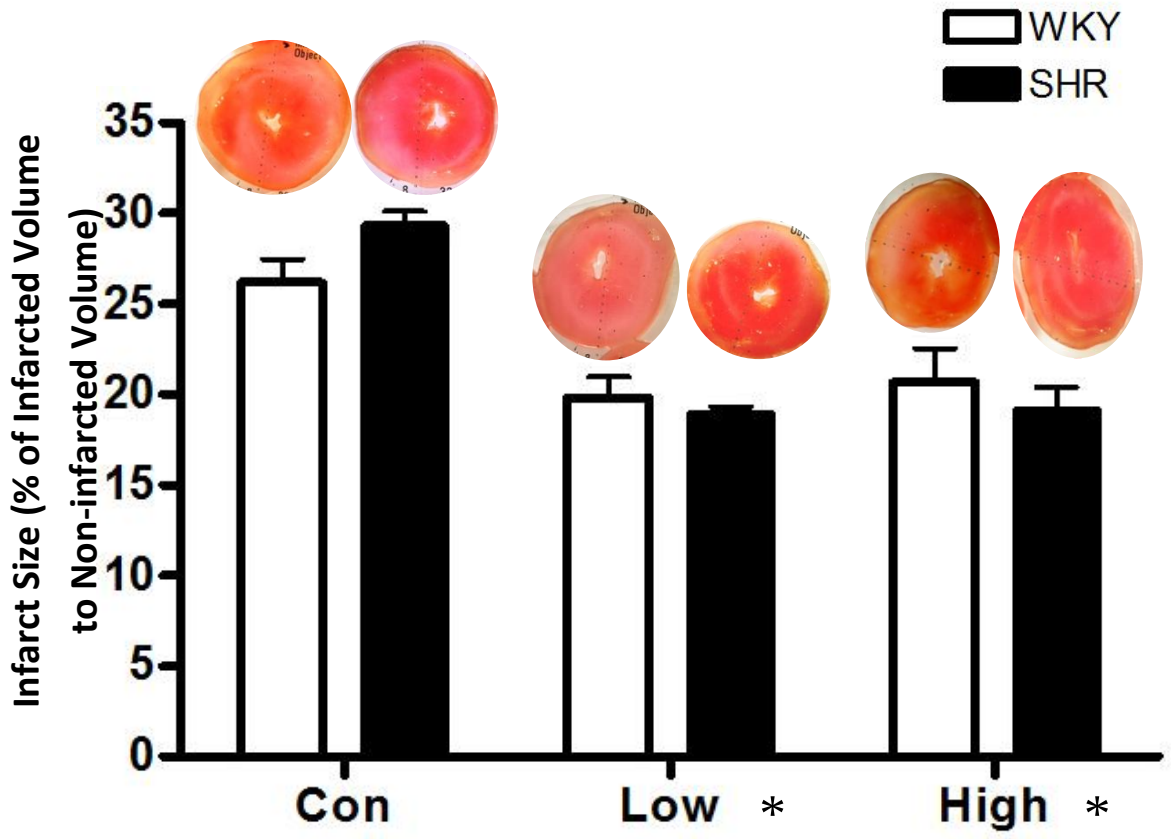

*Figure 9: Myocardial Infarct Size. Calculated as a ratio of infarcted tissue (pale peach/white) to total tissue volume (red), as determined by TTC staining on hearts that underwent 30 min ischemia and 2 hours reperfusion. Values represent means ± S.E.M. *p<0.001 vs control.*

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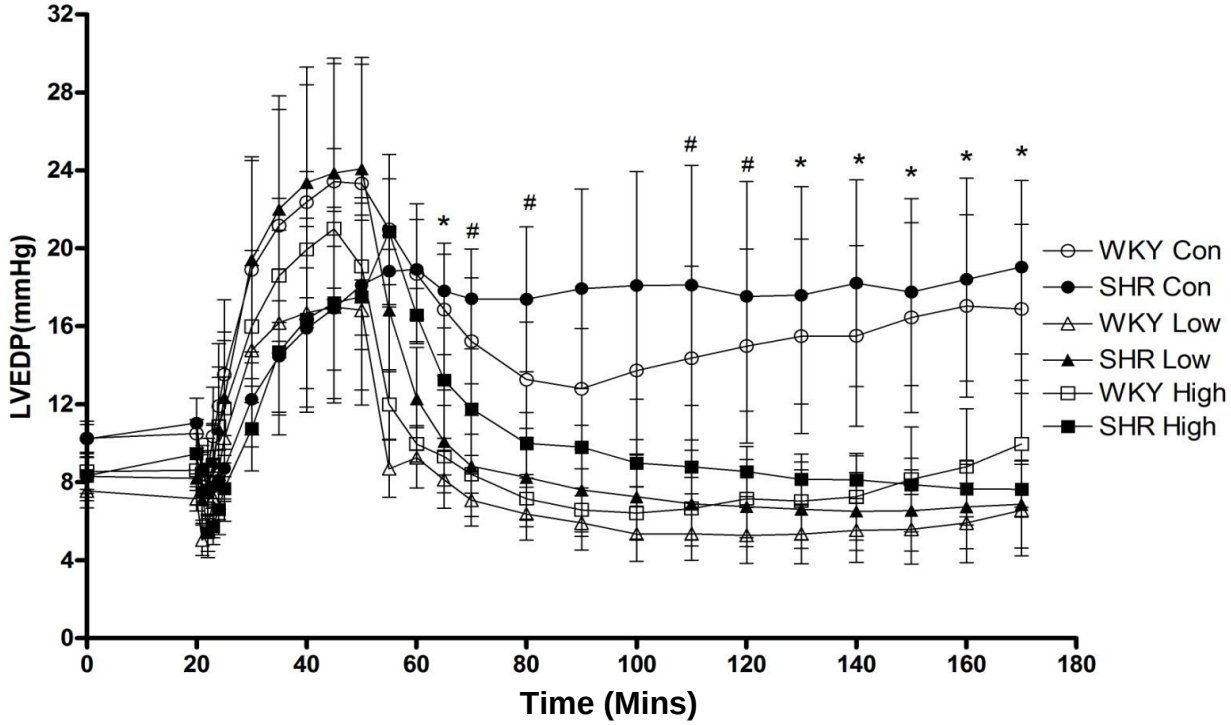

*Figure 10: LVEDP curve during stabilization, ischemia, and reperfusion. Values represent means ± S.E.M.*p<0.05 High and Low vs Con, #p<0.05 Low vs Con. Abbreviations: LVEDP-left ventricular end diastolic pressure; Con-control.*

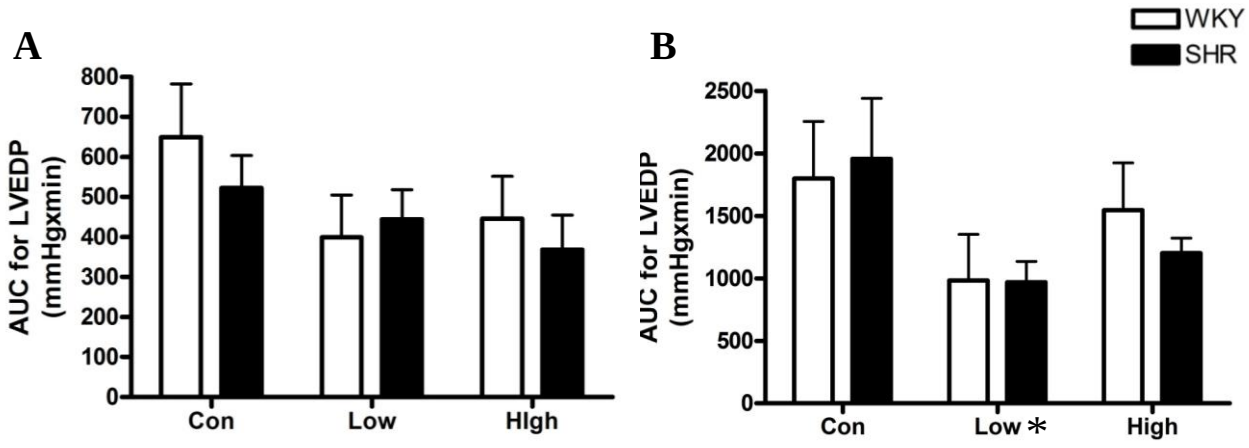

*Figure 11: Area under the curve for LVEDP. A. Area under the curve for LVEDP during 30 mins ischemia. B. Area under the curve for LVEDP during 2 hours reperfusion. Values represent means ± S.E.M. *p<0.05 Low vs Con. Abbreviations: LVEDP-left ventricular end diastolic pressure; Con-control; AUC- area under the curve.*

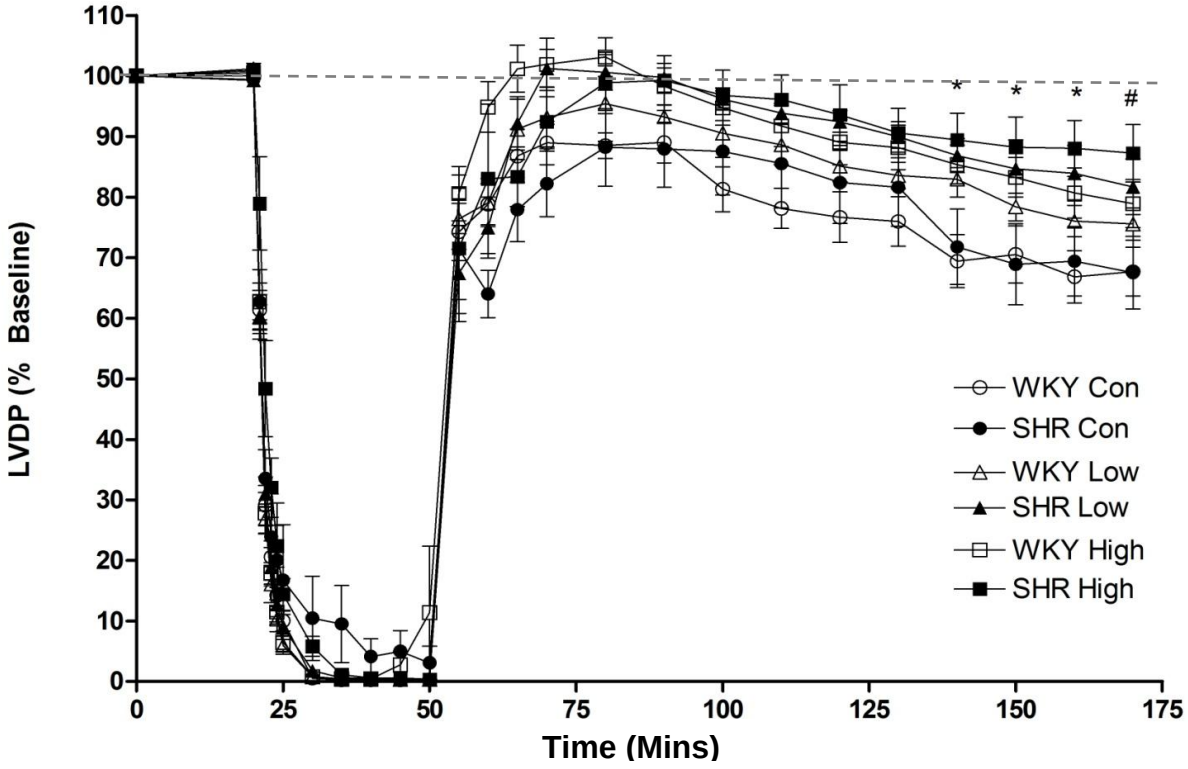

*Figure 12: LVDP during stabilization, ischemia, and reperfusion. All data points are normalized to their respective baseline values, and represent means ± S.E.M. The dashed line represents the baseline in which AUC was calculated from in the subsequent figure. *p<0.05 High and Low vs Con, #p<0.05 High vs Con. Abbreviations: LVDP-left ventricular developed pressure; Con-control.*

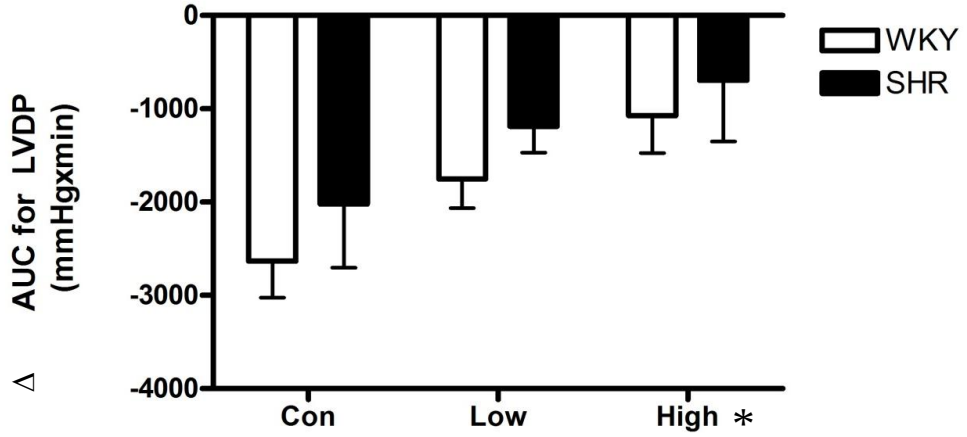

*Figure 13 Change in area under the curve from baseline for LVDP during 2 hours of reperfusion. Baseline area under the curve for 2 hours of reperfusion was subtracted from each condition. Values represent means ± S.E.M *p<0.05 High vs Con. Abbreviations: LVDP-left ventricular end diastolic developed pressure; Con-control; ΔAUC- change in area under the curve from baseline*

