Effects of resveratrol on hypertension induced cardiac remodelling

By

Sijo Joseph (Thandapilly)

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Department of Physiology

University of Manitoba

Winnipeg, Manitoba, Canada

ABSTRACT

The cardioprotective effects of resveratrol have been documented in a few different animal models of pressure overload which mimic clinical situations of hypertension and aortic stenosis. However, no study has examined the effects of resveratrol in preventing/reversing chronic changes in cardiac structure and function due to essential hypertension. Therefore, in this thesis, We have investigated the effects of resveratrol in spontaneously hypertensive rat, a model of essential hypertension, as well in an *in vitro* model of norepinephrine induced adult cardiomyocyte hypertrophy. The initial study with 10 weeks old spontaneously hypertensive rats showed that treatment with resveratrol (2.5mg/kg/day) was beneficial in preventing systolic and diastolic dysfunction in spontaneously hypertensive rats without lowering blood pressure. This effect was mediated by a reduction in oxidative stress. To further elucidate the molecular mechanism underlying the beneficial effects of resveratrol, we induced adult cardiomyocyte hypertrophy by exposing them to high levels of norepinephrine, a pathological trigger of hypertension and hypertrophy. This *in vitro* study revealed that resveratrol was able to prevent adult cardiomyocyte hypertrophy induced by norepinephrine by activating nitric oxide- adenosine monophosphate-activated protein kinase pathway. Consistent with the *in vitro* findings, the anti-hypertrophic effect of resveratrol observed in the spontaneously hypertensive rat model was also associated with increases in nitric oxide- adenosine monophosphate-activated protein kinase activity. In the third study we investigated whether resveratrol alone or in a combination with a blood pressure lowering agent would be beneficial in reversing hypertension-induced cardiac hypertrophy and contractile dysfunction. For this purpose, twenty week old male spontaneously hypertensive rats were treated with resveratrol (2.5 mg/kg/day) and or hydralazine (25 mg/kg/day), an established blood

pressure lowering agent for eight weeks. Resveratrol treatment alone was ineffective in reducing systolic, diastolic blood pressure and diastolic dysfunction in spontaneously hypertensive rat. In contrast resveratrol treatment alone significantly reduced the systolic impairment as well as myocardial fibrosis in SHR. Combination therapy of resveratrol in conjunction with hydralazine significantly reduced blood pressure, improved systolic and diastolic function, reduced fibrosis and improved vascular geometry. Thus, resveratrol may have potential in preventing functional damage to the heart caused by essential hypertension. Accordingly, resveratrol alone or in combination with a blood pressure lowering agent, may be useful in treating patients with essential hypertension.

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ACE	angiotensin-converting enzyme
RAAS	rennin angiotensin aldosterone system
MI	myocardial infarction
LDL	low-density lipoprotein
eNOS	endothelial nitric oxide synthase
CVD	cardiovascular diseases
ARB	angiotensin receptor blocker
SHR	spontaneously hypertensive rat
WKY	wistar-Kyoto rats
LVID	left ventricular internal dimensions
LVPW	left ventricular posterior wall
IVS	interventricular septum
EF	ejection fraction
CO	cardiac output
IVRt	isovolumic relaxation time
TBARS	the thiobarbituric acid reactive substances
TAS	total antioxidant status
ANOVA	one way analysis of variance
NO	nitric oxide
AMPK	adenosine monophosphate kinase
NE	norepinephrine
SIRT	silent information regulator
SNAP	S-nitroso-Nacetylpenicillamine
L-NAME	N_{ω}-Nitro-L-arginine methyl ester hydrochloride
DAG	diacylglycerol
IP3	inositol triphosphate
GSK-3β	glycogen synthase kinase 3 beta
IL-6	interleukin 6
TNF-α	tumor necrosis factor-*alpha*
CO	cardiac output
β-AR	β- adrenergic receptor
SERCA2a	sarcoplasmic calcium ATPase

CHAPTER 1

OVERALL INTRODUCTION

1. 1 INTRODUCTION

Heart failure is a multi-factorial syndrome that is a leading cause of hospitalization and mortality worldwide (Latronico et al., 2008; Mudd and Kass, 2008). According to the World Health Organization, fatalities from heart failure (HF) will rise to more than 20 million per year by 2020. (http://www.who.int/cardiovascular_diseases/en/). Heart failure is a complicated, multifactorial syndrome resulting from the heart's inability to pump sufficient blood to meet the metabolic demand of tissues. The development of heart failure is secondary to diseases such as hypertension, ischemic heart disease, valvular heart disease or cardiomyopathy. Cardiac hypertrophy is the enlargement of the heart in response to stress. It is an adaptation which is beneficial to the stressed heart in the initial stages as it helps the heart to overcome the stress placed on it. However, when the stress placed on the heart is of a chronic nature as that occuring in pathological conditions like the above mentioned diseases, the enlarged heart is unable to maintain normal function, deteriorates with time and subseqently fails (Frey et al., 2004; Frey and Olson, 2003; Latronico et al., 2008; Opie et al., 2006; Selvetella et al., 2004). Current treatments available for treating patients with heart failure include use of β-adrenergic receptor blockers, angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB). Despite the success of these drugs in the treatment of heart failure, the number of deaths due to this ailment is on the rise. In addition, these drugs have significant side effects, which undermine their clinical benefits (Aronson, 2009; Tavares et al., 2008; Tunuguntla, 2007). In this regard, it is of paramount importance to explore alternative strategies to reduce mortality due to heart failure.

Hypertension is a major independent risk factor for the development of heart failure. Although the exact molecular pathway of progression of hypertension to heart failure

is not clear, it is generally believed that chronic blood pressure elevation leads to the development of cardiac hypertrophy as the myocardium increase in size to counter increased stress (Gaddam et al., 2009; Subramaniam and Lip, 2009). Development of cardiac hypertrophy involves progressive changes such as increased cardiomyocyte cell size, enhanced protein synthesis, and sarcomere re-organization. If hypertension is left untreated and cardiac hypertrophy is prolonged, the heart will no longer able to compensate for the stress and the heart will enter into a stage called decompensated cardiac hypertrophy, the beginning of heart failure.

Different plant extracts and phytochemicals have been screened for anti-hypertensive and anti-hypertrophic properties (Ardiansyah et al., 2008; Liu et al., 2005; Lopez-Sepulveda et al., 2008). In this project, I studied the effects of resveratrol (*trans*-3′,4′,5-trihydroxystilbene), a polyphenol found predominantly in grapes and berries, on cardiac remodelling induced by hypertension. The cardioprotective properties of resveratrol have been documented in various *in-vitro* and *in-vivo* experimental settings (Baur and Sinclair, 2006; Li et al., 2012; Wu et al., 2011). A recent study from our lab showed that resveratrol reversed cardiac hypertrophy due to pressure overload induced by abdominal aortic banding in rats (Juric et al., 2007). Although, the aortic banded rat is an experimental model of pressure overload, it does not mimic pressure overload induced by essential hypertension. In this context, this thesis research investigated the potential of resveratrol to prevent or reverse the cardiovascular dysfunction in the spontaneously hypertensive rat (SHR), an excellent model of essential hypertension as well as in an *in vitro* model of norepinephrine induced adult cardiomyocyte hypertrophy. The following section reviews the literature that precedes the current study.

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CHAPTER 2

LITREATURE REVIEW

2.1 Hypertension

Hypertension is one of the leading health concerns worldwide, accounting for about 13% of all mortality and 92 million deaths and disability-adjusted life years globally (Lawes, Vander Hoorn, & Rodgers, 2008; Robitaille, et al., 2012). Its impact on global health is expected to increase over the next 20 years (Kearney, et al., 2005; Lawes, et al., 2008). By definition, hypertension is a systolic blood pressure above 140 mm Hg and/or a diastolic blood pressure above 90 mm Hg. Despite such a large health and societal impact, hypertension remains undiagnosed and/or uncontrolled in a large proportion of patients. Left untreated, hypertension can lead to a spectrum of cardiovascular diseases (CVD) including stroke, coronary artery disease, heart and kidney failure (Robitaille, et al., 2012).

Class		SBP/DBP
Normal	⟹	< 120/80
Prehypertension	⟹	120-139/80-89
Hypertension	⟹	≥ 140/90
Stage 1		140-159/90-99
Stage 2		≥ 160/100

Table 1. A classification system for hypertension for adults. (Source: JAMA 2003;289)

Hypertension is a heterogeneous condition that falls into two major categories; essential (primary) hypertension and nonessential (secondary) hypertension. Essential hypertension is the major type (90-95% of all hypertensive patients) and is of genetic origin influenced by environmental factors (Chen, 2012), whereas nonessential hypertension usually develops in response to an endocrine or renal defect (Binder, 2007; Taler, 2008).

2.2 Pathophysiology

There is still a large amount of uncertainty regarding the pathophysiology of essential hypertension. In most of (90%) cases of human hypertension, no specific mechanism can be identified to account for blood pressure elevation. Theoretically, blood pressure is the product of cardiac output (CO) and systemic vascular resistance. Accordingly, the patients with arterial hypertension will have an increase in cardiac output, an increase in systemic vascular resistance or both (Howell, Sear, & Foex, 2004; Mayet & Hughes, 2003). Many interrelated factors have been reported to contribute to the increase in CO and systemic vascular resistance. These include imbalances in the renin angiotensin aldosterone system (RAAS), endothelial dysfunction, insulin resistance, elevated sympathetic activation, baroreceptor dysfunction, cell membrane alterations, altered renal physiology and other unknown genetic predispositions (Kalil & Haynes, 2011; Sanjay Vikrant, 2001). In patients with non-essential hypertension, increased blood pressure usually develops secondary to specific disorders such as renal parenchymal disease (commonly termed chronic kidney disease), renovascular disease like fibromuscular dysplasia, atherosclerotic renal artery stenosis, endocrine disorders such as primary aldosteronism, pheochromocytoma, cortisol excess, and thyroid or parathyroid abnormalities (Sica, 2008; Taler, 2008).

2.3 End organ targets

Regulation of blood pressure is a complex integrated reaction involving a variety of organ systems including the central nervous system, cardiovascular system, kidneys, and adrenal glands. Accordingly, abnormal elevation of blood pressure (hypertension) can adversely affect these organ systems and can result in the

development of end stage renal disease, stroke and heart failure (Armario, et al., 2009; Gaddam et al., 2009; Khosla et al., 2009) through the following pathological states.

2.3.1 Hypertensive vasculopathy: Hypertensive vasculopathy is caused by endothelial dysfunction and remodeling of the small and large arteries due to persistent hypertension. This leads to a reduced dilation capacity of the high resistance vasculature and further results in vascular stenoses and aneurysms (Schmieder, 2010).

2.3.2 Hypertensive cerebrovascular damage: Hypertension is the most important risk factor which contributes to cerebral damage through arteriolar narrowing or pathological microvascular changes. Hypertension is responsible for silent structural and functional cerebral changes leading to white matter lesions, lacunar infarction, and cognitive impairment (Schmieder, 2010; Sierra, 2009)

2.3.3 Hypertensive Nephropathy: Another important target of chronic hypertension is the kidney in the form of hypertensive nephropathy, which is also known as hypertensive nephrosclerosis. This refers to the hardening of the walls of the small arteries and arterioles (small arteries that carry blood from arteries to the smaller capillaries) of the kidney. The progression of hypertensive nephropathy leads to proteinuria and hematuria and culminates in renal failure.

2.3.4 Hypertensive heart disease: The term hypertensive heart disease refers to a multitude of functional and structural cardiac changes due to persistent hypertension, which finally results in the functional impairment of the myocardium (Cohuet & Struijker-Boudier, 2006).

2.4 From Hypertension to Heart failure

Heart failure is a stage with abnormal myocardial function when the heart is unable to pump enough blood to meet the metabolic demands of the body (Thomas-Kvidera, 2005) Numerous epidemiological studies have already demonstrated that hypertension is a major precursor for the development of heart failure (Kazzam, et al., 2005; Meredith & Ostergren, 2006). The risk of developing heart failure is about two-fold in hypertensive men and three-fold in hypertensive women when compared with their respective normotensive counterparts (Listerman, 2007; Scholze, et al., 2010) Although the exact molecular pathway by which hypertension predisposes to heart failure is not clear, it is generally believed that hypertension can lead to heart failure by one of the two pathways. Firstly it can occur through the development of cardiac hypertrophy, followed by diastolic dysfunction. Secondlyit can promote the process of atherosclerosis and myocardial infarction (MI), which subsequently leads heart failure (Meredith & Ostergren, 2006; Papademetriou, 2004).

In most hypertensive patients, chronic blood pressure elevation leads to the development of cardiac hypertrophy, a compensatory mechanism by which the heart walls increase in size to counter increased stress (Gaddam, et al., 2009; Subramaniam & Lip, 2009). Recently, non-haemodynamic factors (blood pressure-independent) were also implicated in the development of cardiac hypertrophy in hypertension (Kahan & Bergfeldt, 2005; Schmieder, 2005). These include hyper activation of sympathetic nervous system and the renin–angiotensin–aldosterone (RAA) system. Regardless of the stimulus which induces cardiac growth during hypertrophy, it is well established that cardiac hypertrophy is an important intermediate stage in the progression of hypertensive heart disease (Drazner, 2011). Development of cardiac hypertrophy involves progressive changes such as increased cardiomyocyte cell size,

enhanced protein synthesis, and sarcomere re-organization (Colella, et al., 2008). If hypertension is left untreated and cardiac hypertrophy is prolonged, the heart will no longer be able to compensate for the haemodynamic load and will enter into a stage called decompensated cardiac hypertrophy; the beginning of heart failure. The transition from the compensated to the decompensated stage is often characterized by the presence of cardiac fibrosis, arrhythmias and apoptosis (Gaddam, et al., 2009; Subramaniam & Lip, 2009). This deleterious remodeling of the myocardium results in a rapid decline in the contractile performance of the heart, eventually leading to heart failure.

The second pathway by which hypertension leads to heart failure is through the development of atherosclerosis and subsequent MI and systolic dysfunction. Elevated blood pressure triggers the development of atherosclerosis in the arterial system; a condition in which an artery lumen narrows as a result of the accumulation of fats such as cholesterol (Lusis, 2000). The cholesterol accumulation along with inflammation and smooth muscle proliferation progressively leads to the formation of an atherosclerotic lesion. Erosion of this plaque causes intraluminal thrombosis and induces coronary block, which leads to acute myocardial infarction followed by sudden coronary death (Naghavi et al., 2003, Jones et al., 2003). Figure 1 depicts the key events involved in the progression of hypertension to heart failure.