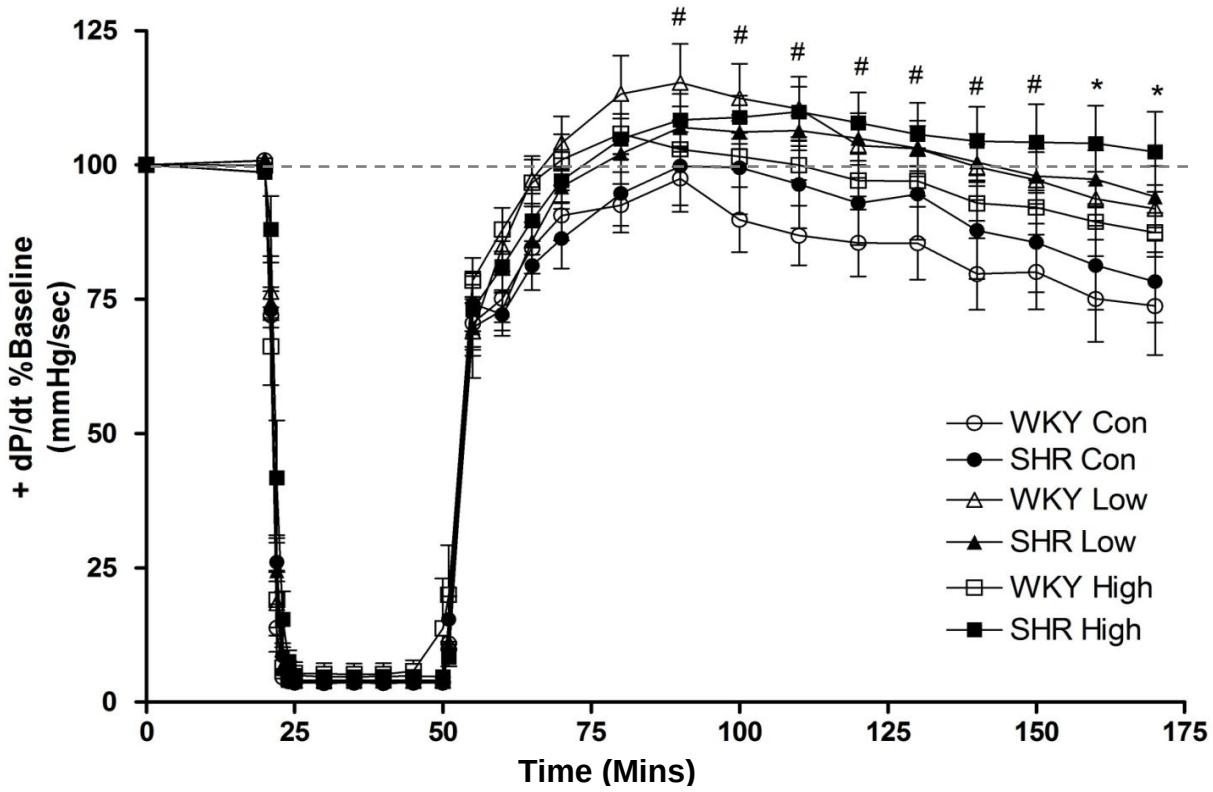

*Figure 14: Maximal rate of pressure development. All data is normalized to its respective baseline value. Values represent means±S.E.M. The dashed line represents the baseline in which AUC was calculated from in the subsequent figure. *p<0.05 High and Low vs Con, #p<0.05 High vs Con.Abbreviations: LVDP-left ventricular developed pressure; Con-control.*

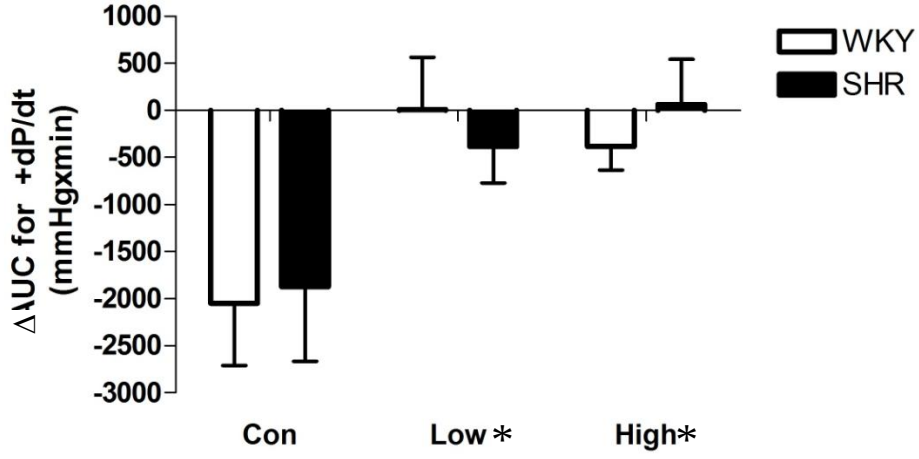

*Figure 15: Area under the curve for rate of pressure development during 2 hours of reperfusion. AUC of baseline across 2 hours of reperfusion was subtracted from each condition. Values represent means ± S.E.M *p<0.01 vs Con. Abbreviations: LVDP-left ventricular end diastolic developed pressure; Con-control; ΔAUC- change in area under the curve from baseline.*

Table 7: Summary Table of Functional and Coronary Parameters During Reperfusion

	WKY			SHR		
	Con	**Low**	**High**	**Con**	**Low**	**High**
30 min Reperfusion						
LVEDP (mmHg)	12.6±2.8	6.7±1.3	9.17±2.2	13.9±4.5	7.38±2.3	9.98±1.1
LVDP (%BL)	88.5±2	95.4±5.9	103.3±3.1	100.3±1.3	100.6±2.8	98.88±4.5
HR (BPM) †	202±10	208±8	191±11	185±12	176±7	188±7
CPP:HW (mmHg/g)	49.9±3.1	47.8±4.3	61.8±6.6	46.7±2.2	53.0±2.7	51.0±3.3
CVR:HW † (mmHg.min/mL/100g)	9.9 ±0.17	8.3±0.13	11.0 ±0.16	14.6 ±0.26	12.1±0.12	11.7±0.31
CDT:HW† (mL/min.mmHg/100g)	10.8.±0.8	12.2±0.9	9.3±0.8	8.4±0.8	8.5±0.9	9.8±0.9
2 Hours Reperfusion						
LVEDP (mmHg)	15.6±3.9	9.08±3.2*	9.96±3.0*	15±4.3	5.6±2.3*	7.6±1.4*
LVDP (%BL)	67.7±3.8	75.5±2.5*	81.1±3.9*	71.3±2.1	81.7±4.3*	87.2±4.2*
HR (BPM)	189±9	200±7	182±10	191±9	171±9	178±4
CPP (mmHg/g)	66.4±8.6	56.9±3.8	76.2±7.9	60.3±4.3	66.5±7.3	61.7±5.4
CVR:HW (mmHg.min/mL/100g)	13.8±3.0	10.1±0.73	15.2±1.4	20.4±0.61	15.2±1.5	15.8 ±0.85
CDT:HW (mL/min.mmHg/100g)	8.9±1.4	10.3±1.1	7.1±0.8	6.7±0.9	7.0±0.71	7.9±1.0

All values reperesent means±S.E.M after 30 minutes of reperfusion, and after 2 hours of reperfusion. Strain effect † $p<0.01$, drug effect * $p<0.05$ vs. Con. Abbreviations: LVEDP-left ventricular end diastolic pressure; LVDP- left ventricular developed pressure; HR- heart rate; bpm-beats per minute; CF- coronary flow; CPP- coronary perfusion pressure; CVR-coronary vascular resistance; HW-heart weight.

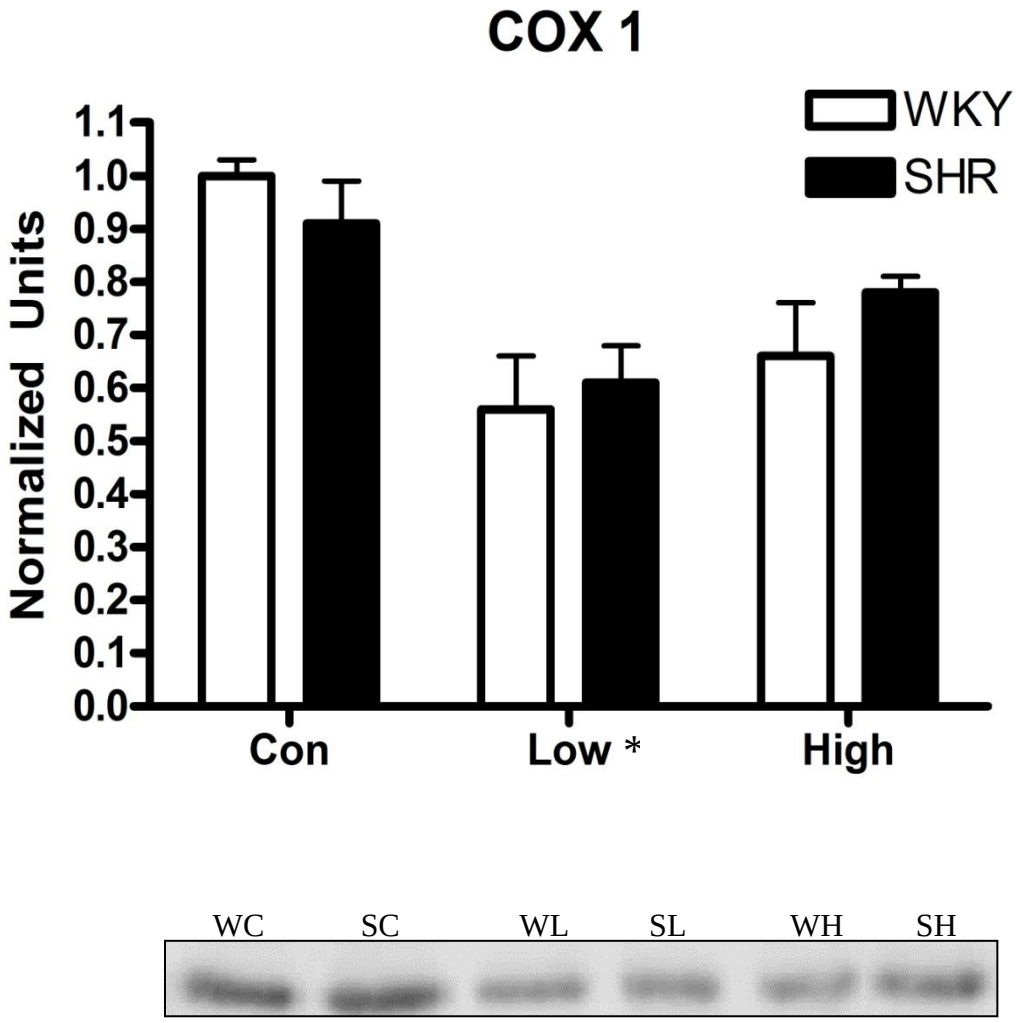

*Figure 16: Western blot analysis of COX-1 protein. All values are normalized to WKY Con. COX-1- cyclooxygenase-1. Values represent means ± S.E.M of densiometry analyses. *p<0.01 vs Con. Abbreviations: COX-1-cyclooxygenase 1; Con- control; SC- SHR Con; SL-SHR Low; SH-SHR High; WC-WKY Con; WL-WKY Low; WH- WKY High.*