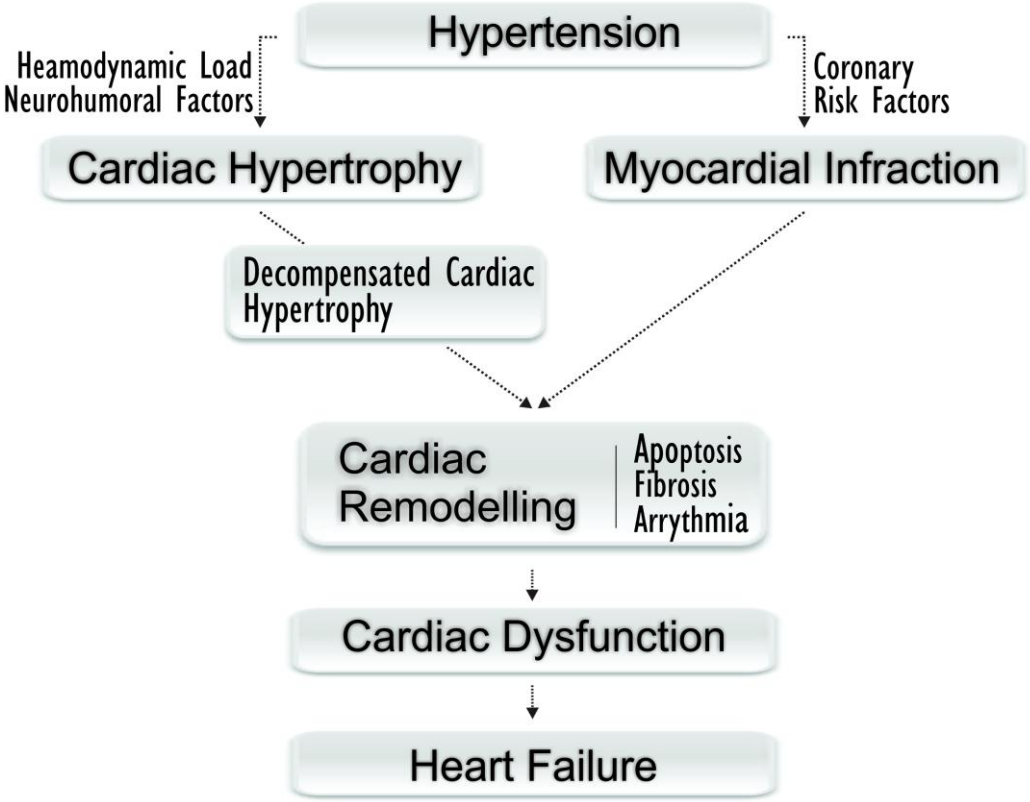


Figure1: Hypertension to heart failure

2.5 Management of hypertension-induced heart disease

2.5.1 Life style and non-pharmacological interventions:

Since hypertension and associated heart disease are caused by multiple defects in the body, all international hypertension guidelines strongly recommend dietary/life style modifications for managing this disease (Williams, 2008). Accordingly, in addition to pharmacological treatment, self-education and understanding the etiology of the hypertension/heart failure is critical. Implementation of a healthy lifestyle can help to keep blood pressure under control and prevent associated cardiovascular events. Lifestyle modifications that effectively lower blood pressure include increased physical activity, weight loss, limited alcohol consumption, and reduced sodium intake (Williams, 2008).

2.5.2 Current pharmacological agents

The selection of therapeutic agent is mainly based on the nature of the hypertensive individual and also depends on the drug's efficacy on reducing blood pressure and cardiovascular events. Some of the well established drugs available for patients with hypertension/heart failure include β-adrenergic receptor (β-AR) blockers, angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor (AR) blockers, calcium channel blockers and diuretics (Aronson JK . Elsevier press, 2009; Gaddam, et al., 2009; Subramaniam & Lip, 2009). These drugs act on different physiological pathways according to the nature and etiology of blood pressure in different hypertensive individuals. β-AR blockers prevent the over-stimulation of sympathetic nervous system to reduce the heart's workload by slowing heart rate. Diuretics act on the kidney and remove excess water and sodium from the body thereby reducing the blood pressure. ACE inhibitors and AR blockers block the activation of RAAS, which is a major contributor of blood pressure elevation in many cases. Calcium channel blockers reduce the entry of calcium into the heart muscle cells and thereby reduce cardiac contractility. Some new therapeutic agents available are aldosterone receptor blockers, renin inhibitors, endothelin receptor antagonists, and dual endopeptidase inhibitors (Armario & de la Sierra, 2009; Gaddam, et al., 2009; Subramaniam & Lip, 2009). Considering the fact that it is not always possible to identify the exact cause of hypertension in some patients, blood pressure control with combination therapy (more than one drug at a time) with the above mentioned agents is also well advised (Gradman, et al., 2011).

2.6 Need for alternative therapies

The current pharmacological agents available in the clinic have proven to be reasonably effective; however, the incidence of mortality due to heart failure is still

high (Tavares, Rezlan, Vostroknoutova, Khouadja, & Mebazaa, 2008; Tunuguntla, 2007). Consequently, there is an important need for alternative therapeutic strategies to prevent or reverse hypertension/heart failure. In this scenario, recent scientific evidence has promoted the prospect of food derived compounds and their efficacy as the future of medicine (Aruoma, 2010). Accordingly, food-based therapies could be applied either as primary treatment or as complementary to the existing strategies. Although, food-based therapies perform better as a preventive strategy, recent research shows that they could be used as a treatment after the onset of the disease (Champagne, 2006). Functional foods and nutraceuticals have thus been now extensively explored for their potential application in the treatment of different diseases. Evidence from epidemiological, *in vivo*, *in vitro*, and clinical studies indicate that plant-based diets and nutraceuticals can reduce the risk of chronic diseases, particularly cancer and cardiovascular diseases (Espin, Garcia-Conesa, & Tomas-Barberan, 2007; Ferrari, 2004; Johnston, 2009). Among these compounds, polyphenols are classes of compounds that have received increased attention in medical research recently (Espin, et al., 2007). Among this group, one of the most studied has been resveratrol, a polyphenol with tremendous health benefit potential (Smoliga, Baur, & Hausenblas, 2012; Yu, Fu, & Wang, 2012).

2.7 Resveratrol

2.7.1 Occurrence and synthesis

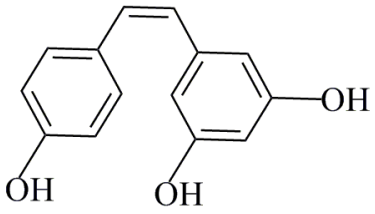
Resveratrol is a polyphenol that is produced by plants in response to environmental stress, such as extreme temperatures, infections and UV radiation (H. Li, Xia, & Forstermann, 2012; Wenzel & Somoza, 2005). Resveratrol is found in abundance in the skin of grapes and in the leaf of epidermis as well as in many fruits and vegetables such as berries and peanuts (Yap, Qin, & Woodman, 2010). The presence of

resveratrol has also been detected in wines, especially red wines and to a much lesser extent in white wines (Pirola & Frojdo, 2008), Reveratrol has also been isolated from the dried roots of Japanese knotweed, *polygonum cuspidetum* (Yap, et al., 2010). The enzyme stilbene synthase is involved in the biosynthesis of resveratrol in response to various stresses in the plants. This biosynthesis happens through a condensation reaction of p-coumaroyl CoA and malonyl CoA precursors in a 1:3 molar ratio (Delaunois et al., 2009; Sydor et al., 2010).

2.7.2 Physicochemical properties

Resveratrol chemically resembles the synthetic estrogen diethylstilbestrol, wherein, two phenolic rings are bonded by a styrene double bond to form 3,4,5-trihydroxystilbene (Pervaiz, 2003; Yu, et al., 2012). It is a solid off-white powder with a molecular formula $C_{14}H_{12}O_3$ (Amri, Chaumeil, Sfar, & Charrueau, 2011). Resveratrol exists in two different isomeric forms, *cis*- (Z) and *tran* (E) –resveratrol. The trans-isomer is biologically more active than the cis form (Amri, 2011). *Cis* resveratrol is highly unstable at room temperature and is not available commercially (Y. B. Chen, Sun, & Chen, 2007). Resveratrol is extremely light sensitive compound and also susceptible to UV-induced isomerization (Amri, et al., 2011; Camont, et al., 2009). Figure 2 shows the chemical structure of *cis* and *trans* resveratrol.

A



B

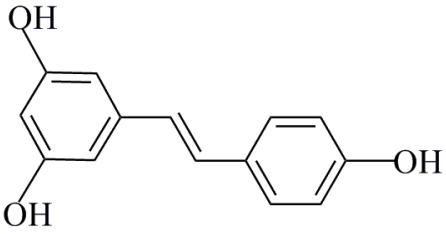


Figure 2: A - Structure of cis-resveratrol
B - Structure of trans-resveratrol

2.7.3 Metabolism and bio-availability

Numerous studies have demonstrated that resveratrol has very low bio-availability, leading to concern that many of the beneficial effects exerted by resveratrol in *in-vitro* experiments may not be observed in animals and humans (Smoliga, Baur, & Hausenblas; Walle, Hsieh, DeLegge, Oatis, & Walle, 2004). Earlier studies have demonstrated that the oral absorption of resveratrol in human is about 75% and it occurs mainly through trans-epitheial diffusion. Metabolic studies using both plasma and urine revealed that resveratrol is metabolized into glucuronides and sulfates, which might have similar biological activity (Delmas, et al., 2011). Total sulfates conjugates accounted for 37% of the metabolites and 19% of were glucuronides. Only trace amounts of unchanged resveratrol were detected in the blood plasma after a 25mg oral dosage (Boocock, et al., 2007). Intestine and liver have been identified as the main sites of resveratrol metabolism. The hepatic uptake of resveratrol was also investigated by Lancon et al (2004), where they showed that resveratrol influx does not occur only by passive diffusion but also involves a carrier mediated process. This

process allows the efficient hepatic uptake of resveratrol from the blood. However, it is not yet clear whether native resveratrol itself or derivates or metabolites have therapeutic *in vivo* effects. Identification of the role of resveratrol's active metabolites will make possible correct interpretation of the pharmacodynamic data obtained in preclinical studies and extrapolation of the data to humans (Santos, Veiga, & Ribeiro, 2011). A better understanding of resveratrol metabolites and quantification of their level of activity will allow a better delivery system for resveratrol in the future. Currently, several active studies are ongoing to develop novel formulations to stabilize and protect resveratrol from degradation and to enhance its bioavailability. However, despite all these concerns regarding resveratrol bioavailability, abundant *in vivo* and clinical trial evidence indicates that resveratrol treatment has protective effects against various pathologies.

2.8 Resveratrol and cardioprotection

The research on resveratrol and cardioprotection gained its momentum after the results from epidemiological studies, where an inverse correlation was revealed between regular wine consumption and the incidence of cardiovascular disease, a phenomenon that became known as "French Paradox. According to this theory, the incidence of cardiovascular disease in French population is approximately 40% lower than in the rest of the Europe; despite consumption of a saturated fat rich diet (Renaud & de Lorgeril, 1992). This led to the suggestion that resveratrol might be the active ingredient which imparts these beneficial effects. Later on, various studies from different parts of the world started reporting strong cardioprotection with resveratrol in different *in vitro* and *in vivo* models of cardiovascular disease (Li, et al., 2012; Wu, Hsieh, & Wang, 2011). The experimental evidence showing the potential of resveratrol in preventing the development and progression of cardiovascular diseases

is discussed below. Figure 3 provides an overview of the cardiovascular effects of resveratrol.

Mechanisms of cardioprotection

Resveratrol exhibits diverse cardioprotective effects in animal studies as well as in human clinical trials. This versatileeffects may be attributable to the ability of resveratrol to act on many pathological factors at the same time. Some of the direct and indirect cardiovascular effects of resveratrol are depicted below.

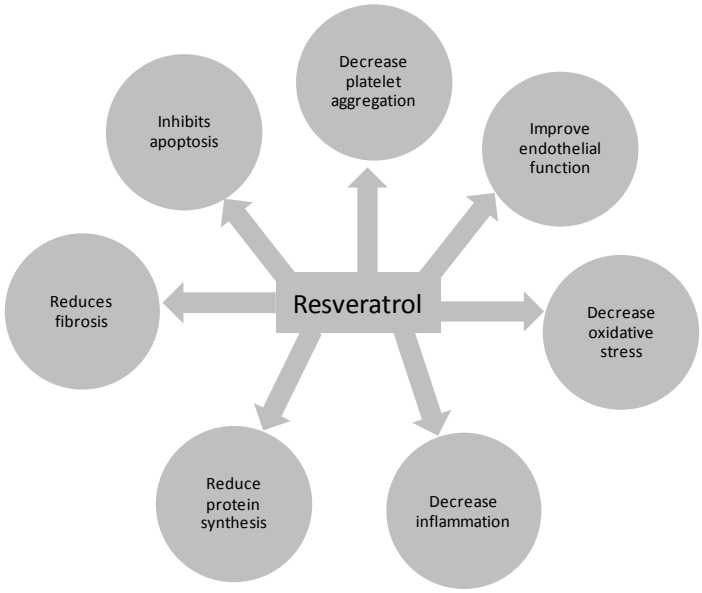


Figure: 3 Cardiovascular targets of resveratrol

2.8.1 Hypertension and cardiac hypertrophy

Resveratrol has been well studied for its anti-hypertensive and anti-hypertrophic activities in different animal models. Liu et al. (2005) reported that daily administration of partially nephrectomized rats with 10 or 50 mg/kg body weight

resveratrol for four weeks significantly attenuated the increased systolic blood pressure and arrested the development of cardiac hypertrophy. This effect was in a dose-dependent manner and was attributed to an up-regulation in the levels of nitric oxide, an anti-hypertrophic molecule. Li et al. (2005) also reported similar effects with isorhapontigenin, a resveratrol analog at a dosage of 50 mg/kg body weight for 3 weeks following transverse aortic constriction surgery. This study showed that the resveratrol analog attenuated the progression of cardiac hypertrophy and improved diastolic function in rats; this effect was linked with a reduction in oxidative stress. The regression of cardiac hypertrophy and diastolic dysfunction in rats subjected to abdominal aortic banding surgery was also reported by Juric et al. (Juric et al., 2007). In this study, resveratrol treatment (at a lower dose of 2.5 mg/kg body weight/day) was started after surgery for a period of two weeks. In ovariectomized, stroke prone SHR, a diet supplemented with resveratrol (5mg/kg/day) improved endothelium dependent vascular relaxation and lowered systolic blood pressure significantly after 3 weeks of administration (Mizutani et al., 2000). In another study, resveratrol (10 mg/kg/day) prevented the increase in systolic blood pressure and cardiac hypertrophy in fructose-fed rats through the activation of eNOS thereby alleviating oxidative stress (Miatello, et al., 2005).

Most recently, a lower dose of resveratrol (2.5 mg/kg/day) prevented the development of concentric hypertrophy and contractile abnormalities in SHR without lowering the blood pressure in two different studies (Dolinsky, et al., 2009; Thandapilly, et al., 2010). This indicated that the beneficial effects observed in the above studies are not only mediated by reducing blood pressure, but also involve factors other than haemodynamic load. These include the activation of AMP-activated

protein kinase (AMPK) and the reduction of oxidative stress (Dolinsky, et al., 2009; Thandapilly, et al., 2010).

In vitro studies using cardiomyocytes have also reported beneficial effects of resveratrol on cardiomyocyte hypertrophy stimulated by potent hypertrophic agents such as angiotensin II, phenylephrine and norepinephrine. A study, (Cheng, et al., 2004), showed that treatment with 10 μM resveratrol attenuated the angiotensin-II induced cardiomyocyte hypertrophy in neonatal cardiomyocytes. This effect was linked to an attenuation of reactive oxygen species generation. Chan et al. (2008) also reported similar effects with 50 μM resveratrol in cardiomyocyte exposed with phenylephrine; this effect was associated with the up-regulation of AMPK, an endogenous inhibitor of cardiac hypertrophy. Recently, we (Thandapilly, et al., 2011) have also reported that resveratrol could prevent adult cardiomyocyte hypertrophy induced by norepinephrine via activation of the NO-AMPK pathway.

2.8.2 Diabetes

Resveratrol has been well documented in the scientific literature for its beneficial effects in preventing and treating type 1 and 2 diabetes by reducing blood glucose, preserving β cells, and improving insulin sensitivity (Szkudelski & Szkudelska, 2011). Palsamy & Subramanian (2009) earlier reported that resveratrol administration to type 1 diabetic rat, improved hepatic glycogen content by reducing oxidative stress and inflammation. The anti-hyperglycemic effect of resveratrol has also been confirmed by Su et al. (2006) in streptozotocin-induced (Type 1) as well as in nicotinamide induced (Type 2) diabetic rats. In the above studies, the glucose reduction observed with resveratrol treatment is likely to result from the enhanced glucose uptake by peripheral tissues. In addition, resveratrol has also showed promising results in *in vitro* experiments where it enhanced glucose uptake in isolated

cells in the absence of insulin (Su, et al., 2006). Moreover, recently resveratrol has been reported to induce the glucose stimulated insulin secretion in beta-cells via sirtuin mediated pathways (Vetterli L, 2011). Another study from Sulaiman et al. (2010) gave insight to a possible involvement of resveratrol as an activator of SIRT-1, and sarcoplasmic calcium ATPase (SERCA2a), thus improving cardiac function in chronic Type 1 diabetes.

Since insulin resistance is a key factor in the development of type 2 diabetes, molecules that can improve insulin sensitivity will have a great impact on glucose management (H. Li, et al., 2012). It has been earlier reported that resveratrol enhances insulin sensitivity in *in vitro* and *in vivo* models of diabetes (Sun, et al., 2007).

2.8.3 Ischemic heart disease

Resveratrol has showed great potential in protecting the myocardium from acute and chronic ischemia through various mechanisms, which include inhibition of platelet aggregation and thereby myocardial infarction, protection of myocardium from ischemia reperfusion injury by preconditioning, and triggering the regeneration of infarcted myocardial tissue (Sun, et al., 2007). Hung et al. (2004) reported that resveratrol renders cardioprotection against ischemia reperfusion injury in rats with left coronary artery ligation, where it reduced cardiac arrhythmia and infarct size. The effects of resveratrol have also been documented in other *in vivo* experimental models of myocardial infarction. Chen et al. (2008) reported that resveratrol administration (5 mg/kg body weight/day) for one week before coronary artery ligation surgery and continuing for 14 weeks after surgery, significantly arrested the progression of cardiac hypertrophy induced by myocardial infarction; this effect was associated with a decrease in infarct size. Similar findings were also reported by Lin et al. (Lin, et al., 2008), where they observed a significant reduction in infarct size and an improvement

in left ventricular systolic and diastolic function with resveratrol treatment (1 mg/kg) in rats with myocardial infarction. In another study, resveratrol treatment (100 mg/kg/d) improved myocardial perfusion in a swine model of chronic myocardial ischemia by enhancing the angiogenesis (Robich, et al., 2010). Xi J et al. (2009) also examined the effect of resveratrol on myocardial infarct size on isolated rat hearts subjected to ischemic reperfusion and investigated the mechanism underlying the beneficial effect. This study revealed that resveratrol rendered cardioprotection against myocardial reperfusion injury by targeting the mitochondrial permeability transition pore via the translocation of GSK-3β from cytosol to mitochondria.

2.8.4 Cardiomyopathy

The cardioprotective effect of resveratrol has also been investigated in different models of cardiomyopathies. Yoshida et al. (2007) investigated the cardio-protective effects of resveratrol in an animal model of autoimmune myocarditis. This study showed that treatment with resveratrol (50 mg/kg/day) for 1 day prior to immunization and for 14 days after immunization with cardiac myosin significantly reduced left ventricular hypertrophy and improved systolic function in rats with cardiac myocarditis. These beneficial effects were associated with the anti-oxidant and anti-inflammatory properties of resveratrol. Wang et al. (Z. P. Wang, et al., 2009) have also reported that resveratrol can inhibit myocardial collagen synthesis and thereby improve cardiac performance in a mouse model of chronic viral myocarditis. In another study conducted by Zhao et al. (2008), resveratrol was able to improve the abnormalities in electrical activity and cardiac structure in a mouse model of arsenic trioxide-induced cardiomyopathy by alleviating oxidative stress. Similar findings were also reported by Tatlidede et al. (2009), who observed an improvement in cardiac function with resveratrol treatment (10 mg/kg) in a model of doxorubicin-

induced cardiomyopathy; this effect was also associated with a reduction in oxidative stress damage. More recently, a high dose of resveratrol (4 g/kg) suppressed fibrosis, preserved cardiac function, and significantly improved survival in a mice deficient in mitochondrial manganese superoxide dismutase, an experimental model of dilated cardiomyopathy and chronic heart failure (Tanno, et al., 2010).

2.8.5 Myocardial remodeling

Loss of cardiomyocytes through fibrosis as well as apoptosis has been proposed as a cause of ventricular remodeling during heart failure (Chen, et al., 2009). In this context, a few studies have examined the potential of resveratrol in preventing these key events in *in vitro* settings. Olson et al. (2005) reported that treatment with resveratrol (25 µM) prevented angiotensin II (a potent inducer of fibrosis) induced cardiac fibroblast proliferation and differentiation; this effect was associated with attenuation of extracellular signal-regulated kinase activation. Wang et al. (2007) also reported the inhibition of angiotensin II-induced cardiac fibroblast proliferation with resveratrol (25-100 µM) treatment through activation of the NO-cGMP pathway. Considering the role of ischemia- and hypoxia-induced apoptosis of cardiomyocytes in many of the cardiac pathologies, Zhang et al. (2012) reported the efficacy of resveratrol in protecting cardiomyocytes against anoxia/reoxygenation injury via attenuation of inflammatory response. Chen et al. (2009) also reported similar effects in cardiomyocytes; they observed cytoprotective effects of resveratrol against ischemia/hypoxia induced apoptosis through the activation of SIRT-1. Table 2 list the evidence of cardioprotection rendered by resveratrol from animal and human trials.

Table 2 –Cardioprotection with resveratrol- Evidence from animal studies and clinical trials

Animal model	Dosage used (treatment period)	Result
Hypertension and cardiac hypertrophy:		
Ovariectomized, stroke prone SHR	5mg/kg/day in diet (3 weeks post-surgery)	Improved endothelial dependent vascular relaxation and lowered systolic blood pressure significantly
Transverse aortic (TAC) constriction in rat	** 50 mg/kg/d i.p. injection (24 h post-TAC, 21 d post-TAC)*	Inhibited hypertrophy through antioxidant mechanism
Abdominal aortic banding in rat	2.5 mg/kg/d oral gavage (2 wk post-banding, 4 wk post-banding)	Attenuation of hypertrophy & diastolic dysfunction with upregulation of eNOS & iNOS
SHR rat (10 week old)	2.5 mg/kg/d oral gavage (for 10 weeks)	Prevented cardiac hypertrophy and improved systolic and diastolic cardiac functions by alleviating oxidative stress
SHR rat (12 week old)	2.5 mg/kg/d oral gavage (for 2 weeks)	Prevented cardiac hypertrophy and significantly improved cardiac functions through the activation of AMPK
Diabetes:		
Streptozotocin-induced diabetic (type 1) rats	0.5 mg/kg three times a day, oral gavage (for 14 days)	Delayed the onset of insulin resistance, improved the glucose uptake by peripheral tissues.
Nicotinamide induced diabetic (type 2) rats	0.5 mg/kg three times a day, oral gavage (for 14 days)	Delayed the onset of insulin resistance, improved the glucose uptake by peripheral tissues.
Streptozotocin-nicotinamide-induced diabetic rats	5 mg/kg body weight, orally (for 30 days)	Improved the activities of the key enzymes of carbohydrate metabolism and hepatic glycogen content.
Streptozotocin induced diabetic mice	diet enriched with resveratrol at 0.067% (12 weeks)	Improved cardiac function by activating SERCA2a expression
C57BL/6J mice fed with high fat diet	2.5 mg/kg/d in drinking water (for 12 weeks)	Attenuated insulin resistance through SIRT-1 dependent mechansims
Ischemic heart disease:		
LAD coronary artery ligation in rat	5 mg/kg/d orally (7 d pre-treatment, 3 wk post-ligation)	Attenuated ventricular arrhythmias & hypertrophy; improved long-term survival at 14 wk post-ligation
Left main coronary occlusion in rat	10 μM transjugular infusion (15 min pretreatment, cessation at occlusion)	Improved cardiac function & reduced infarct size via reduced myocardial oxidative stress associated with NO
Left main coronary occlusion in rat	2.3 $x10^{-7}$,$x10^{-6}$, $x10^{-5}$ g/kg (15 min pretreatment, cessation at occlusion)	Prevented ischemia/reperfusion associated arrhythmias & mortality, with increased production of NO
aconitine-, oubain- & coronary artery ligation-induced in rat	5-45 mg/kg i.v. injection (one time 10 min prior to measurements)	Dose dependent anti-arrhythmic effect due to shortened action potential duration by enhancement of I_{ks} without change in I_{kr}
Yorkshire miniswine fed with high fat diet	diet supplemented with 100 mg/kg/d (for 4 weeks)	Improved myocardial perfusion in the collateral dependent region and unregulated markers of angiogenesis
Cardiomyopathy:		
Rats with Myosin-induced autoimmune myocarditis	50 mg/kg/d ip injections (for 15 days)	Significantly ameliorated myocardial injury and preserved cardiac function
Mice inoculated with Coxsackievirus (viral myocarditis)	100-1000mg/kg/day (for 30 days)	Inhibited hyperplasia of myocardial collagen
Mouse model of Arsenic-induced cardiomyopathy	3mg/kg/day ip injections (for 5 days)	Improved electrical activity and cardiac structure through the alleviation of oxidative stress
Rats doxorubicin-induced cardiomyopathy	10 mg/kg/day (for 7 weeks)	Improved cardiac function via reducing oxidative stress
TO-2 hamsters (model of dilated cardiomyopathy)	4g/kg/day (for 29 weeks)	Suppressed fibrosis, preserved cardiac function, and significantly improved survival
Type of patients	**Dosage used (start time, end point)**	**Result**
Healthy obese men	150mg/day (for 30 days)	Improvement in metabolic parameters
Type 2 diabetic patients	2 × 5 mg/day (for 4 weeks)	Improved insulin sensitivity via reducing oxidative stress damage
Patients with myocardial infarction	10mg/day (for 3 months)	Improved diastolic heart function, endotheial function and lowered LDL cholesterol levels

** isorhapontigenin – an analog of resveratrol was used*;SHR, spontaneously hypertensive rat; AMP, adenosine monophosphate; i.p. intraperitoneal; NO, nitric oxide synthase; LAD, left anterior descending; ip., intraperitonial; I_{ks}, slow delayed rectifying potassium current; I_{kr}, rapid delayed rectifying potassium current; LDL, low density lipoprotein

2.8.6 Clinical trial evidence

Despite the increasing body of evidence from preclinical studies suggesting cardio-protection rendered by resveratrol, the evidence from appropriately designed clinical trials is limited (Patel, et al., 2011). The clinical trial database shows a total of 16 studies involving resveratrol that are ether active or are recruiting, plus six more that have recently been completed (http://clinicaltrials.gov). Most of the published clinical trials have largely investigated the pharmacokinetics and metabolism of resveratrol (Smoliga, et al., 2012). In a recent randomized double-blind crossover study, resveratrol treatment (150 mg/day) for 30 days induced modest metabolic improvement in 11 healthy obese men (Timmers, et al., 2011). Another human study conducted by Brasnyo et al. (Brasnyo, et al., 2011) reported that resveratrol improves insulin sensitivity in Type 2 diabetic patients through a reduction in oxidative stress to thereby improve insulin signaling. However, the first human study to investigate the potential of resveratrol in treating heart disease was recently published by Magyar et al. (Magyar, et al., 2012), where they administered resveratrol in patients after myocardial infarction. This was a double-blind, placebo controlled trial, involved 40 post-infarction Caucasian patients in which the patients received 10 mg resveratrol capsule daily for 3 months. The study showed promising results in which resveratrol improved ventricular diastolic function, endothelial performace, lowered LDL-cholesterol level and protected against unfavorable hemorheological changes. The results from future clinical studies will confirm the efficacy of resveratrol in different human heart diseases and may help to translate this molecule from bench to bedside.

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OBJECTIVE: 1

To examine the effects of resveratrol in preventing cardiovascular abnormalities spontaneously hypertensive rat

RATIONALE

Resveratol mediated cardioprotection has been well documented in different experimental models of concentric hypertrophy and cardiac dysfunction. However, no study has investigated the effects of resveratrol in preventing cardiovascular abnormalities an animal model of essential hypertension. Therefore, we examined the effects of resveratrol in spontaneously hypertensive rats (SHR), an *in vivo* model of essential hypertension.

Manuscript: 1

Title: Resveratrol prevents the development of pathological cardiac hypertrophy and contractile dysfunction in the SHR without lowering blood pressure

Access to the publication online is available via the following link:

http://www.nature.com/ajh/journal/v23/n2/full/ajh2009228a.html

My contributions to the work

I was involved in planning and designing the study. I conducted the animal study which includes weighing the animals, preparing resveratrol solutions, gavaging, etc. I was involved in the measurement of blood pressure on animals. I performed the echocardiographic measurements and analyzed all echocardiography data. I also did TBARS and total antioxidant activity assays on both tissues as well as blood plasma. Finally, I wrote the manuscript with the help of Dr. Thomas Netticadan and did the necessary revisions for the publication.