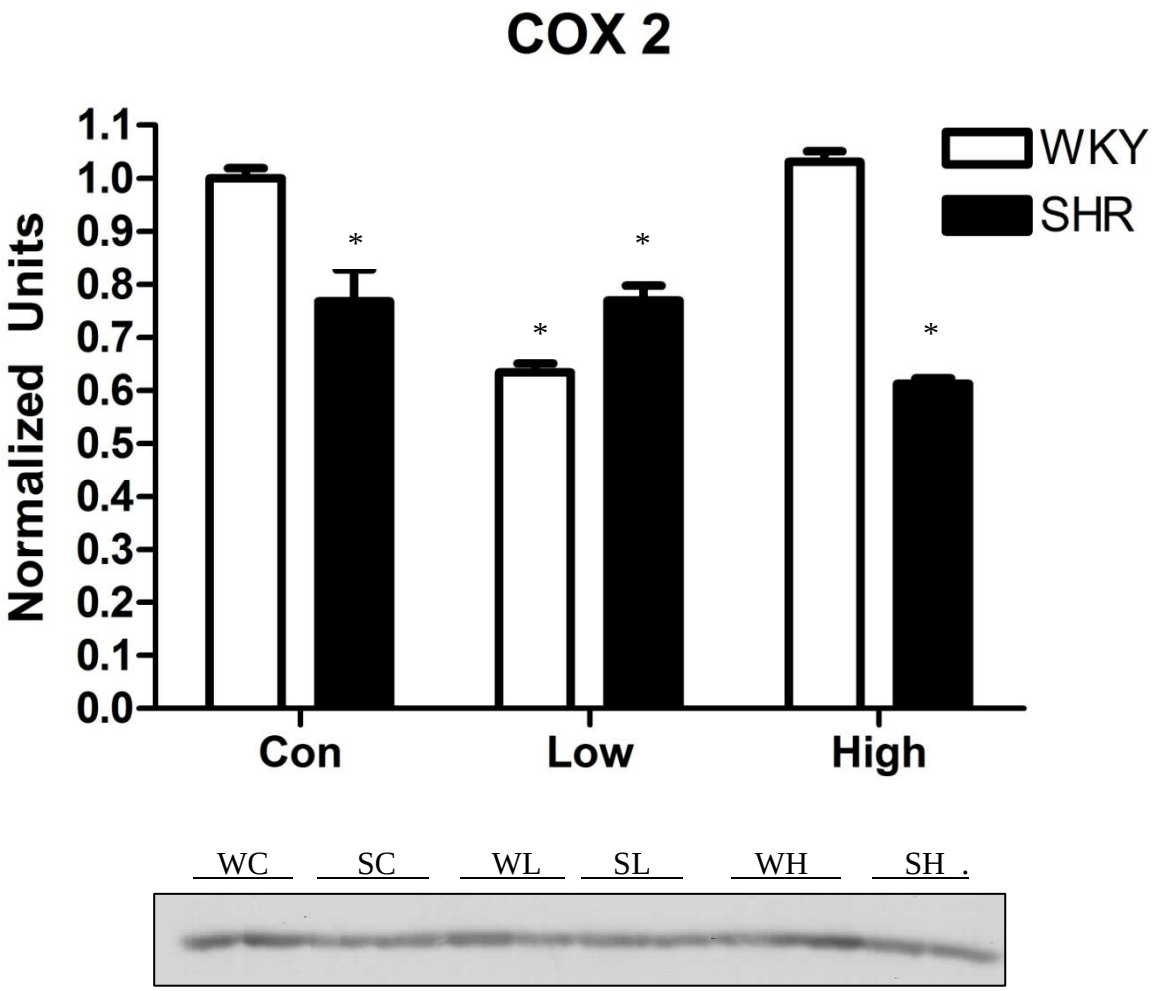

*Figure 17: Western blot analysis of baseline COX-2 protein. All values are normalized to WKY Con. Values represent means ± S.E.M of densiometry analyses. *p<0.01 vs WKY Con or WKY High. Abbreviations: COX-2-cyclooxygenase 2; Con- control; SC- SHR Con; SL-SHR Low; SH-SHR High; WC-WKY Con; WL-WKY Low; WH- WKY High.*

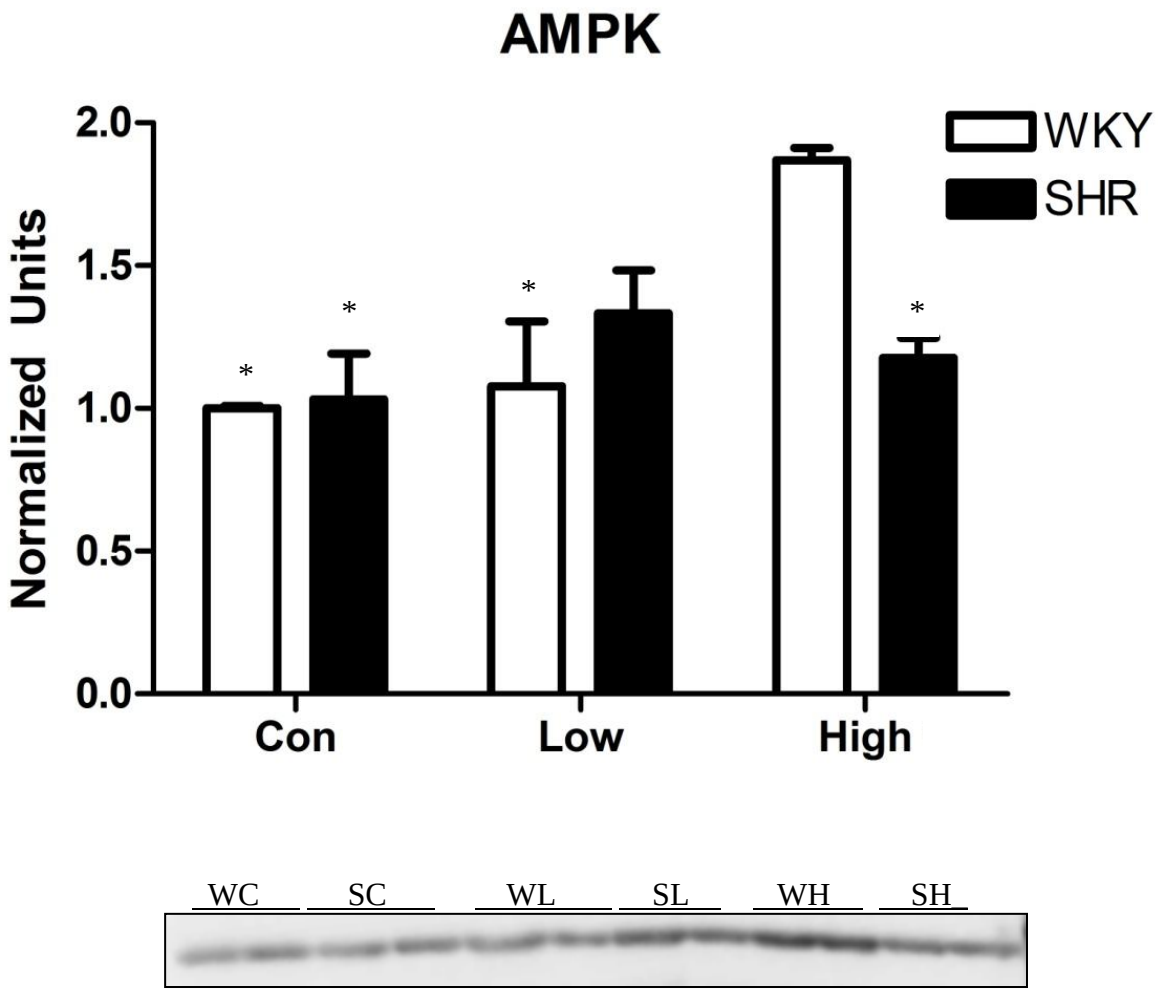

*Figure 18 Western blot analysis of AMPK protein. All values are normalized to WKY Con. Values represent means ± S.E.M of densiometry analyses. *p<0.05 vs WKY High. Abbreviations: AMPK- AMP activated protein kinase; Con- control; SC- SHR Con; SL-SHR Low; SH-SHR High; WC-WKY Con; WL-WKY Low; WH- WKY High.*

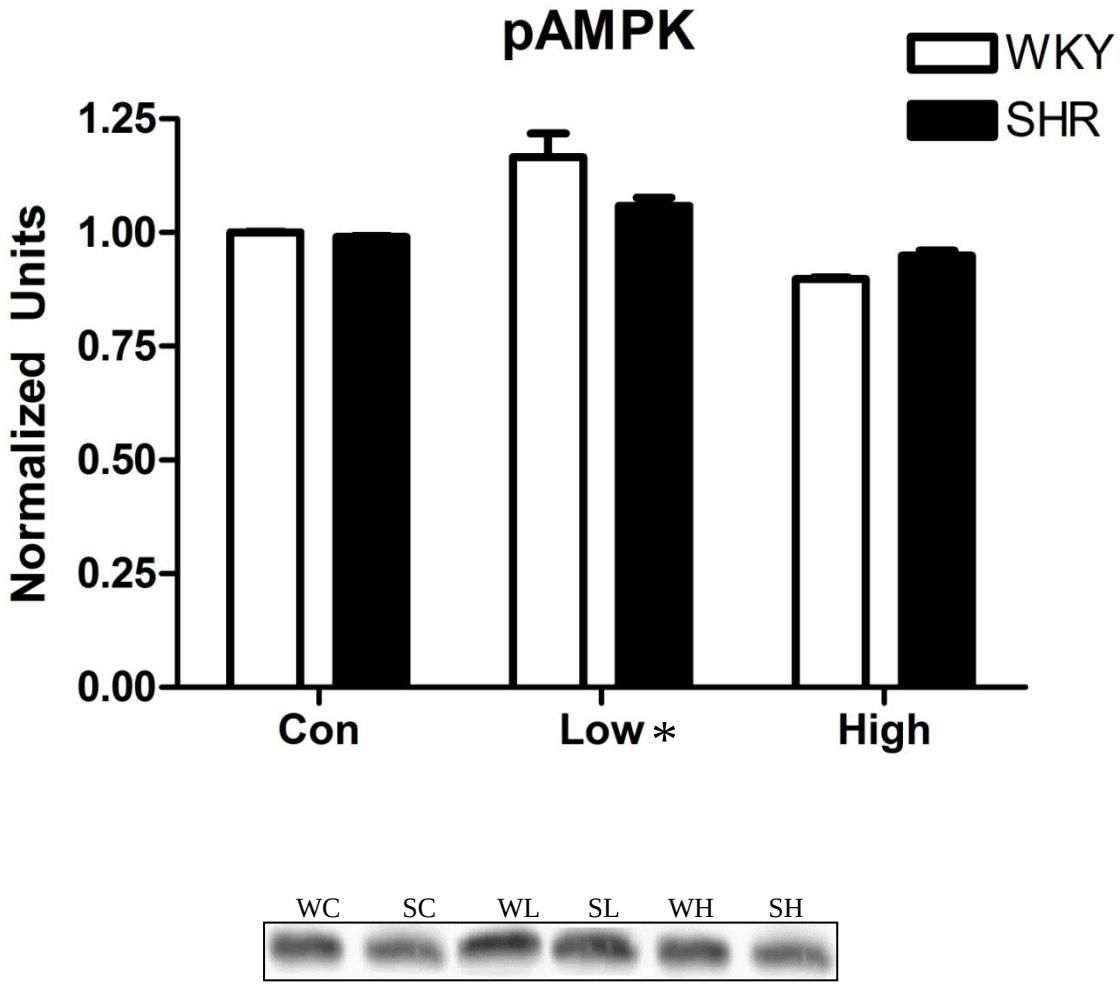

*Figure 19: Western blot analysis of phosphorylated AMPK protein at Threonine175. All values are normalized to WKY Con. Values represent means ± S.E.M of densiometry analyses. *p<0.01 vs High. Abbreviations: pAMPK- phosphorylated AMP activated protein kinase; Con- control; SC- SHR Con; SL-SHR Low; SH-SHR High; WC-WKY Con; WL-WKY Low; WH- WKY High.*

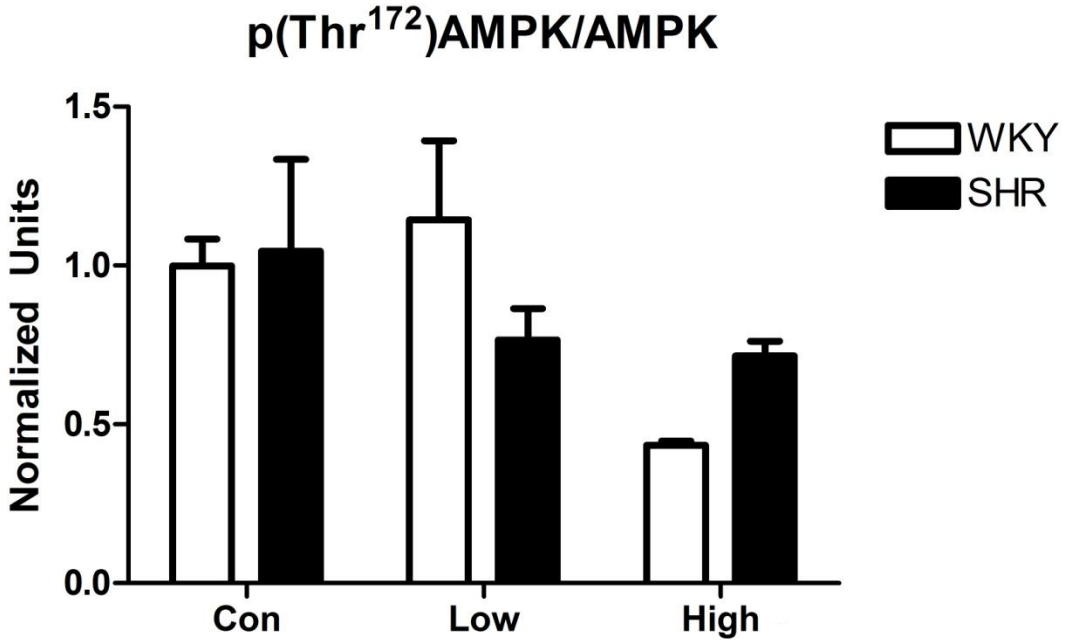

Figure 20: Ratio of phosphorylated AMPK to total AMPK protein. All values are normalized to WKY Con ratios. Values represent means ± S.E.M of densiometry analyses. Abbreviations: pAMPK- phosphorylated AMP activated protein kinase; Con-control; SC- SHR Con; SL-SHR Low; SH-SHR High; WC-WKY Con; WL-WKY Low; WH- WKY High.