CHAPTER 3

Resveratrol prevents the development of pathological cardiac hypertrophy and contractile dysfunction in the SHR without lowering blood pressure

3.1 ABSTRACT

Cardiac hypertrophy is a compensatory enlargement of the heart in response to stresses such as hypertension. It is initially beneficial in reducing stress placed on the heart. However, when the stress is of a chronic nature, it becomes pathological and leads to cardiac dysfunction and heart failure. Current treatments for hypertension and heart failure have proven beneficial but are not highly specific and are associated with side effects. Accordingly, there is an important need for alternative strategies to provide safe and effective treatment. Ten weeks old male spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats were treated with resveratrol (2.5 mg/kg/day) for a period of 10 weeks. Systolic blood pressure, cardiac structure and function were measured in all groups at different time points of resveratrol treatment. Oxidative stress was also determined in all groups after 10 weeks of resveratrol treatment. SHR were characterized as having high blood pressure and concentric hypertrophy from 15 weeks of age. Cardiac functional abnormalities were also evident in SHR from 15 weeks onwards. Resveratrol treatment significantly prevented the development of concentric hypertrophy and systolic and diastolic dysfunction in SHR without lowering blood pressure. Resveratrol also significantly reduced the oxidative stress levels of cardiac tissue in SHR. Resveratrol treatment was beneficial in preventing the development of concentric hypertrophy and cardiac dysfunction in SHR. The cardioprotective effect of resveratrol in SHR may be partially mediated by a reduction in oxidative stress. Thus, resveratrol may have potential in preventing cardiac impairment in patients with essential hypertension.

3.2 INTRODUCTION

Heart failure is a leading cause of mortality worldwide (Mudd et al., 2008; Latronico et al., 2008). It is a complicated, multifactorial syndrome resulting from the heart's inability to pump sufficient blood to meet the metabolic demands of tissues. The development of heart failure is secondary to diseases such as hypertension, ischemic heart disease, valvular heart disease or cardiomyopathy. Heart failure is always preceded by cardiac hypertrophy, which is the enlargement of heart in response to stress (pressure or volume overload). It is an adaptation which is beneficial to the stressed heart in the initial stages as it helps to overcome the stress placed on it. If hypertrophy prolongs, the heart will no longer able to compensate the stress and will enter into a decompensated stage. This transition from compensated to decompensated stage is often characterized by the presence of cardiac fibrosis, arrhythmia and apopotosis. This will eventually lead to irreversible functional deterioration and heart failure (Latronico et al., 2008; Opie et al., 2006). Current treatments available for patients with heart failure include use of β-adrenergic receptor blockers, angiotensin-converting enzyme inhibitors and angiotensin receptor blockers (Tavares et al., 2008; Tunuguntla et al., 2007). These therapies are not highly specific and often result in a number of side effects (Aronson et al., 2009). Accordingly, it is imperative to explore alternate strategies to provide safe and effective treatment for heart failure.

Hypertension induces pressure overload resulting in cardiac hypertrophy. Hypertension is a major independent risk factor for the development of heart failure (Subramaniam et al., 2009; Arguedas et al., 2009). Different plant extracts and phytochemicals have been recently screened for anti-hypertensive and anti-hypertrophic properties (Seymour et al., 2008; Ardiansyah et al., 2008).

Cardioprotection by resveratrol (*trans*-3′, 4′, 5-trihydroxystilbene), a polyphenol found predominantly in grapes and berries, or its analogue has been documented in different experimental settings of pressure overload (Liu et al., 2005; Juric et al., 2007). However, no study has examined the effects of resveratrol in preventing chronic changes in cardiac structure and function due to essential hypertension. Therefore, we examined the effects of resveratrol in spontaneously hypertensive rats (SHR), an appropriate model of essential hypertension.

3.3 MATERIALS ANDF METHODS

The experimental protocols used in this paper were approved by the University of Manitoba Animal Care Committee and are in agreement with the *Canadian Council on Animal Care and Use of Experimental Animals* (vol. 1st, 2nd ed., 1993).

3.3.1 Animal model

Ten week old male SHR and their normotensive controls Wistar-Kyoto rats (WKY) obtained from Charles River Inc. Canada were used in this study. Animals were acclimatized in temperature and humidity controlled rooms with a 12 hour dark and 12 hour light period cycle throughout the study.

3.3.2 Treatment and examinations

Ten week SHR and WKY were treated daily by oral gavage with resveratrol (2.5 mg/kg/day), a dose previously established by us (Juric et al., 2007), for a period of 10 weeks.

3.3.3 Measurement of blood pressure

Systolic blood pressures were measured in SHR and WKY rats starting at 0 week, 5 week and 10 week, of resveratrol treatment using, the Non-Invasive Blood Pressure Measurement System (CODA) equipped with volume pressure recording

(Kent Scientific Corporation, CT, USA). A total of 4 groups were examined – WKY, resveratrol treated WKY, SHR, and resveratrol treated SHR.

3.3.4 Assessment of cardiac structure and function

Cardiac structure and function were assessed by echocardiography in 10 week SHR and WKY rats starting at 0 week, 5 week and 10 weeks, of resveratrol treatment.

3.3.5 Examination of cardiac structure in vivo

Two-dimensional-guided (2D) M-Mode echocardiography was used to examine cardiac structure as described earlier (Cantor et al., 2005). The following parameters were measured: left ventricular internal dimensions (LVID) at diastole and systole, left ventricular posterior wall (LVPW) thickness at diastole and systole, and interventricular septum (IVS) thickness at diastole and systole.

3.3.6 Measurement of cardiac function in vivo

Two-dimensional-guided M-Mode echocardiography and Pulse Wave Doppler echocardiography were used to assess cardiac function as described earlier (Cantor et al., 2005). Contractile parameters of systolic function such as left ventricular ejection fraction (EF) and cardiac output (CO) were assessed by 2D-guided M-Mode echocardiography. Diastolic function was assessed by measuring the isovolumic relaxation time (IVRt) using Pulse Wave Doppler echocardiography.

3.3.7 Measurement of lipid peroxidation levels

The degree of lipid peroxidation was assessed by measuring malondialdehyde (MDA) levels in the homogenized left ventricular (LV) tissue and blood plasma using the OxiSelect TBARS Assay Kit (Cell Biolabs, San Diego, California, USA). The thiobarbituric acid reactive substances (TBARS) values in tissue and plasma were expressed as nmol/mg of protein and nmol/ml of plasma respectively.

3.3.8 Measurement of Total Antioxidant status (TAS)

The Total antioxidant status (TAS) of the blood plasma was determined using the TAS assay kit (Randox Lab, Crumlin, UK) according to the manufactures protocol. Plasma total anti-oxidant status values were expressed as µmol/ml of plasma.

3.3.9 Statistical Analysis

All values are expressed as means ± SE. One way analysis of variance (ANOVA) was used to analyze variations between the means of the groups. Significant values are defined as $P \leq 0.05$. When significance was obtained, one-way ANOVA was followed by Tukey post-hoc test.

3.4 RESULTS

3.4.1 General Observations

There was a significant increase in the heart Weight to tibia length ratio in the 20 week old SHR in comparison to their age matched WKY controls; this increase was significantly prevented after 10 weeks of resveratrol treatment (Figure 1 A). Treatment with resveratrol did not affect heart to tibia length ratio in 20 week WKY.

3.4.2 Blood pressure

Blood pressure measurements were conducted in 10, 15, and 20 week SHR and their age-matched WKY, treated with and without resveratrol. Systolic blood pressure was significantly increased in SHR at all time points, when compared to their age-matched WKY controls. Treatment with resveratrol did not significantly lower systolic blood pressure in SHR or WKY rats (Figure 1 B).

3.4.3 Cardiac Structure

Echocardiographic analysis of cardiac structure was carried out in 10, 15 and 20 week SHR and their age-matched WKY, treated with and without resveratrol. M-mode echocardiography showed no changes in cardiac structure in 10 and 15 week SHR (in comparison to their age-matched WKY controls). However, 20 week SHR

exhibited a significant increase in the IVS and LVPW when compared to their age-matched WKY controls; this increase was significantly reduced after 10 weeks of resveratrol treatment (Figure 1 C, Figure 1 D). Twenty week old SHR did not exhibit any change in LVID (in comparison with WKY controls) (Figure 1 E).

3.4.4 Cardiac Function

Echocardiographic analysis of cardiac function was carried out in 10, 15 and 20 week SHR and their respective age-matched WKY, treated with and without resveratrol. A significant increase in the diastolic functional parameter, IVRt was observed in 20-week SHR but not in 10 and 15 week SHR (in comparison with their respective WKY controls); resveratrol treatment prevented this elevation (Figure 1 F). There was also a significant decrease in the systolic functional parameter EF in 20 week SHR; resveratrol treatment significantly prevented this decrease (Figure 1 E). Treatment with resveratrol did not affect IVRt and EF in 20 week WKY. CO was unchanged in all groups at all time points of the study. (Figure 1 G).

3.4.5 Oxidative stress

Concentrations of TBARS were significantly elevated in the in the blood plasma of 20 week SHR when compared to the age matched WKY controls (Figure 2 A). Resveratrol treatment significantly reduced the elevation in TBARS in the blood plasma of SHR (Figure 2 A).

On the other hand, a significant decrease was observed in the total antioxidant status (TAS) of blood plasma in 20 week SHR when compared to age matched WKY controls (Figure 2 B). The treatment with resveratrol significantly recovered the total anti-oxidant capacity in 20 week SHR (Figure 2 B).

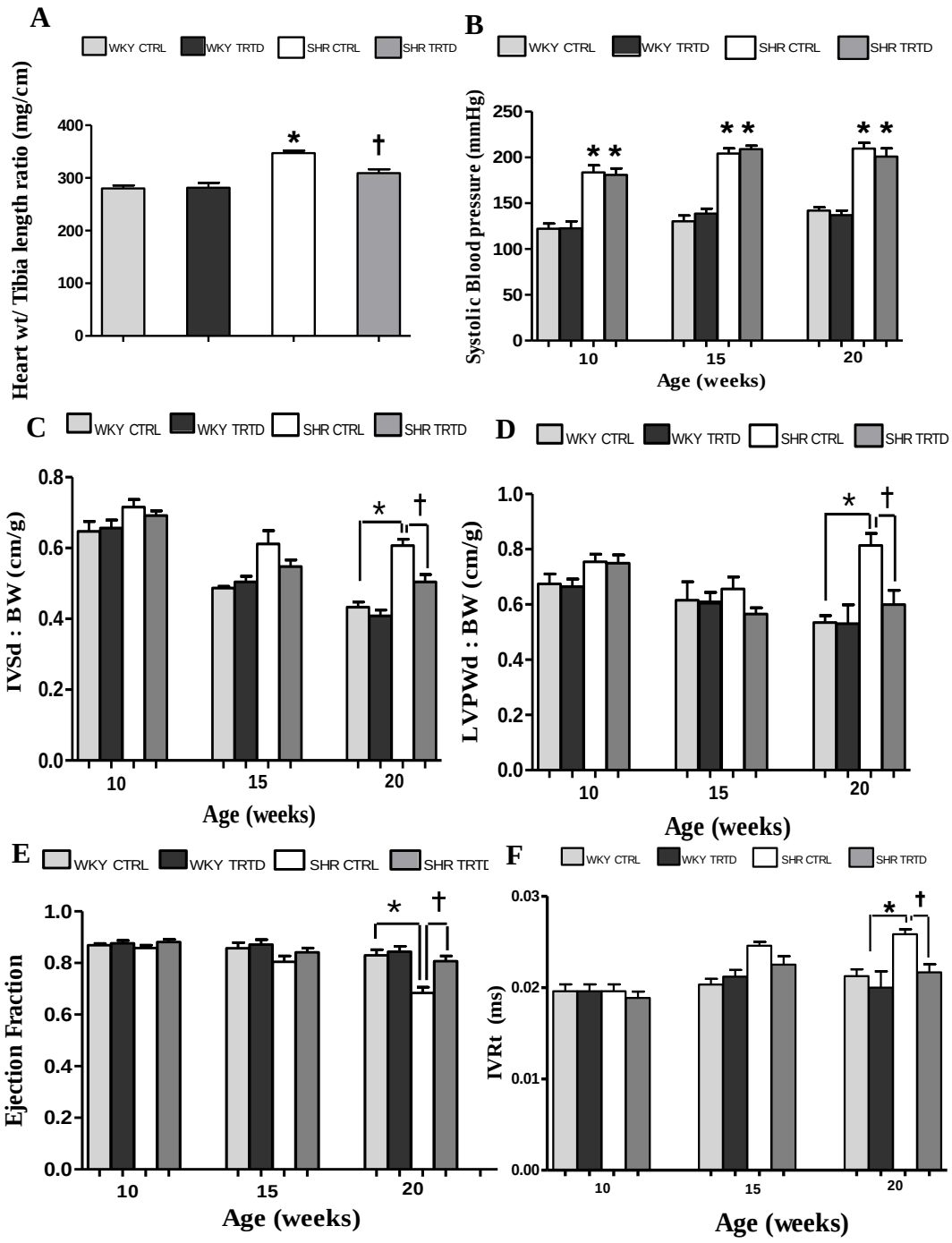

Figure 1. Effect of resveratrol treatment on cardiovascular structure and function. Heart weight/tibia length ratio in 20 week WKY and SHR treated with and without resveratrol (A). Analysis of blood pressure (B), left ventricular posterior wall

thickness at diastole (LVPWd) (C), interventricular septal wall thickness at diastole (IVSd) (D), Left ventricular internal dimension at diastole (LVIDd) (E), ejection fraction (F), cardiac output (CO) (G) and isovolumetric relaxation time (IVRt) (H) in 10, 15 and 20 weeks old WKY and SHR with or without resveratrol treatment. CTRL: control; TRTD: treated. Data are mean ± SE. n = 4-8. * $P < 0.05$ Vs WKY; † $P < 0.05$ Vs. SHR CTRL.