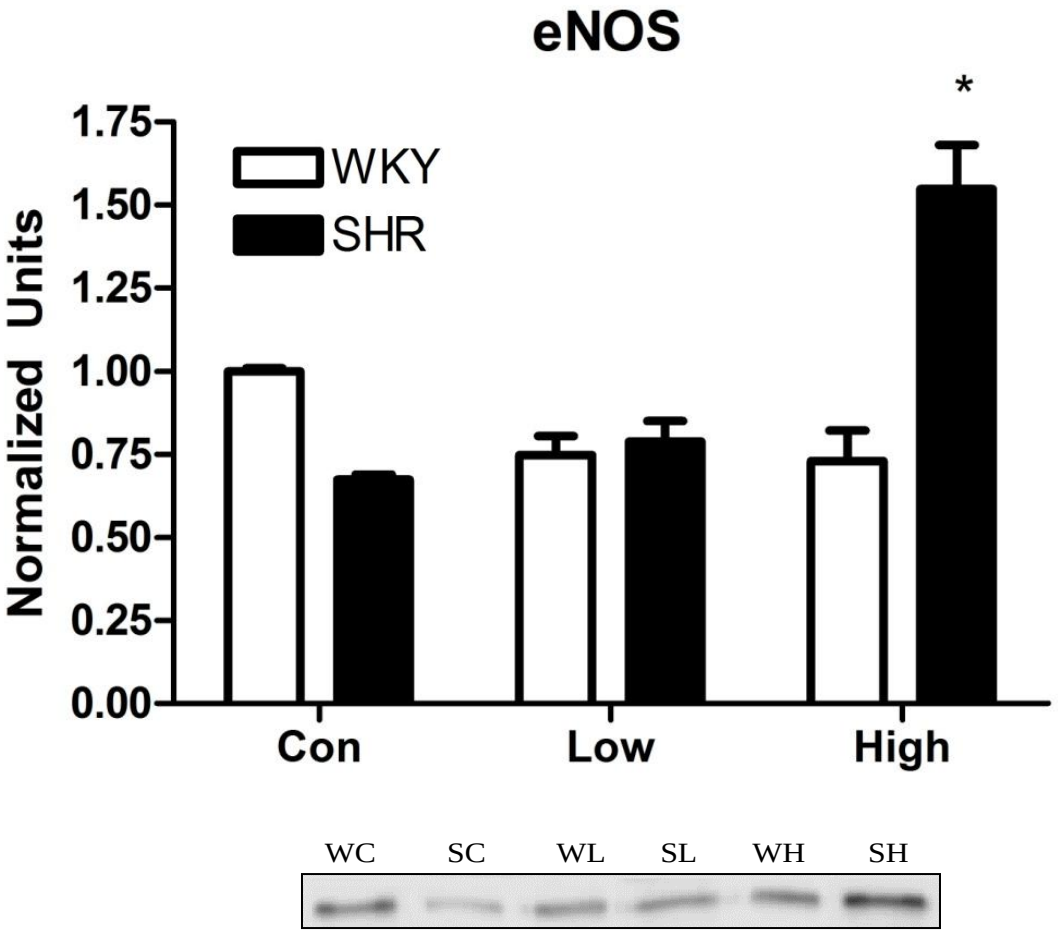

*Figure 21: Western blot analysis of eNOS protein. All values are normalized to WKY Con. Values represent means ± S.E.M of densiometry analyses. *p<0.01 vs all groups. Abbreviations: eNOS- endothelial nitric oxide synthase; Con-control; SC- SHR Con; SL-SHR Low; SH-SHR High; WC-WKY Con; WL-WKY Low; WH- WKY High.*

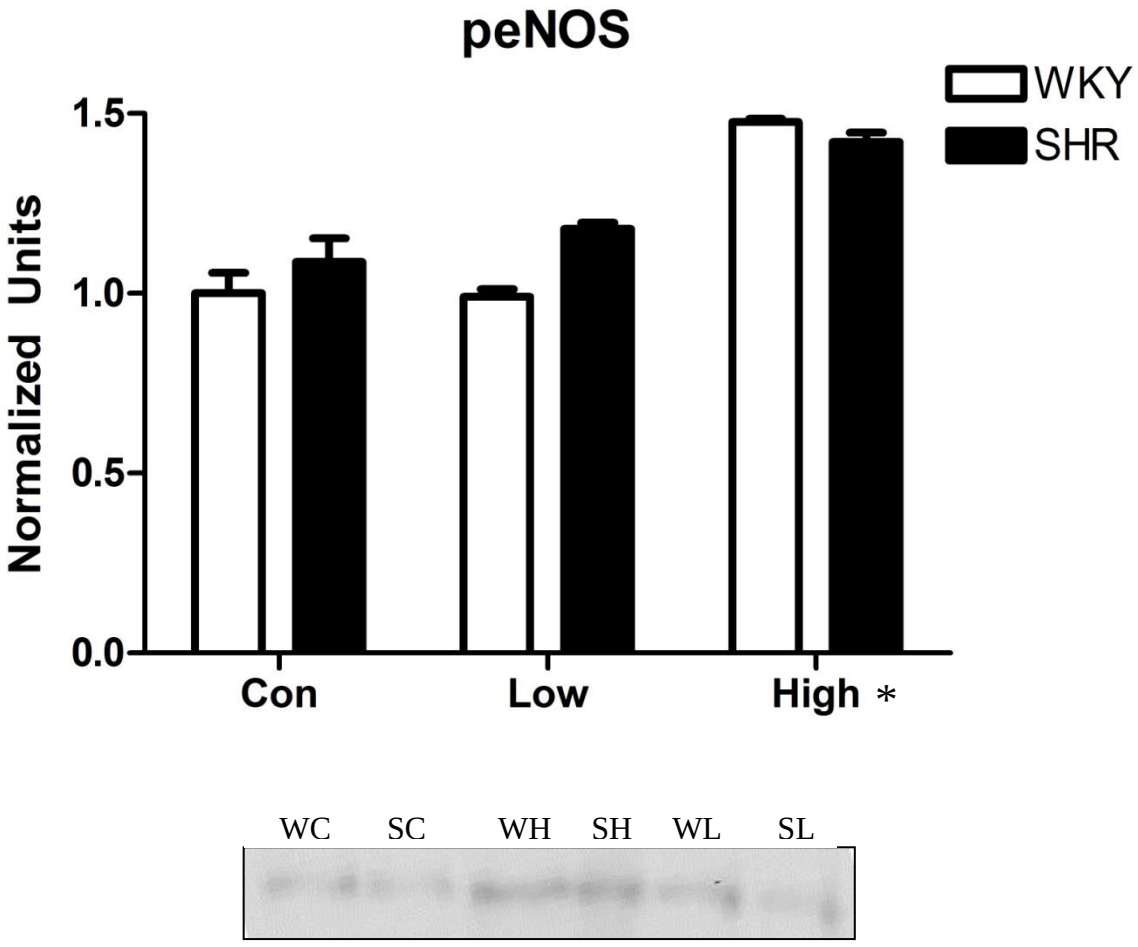

*Figure 22: Western blot analysis of phosphorylated eNOS protein at Serine1177. All values are normalized to WKY Con. Values represent means ± S.E.M of densiometry analyses. *p<0.0001 vs Con and Low. Abbreviations: peNOS- phosphorylated endothelial nitric oxide synthase; Con-control; SC- SHR Con; SL-SHR Low; SH-SHR High; WC-WKY Con; WL-WKY Low; WH- WKY High.*

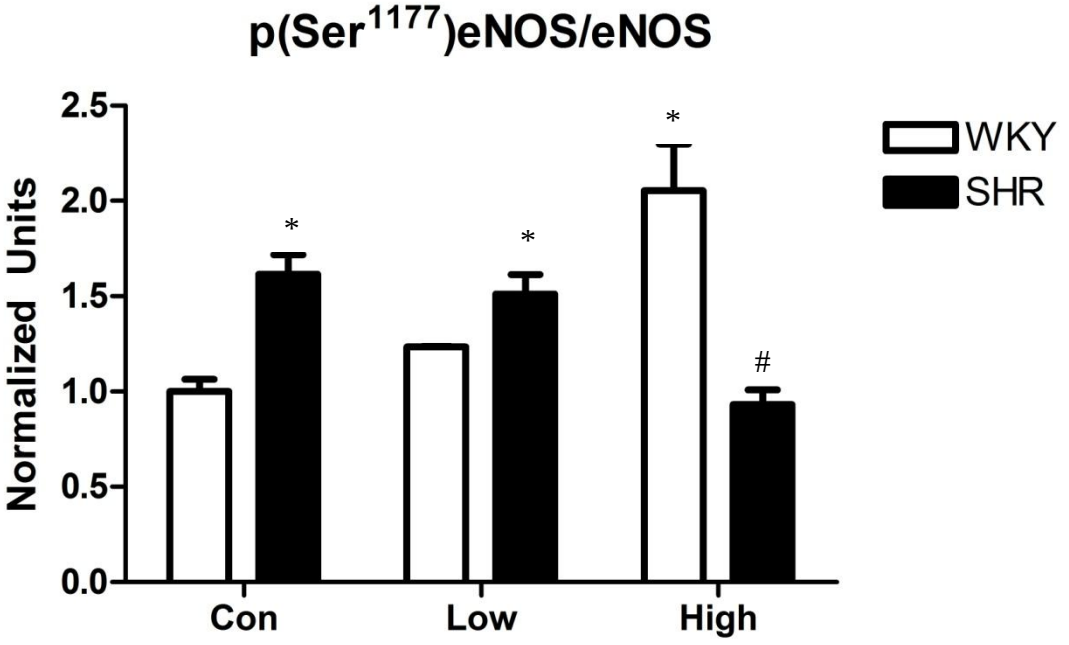

*Figure 23: Ratio of phosphorylated eNOS to total eNOS protein. All values are normalized to WKY Con ratios. Values represent means ± S.E.M of densiometry analyses. *p<0.01 vs WKY Con, #p<0.01 vs SHR Con. Abbreviations: peNOS-phosphorylated endothelial nitric oxide synthase; Con-control; SC- SHR Con; SL-SHR Low; SH-SHR High; WC-WKY Con; WL-WKY Low; WH- WKY High.*

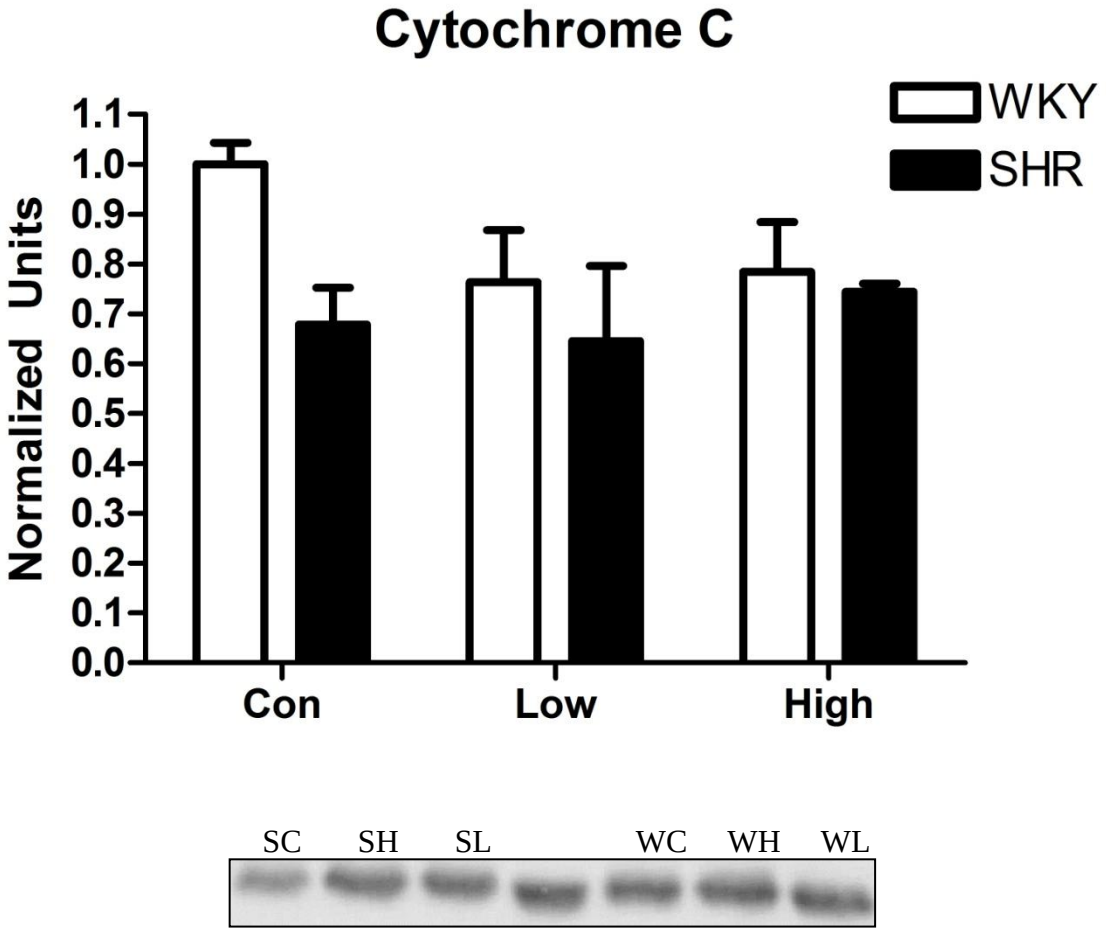

Figure 24: Western blot analysis of cytochrome C protein. All values are normalized to WKY Con. All values are normalized to WKY Con. Values represent means ± S.E.M of densiometry analyses. Abbreviations: Con-control; SC- SHR Con; SL-SHR Low; SH-SHR High; WC-WKY Con; WL-WKY Low; WH- WKY High.