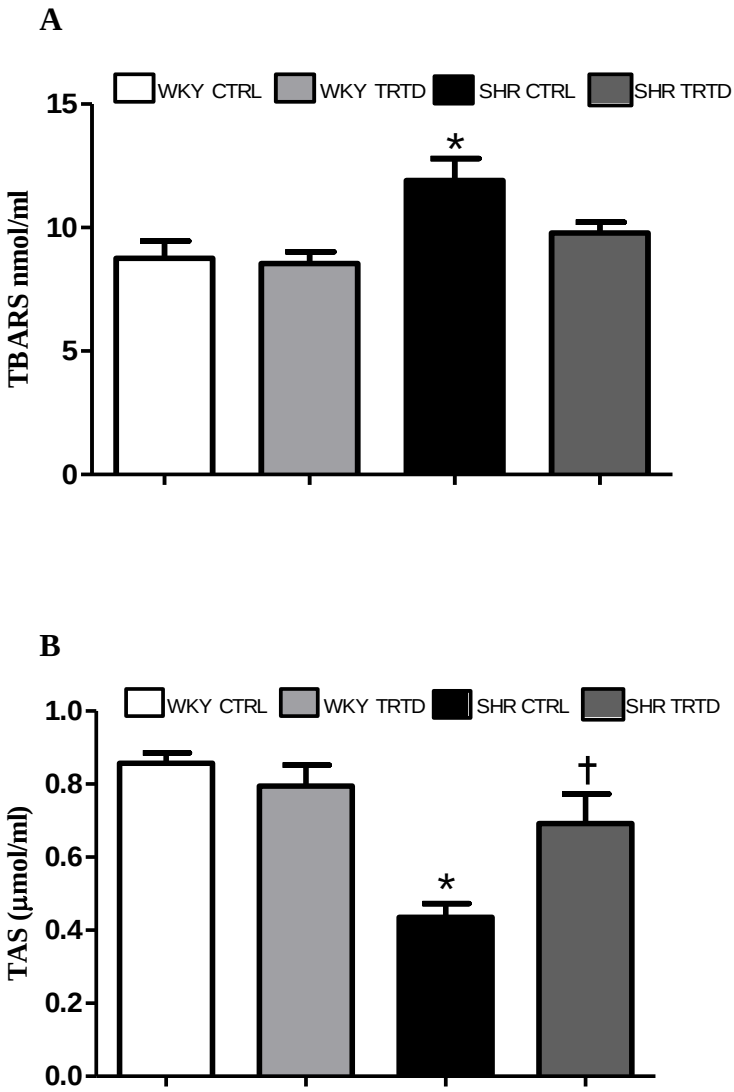

Figure 2. Effect of resveratrol treatment on oxidative stress and anti-oxidant activity. TBARS levels in the heart tissues of 20 week WKY and SHR treated with and without resveratrol (A). TBARS concentration in blood plasma of 20 week WKY and SHR treated with and without resveratrol (B). Total anti-oxidant status (TAS) in blood plasma of 20 week WKY and SHR treated with and without resveratrol (C). CTRL: control; TRTD: treated. Data are mean ± SE. n = 3-5. * $P < 0.05$ Vs WKY; † $P < 0.05$ Vs. SHR CTRL.

3.5 DISCUSSION

Regression of pressure overload induced cardiac hypertrophy and its deleterious consequences on heart function have been reported by us previously in resveratrol treated abdominal aortic banded rats (Juric et al., 2007). Li *et al*. (2005) also reported prevention of cardiac structural and functional alterations by resveratrol treatment in another experimental model of pressure overload created by transverse aortic constriction (Li et al., 2005). Thus, it appears that resveratrol is beneficial in treating pathological pressure overload conditions which include hypertension.

Although the abdominal aortic banded rat and the transverse aortic constricted rat are experimental models of pressure overload, they do not mimic pressure overload induced by essential hypertension. In this study, we used a suitable model of essential hypertension, the SHR which develop hypertension in early stages of life as a consequence of genetic, hemodynamic, vascular, renal and neurohormonal alterations (Trippodo et al., 1981). These rats also develop cardiac hypertrophy gradually in response to the progressive hypertensive disease and not abruptly as a consequence of the surgical procedure. Thus, the occurrence and nature of left ventricular hypertrophy in SHR may resemble development of left ventricular hypertrophy in human, secondary to systemic hypertension (Bing et al., 2002; Doggrell et al., 1998). In the present study, we demonstrate that resveratrol treatment is beneficial in preventing the development of cardiac hypertrophy and cardiac dysfunction due to essential hypertension.

Recently, Dolinsky *et al.* (Dolinsky et al., 2009) reported that short-term (2 week) administration of resveratrol prevented cardiac structural and functional alterations in 14 week SHR. It is very important to point out that Dolinsky *et al.* (Dolinsky et al., 2009) presented data on SHR rats (treated with and without

resveratrol) ONLY, but not on control wistar rats (treated with and without resveratrol), making it impossible to determine the development of cardiac structural and functional alterations in SHR, as well as the effects of resveratrol treatment on these parameters in SHR. Furthermore, there was no significant difference observed (in Dolinsky *et al.*'s study (2009)) in most of the functional parameters (ejection fraction and IVRt) in SHR treated with resveratrol (when compared to untreated SHR). In view of these serious limitations in Dolinsky *et al.*'s study, our study is the first to report that administration of resveratrol prevented development of pathological cardiac hypertrophy and overt cardiac dysfunction in 20 week old SHR.

In the present study, systolic blood pressure was significantly elevated in the SHR group starting from 10 weeks of age. Development of concentric hypertrophy was evident from 15 weeks of age (in SHR), as characterized by increased thickness of the ventricular walls (IVS and LVPW) with no change in chamber dimension (LVID). Systolic and diastolic dysfunctions appeared from 15 weeks of age (in SHR), as characterized by a decline in fractional shortening and an elevation of IVRt, respectively. However, cardiac output was not altered at all time points despite increased blood pressure in SHR; this may be due to the relative increase in arterial peripheral resistance. These results are consistent with the findings of previous studies (Kokubo et al., 2005; Pfeffer et al., 1976) and validate the model used in this study.

In the present study, treatment with resveratrol prevented the development of pressure overload induced cardiac hypertrophy and cardiac dysfunction in SHR without removing the actual stress (hypertension) placed on the heart. The ineffectiveness of resveratrol in lowering blood pressure is consistent with previously reported studies in spontaneously hypertensive rats (Dolinsky et al., 2009; Rush et al., 2007).

In other models of pressure overload such as transverse aortic constriction and partially nephrectomized rats, it has been reported that the anti-hypertrophic effects of resveratrol is partially mediated by a reduction in blood pressure (Li et al., 2005; Liu et al., 2005). One of the reasons for the observed discrepancy of the effects of resveratrol in lowering blood pressure may be the nature of hypertension in these models - local (transverse aortic constriction and abdominal aortic constriction) versus systemic (SHR), as well as, the strong genetic predisposition in the latter group. On the basis of the results obtained in the present study we speculate that resveratrol has a direct anti-hypertrophic effect on the heart. This view is consistent with other reports showing reversal of cardiac hypertrophy in SHR without lowering blood pressure by Matsuoka *et al*. (Matsuoka et al., 1996) and Tsutsui *et al.* (Tsutsui et al., 1999) also suggesting a direct effect of resveratrol on the heart. The underlying mechanisms already reported include a reduction of oxidative stress (Tatlidede et al., 2009), stimulation of nitric oxide (Li et al., 2005) or activation of SIRT1 (Yoshida et al., 2007). It is well established that oxidative stress is one of the main causes of development of pathological cardiac hypertrophy in SHR (Alvarez et al., 2008) In this study we found that resveratrol significantly reduced oxidative stress in SHR; this reduction was consistent with a recovery in the total antioxidant levels in SHR. Thus the alleviation of the oxidative stress may be one of the mechanisms by which resveratrol prevents the development of pathological hypertrophy and cardiac dysfunction in SHR.

In conclusion, we report for the first time that resveratrol is beneficial in preventing the development of concentric hypertrophy and contractile dysfunction in SHR without affecting blood pressure. Thus, resveratrol may have potential in preventing functional damage to the heart caused by essential hypertension.

Accordingly, resveratrol in combination with a blood pressure lowering agent, may be useful in treating patients with essential hypertension.

3.6 ACKNOWLEDGEMENT

Mr. Sijo Joseph Thandapilly and Mr. Xavier Louis are recipients of the International Graduate Student Entrance Scholarship from University of Manitoba. Mr. Peter Wojciechowski is a recipient of the CIHR Graduate Scholarship Master's Award and Manitoba Health Research Council Graduate Fellowship. Dr. Hope Anderson is the recipient of the New Investigator Award from the CIHR, Canada. Dr. Thomas Netticadan was the recipient of the Target Obesity New Investigator Award from Heart and Stroke Foundation of Canada and CIHR. This study was supported by operating grants from Heart and Stroke Foundation and Agriculture and Agri-Food Canada held by Dr. Netticadan.

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Yoshida Y, Shioi T, Izumi T. Resveratrol ameliorates experimental autoimmune myocarditis. *Circ J. 2007*; 71(3):397-404.

OBJECTIVE: 2

To understand the cellular mechanisms underlying the beneficial effects of resveratrol on the heart

RATIONALE

Various *in vitro* and *in vivo* have demonstrated cardioprotection with resveratrol in different models of cardiovascular disease. Some of these studies, including our previous study showed that resveratrol-mediated cardioprotection may be due to a direct action of resveratrol on the heart. Accordingly, it is of great significance to study the effect of this polyphenol on heart muscle cells, cardiomyocytes and the signalling molecules involved in the resveratrol mediated cardioprotection.

Manuscript: 2

Title: Resveratrol prevents norepinephrine induced hypertrophy in adult rat cardiomyocytes, by activating NO-AMPK pathway

Access to the publication online is available via the following link:

http://www.sciencedirect.com/science/article/pii/S0014299911007497

My contributions to the work

I was involved in planning and designing the study. I performed the drug treatments on cardiomyocyte culture. I did the morphometric analysis and other biochemical assays in this study. I performed the western blot analysis of cardiomyocytes and animal heart tissues. Finally, I wrote the manuscript with the help of Dr. Thomas Netticadan and did the necessary revisions for the publication.

CHAPTER 4

Resveratrol prevents norepinephrine induced hypertrophy in adult rat cardiomyocytes, by activating NO-AMPK pathway

4.1 ABSTRACT

Increased adrenergic drive is a major factor influencing the development of pathological cardiac hypertrophy, a stage which precedes overt heart failure. We examined the effect of resveratrol, a polyphenol (found predominantly in grapes), in preventing norepinephrine induced hypertrophy of adult cardiomyocyte, and the role of nitric oxide (NO) and adenosine monophosphate kinase (AMPK) in mediating the effects of resveratrol. Cardiomyocytes isolated from adult rats were pretreated, or not, with resveratrol and then exposed to norepinephrine for 24 hours. In other experiments cardiomyocytes were also treated with different pharmacological inhibitors of NO synthase, AMPK and sirtuin for elucidating the signaling pathways underlying the effect of resveratrol. In order to validate the role of these signaling molecules in the *in vivo* settings, we also examined hearts from resveratrol treated spontaneously hypertensive rats (SHR), a genetic model of essential hypertension. Cardiomyocyte hypertrophy was determined by morphometry and ^{3}H-phenylalanine incorporation assay. NO levels and AMPK activity were measured using a specific assay kit and western blot analysis respectively. *In vitro,* resveratrol prevented the norepinephrine-induced increase in cardiomyocytes size and protein synthesis. Pharmacological inhibition of NO-AMPK signalling effectively abolished the anti-hypertrophic action of resveratrol. Consistent with the *in vitro* findings, the anti-hypertrophic effect of resveratrol in the SHR model was associated with increases in NO and AMPK activity. This study demonstrates that NO-AMPK signaling is linked to the anti-hypertrophic effect of resveratrol in adult cardiomyocytes *in vitro*, and in the SHR model *in vivo*.

4.2 INTRODUCTION

Cardiac hypertrophy is an adaptation of the heart in which the heart muscle cells enlarge in size to adapt to a stress (Hill and Olson, 2008). Activation of the sympathetic nervous system is critical in this adaptation process. It helps the enlarged heart to overcome the stress and maintain normal function. However, chronic activation of the sympathetic nervous system is detrimental and will eventually lead to heart failure. Catecholamines such as norepinephrine (NE) and epinephrine are the major stimulators of sympathetic activity in the heart (Grassi et al., 2009). These molecules bind to α and β-adrenergic receptors. Activation of β-receptors increases cardiac contractility and heart rate thus maintaining cardiac output and systemic blood pressure; whereas binding to α receptors elicits cardiac hypertrophy (Lee and Tkacs, 2008). Elevated plasma levels of norepinephrine are considered to be a clinical marker of heart failure (Tsutamoto et al., 2008).

Heart failure is the number one cause of death worldwide. The morbidity associated with this ailment is an increasing challenge to the economy and health care despite remarkable development in diagnosis and treatment of this disease (Mudd and Kass, 2008; Rosamond et al., 2008). The multifactorial nature of the etiology of heart failure makes it very difficult to develop a highly effective medical intervention. Current pharmacological approaches that counter the progression of diseases (hypertension, ischemic heart disease, valvular heart disease or cardiomyopathies) into heart failure (Tavares et al., 2008; Tunuguntla, 2007) include calcium channel blockers, beta-blockers and renin-angiotensin-aldosterone system inhibitors. Despite impressive gains with existing pharmacological therapies, patients still experience a poor quality of life and suffer unpredictable deleterious side effects (Cheng and Nayar, 2009; Messerli et al., 2009; Nolin and Himmelfarb, 2010). Accordingly, the

need to explore novel strategies remains. Recently, resveratrol (*trans*-3′, 4′, 5-trihydroxystilbene), a polyphenol found predominantly in grapes, has been reported to be cardioprotective in animal models of heart disease (Cucciolla et al., 2007; Leifert and Abeywardena, 2008). In this context we reported that resveratrol attenuates the development of pathological cardiac hypertrophy and contractile dysfunction in two different pressure overload models used in the study (Juric et al., 2007; Thandapilly et al., 2010). Although other investigators have also reported similar findings (Dolinsky et al., 2009; Liu et al., 2005), there is limited information on the mechanisms underlying the cardioprotective effects of resveratrol in pressure overload models; furthermore all previous studies utilized neonatal cardiomyocytes.

It has been reported that resveratrol directly induces NO production in human endotheial cells (Wallerath et al., 2002; Klinge et. al., 2008). In addition, resveratrol was also shown to restore the eNOS expression and NO availability in the hypertrophied as well as diabetic myocardium in rats (Liu et al., 2005; Zhang et al., 2010) indicating the potential of resveratrol in activating NOS and enhancing NO production. It has also been shown that resveratrol directly activates AMPK via phosphorylation at threonine residue in neuronal cells (Dasgupta et al., 2007). Moreover, resveratrol also prevented cardiomyocyte hypertrophy by restoring the impaired AMPK activity in phenyleprhine exposed cardiomyocytes as well as in SHR rats (Chan et al., 2008; Dolinsky et al., 2009), suggesting an important role for AMPK in mediating the effects of resveratrol. Sirtuins (silent information regulator 2 (Sir2) family deacetylase) have also been widely studied as a potential target of resveratrol. Earlier, resveratol was shown to directly activate sirtuins in a substrate specific manner (Keberlein et al., 2005). In addition, resveratrol was able to restore the activity of sirtuin, and thereby improve cardiac function in rats with diabetic

cardiomyopathy (Sulaiman et al., 2010). Breen et al 2010, studied the interaction between AMPK and sirtuin in resveratrol mediated signaling in skeletal muscle cells. In this study (Breen et al., 2010) increased skeletal muscle glucose uptake was observed upon resveratrol treatment which was mediated by the sirtuin-AMPK dependent pathway In the present study, we decided to examine the effects of resveratrol on neorepinephrine induced hypertrophy in adult cardiomyocytes. In particular, the role of NO, sirtuins, and AMPK was examined in resveratrol mediated anti-hypertrophic effect by using pharmacological inhibitors of these molecules. The results obtained from *in vtro* experiments were further validated *in vivo* by studying heart tissues obtained from resveratrol treated spontaneously hypertensive rat (SHR), an established model of essential hypertension.

4.3 MATERIALS AND METHODS

4.3.1 Animal ethics

All experimental protocols used in this study were approved by the University of Manitoba Animal Care Committee and are in agreement with the *Canadian Council on Animal Care and Use of Experimental Animals* (vol. 1, 2nd ed., 1993).

4.3.2 Chemicals and reagents

All chemicals used in this study were purchased from Sigma-Aldrich, Ontario, Canada.

4.3. 3 Adult cardiomyocyte isolation and culture

Ventricular myocytes were isolated from 12 week old male Sprague Dawley rats (200-250 g) as described previously (Netticadan et al., 1999). In brief, hearts were excised from anesthetized animals, transferred to a Langendorf apparatus and perfused with Ca^{2+} free buffer containing (in mM); 90 NaCl, 10 KCl, 1.2 KH_2PO_4, 5 $MgSO_4.7H_2O$, 15 $NaHCO_3$, 30 taurine and 20 glucose for 5 min. The perfusion

medium was then switched to a Ca^{2+} free buffer containing collagenase (0.05%) and bovine serum albumin (0.2%). After 30 min, the perfused heart was taken off the Langendorff apparatus; ventricles were cut into small pieces, incubated in a 37^{o}C waterbath and separated into individual cardiomyocytes by slow pipetting. Cardiomyocytes were then suspended in a buffer containing 200 µmol Ca^{2+} and the cells were allowed to settle. The supernatant buffer was then replaced stepwise with Ca^{2+} buffers containing higher concentration of calcium. This step was repeated thrice to increase the extracellular Ca^{2+} concentration from 0.5 mM to 1 mM and then to 1.5 mM. Cells were finally resuspended in Medium-199 containing 10% fetal bovine serum and transferred to laminin coated culture dishes. After 2 hours of incubation in a CO_2 incubator, the existing medium was replaced with serum free Medium-199 supplemented with 5 mM taurine, 2 mM carnitine, 1 mM creatine and 1 µmol insulin. All cells were then incubated for 24 hours in a CO_2 incubator (5% CO_2 and 95% air) at 37^{o}C before starting any experimental procedures.

4.3.4 Cell treatments

In one set of experiments, cardiomyocytes were stimulated with different concentrations of NE (0.1 to 1 µmol) for 24 hours; unstimulated cardiomyocytes served as controls. In the second set of experiments, cardiomyocytes were pre-treated with different doses of resveratrol (15, 30 and 60 µmol) for 30 min and then co-incubated with NE (0.25 µmol) for 24 hours. In the third set of experiments cardiomyocytes were pre-treated with different concentrations (10, 100 and 250 µmol) of L-NAME, an inhibitor of NO synthase, for 1 hour. Resveratrol (30 µmol) was added to the medium and incubated for another 30 min. The cells were then exposed to NE (0.25 µmol) for 24 hours and used for further examinations. In the fourth set of experiments cardiomyocytes were either treated with 1 µmol

dorsomorphin (a specific inhibitor of AMPK) or with 40 mM nicotinamide (a sirtuin inhibitor) 1 hour prior to the addition of resveratrol. The cells were then exposed with NE (0.25 μmol) for 24 hours and used for further experiments. In the last set of experiments cardiomyocytes were pretreated with a NO donor S-nitroso-Nacetylpenicillamine (SNAP) (100 μM) for 30 minutes. The cells were then exposed with NE (0.25 μmol) for 24 hours and used for for morphometric analysis. The effective concentrations of dorsomorphin, nicotinamide and SNAP were taken from the previous studies carried out on cardiomyocytes (Chen et al., 2009; Florian et al., 2010; Hunter et al., 2009).

4.3.5 Measurement of cardiomyocyte surface area

Phase contrast images of cardiomyocytes were randomly taken from four different fields of the culture dish using a Zeiss LSM 5 Pascal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). The surface area of individual cardiomyocytes was measured in a double blinded fashion using ImageJ software. A total of 100 cells from 3 different animal isolations were used for each analysis.

4.3.6 Measurement of cardiomyocyte protein synthesis

Protein synthesis was determined by measuring as the incorporation of ^{3}H-phenylalanine (Amersham Biosciences, NJ, USA) into the adult cardiomyocytes as previously described (Louis et al., 2009). Cardiomyocytes were treated with resveratrol for 30 min before addition of 0.5 μCi/mL ^{3}H-phenylalanine and 0.3 mM non-radioactive phenylalanine and further stimulated with NE for 1 hour. After incubation, the medium was removed and wells were washed with ice-cold 1X phosphate buffered saline (PBS). One mL ice-cold 10% trichloroacetic acid (TCA) was added and incubated at 4^{o}C for overnight. TCA was removed and rinsed again with 1X PBS. One mL 0.1 M NaOH/0.01% SDS was added and incubated overnight

at 37^{o}C to neutralize the reaction. Five thousand µL from each well was transferred to a scintillation vial and radioactivity was determined by liquid scintillation counting in 3 mL Ecolume. All readings were normalized to the DNA concentration which was quantified using Hoechst 33342 stain (Sigma-Aldrich, ON, Canada).

4.3.7 Immunostaining

After treatments, cells were washed with phosphate buffered saline (PBS) and fixed with 4% formaldehyde. Triton X-100 permeabilized cells were incubated with anti-α-actinin antibody. Cells were then incubated with Alexa Fluor goat anti mouse secondary antibody and viewed under a fluorescent microscope.

4.3.8 Animal model

Ten-week old male SHR and their control Wistar–Kyoto (WKY) rats (Charles River, St Constant, Quebec, Canada) were used in this study. Animals were acclimatized in temperature and humidity-controlled rooms with a 12-h dark and 12-h light period cycle throughout the study.

4.3.9 Treatment regime

Ten-week old SHR and WKY were treated daily by oral gavage with resveratrol (2.5 mg/kg/day), a dose we have previously established as being effective (Thandapilly et al., 2010) in 50% ethanol as vehicle for a period of 10 weeks. Control groups received 1 mL of 50% ethanol for the same period of time.

4.3.10 Tissue collection

At the end of the study, all rats were anesthetized by ketamine/xylazine injection. The heart tissues were removed, weighed, flash frozen in liquid nitrogen and stored at -80^{o}C for further analyses. Blood samples were collected, centrifuged at 2500 rpm for 20 min to obtain serum and plasma which were stored at -80^{o}C for further analyses.

4.3.11 Nitric oxide (NO) assay

Nitrate (NO_3^-) + nitrite (NO_2^-) were measured in the cardiomyocyte culture medium as well as in the blood plasma from the SHR and WKY rats treated with and without resveratrol using the Cayman Chemical Nitrate/Nitrite Colorimetric Assay kit (Cayman Chemical Co., Ann Arbor, MI). The samples were analyzed according to the manufactures instructions. The samples were thawed and filtered through a 30 kDa molecular weight cut-off filter (Millipore Corporation, Bedford, MA), which were pre-rinsed with ultra pure water prior to the filtration process and centrifuged to remove any background absorbance. The assays were analyzed in a 96-well plate at a wavelength of 540 nm. A nitrite standard curve was generated in each plate to determine total nitrate/nitrite (μmol).

4.3.12 Western blot analysis

AMPK activity was measured in cardiomyocytes as well as in heart tissues by assessing phosphorylation of AMPK at its activation site, threonine 172 (T172) (phosphorylated AMPK (P-AMPK)) by using western blot analysis as described previously (Juric et al., 2007). Protein samples (20–25 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane, which were probed with either rabbit monoclonal anti-AMPK (1: 1,000 dilution) or rabbit monoclonal anti-P-AMPK (1: 1,000 dilution) purchased from Cell Signaling Technology (Danvers, MA). Appropriate secondary antibodies were used, and the antibody-antigen complexes in all membranes were detected by the ECL PLUS kit (Amersham Life Science, Oakville, ON, Canada). An Imaging Densitometer model GS-800 (Bio-Rad, Hercules, CA) was used to scan the protein bands and they were quantified using the Quantity One 4.4.0 software from Bio-Rad.

4.3.13 Statistical analysis

Data were expressed as mean + standard error (SEM). Statistical analysis of data was performed by applying one-way analysis of variance (ANOVA) followed by a post-hoc test. P value <0.05 was considered statistically significant.

4.4 RESULTS

4.4.1 Effect of NE on adult rat cardiomyocyte morphology

Incubation of adult rat cardiomyocytes for 24 hours with different concentrations of NE induced a significant (P<0.05) dose-dependent increase in cell surface area as compared to unstimulated control cardiomyocytes **(Fig. 1A)**. The minimum dose required to elicit a maximal response (0.25 µmol) was used for all subsequent experiments.

4.4.2 Effect of resveratrol on NE induced increase in cardiomyocyte size

Pre-treatment with different concentrations of resveratrol for 30 minutes prevented the increase in surface area induced by NE (in comparison to untreated control cardiomyocytes stimulated with NE), **(Fig. 1B)**; with the effects of 30 and 60 µmol resveratrol concentrations being statistically significant (p<0.05). The effects of 30 and 60 µmol resveratrol were comparable and therefore 30 µmol resveratrol was used in subsequent experiments. Resveratrol treatment (30 µmol) alone did not have any effect on size of control cardiomyocytes **(Fig. 1B)**. **Fig. 1C** shows representative anti-actinin immunostained (immunofluorescenec) cardiomyocytes images showing that 30 µmol resveratrol prevent the increase in cardiomyocyte surface area induced by NE.

4.4.3 Effect of resveratrol on NE induced increase in protein synthesis

Exposure of cardiomyocytes with 0.25 µmol of NE induced a significant (P<0.05) increase in protein synthesis in comparison with unstimulated control cardiomyocytes **(Fig. 1D)**. Pre-treatment of cardiomyocytes with 30 µmol resveratrol significantly

($p<0.05$) prevented the increase in protein synthesis (in comparison to untreated control cardiomyocytes stimulated with NE). Unstimulated control cardiomyocytes showed no change in protein synthesis when treated with 30 µmol resveratrol **(Fig. 1D)**.

4.4.4 Effect of L-NAME, dorsomorphin and nicotinamide on resveratrol action in NE stimulated cardiomyocytes

Pre-treatment with L-NAME abolished the effects of resveratrol on NE stimulated cardiomyocytes; the observed effect of L-NAME was concentration-dependent **(Fig. 2A)**. L-NAME at concentrations of 100 µmol or higher significantly inhibited the resveratrol mediated prevention of cardiomyocyte surface area increase induced by NE. As observed with L-NAME, pre-treatment with 1 µmol dorsomorphin also completely abolished the anti-hypertrophic effect of resveratrol on NE stimulated cardiomyocytes **(Fig. 2B)**. On the other hand, pre-treatment with a sirtuin inhibitor nicotinamide (40mM) had no effect on the anti-hypertrophic action of resveratrol in NE stimulated cells **(Fig. 2B)**. L-NAME, dorsomorphin and nicotinamide did not have any effect on control cardiomyocytes **(Fig. 2B)**.

4.4.5 Effect of NO donor SNAP on NE induced cardiomyocyte hypertrophy

Pre-treatment with the NO donor SNAP prevented the increase in surface area induced by NE (in comparison to untreated control cardiomyocytes stimulated with NE), **(Table. 1).** SNAP treatment (100 µmol) alone did not affect the size of control cardiomyocytes (**Supplementary figure:2**)

4.4.6 Effect of resveratrol on cardiac hypertrophy in SHR

There was a significant increase in the heart-to-body weight ratio in the 20 week-old SHR in comparison to their age matched WKY controls; this increase was significantly prevented after 10 weeks of resveratrol treatment **(Supplementary**

Fig.1). Treatment with resveratrol did not affect the heart to body weight ratio of 20 week old WKY rats.

4.4.7 Effect of resveratrol on nitric oxide production

Nitrate/nitrite levels were downregulated in cardiomyocyte culture medium after 24 hours of NE treatment, and resveratrol treatment significantly restored NO production in cardiomyocytes **(Fig. 3A)**. Dorsomorphin treatment did not affect the NO levels in resveratrol treated cardiomyocytes, whereas L-NAME treatment prevented the increase NO production in resveratrol treated as well as control cardiomyocytes **(Fig. 3A)**. NO levels were also reduced in plasma from SHR when compared to their normotensive counterparts WKY rats. Ten weeks of resveratrol treatment significantly improved the NO levels in plasma of SHR **(Fig. 3B)**.

4.4.8 Effect of resveratrol on AMPK activity

AMPK activity was significantly reduced in NE exposed cardiomyocytes as assessed by P-AMPK; resveratrol treatment was able to restore AMPK activity of cardiomyocytes exposed to NE for 2 hours **(Fig. 4A).** Dorsomorphin and L-NAME pre-treatment downregulated AMPK activity both in resveratrol treated as well as in control cardiomyocytes **(Fig. 4A).** AMPK activity was also significantly reduced in 20 week old SHR when compared to control WKY rats. Interestingly, P-AMPK was significantly increased in the hearts from resveratrol treated SHRs when compared to untreated SHRs **(Fig. 4B)**.