DISCUSSION

In this study we evaluated the cardioprotective effects of resveratrol treatment on hypertensive and normotensive animals by providing a dose of resveratrol in the drinking water that mimicked the resveratrol content one could ingest through moderate red wine consumption (Low), and a second dose that mimicked the resveratrol amount that one could ingest through daily supplemental consumption (High). Secondary to this we investigated protein expression in treated and untreated hearts.

Main Findings:

1. *Chronic* resveratrol treatment does not affect animal characteristics, baseline cardiac functional parameters, or indices of hypertrophy.
2. Coronary parameters are not affected by resveratrol treatment at baseline, but are affected by strain when normalized to BW or HW.
3. *Chronic* resveratrol treatment reduces infarct size, and a High dose protects the myocardium against infarction to the same extent as a Low dose. Strain differences are not evident.
4. Coronary parameters during reperfusion are not affected by resveratrol treatment.
5. *Chronic* resveratrol treatment improves functional recovery of contractility (LVDP and +dP/dt) to the same extent in WKY and SHR, and a Low dose is as effective as a High dose.
6. *Chronic* resveratrol treatment at both a High and Low dose protects against contracture (LVEDP) during reperfusion to the same extent in WKY and SHR.
7. Strain differences do not exist for recovery of cardiac functional parameters during reperfusion

Animal Characteristics

Similarly to the literature (Bradamante et al., 2003; Hung et al., 2004; Rush et al., 2007), resveratrol treatment did not affect animal characteristics including body weight and food consumption, though strain differences were apparent. SHR animals weighed more than WKY at the beginning and end of the treatment protocol, and this corresponds with greater food and water intake. It is typical for SHR weight gain to diverge from WKY between the ages of 13 to 25 weeks (Pfeffer et al., 1979). Due to increased water intake, SHR High animals consumed greater amounts of resveratrol than WKY High, although WKY Low and SHR Low groups did not differ. Although differences existed between the resveratrol intake in SHR and WKY in the High groups, this is likely not a limiting factor in our study as the functional data does not suggest a dose dependant increase in protection in SHR versus WKY. As expected resveratrol intake was 100 times greater in the High group versus the Low group.

As planned, when weight-adjusted to the size of a 70Kg human, consumption of resveratrol in the Low group (5.5mg/day) mimicked the amount resveratrol attainable through daily moderate red wine intake (Faustino et al., 2003), and in the High group (508mg/day) mimicked the amount of resveratrol present in supplemental form (Smoliga et al., 2011).

Strain differences that were expected emerged for indices of hypertrophy. Hypertrophy of the left ventricle is evidenced by 13 weeks of age in SHR (Pfeffer et al., 1979), and was evident in our study as demonstrated by increased indices of hypertrophy including HW:BW and LVW:BW at 20-22 weeks of age. Low resveratrol treatment reduced HW across treatment groups, as compared to Control, but is simply an artifact of a lesser body weight in this group, and not a reduction in hypertrophy.

Resveratrol treatment did not affect indices of hypertrophy in our study, which contrasts with a previous study showing the anti-hypertrophic effects of resveratrol treatment in SHR (Thandapilly et al., 2010). In contrast to our methods, this study used resveratrol injections at a much higher concentration than ours, and began treatment at 10 weeks of age, for a 10 week period (Thandapilly et al., 2010). Our treatment differed by lower,

physiologically relevant doses, and began later in the developmental phase of hypertension- at a time when clinical treatment would be administered to a patient presenting with hypertension. It is possible that with a longer treatment period our dosages could produce anti-hypertrophic effects.

Baseline Cardiac Function and Coronary Parameters

As confirmed by others (Bradamante et al., 2003; Hung et al., 2004; Rush et al., 2007), resveratrol treatment at either dose did not affect baseline cardiac function (LVDP, LVEDP, +dP/dt).

Coronary parameters at baseline (CPP, CVR, CDT) were not affected by resveratrol treatment in our study; however, strain differences exist. Recent studies confirm our observations that flow per gram in the hypertrophied myocardium of SHR is similar to that of a non hypertrophied heart at 22 weeks of age (Susic et al., 2008).

When normalized to either BW or HW, higher CVR and lower CDT are apparent in SHR versus WKY. Previous studies have confirmed that resistance in coronary arteries is increased as a result of hypertensive left ventricular hypertrophy, and that conductance is reduced (Kelm et al., 1995; Edoute et al., 1986; Susic et al., 2008). *Acute* Resveratrol treatment has been shown to elicit a vasodilatory response in isolated porcrine coronary arteries (Jäger et al., 1999), and *acute* incubation of human coronary arteries with red wine extract elicits an endothelium dependant dilation (Flesch et al., 1998), though whether these effects hold true with *chronic* resveratrol treatment in the coronary arteries has yet to be determined. Using CVR as a measure of vasomotor state, we did not find greater basal vasodilation with resveratrol treatment in our study. The differences are likely attributable to the treatment model (*acute* vs *chronic*), and artery preparation (isolated from the heart versus intact on an isolated heart). The *acute* effects are a result of direct stimulation of the endothelium and/or smooth muscle with resveratrol incubation. In contrast to this, any effects of resveratrol on the

coronary arteries in our study could only to be due to stored resveratrol within a cell, or adaptations to protein expression within a cell.

Infarct Size

In a recent study, 27 week old SHR animals with LV hypertrophy demonstrate a greater infarct size post ischemia reperfusion than do WKY (~38% vs. ~30% infarcted tissue/total area at risk) (Ravingerová et al., 2011). We found no difference between the infarct size of SHR and WKY animals. Our infarct size in WKY Con is similar to that in the literature, however our infarct size in SHR is ~8% less (Ravingerová et al., 2011). This difference may be due to the stage of hypertrophy. In SHR at and beyond 25 weeks there is dissociation between the increase in left ventricular hypertrophy and increasing ventricle diameter such that the widening of diameter begins to decline (Pfeffer et al., 1979). These animals may begin to show dysfunction of the myocardium due to altered pumping characteristics, and this may affect their tolerance to ischemia. Our animals were sacrificed around 20 weeks of age, a point at which they may not yet show dysfunctional hypertrophy of the myocardium.

Resveratrol treatment protects against infarction to the same extent in WKY and SHR. It appears that there isn't a dose dependant increase in protection against infarct size. Other research has revealed a dose dependant effect of resveratrol treatment on infarct size in Sprague Dawley rats (Hung et al., 2004), though the dosages used in this study were beyond the range that our dosages were in (0.5-25mg/Kg/day-i.p.). Our study was designed to use a physiologically relevant dose of resveratrol, and to contrast a realistic dietary dose with a supplemental dose. As compared to the literature, our normotensive control and resveratrol treated hearts showed ~10% less infarcted tissue (Hung et al., 2000).

The reduction in cardiomyocyte death by resveratrol treatment may be a factor of reduced oxidative damage, or reduction in apoptotic signaling and an induction of survival signaling (Das et al., 2006). Most importantly, our results demonstrate that the amount of

resveratrol present in moderate red wine consumption is as effective as the amount of resveratrol in a supplemental dose at providing protection against infarction.

Ischemia Reperfusion Injury- Functional and Coronary Parameters

Resveratrol treatment did not affect coronary parameters including CDT, CVR, and CPP at 30 minutes, and 2 hours of reperfusion. Heart rate was not affected at baseline, or during reperfusion by resveratrol treatment. Previous research has demonstrated that *chronic* resveratrol treatment at 2.5, 10, and 25mg/Kg/day-i.p. for 7 days can maintain heart rate near baseline values during reperfusion (Mokni et al., 2007), but again most of these concentrations are beyond an achievable amount from dietary sources.

For the first time, our results reveal that both a High and Low dose of resveratrol protects against IRI in hypertensive animals. As resveratrol is a major cardioprotective constituent of red wine extract, we expected to find protection against IRI in SHR based on previous findings that *chronic* treatment with red wine extract protects SHR against IRI (Fantinelli et al., 2007).

In our study, cardiac functional tolerance to IRI was not lower in SHR animals as compared to WKY during reperfusion, which is contradictory to the literature (Golden et al., 1994; Fantinelli et al., 2007; Snoeckx et al., 1986; Ravingerová et al., 2011). This inconsistency may be due to different breeding strains from which the animals were born and therefore may have dissimilar genetics. In SHR, beyond 25 weeks of age, ventricular diameter does not increase proportionally to LV hypertrophy, which can cause dysfunctional hemodynamics and impaired functional ability (Pfeffer et al., 1979). This may also explain the difference in our study as compared to the literature, as the rats used in other studies were two to fourty-two weeks older than ours. During the progression of hypertension-induced left ventricular hypertrophy in Dahl salt sensitive rats a period exists where cardiac response to ischemia is improved (Saupe et al., 2000). If differences in tolerance along the progression of hypertrophy in SHR are teased out, we may see similar results to those in Dahl salt sensitive

rats, and it may have been during a protective period when our animals were sacrificed. Lastly, cardiac myocytes from SHR show increased contractile force in the early stages of hypertrophy, and this may act as protective adaptation in our animals (Brooksby et al., 1992).

Overall, tolerance to IRI was improved in High and Low resveratrol treated hearts, as measured by functional parameters including LVDP, LVEDP, and +dP/dt. Importantly, we did not find a significant difference between High and Low treatment on functional recovery. Other research has shown dose dependant effects of resveratrol treatment on infarct size in Sprague Dawley rats though these dosages were well beyond the range that our dosages were in (Mokni et al., 2007). Most interesting is that a small daily dose of resveratrol that is achievable from moderate red wine consumption provides the same cardiac function protection as a supplement containing 100 fold the amount of resveratrol.

Resveratrol treatment did not significantly affect LVDP until the late stages of reperfusion. Cell death is a dynamic process and although it may begin during ischemia or at the onset of reperfusion, it requires time to complete. The lack of immediate effect by resveratrol treatment on LVDP, but emergence of protection near the end of reperfusion is most likely due to reversible injury, or myocardial cells reaching end-point death in Control animals, and the sustainment of tissue viability by resveratrol treatment. Recovery from reversible injury is likely a result of repairing oxidative damage and/or resynthesis of contractile proteins (Bolli et al., 1999), and in our study, may be an effect of resveratrol's ability to stimulate NO or other antioxidant production, and thereby reduce oxidative stress in the myocardium.

Resveratrol treatment at both a High and a Low dose significantly improved +dP/dt, during reperfusion. This effect was apparent in both the time-lapse curves, and ΔAUC measurements. Interestingly, when analyzing on a point-by-point basis, High resveratrol treatment improved +dP/dt during the entire reperfusion period, whereas Low resveratrol treatment did not improve +dP/dt until the late stages of reperfusion. Since Low resveratrol treatment protects against a reduction in LVDP during the entire phase of reperfusion, but does

not protect against a reduction in +dP/dt until the late stages of reperfusion, the Low dose must either require a greater length of time to reverse damage to contractile apparatus that are involved with the time-dependant response of pressure development, or is not high enough to protect against cellular changes at the early stages of reperfusion as the High dose does.

Resveratrol treatment at a High and Low dose significantly maintained LVEDP around baseline values during reperfusion, representing a protection by resveratrol against myocardial contracture. Contracture is caused by formation of actin-myosin crossbridges, with the inability to deattach (Yellon et al., 2007). It's plausible that the reduction in contracture by resveratrol is related to either a reduction in Ca^{2+} overload, or preservation of ATP. This is likely an effect of resveratrol's ability to upregulate antioxidant enzymes within a cell, thereby providing protection to membranes, Ca^{2+} transporters, and energy producing apparatus in response to IRI (Yellon et al., 2007).