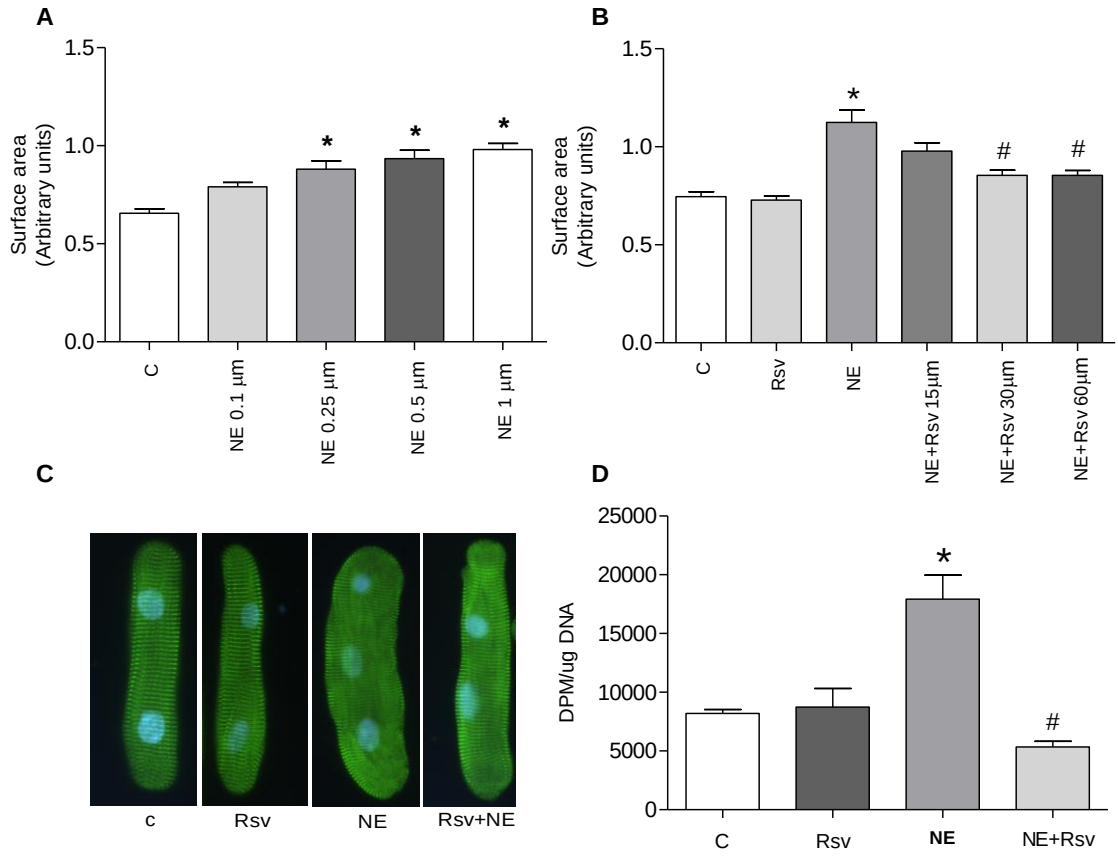


Figure 1: Effect of resveratrol on NE induced adult cardiomyocyte hypertrophy. **A.** Adult rat cardiomyocytes treated with different doses of norepinephrine have increased cell surface area (n=100 cells). Data are mean ± s.e. **P* < 0.05 vs. Control. **B.** Data showing resveratrol pre-treatment prevents NE induced increase in cell surface area. Data are mean ± s.e. *n* = 3. * P<0.05 vs control, $^{\#}$P<0.05 vs NE. **C.** Immunostaining of cardiomyocytes treated with and without resveratrol and NE. **D.** [^{3}H] phenylalanine incorporation assay data showing resveratrol (30 µmol) pre-treatment prevents NE (0.25µmol) induced increase in protein synthesis. Data are mean ± s.e. *n* = 3–5.*P<0.05 control vs NE, #P<0.05 NE vs NE + Rsv. C, control; NE, norepinephrine; Rsv, resveratrol. DPM, Disintegrations per minute.

Figure 2: Effect of different pharmacological inhibitors on anti-hypertrophic effect of resveratrol A. Data showing dose dependant inhibitory effect of L-NAME on resveratrol action on cardiomyocytes stimulated with NE (n=100 cells). Data are mean ± s.e. *P<0.05 control vs NE, #p<0.05 NE vs NE +Rsv, $^{\Phi}$P<0.05 NE+Rsv vs L –NAME 100µmol+Rsv+NE. **B.** Data showing the effect of L-NAME, dorsomorphin and nicotinamide on resveratrol action on cardiomyocytes stimulated with NE. Data are mean ± s.e. *n* = 3–5. *P<0.05 vs control, $^{\#}$P<0.05 vs NE, $^{\Phi}$P<0.05 control vs NE+Rsv; C, control; Rsv, resveratrol; NE, norepinephrine; L-NAME, NG-nitro-*L*-arginine methyl ester.

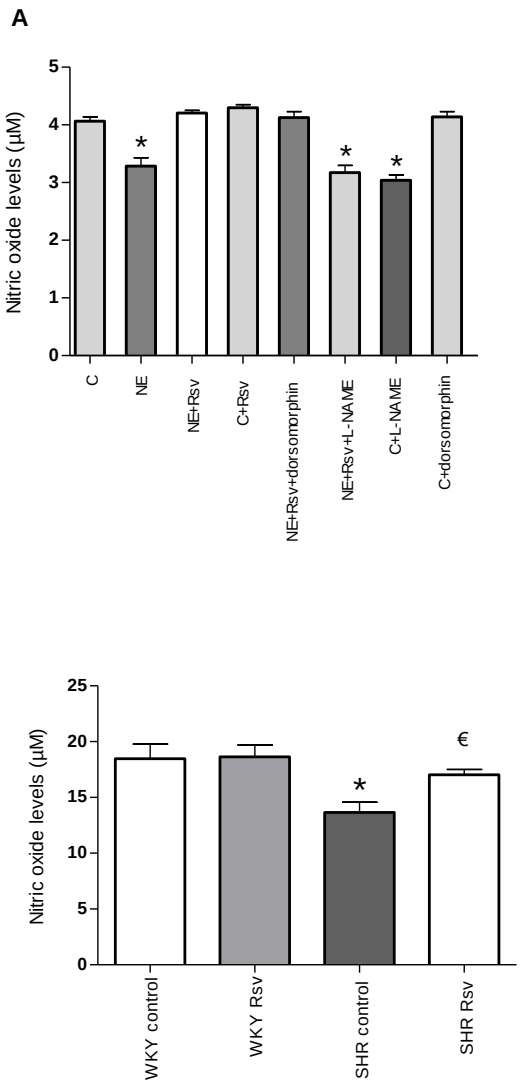


Figure 3: Effect of resveratrol on nitric oxide production. A. Data showing the effect of NE, resveratrol, L-NAME and dorsomorphin on nitric oxide production in adult cardiomyocytes. Data are mean ± s.e (n=3-4). *P<0.05 vs control; B. Effect of resveratrol treatment on nitric oxide levels in plasma of 20-week old WKY and SHR treated with and without resveratrol. Data are mean ± s.e. n = 3–5. *P < 0.05 vs. WKY; $^{€}P$ < 0.05 vs. SHR control; SHR, spontaneously hypertensive rat; WKY, Wistar–Kyoto. Rsv, resveratrol; NE, norepinephrine; L-NAME, NG-nitro-*L*-arginine methyl ester.

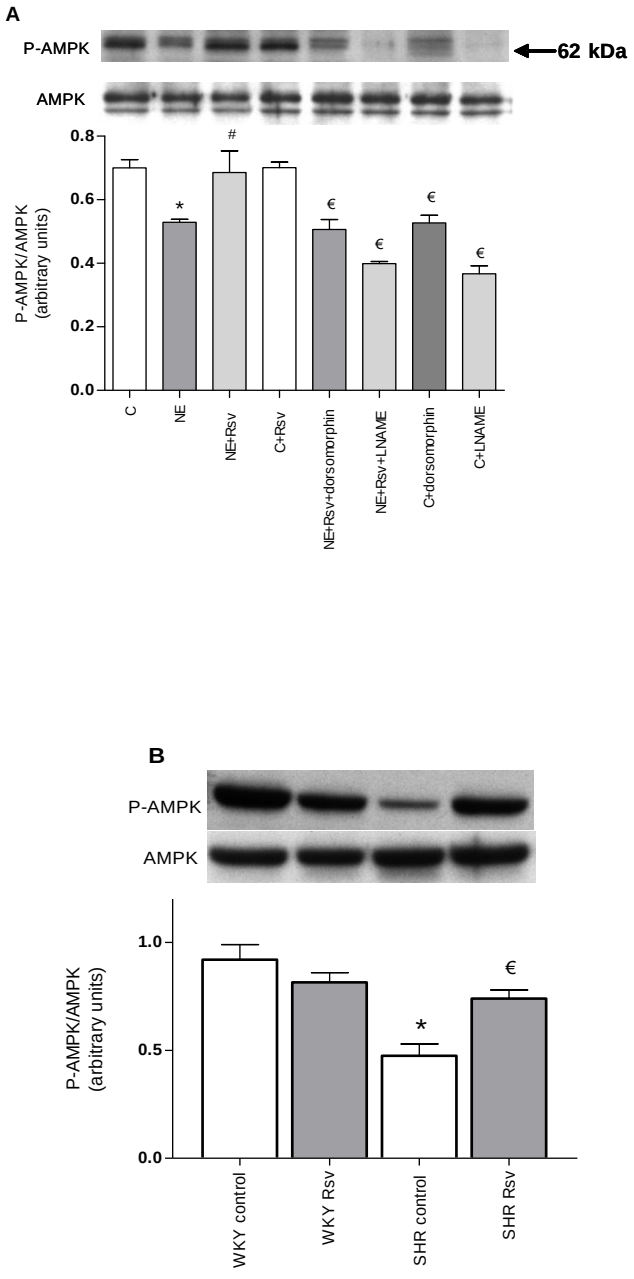
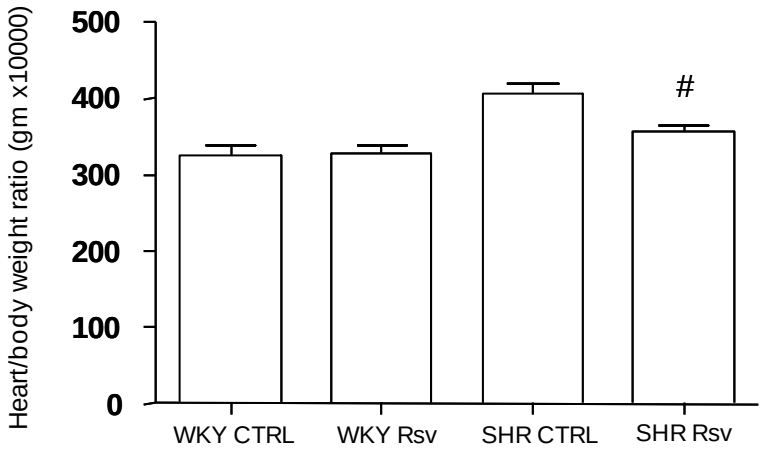
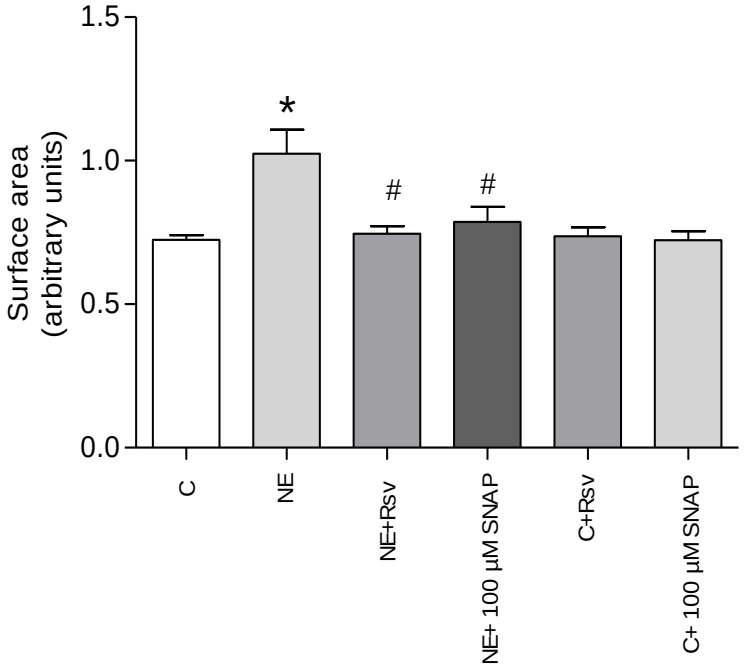


Figure 4: Effect of resveratrol on AMPK activity. A. Data showing the effect of resveratrol, L-NAME and dorsomorphin on P-AMPK/AMPK ratio in adult cardiomyocytes stimulated with NE (n=20-30 cells). Data are mean ± s.e. *n* = 3–5. *P<0.05 vs control, $^{\#}$P<0.05 vs NE, $^{€}$P<0.05 vs NE+Rsv, $^{£}$P<0.05 vs all other groups **B.** Analysis of cardiac AMPK expression in 20 -week-old SHRs and WKY rats

treated with and without resveratrol. Data are mean ± s.e. n = 3–5. *P < 0.05 vs. WKY; $^{€}P$ < 0.05 vs. SHR control. Immunoblot analysis was performed on homogenates from ventricles and levels of P-AMPK were quantified by densitometry and normalized against total AMPK to determine AMPK activity. C, control; Rsv, resveratrol; NE, norepinephrine; L-NAME, NG-nitro-*L*-arginine methyl ester; AMPK, adenosine monophosphate kinase; P-AMPK, phosphorylated AMPK.



Supplementary Figure 1: Effect of resveratrol on cardiac hypertrophy. Heart/body weight ratio in 20-week WKY and SHR treated with and without resveratrol. CTRL, control; SHR, spontaneously hypertensive rat; WKY, Wistar–Kyoto; Rsv, Resveratrol. Data are mean ± S.E.M. n = 4–8. * P < 0.05 vs. WKY; $^{\#}P$ < 0.05 vs. SHR CTRL



Supplementary Figure 2: Effect of NO donor SNAP on NE induced cardiomyocyte hypertrophy. Data showing the anti hypertrophic effect of SNAP on cardiomyocytes stimulated with NE (n=100 cells). Data are mean ± S.E.M. *n* = 3–4. *P<0.05 vs control, $^{\#}$P<0.05 vs NE. C, control; Rsv, resveratrol; NE, norepinephrine; SNAP, S-nitroso-Nacetylpenicillamine.

4.5 DISCUSSION

Several studies have demonstrated cardioprotection with resveratrol in different *in vivo* models of heart disease (Dolinsky et al., 2009; Juric et al., 2007; Liu et al., 2005; Thandapilly et al., 2010). Some of these studies (Dolinsky et al., 2009; Thandapilly et al., 2010) indicate that resveratrol-mediated cardioprotection may be due to a direct action of resveratrol on the heart. Thus, it is important to explore the effect of this polyphenol on heart muscle cells. In this regard, a few groups have examined the mechanisms underlying the effect of resveratrol on neonatal cardiomyocytes subjected to different stresses (Chan et al., 2008; Cheng et al., 2004). Earlier Cheng *et al.* (Cheng et al., 2004) reported that resveratrol inhibits angiotensin II-induced neonatal cardiomyocyte hypertrophy by attenuating reactive oxygen species generation. In another study using neonatal cardiomyocytes, Anita *et al.* showed that resveratrol inhibits phenylephrine (PE) induced hypertrophy (Chan et al., 2008). Although neonatal cardiomyocytes have been widely used to study heart disease at the molecular level, they have significant limitations despite being the best model to study cardiac abnormalities in the newborn. The majority of the cardiac problems are associated with age and emerge in the later stages of life (Fares and Howlett, 2010). In addition, it has been reported that there are differences in intra-cellular signaling modulated by hypertrophic stimuli between the neonatal and adult cardiomyocytes (Schluter et al., 1999). Therefore, it is more appropriate to use adult cardiomyocytes rather than neonatal cardiomyocytes to study the pathology of an adult failing heart. Furthermore, no study has examined the effects of resveratrol on adult cardiac myocytes subjected to stress.