In comparison to the literature, our hearts of both WKY and SHR showed greater functional recovery at the relative time points. LVDP was ~30% higher in our SHR and WKY hearts as compared to the literature (Fantinelli et al., 2007), and LVEDP was ~20mmHg lower in our hearts as compared to the literature (Fantinelli et al., 2007). It is difficult to compare the outcome of our resveratrol treated hearts to the literature as the bulk of these studies use i.p. injection or *acute* application of resveratrol, and do not take into account digestion. However; our normotensive hearts that were treated with resveratrol show greater functional recovery than in the literature, which is likely due to the reduced susceptibility our control animals showed as compared to the literature (Ray et al., 1999; Mokni et al., 2007; Dernek et al., 2004).

Secondary Protein Analysis Findings

1. Resveratrol treatment at a Low dose effectively reduces COX-1 protein expression in both strains.
2. Resveratrol treatment at a Low dose reduces COX-2 protein expression in WKY. COX-2 protein expression was reduced in SHR Control compared to WKY Control.

3. Resveratrol treatment at a High dose increases AMPK protein expression in WKY animals. Resveratrol treatment at a Low dose significantly increases phosphorylation of AMPK as compared to the High dose. There are no main or interaction effects of the phosphorylated AMPK (p-AMPK) to AMPK ratio.
4. High resveratrol treatment significantly increases eNOS protein expression in SHR animals, but does not have an effect on WKY animals. Activated eNOS is significantly increased by High resveratrol treatment. No strain differences exist. An interaction exists between strain and treatment reflected in the ratio of phosphorylated eNOS (p-eNOS) to eNOS.
5. Resveratrol treatment did not affect cytochrome C protein expression in either strain.

From this study we are unable to determine the similarities and differences in mechanisms for protection against IRI in SHR and WKY, though our baseline protein analysis will provide insight as to which pathways may be contributing to resveratrol induced adaptations, and cardioprotection.

A large body of evidence indicates that myocardial COX-2 can confer protection against IRI (Camitta et al., 2001; Yuhki et al., 2011; Bolli et al., 2002). This is contrary to the negative effects of COX derived prostanoids in the vasculature that includes signaling of platelet aggregation and resultant cytotoxicity (Yuhki et al., 2010). This protection exists when COX-2 is stimulated by NO under ischemia reperfusion conditions (Bolli et al., 2002), and when COX-2 is constitutively expressed (Inserte et al., 2009). Additionally COX-1 and COX-2 null mice demonstrate reduced tolerance to IRI (Camitta et al., 2001; Yuhki et al., 2011), though the protective effects of COX derived protection remains under examination. Differences between COX protein expression in SHR and WKY have yet to be determined, though COX plays a role in hypertension, ischemia reperfusion, and LV hypertrophy, suggesting that COX protein would be altered in SHR.

Our results show a reduction in COX-1 protein in Low treated animals. This is contrary to research demonstrating no effect of resveratrol on COX-1 protein expression in wild type rats, undergoing 14 days of 2.5mg/Kg-i.p. resveratrol treatment (Dudley et al., 2008). Our resveratrol treatments are at a slightly lower dose than the literature and are dietary doses, and may account for the differences.

A reduction in COX-2 protein expression in WKY animals treated with a Low resveratrol dose was found. This result was not apparent with High resveratrol treatment, which resulted in no change from baseline in WKY animals. Research applying a resveratrol (2.5mg/Kg-i.p.) for two weeks have greatly increased COX-2 protein in Sprague Dawley animals (Dudley et al., 2008). Potentially with a higher dose of resveratrol then our High dose a further increase in COX-2 protein would have been found in our WKY animals. This would suggest a 'J' or 'U' shaped curve of COX-2 protein in response to doses of resveratrol as our Low dose reduced expression but the High dose did not. Resveratrol treatment in SHR did not affect COX-2 protein at either dose. COX-2 expression in SHR myocardium has not previously been reported. We found reduced COX-2 expression in SHR Con as compared to WKY Con.

AMP- activated protein kinase (AMPK) is an energy sensing protein kinase, and plays a protective role in metabolic stress situations such as ischemia. Resveratrol reduces damage and cell death in H9c2 cardiac muscle cells by activating AMPK, when the ROS production of ischemic conditions are mimicked using hydrogen peroxide (Hwang et al., 2008). The effects of resveratrol on AMPK during IRI, and the contribution to the overall level of cardioprotection has yet to be uncovered, and is an area of interest as activated AMPK is lower in the SHR myocardium at 15 weeks (Dolinsky et al., 2009). AMPK expression in SHR at 20-22 weeks was not reduced as compared to WKY in our study. Interestingly our results show that resveratrol treatment affects the expression of AMPK differently in SHR and WKY. AMPK was only increased in WKY High treated hearts. This suggests that in the diseased, hypertensive state, our concentrations of resveratrol are unable to affect protein expression of AMPK.

As mentioned, pAMPK activity is severely reduced in the SHR myocardium at 15 weeks (Dolinsky et al., 2009). Our protein expression analysis for pAMPK revealed no strain differences. Across treatment groups, High resveratrol treatment did not affect pAMPK protein as compared to Control, though Low resveratrol treatment slightly increased pAMPK content as compared to High treatment. While studies have demonstrated the activation of AMPK by resveratrol under stressed conditions, our analysis was completed under resting conditions. Interestingly activation of AMPK by resveratrol inhibits phenylephrine induced hypertrophy (Chan et al., 2004), and with further research may prove to be a potential mechanism for the inhibition of hypertrophy in the SHR rat as previously observed (Thandapilly et al., 2010). We did not find a large change in activation of AMPK by resveratrol treatment, though our measurements were at baseline conditions, and it's possible that we would see greater activation of AMPK in our High treated hearts under stressed conditions as AMPK protein expression was increased in these hearts. The ratio of phosphorylated AMPK to AMPK revealed no main or interaction effects. Despite not reaching significance, the ratio of p-AMPK to AMPK in WKY High follows the trends of unchanged p-AMPK, and increased AMPK.

The protective role of eNOS in IRI is well recognized, as are the effects of eNOS regulation by resveratrol. In the left ventricle of 18 week old SHR, there is a ~25% reduction in eNOS expression as compared to WKY (Lee et al., 2006; Piech et al., 2003), and this was confirmed by our results. Resveratrol treatment at a Low dose did not affect protein levels, but at a High dose significantly increased eNOS of SHR, to a level that was nearly double SHR Con. Upregulation of eNOS protein in normotensive animals is seen after 7, and 14, days of 2.5mg/Kg/day resveratrol treatment (Das et al., 2005; Dudley et al., 2008). Our research suggests resveratrol administered orally increases eNOS expression in hypertensive animals, but is too low of a dose to affect eNOS expression in the hearts of normotensive animals. Due to the improvement in functional recovery post IRI being partially NO dependant, and the reduced

basal eNOS content in SHR, we would expect upregulation of eNOS by resveratrol in SHR to produce protective effects.

Phosphorylation of eNOS induces NO production, a key factor in the cardioprotective abilities of eNOS, and resveratrol treatment (Das et al., 2006). Previous research indicates increased activation of eNOS by resveratrol treatment in endothelial cells (Wallerath et al., 2002). Interestingly, resveratrol treatment did not affect levels of p-eNOS in our study. Most interesting to this area of study is the evidence that although eNOS expression is altered in response to hypertension and resveratrol treatment, the myocardium compensates in order to maintain the level of p-eNOS, most likely by the alteration of phosphotase and kinase activity. This effect is clearly demonstrated by a higher p-eNOS to eNOS ratio, and the reduced eNOS expression in SHR Con compared to WKY Con despite no differences in p-eNOS. Additionally, this effect is demonstrated by a reduced p-eNOS to eNOS ratio, and a large increase in eNOS protein expression SHR High as compared to WKY High, despite yet again no differences in p-eNOS.

AMPK is an upstream activator of eNOS (Chen et al., 1999). We found increased AMPK content in WKY High hearts, which mimics the increase in p-eNOS protein expression in WKY High hearts, but we did not find any significant increases in phosphorylated AMPK suggesting that the increased activation of eNOS may be directly through resveratrol, or by means of a secondary pathway. Overall we cannot directly assume that upregulation of eNOS activity is producing an increase in NO bioavailability, but this may be an important factor, for further study, in the protection afforded by resveratrol against IRI.

In the current study cytochrome C immunohistochemistry was used to provide a crude understanding of resveratrol effects on mitochondrial biogenesis. Resveratrol can elicit mitochondrial biogenesis in endothelial cells (Csiszar et al., 2009) though the effects of resveratrol on mitochondrial content in other cells is lacking. In our study, resveratrol treatment did not affect cytochrome C protein expression in the myocardium, and no strain differences exist. Measuring mitochondrial DNA content, or analyzing mitochondrial

biogenesis factors would be a more accurate assessment, and may provide better insight into the effects of resveratrol on mitochondrial content in the myocardium.

LIMITATIONS

Although we compare our Low resveratrol treatment to the resveratrol content attainable through moderate red wine consumption, our results cannot be directly extended to studies using red wine or red wine extract as other components of red wine such as ethanol and other phenolic compounds, may have confounding effects to those of resveratrol.

Our calculation of resveratrol consumption requires that all water removed from the bottles was consumed by the animal, when infact it is known that a significant level of dripping occurs as a result of human or animal movement of the container, or animals playing with the spigots. IP injections are a more accurate way of defining the amount of resveratrol that is given to animals, but is a known cause of stress- a factor which can affect the cardiovascular function by increasing HR and blood pressure, and affect response to IRI (Ravingerová et al., 2011). When treated daily by injection or gavage for 4 weeks, an animals' cardiovascular state may be influenced which is why we chose to apply resveratrol treatment by way of drinking water.

Kidney function in SHR is altered, and may act to impair the clearance of resveratrol as compared to the WKY animals. To assess whether altered kidney function can affect the amount of resveratrol that is taken into the blood we would need to assess plasma levels of resveratrol in the different strains and treatments.

Sodium pentobarbital was the anesthetic used in this study, and it is known to depress cardiac function while circulating in the bloodstream. A study using sodium pentobarbital anesthesia before heart excision showed a reduction in maximal contractility to increasing Ca^{2+} in the perfusion medium (Segal et al., 1990). Other research recommends using barbiturates for anesthesia as no complicated equipment is required for administration (Döring, 1990). We chose to use pentobarbital as invasive hemodynamic measurements were taken prior to heart excision, and inhalation of volatile agents can stimulate sympathetic nervous system activity, and act as a cardioprotective agent (Döring, 1990; Fantinelli et al., 2007). Ultimately, an ideal anesthetic does not exist.

The Langendorff isolated heart is void of neural and hormonal control of myocardial and coronary function. Additionally, other circulating factors that are central to ischemia reperfusion injury such as platelets do not play a role in this model. These factors can contribute to the myocardium's response to ischemia reperfusion injury, making our model less comprehensive. In order to include these factors one would need to use a model of *in vivo* coronary artery ligation, which is not a procedure available to our laboratory. Furthermore, interaction between myocytes of ischemic and non ischemic myocardium in a clinical setting of thrombotic occlusion is eliminated in our study of global ischemia.

Our protocol of infarct size staining assumes the infarcted area propagates in a 3-dimensional manner down through the 1mm slice in the same dimensions as the 2-dimensional outline. This can cause the calculation of infarcted volume of a heart slice to be somewhat inaccurate, though the slices are thin and imaging of both sides of the slices allows us to check the planimetry for propogation of the infarct through the slice.

The results of the secondary analysis of protein expression cannot aid in interpretation of our functional results as we did not study their activities or change in activation during IR. We were; however, able to assess the effects of strain and resveratrol treatment on proteins that play a key role in cardioprotection against IRI.

CONCLUSIONS

This study demonstrates the cardioprotective nature of *chronic* resveratrol treatment at a dose that mimicks the resveratrol content achievable through moderate red wine intake and a second dose that mimicks resveratrol supplementation. Resveratrol treatment protects against a depression in cardiac function, and reduces infarct size caused by ischemia reperfusion injury. The cardioprotective effects may have been through resveratrol's ability to act as an anti-oxidant, anti-inflammatory, or anti-apoptotic signaling agent, though further research is required to elucidate the cellular mechanisms by which resveratrol exerts these effects in the current hypertensive model.

We found that SHR, between 20-22 weeks, with established LV hypertrophy do not have increased susceptibility to ischemia reperfusion injury compared to WKY, contrary to the literature (Golden et al., 1994; Snoeckx et al., 1989; Ravingerová et al., 2011). Our results may reflect a protected stage during the development of LV hypertrophy.

We have shown that resveratrol treatment at doses achievable from the diet can induce changes to the expression of myocardial proteins that are part of various pathways that play cardioprotective roles against IRI. These protein expression responses to resveratrol differ in SHR and WKY, and with future research using isolated hearts and protein antagonists, may provide a clearer understanding of mechanisms for resveratrol induced cardioprotection in SHR versus WKY. Future research should attempt to test whether the adaptations to protein expression by resveratrol treatment translates into altered activity during IRI, and whether the upregulation or downregulation of activity or activation contributes to cardioprotection.

As little is known about resveratrol induced-protection against IRI in SHR, the next reasonable research direction stemming from this study would be to study specific pathways, and their relative contributions to the overall protection. Research has uncovered the dependency on NOS of resveratrol induced cardioprotection in normotensive animals, though this pathway does not account for the total protective response (Hung et al., 2004), and may play a less significant role in adult hypertensive animals as NOS protein expression is reduced

(Piech et al., 2003). The COX pathway is a novel area for future research as little is understood about the effects of resveratrol on COX in the myocardium, let alone a global opinion as to the overall effect of COX on IRI. Additionally, in SHR myocardium of COX is understudied as its role in the vasculature has been the forefront of COX research.

Future studies should attempt to understand the progression of LV hypertrophy and the effects of progression on ischemia reperfusion injury, as previously done in the Dahl salt sensitive rats. Additionally, suggested by the results of our protein analysis showing resveratrol affects protein expression differently in SHR and WKY, the mechanisms of protection by resveratrol during IRI may prove to be different in SHR and WKY. Studies should attempt to tease out these differences by assessing the contribution of specific pathways to the overall response of the heart to IRI, and the influence on these pathways by resveratrol during IRI. This would require Western analysis of protein expression and activation during ischemia and reperfusion, in addition to inhibiting certain pathways and analyzing the functional outcome in response to IRI.

In the future it would be interesting to treat hypertensive animals with our physiologically relevant doses of resveratrol before LV hypertrophy begins to develop, in order to assess the potential for resveratrol to prevent LV hypertrophy. This would be similar to the study by *Thandapilly et al.,* with the exception of lower, more relevant doses (Thandapilly et al., 2010).

Lastly, our results provide good evidence of a protective effect of resveratrol treatment in SHR, though these methods must be tested on humans to fully understand the possibility of clinical application against hypertension and IRI.

REFERENCES

Anderson, P G, Allard, M. F., Thomas, G. D., Bishop, S. P., & Digerness, S. B. (1990). Increased ischemic injury but decreased hypoxic injury in hypertrophied rat hearts. *Circulation Research, 67*(4), 948-959.

Anderson, P G, Bishop, S. P., & Digerness, S. B. (1987). Transmural progression of morphologic changes during ischemic contracture and reperfusion in the normal and hypertrophied rat heart. *The American Journal of Pathology, 129*(1), 152-167.

Ballinger, S W (2005). Mitochondrial dysfunction in cardiovascular disease. *Free Radical Biology & Medicine, 38*(10), 1278-1295.

Batist, G, Mersereau, W., Malashenko, B. A., & Chiu, R. C. (1989). Response to ischemia-reperfusion injury in hypertrophic heart. Role of free-radical metabolic pathways. *Circulation, 80*(5 Pt 2), III10-III13.

Becker, L B (2004). New concepts in reactive oxygen species and cardiovascular reperfusion physiology. *Cardiovascular Research, 61*(3), 461-470.

Besík, J, Szárszoi, O., Kunes, J., Netuka, I., Malý, J., & Kolár, F., et al. (2007). Tolerance to acute ischemia in adult male and female spontaneously hypertensive rats. *Physiological Research / Academia Scientiarum Bohemoslovaca, 56*(3), 267-274.

Bolli, R (2001). Cardioprotective function of inducible nitric oxide synthase and role of nitric oxide in myocardial ischemia and preconditioning: an overview of a decade of research. *Journal of Molecular and Cellular Cardiology, 33*(11), 1897-1918.

Bolli, R, & Marbán, E. (1999). Molecular and cellular mechanisms of myocardial stunning. *Physiological Reviews, 79*(2), 609-634.

Bolli, R, Shinmura, K., Tang, X. L., Kodani, E., Xuan, Y. T., & Guo, Y., et al. (2002). Discovery of a new function of cyclooxygenase (COX)-2: COX-2 is a cardioprotective protein that alleviates ischemia/reperfusion injury and mediates the late phase of preconditioning. *Cardiovascular Research, 55*(3), 506-519.

Bradamante, S, Barenghi, L., Piccinini, F., Bertelli, A. A. E., De Jonge, R., & Beemster, P., et al. (2003). Resveratrol provides late-phase cardioprotection by means of a nitric oxide- and adenosine-mediated mechanism. *European Journal of Pharmacology, 465*(1-2), 115-123.

Bradamante, S, Barenghi, L., & Villa, A. (2004). Cardiovascular protective effects of resveratrol. *Cardiovascular Drug Reviews, 22*(3), 169-188.

Brooksby, P, Levi, A. J., & Jones, J. V. (1992). Contractile properties of ventricular myocytes isolated from spontaneously hypertensive rat. *Journal of Hypertension, 10*(6), 521-527.

Buja, L M (2005). Myocardial ischemia and reperfusion injury. *Cardiovascular Pathology : the Official Journal of the Society for Cardiovascular Pathology, 14*(4), 170-175.

Cameron, J S, Kimura, S., Jackson-Burns, D. A., Smith, D. B., & Bassett, A. L. (1988). ATP-sensitive K+ channels are altered in hypertrophied ventricular myocytes. *The American Journal of Physiology, 255*(5 Pt 2), H1254-H1258.

Camitta, M G, Gabel, S. A., Chulada, P., Bradbury, J. A., Langenbach, R., & Zeldin, D. C., et al. (2001). Cyclooxygenase-1 and -2 knockout mice demonstrate increased cardiac ischemia/reperfusion injury but are protected by acute preconditioning. *Circulation, 104*(20), 2453-2458.

CANISM (2010). CANSIM Table 102-0529:Deaths, by cause, Chapter IX: Diseases of the circulatory system (I00 to I99), age group and sex, canada, annual (number), 2000 to 2006.

Chan, A Y M, Soltys, C. L. M., Young, M. E., Proud, C. G., & Dyck, J. R. B. (2004). Activation of AMP-activated protein kinase inhibits protein synthesis associated with hypertrophy in the cardiac myocyte. *The Journal of Biological Chemistry, 279*(31), 32771-32779.

Chen, Y R, Yi, F. F., Li, X. Y., Wang, C. Y., Chen, L., & Yang, X. C., et al. (2008). Resveratrol attenuates ventricular arrhythmias and improves the long-term survival in rats with myocardial infarction. *Cardiovascular Drugs and Therapy / Sponsored by the International Society of Cardiovascular Pharmacotherapy, 22*(6), 479-485.

Chen, Z P, Mitchelhill, K. I., Michell, B. J., Stapleton, D., Rodriguez-Crespo, I., & Witters, L. A., et al. (1999). AMP-activated protein kinase phosphorylation of endothelial NO synthase. *Febs Letters, 443*(3), 285-289.

Cokkinos, D. V., Pantos, C., & Heusch, G. (2006). *Myocardial ischemia.* Springer Verlag.

Cole, W C, McPherson, C. D., & Sontag, D. (1991). ATP-regulated K+ channels protect the myocardium against ischemia/reperfusion damage. *Circulation Research, 69*(3), 571-581.

Csiszar, A, Labinskyy, N., Pinto, J. T., Ballabh, P., Zhang, H., & Losonczy, G., et al. (2009). Resveratrol induces mitochondrial biogenesis in endothelial cells. *American Journal of Physiology. Heart and Circulatory Physiology, 297*(1), H13-H20.

Das, D K, & Maulik, N. (2006). Resveratrol in cardioprotection: a therapeutic promise of alternative medicine. *Molecular Interventions, 6*(1), 36-47.

Das, S, Alagappan, V., Bagchi, D., Sharma, H., Maulik, N., & Das, D., et al. (2005). Coordinated induction of iNOS-VEGF-KDR-eNOS after resveratrol consumption: a potential mechanism for resveratrol preconditioning of the heart. *Vascular Pharmacology, 42*(5-6), 281-289.

Das, S, Cordis, G. A., Maulik, N., & Das, D. K. (2005). Pharmacological preconditioning with resveratrol: role of CREB-dependent Bcl-2 signaling via adenosine A3 receptor activation. *American Journal of Physiology. Heart and Circulatory Physiology, 288*(1), H328-H335.

Das, S, & Das, D. K. (2007). Anti-inflammatory responses of resveratrol. *Inflammation & Allergy Drug Targets, 6*(3), 168-173.

Das, S, Falchi, M., Bertelli, A., Maulik, N., & Das, D. K. (2006). Attenuation of ischemia/reperfusion injury in rats by the anti-inflammatory action of resveratrol. *Arzneimittel-forschung, 56*(10), 700-706.

Das, S, Fraga, C. G., & Das, D. K. (2006). Cardioprotective effect of resveratrol via HO-1 expression involves p38 map kinase and PI-3-kinase signaling, but does not involve NFkappaB. *Free Radical Research, 40*(10), 1066-1075.

Das, S, Tosaki, A., Bagchi, D., Maulik, N., & Das, D. K. (2006). Potentiation of a survival signal in the ischemic heart by resveratrol through p38 mitogen-activated protein kinase/mitogen- and stress-activated protein kinase 1/cAMP response element-binding protein signaling. *The Journal of Pharmacology and Experimental Therapeutics, 317*(3), 980-988.

Dernek, S, Ikizler, M., Erkasap, N., Ergun, B., Koken, T., & Yilmaz, K., et al. (2004). Cardioprotection with resveratrol pretreatment: improved beneficial effects over standard treatment in rat hearts after global ischemia. *Scandinavian Cardiovascular Journal : Scj, 38*(4), 245-254.

Dolinsky, V W, Chan, A. Y., Robillard Frayne, I., Light, P. E., Des Rosiers, C., & Dyck, J. R., et al. (2009). Resveratrol prevents the prohypertrophic effects of oxidative stress on LKB1. *Circulation, 119*(12), 1643-1652.

Döring, H J (1990). The isolated perfused heart according to Langendorff technique--function--application. *Physiologia Bohemoslovaca, 39*(6), 481-504.

Dudley, J I, Lekli, I., Mukherjee, S., Das, M., Bertelli, A. A. A., & Das, D. K., et al. (2008). Does white wine qualify for French paradox? Comparison of the cardioprotective effects of red and white wines and their constituents: resveratrol, tyrosol, and hydroxytyrosol. *Journal of Agricultural and Food Chemistry, 56*(20), 9362-9373.

Edoute, Y, Luscher, T. F., & Rubanyi, G. M. (1986). Autoregulation and vascular reserve in the coronary circulation of the spontaneously hypertensive rat. *Journal of Hypertension. Supplement : Official Journal of the International Society of Hypertension, 4*(5), S290-S292.

Fantinelli, J, & Mosca, S. (2007). Cardioprotective effects of a non-alcoholic extract of red wine during ischaemia and reperfusion in spontaneously hypertensive rats. *Clinical and Experimental Pharmacology & Physiology, 34*(3), 166-169.

Fantinelli, J C, & Mosca, S. M. (2007). Comparative effects of ischemic pre and postconditioning on ischemia-reperfusion injury in spontaneously hypertensive rats (SHR). *Molecular and Cellular Biochemistry, 296*(1-2), 45-51.

Faustino, R S, Sobrattee, S., Edel, A. L., & Pierce, G. N. (2003). Comparative analysis of the phenolic content of selected Chilean, Canadian and American Merlot red wines.

Molecular and Cellular Biochemistry, 249(1-2), 11-19.

Ferdinandy, P, Schulz, R., & Baxter, G. F. (2007). Interaction of cardiovascular risk factors with myocardial ischemia/reperfusion injury, preconditioning, and postconditioning. *Pharmacological Reviews, 59*(4), 418-458.

Flesch, M, Schwarz, A., & Böhm, M. (1998). Effects of red and white wine on endothelium-dependent vasorelaxation of rat aorta and human coronary arteries. *The American Journal of Physiology, 275*(4 Pt 2), H1183-H1190.

Frankel, E N, Waterhouse, A. L., & Kinsella, J. E. (1993). Inhibition of human LDL oxidation by resveratrol. *Lancet, 341*(8852), 1103-1104.

Friehs, I, & del Nido, P. J. (2003). Increased susceptibility of hypertrophied hearts to ischemic injury. *The Annals of Thoracic Surgery, 75*(2), S678-S684.

Galiñanes, M, & Fowler, A. G. (2004). Role of clinical pathologies in myocardial injury following ischaemia and reperfusion. *Cardiovascular Research, 61*(3), 512-521.

Goh, S, Woodman, O., Pepe, S., Cao, A., Qin, C., & Ritchie, R., et al. (2007). The red wine antioxidant resveratrol prevents cardiomyocyte injury following ischemia-reperfusion via multiple sites and mechanisms. *Antioxidants & Redox Signaling, 9*(1), 101-113.

Golden, A L, Bright, J. M., Pohost, G. M., & Pike, M. M. (1994). Ischemic dysfunction and impaired recovery in hypertensive hypertrophied hearts is associated with exaggerated intracellular sodium accumulation. *American Journal of Hypertension, 7*(8), 745-754.

Grover, G J, & Garlid, K. D. (2000). ATP-Sensitive potassium channels: a review of their cardioprotective pharmacology. *Journal of Molecular and Cellular Cardiology, 32*(4), 677-695.

Gutiérrez-Pérez, A, Cortés-Rojo, C., Noriega-Cisneros, R., Calderón-Cortés, E., Manzo-Avalos, S., & Clemente-Guerrero, M., et al. (2011). Protective effects of resveratrol on calcium-induced oxidative stress in rat heart mitochondria. *Journal of Bioenergetics and Biomembranes, 43*(2), 101-107.

Hattori, R, Otani, H., Maulik, N., & Das, D. K. (2002). Pharmacological preconditioning with resveratrol: role of nitric oxide. *American Journal of Physiology. Heart and Circulatory Physiology, 282*(6), H1988-H1995.

Ho, K K, Pinsky, J. L., Kannel, W. B., & Levy, D. (1993). The epidemiology of heart failure: the Framingham Study. *Journal of the American College of Cardiology, 22*(4 Suppl A), 6A-13A.

Hoffman, J W, Gilbert, T. B., Poston, R. S., & Silldorff, E. P. (2004). Myocardial reperfusion injury: etiology, mechanisms, and therapies. *The Journal of Extra-corporeal Technology, 36*(4), 391-411.

Holvoet, P (2004). Oxidized LDL and coronary heart disease. *Acta Cardiologica, 59*(5), 479-484.

Hung, L M, Su, M. J., & Chen, J. K. (2004). Resveratrol protects myocardial ischemia-reperfusion injury through both NO-dependent and NO-independent mechanisms. *Free Radical Biology & Medicine, 36*(6), 774-781.

Hwang, J T, Kwon, D. Y., Park, O. J., & Kim, M. S. (2008). Resveratrol protects ROS-induced cell death by activating AMPK in H9c2 cardiac muscle cells. *Genes & Nutrition, 2*(4), 323-326.

Imamura, G, Bertelli, A. A., Bertelli, A., Otani, H., Maulik, N., & Das, D. K., et al. (2002). Pharmacological preconditioning with resveratrol: an insight with iNOS knockout mice. *American Journal of Physiology. Heart and Circulatory Physiology, 282*(6), H1996-H2003.

Inserte, J, Molla, B., Aguilar, R., Través, P. G., Barba, I., & Martín-Sanz, P., et al. (2009). Constitutive COX-2 activity in cardiomyocytes confers permanent cardioprotection Constitutive COX-2 expression and cardioprotection. *Journal of Molecular and Cellular Cardiology, 46*(2), 160-168.

Jäger, U, & Nguyen-Duong, H. (1999). Relaxant effect of trans-resveratrol on isolated porcine coronary arteries. *Arzneimittel-forschung, 49*(3), 207-211.

Kelm, M, Feelisch, M., Krebber, T., Deussen, A., Motz, W., & Strauer, B. E., et al. (1995). Role of nitric oxide in the regulation of coronary vascular tone in hearts from

hypertensive rats. Maintenance of nitric oxide-forming capacity and increased basal production of nitric oxide. *Hypertension, 25*(2), 186-193.

Klabunde, R. E. (2005). *Cardiovascular physiology concepts.*

Kopp, P (1998). Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the 'French paradox'? *European Journal of Endocrinology / European Federation of Endocrine Societies, 138*(6), 619-620.

Lassègue, B, & Griendling, K. K. (2004). Reactive oxygen species in hypertension; An update. *American Journal of Hypertension, 17*(9), 852-860.

Lee, Y I, Cho, J. Y., Kim, M. H., Kim, K. B., Lee, D. J., & Lee, K. S., et al. (2006). Effects of exercise training on pathological cardiac hypertrophy related gene expression and apoptosis. *European Journal of Applied Physiology, 97*(2), 216-224.

Lin, J F, Lin, S. M., Chih, C. L., Nien, M. W., Su, H. H., & Hu, B. R., et al. (2008). Resveratrol reduces infarct size and improves ventricular function after myocardial ischemia in rats. *Life Sciences, 83*(9-10), 313-317.

Lu, Z, Zhang, Y., Liu, H., Yuan, J., Zheng, Z., & Zou, G., et al. Transport of a Cancer Chemopreventive Polyphenol, Resveratrol: Interaction with Serum Albumin and Hemoglobin. *Journal of Fluorescence, 17*(5), 580-587.

Machado, N G, Alves, M. G., Carvalho, R. A., & Oliveira, P. J. (2009). Mitochondrial involvement in cardiac apoptosis during ischemia and reperfusion: can we close the box? *Cardiovascular Toxicology, 9*(4), 211-227.

Mokni, M, Limam, F., Elkahoui, S., Amri, M., & Aouani, E. (2007). Strong cardioprotective effect of resveratrol, a red wine polyphenol, on isolated rat hearts after ischemia/reperfusion injury. *Archives of Biochemistry and Biophysics, 457*(1), 1-6.

Mosca, S M, & Cingolani, H. E. (2002). Cardioprotection from ischemia/reperfusion induced by red wine extract is mediated by K(ATP) channels. *Journal of Cardiovascular Pharmacology, 40*(3), 429-437.

Netticadan, T, Temsah, R., Osada, M., & Dhalla, N. S. (1999). Status of Ca2+/calmodulin protein kinase phosphorylation of cardiac SR proteins in ischemia-reperfusion. *The American Journal of Physiology*, *277*(3 Pt 1), C384-C391.

Ogita, H, & Liao, J. (2004). Endothelial function and oxidative stress. *Endothelium : Journal of Endothelial Cell Research, 11*(2), 123-132.

Peng, L, Bradley, C. J., Zulli, A., & Liu, J. J. (1999). The relationship between apoptosis and spontaneous hypertension. *Chinese Medical Journal, 112*(6), 562-568.

Peyton, R B, Van Trigt, P., Pellom, G. L., Jones, R. N., Sink, J. D., & Wechsler, A. S., et al. (1982). Improved tolerance to ischemia in hypertrophied myocardium by preischemic enhancement of adenosine triphosphate. *The Journal of Thoracic and Cardiovascular Surgery, 84*(1), 11-15.

Pfeffer, J M, Pfeffer, M. A., Fishbein, M. C., & Frohlich, E. D. (1979). Cardiac function and morphology with aging in the spontaneously hypertensive rat. *The American Journal of Physiology*, *237*(4), H461-H468.

Piech, A, Dessy, C., Havaux, X., Feron, O., & Balligand, J. L. (2003). Differential regulation of nitric oxide synthases and their allosteric regulators in heart and vessels of hypertensive rats. *Cardiovascular Research, 57*(2), 456-467.

Piper, H M, Abdallah, Y., & Schäfer, C. (2004). The first minutes of reperfusion: a window of opportunity for cardioprotection. *Cardiovascular Research, 61*(3), 365-371.

Piper, H M, Meuter, K., & Schäfer, C. (2003). Cellular mechanisms of ischemia-reperfusion injury. *The Annals of Thoracic Surgery, 75*(2), S644-S648.

Quinn, R R, Hemmelgarn, B. R., Padwal, R. S., Myers, M. G., Cloutier, L., & Bolli, P., et al. (2010). The 2010 Canadian Hypertension Education Program recommendations for the management of hypertension: part I - blood pressure measurement, diagnosis and assessment of risk. *The Canadian Journal of Cardiology*, *26*(5), 241-248.

Ravingerová, T, Bernátová, I., Matejíková, J., Ledvényiová, V., Nemčeková, M., & Pecháňová, O., et al. (2011). Impaired cardiac ischemic tolerance in spontaneously hypertensive rats is attenuated by adaptation to chronic and acute stress. *Experimental*

and Clinical Cardiology, 16(3), e23-e29.

Ray, P S, Maulik, G., Cordis, G. A., Bertelli, A. A., Bertelli, A., & Das, D. K., et al. (1999). The red wine antioxidant resveratrol protects isolated rat hearts from ischemia reperfusion injury. *Free Radical Biology & Medicine, 27*(1-2), 160-169.

Rush, J W E, Quadrilatero, J., Levy, A. S., & Ford, R. J. (2007). Chronic resveratrol enhances endothelium-dependent relaxation but does not alter eNOS levels in aorta of spontaneously hypertensive rats. *Experimental Biology and Medicine (maywood, N.j.), 232*(6), 814-822.

Ruuge, E K, Ledenev, A. N., Lakomkin, V. L., Konstantinov, A. A., & Ksenzenko MYu (1991). Free radical metabolites in myocardium during ischemia and reperfusion. *The American Journal of Physiology, 261*(4 Suppl), 81-86.

Saini, H K, & Dhalla, N. S. (2005). Defective calcium handling in cardiomyocytes isolated from hearts subjected to ischemia-reperfusion. *American Journal of Physiology. Heart and Circulatory Physiology, 288*(5), H2260-H2270.

Saupe, K W, Lim, C. C., Ingwall, J. S., Apstein, C. S., & Eberli, F. R. (2000). Comparison of hearts with 2 types of pressure-overload left ventricular hypertrophy. *Hypertension, 35*(5), 1167-1172.

Schwarz, E, Somoano, Y., Hale, S., & Kloner, R. (2000). What is the required reperfusion period for assessment of myocardial infarct size using triphenyltetrazolium chloride staining in the rat? *Journal of Thrombosis and Thrombolysis, 10*(2), 181-181.

Segal, J, Schwab, H., Smorak, V., & Urtzkey, G. (1990). Effects of anesthesia on cardiac function and response in isolated rat hearts. *Journal of Molecular Cell Cardiology, 22*, 1317-1324.

Shigematsu, S, Ishida, S., Hara, M., Takahashi, N., Yoshimatsu, H., & Sakata, T., et al. (2003). Resveratrol, a red wine constituent polyphenol, prevents superoxide-dependent inflammatory responses induced by ischemia/reperfusion, platelet-activating factor, or oxidants. *Free Radical Biology & Medicine, 34*(7), 810-817.

Smoliga, J M, Baur, J. A., & Hausenblas, H. A. (2011). Resveratrol and health--a comprehensive review of human clinical trials. *Molecular Nutrition & Food Research,*

55(8), 1129-1141.

Snoeckx, L H, van der Vusse, G. J., Coumans, W. A., Willemsen, P. H., van der Nagel, T., & Reneman, R. S., et al. (1986). Myocardial function in normal and spontaneously hypertensive rats during reperfusion after a period of global ischaemia. *Cardiovascular Research, 20*(1), 67-75.

Snoeckx, L, van, D., Coumans, W., & Reneman, R. (1989). Recovery of hypertrophied rat hearts after global ischemia and reperfusion at different perfusion pressures. *Pflügers Archiv : European Journal of Physiology, 413*(3), 303-312.

Susic, D, Varagic, J., & Frohlich, E. D. (2008). Coronary circulation in hypertension and aging: an experimental study. *The Ochsner Journal, 8*(1), 5-10.

Thandapilly, S J, Wojciechowski, P., Behbahani, J., Louis, X. L., Yu, L., & Juric, D., et al. (2010). Resveratrol prevents the development of pathological cardiac hypertrophy and contractile dysfunction in the SHR without lowering blood pressure. *American Journal of Hypertension, 23*(2), 192-196.

Torii, M, Ito, H., & Suzuki, T. (1992). Lipid peroxidation and myocardial vulnerability in hypertrophied SHR myocardium. *Experimental and Molecular Pathology, 57*(1), 29-38.

Trialists' Collaboration, A (1994). Collaborative overview of randomised trials of antiplatelet therapy Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. *Bmj, 308*(6921), 81.

Turer, A T, & Hill, J. A. (2010). Pathogenesis of myocardial ischemia-reperfusion injury and rationale for therapy. *The American Journal of Cardiology, 106*(3), 360-368.

Wallerath, T, Deckert, G., Ternes, T., Anderson, H., Li, H., & Witte, K., et al. (2002). Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. *Circulation, 106*(13), 1652-1658.

Wenzel, E, & Somoza, V. (2005). Metabolism and bioavailability of trans-resveratrol. *Molecular Nutrition & Food Research, 49*(5), 472-481.

Wilkins, K, Campbell, N. R. C., Joffres, M. R., McAlister, F. A., Nichol, M., & Quach, S., et al. (2010). Blood pressure in Canadian adults. *Health Reports / Statistics Canada, ..//Canadian Centre for Health Information = Rapports Sur La Sante / Statistique Canada, Centre Canadien D'information Sur La Sante, 21*(1), 37-46.

Yamori, Y, & Okamoto, K. (1973). Spontaneous hypertension in rats versus essential hypertension in man. *Singapore Medical Journal, 14*(3), 393-394.

Yellon, D M, & Hausenloy, D. J. (2007). Myocardial reperfusion injury. *The New England Journal of Medicine, 357*(11), 1121-1135.

Yuhki, K, Kashiwagi, H., Kojima, F., Kawabe, J., & Ushikubi, F. (2010). Roles of prostanoids in the pathogenesis of cardiovascular diseases. *International Angiology : a Journal of the International Union of Angiology, 29*(2 Suppl), 19-27.

Yuhki, K I, Kojima, F., Kashiwagi, H., Kawabe, J. I., Fujino, T., & Narumiya, S., et al. (2011). Roles of prostanoids in the pathogenesis of cardiovascular diseases: Novel insights from knockout mouse studies. *Pharmacology & Therapeutics, 129*(2), 195-205.

Yusuf, S, Hawken, S., Ounpuu, S., Dans, T., Avezum, A., & Lanas, F., et al. (2004). Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet, 364*(9438), 937-952.

Zhao, Z Q (2004). Oxidative stress-elicited myocardial apoptosis during reperfusion. *Current Opinion in Pharmacology, 4*(2), 159-165.