The presence of high levels of NE has been considered as a pathological marker of heart failure (Tavares et al., 2008). Accordingly, we explored the

cardioprotective role of resveratrol in adult cardiomyocytes exposed to high levels of NE. It is proposed that NE binds with the alpha adrenergic receptor on the cardiac cell membrane, the sarcolemma, and activates phospholipase C resulting in the formation of 1,2-diacylglycerol (DAG) and inositol triphosphate (IP3). In turn,DAG stimulates cytosolic protein kinase activity resulting in increased protein synthesis leading to the development of cardiac hypertrophy (Hefti et al., 1997; Jalili et al., 1999; Eskildsen-Helmond et al., 1997) We and others (Dolinsky et al., 2009; Thandapilly et al., 2010) have recently reported that resveratrol prevented the development of pathological cardiac hypertrophy in genetically hypertensive rats without any effect on blood pressure, which is considered as the pathological stimulus for the development of hypertrophy (Thandapilly et al., 2010). This observation suggested a direct action of resveratrol on the heart rather than on the vasculature. These interesting *in vivo* results directed our focus to examine the effect of resveratrol on cardiomyocytes which are hypertrophied when exposed to high levels of NE.

In this study we show that resveratrol prevented the development of NE induced hypertrophy in adult cardiomyocytes. The inhibition of NO synthase and AMPK by L-NAME and dorsomorphin completely abolished the anti-hypertrophic action of resveratrol, suggesting that these molecules are involved in the anti-hypertrophic action of resveratrol. It must be noted that L-NAME and dorsomorphin did not induce hypertrophy in the control cardiomyocyte. This may be because inhibition of NO and/or AMPK alone is not sufficient to trigger the alpha/beta adrenergic receptor mediated hypertrophic signaling. Despite evidence suggesting the involvement of sirtuins in resveratrol mediated cardioprotection (Chen et al., 2009; Sulaiman et al., 2010), our results showed that the anti-hypertrophic effects of resveratrol were not mediated by sirtuin activation. This discrepancy in the results

may be due to either the cell type (neonatal vs. adult cardiomyocytes), species (mice vs. rat), or the stimulus used to induce hypertrophy (PE vs. NE). The lack of effect with a sirtuin inhibitor on this resveratrol-mediated effect was consistent with a previous study where the resveratrol mediated neuroprotection was not abolished by the sirtuin inhibitor, nicotinamide. (Okawara et al., 2007))

In this study we also found that blocking NO production with the blocker L-NAME significantly prevented the resveratrol induced activation of AMPK in cardiomyocytes. In order to verify whether the action of resveratrol could be mimicked with a NO donor, cardiomyocytes were pre-treated with NO donor SNAP before exposing it to NE. Interestingly, we observed a resveratrol mimicking anti-hypertrophic effect with the NO donor, SNAP. In addition, the AMPK inhibitor dorsomorphin did not block resveratrol-mediated NO production suggesting that NO acts as an upstream molecule to AMPK in the anti-hypertrophic signaling pathway of resveratrol.

Consistent with our *in vitro* results, we observed that treating hypertensive rats with resveratrol for 10 weeks restored the impaired NO-AMPK signaling as evident from the improved NO levels in the plasma as well as the increased AMPK activity in the heart tissues from SHR. These *in vivo* results validate the role of NO-AMPK signaling observed in our *in vitro* studies.

Although the anti-hypertrophic effect of both molecules (NO and AMPK) is established (Ruiz-Hurtado., 2010; Meng., 2009), no study had examined the role of NO-AMPK signaling axis in the anti-hypertrophic effects of resveratrol. In view of the limitations of studying the effect of resveratrol on hypertrophy in neonatal cardiomyocytes, this study is the first to report the mechanism underlying the anti-hypertrophic effects of resveratrol using adult cardiomyocytes. Along with the recent

evidence that NO acts as an endogenous AMPK activator in vascular endothelial cells (Zhang et al., 2008), our data in adult cardiomyocytes and in SHR indicate that the effect of resveratrol in preventing cardiac hypertrophy is through the activation of NO-AMPK signaling, and not via the sirtuin pathway.

Oxidative stress is considered to be a major cause for the development of pathological cardiac hypertrophy (Takimoto et al., 2007; Alvarez et al., 2008), and it has been well-documented that resveratrol has strong antioxidant properties (Ozkan et al., 2009). Accordingly, it is important to propose that the anti-hypertrophic effect of resveratrol might be due to the reduction of oxidative stress via the activation of anti oxidant systems as previously shown by us and other groups in animal models of cardiac hypertrophy as well as in hypertrophic cardiomyocytes treated with resveratrol (Thandapilly et al., 2010; Wojciechowski et al., 2010; Li et al., 2005). Furthermore, it has been reported that oxidative stress affects NO availability by depleting the free NO by the formation of peroxynitirite ion, and also by directly inactivating endothelial nitric oxide synthase (eNOS) activity (Kline et al., 2008; Tang et al., 2009). It is also well documented that NE causes oxidative stress in cardiomyocytes (Qin et al., 2001; Liu et al., 2004), and in the present study we have showed that exposure to it (NE), results in a downregulation of basal levels of NO. In view of the above facts, we speculate that resveratrol may prevent the NE induced NO depletion and eNOS inactivation by reducing oxidative stress.

In summary, our results show that exposure of cardiomyocytes to NE induces cardiomyocyte hypertrophy, which is prevented by treatment with resveratrol. Our results also show that the mechanism by which resveratrol acts could be by preventing the impairment of endogenous NO production in cardiomyocytes. NO in turn activates AMPK via phosphorylation of AMPK at Thr-172. Activation of AMPK may

be through $Ca2^{+}$ dependent, guanylyl cyclase-mediated pathway (Zhang et al., 2008). Since AMPK is known to increase the release of NO through eNOS phosphorylation, activation of AMPK by NO may stimulate the positive feed back loop to further induce NO production in cardiomyocytes thus resulting in additional anti-hypertrophic effect observed in resveratrol treated cardiomyocytes. We also further speculate that inhibition of Glycogen synthase kinase 3 beta (GSK-3β) may be one of the downstream effectors of NO-AMPK signaling as it has been reported that resveratrol mediated GSK-3β inhibition conferred protection against oxidative stress in heptocytes through the inhibitory phosphorylation of GSK-3β mediated by AMPK (Shin et al., 2009), as well as it protected the rat hearts from ischemic reperfusion injury by inhibiting the activity of GSK-3β (Xi et al., 2009) .

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OBJECTIVE: 3

To examine whether resveratrol alone or in combination with a blood pressure lowering agent can reverse cardiovascular abnormalities spontaneously hypertensive rat (SHR) in advanced hypertension.

RATIONALE

Although we showed in the previous study that resveratrol treatment prevented the development of pathological cardiac hypertrophy and cardiac dysfunction in SHR, it did not lower the blood pressure. In view of this finding, reducing hemodynamic load with an established blood pressure lowering agent might be more beneficial in reversing the cardiovascular abnormalities in SHR in advanced hypertension by enhancing the efficacy of resveratrol.

Manuscript: 3

Title: Resveratrol in combination with hydralazine reverses cardiovascular dysfunction in hypertensive rat

My contributions to the work

I was involved in planning and designing the study. I conducted the animal study which includes weighing the animals, preparing resveratrol solutions, gavaging etc. I performed the blood pressure measurements and echocardiographic analysis on rats. I analyzed the blood pressure and echocardiographic data. I also did TBARS and interleukins assays on animal tissues. Finally, I wrote the manuscript with the help of Dr. Thomas Netticadan and did the necessary revisions for the publication.

CHAPTER 5

Resveratrol in combination with hydralazine reverses cardiovascular dysfunction in hypertensive rat

5.1 ABSTRACT

Cardiac hypertrophy and associated myocardial remodelling is one of the main complications of hypertension resulting in the development of heart failure. It is of great significance to investigate the potential of novel treatments which can revert cardiac hypertrophy in hypertensives with or without affecting blood pressure. In this context, we have earlier reported that reveratrol was beneficial in preventing cardiac hypertrophy and cardiac dysfunction in different animal models of hypertension without lowering blood pressure. In the present study, we investigated whether resveratrol alone or in a combination with a widely used blood pressure lowering agent will be beneficial in reversing hypertension-induced cardiac hypertrophy and contractile dysfunction. For this purpose, twenty week old male spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats were treated with resveratrol (2.5 mg/kg/day) and or hydralazine (25 mg/kg/day) for eight weeks. Systolic and diastolic blood pressure, cardiac structure and function, electrocardiogram measurements were carried out in all groups at different time points. Pressure myography of resistance arteries, histological examinations, oxidative stress, inflammatory measurements were also preformed to assess the efficacy of the treatment. SHR were characterized with high blood pressure and concentric hypertrophy and diastolic dysfunction at 20 weeks of age. Resveratrol treatment alone was ineffective in reducing systolic blood pressure, diastolic blood pressure and diastolic dysfunction in SHR. However resveratrol treatment alone significantly reduced the systolic impairment as well as myocardial fibrosis in SHR. Combination therapy of resveratrol in conjunction with hydralazine significantly reduced blood pressure, improved systolic and diastolic function, reduced fibrosis and improved vascular geometry. Resveratrol alone or in combnation with hydralazine significantly reduced the oxidative stress and

inflammation in SHR. In summary, resveratrol itself was able to reverse cardiac fibrosis and some of the other functional abnormalities in 28 week old SHR. However, resveratrol in combination with hydralazine was more effective in improving cardiovascular parameters when compared to either resveratrol or hydralazine alone.

5.2 INTRODUCTION

Despite advances in the management and treatment of high blood pressure, cardiovascular complications and associated mortality due to hypertension continue to be on the rise (Roger et al., 2010). Cardiac hypertrophy is one of the main complications of hypertension resulting in the development of heart failure (Purushothaman et al.) Considering the fact that lowering hemodynamic load (blood pressure) is an important factor, but not the sole factor for the treatment of cardiac hypertrophy (Sen et al., 1977), it is of great importance to explore the potential of novel treatments which can regress cardiac hypertrophy in hypertensives with or without affecting blood pressure.

Resveratrol, a polyphenol found predominantly in grapes, has been reported to render strong cardioprotection against various diseases such as obesity, diabetes, hypertension, and ischemic heart disease (Smoliga et al.; Wu et al., 2011; Yu et al., 2012). We (Juric et al., 2007; Thandapilly et al., 2010; Wojciechowski et al., 2010) and others (Dolinsky et al., 2009; Li et al., 2005; Liu et al., 2005)) have earlier reported that reveratrol was beneficial in preventing cardiac hypertrophy and contractile dysfunction in different models of hypertension. However, in all the studies mentioned above, the cardioprotection observed with resveratrol treatment was independent of blood pressure lowering effect suggesting that resveratrol might act directly on the myocardial tissue level than on the vasculature. This notion was consistent with a recent study conducted in our laboratory which demonstrated the effectiveness of resveratrol in preventing the development of hypertrophy in adult rat cardiomyocytes, (Thandapilly et al., 2011).

Although, previous studies have examined the potential of resveratrol in **preventing** cardiac hypertrophy and associated abnormalities in function in different animal models of hypertension, no study has examined whether resveratrol alone or in a combination with a blood pressure lowering agent would be beneficial in **reversing** hypertension-induced cardiac hypertrophy and contractile dysfunction. Accordingly, in this study we addressed this gap in the literature by testing the effectiveness of resveratrol alone and in combination with hydralazine in a well established model of hypertension, the spontaneously hypertensive rat (SHR).

5.3 MATERIALS AND METHODS

The experimental protocols used in this project were approved by the University of Manitoba Animal Care Committee and are in agreement with the Canadian Council on Animal Care and Use of Experimental Animals (Olfert ED, 1993).

5.3.1 Animal model: Twenty week-old male SHR and their controls Wistar–Kyoto (WKY) rats obtained from Charles River, St Constant, Quebec, Canada were used in this study. Animals were acclimatized in temperature and humidity-controlled rooms with a 12-h dark and 12-h light period cycle throughout the study.

5.3.2 Treatment and examinations: Twenty-week SHR and WKY were treated with resveratrol and or hydralazine for eight weeks. Both resveratrol and hydralazine (Sigma-Aldrich Ltd, Ontario, Canada) were dissolved in 50% ethanol (vehicle) were administered daily by oral gavage (1mL/rat) at a dosage of 2.5 mg/kg body weight (an effective concentration taken from our previous studies (Thandapilly et al., 2010; Wojciechowski et al., 2010) and 25 mg/kg body weight (Onaka et al., 1998), respectively. Control groups received 1mL of 50% ethanol daily by oral gavage. The study was terminated at the end of 8 weeks of treatment.

5.3.3 Blood pressure measurements: Blood pressure measurement was carried out on all groups of animals at 0 and 8 weeks of treatment, as described previously (Cipolla et al., 2008). A CODA multi-channel, computerized Non-invasive blood pressure system (Kent Scientific, Torrington, CT) with a tail-cuff sphygmomanometer was used to measure systolic and diastolic blood pressure on conscious rats.

5.3.4 Echocardiography: Cardiac structure and function were measured in all groups of animals using echocardiography at 0 and 8 weeks of treatment; transthoracic two-dimensionally (2D) guided M-mode and Pulse-Wave Doppler measurements were performed using a Sonos 5500 ultrasound system (Agilent Technologies, Andover,

MA, USA) equipped with a 12MHz (s12) transducer as described by us earlier (Cantor et al., 2005). 2D M-mode measurements include percentage of left ventricular fractional shortening, left ventricular ejection fraction (EF), cardiac output (CO), left ventricular mass, heart rate, interventricular septal wall thickness at diastole (IVSd) and systole, left ventricular posterior wall thickness at diastole (LVPWd) and systole and left ventricular internal dimensions at diastole (LVIDd) and systole. Doppler measurements included isovolumetric relaxation time (IVRt).

5.3.5 Electrocardiogram (ECG) measurements: ECG recordings were taken at 0 and 8 weeks of treatment on lightly anesthetized rats using a BioPac MP100 system. The ECG signal was analyzed using Acqknowledge 3.7.3 software (Biopac Systems Inc.).

5.3.6 Pressure myography measurements:

5.3.6.1 Small Arteries: Rats were sacrificed at 28 weeks of age by decapitation, and the mesenteric vasculature was isolated. The use of mesenteric arteries was predicated on consideration that, (a) mesenteric arteries remodel in human hypertensives (Short, 1966); (b) a large percentage of cardiac output flows through the mesenteric circulation, and therefore it contributes to peripheral resistance, and (3) though coronary, renal, femoral and mesenteric resistance arteries remodel and respond to treatment similarly in rat models of hypertension (Sharifi et al., 1998), minimal branching in mesenteric small arteries renders them suitable for study by pressure myography. A segment of the mesenteric artery was mounted in a pressure myograph (Living Systems Instrumentation) such that vessel walls were parallel without stretch (Falloon et al., 1993). To ensure unbiased sampling consistency, all segments were from arterial branches of the third-order. Vessels were equilibrated for

1 h at 37°C at 45 mm Hg(Falloon et al., 1993) with aerated Krebs solution (pH ~7.4). Vessels were considered viable if KCl (125 mmol/L) elicited >50% constriction.

5.3.6.2 Vascular mechanics: Vessels were deactivated with Ca^{2+}-free Krebs solution containing 10 mmol/L EGTA. To obtain pressure-lumen diameter relationships, intraluminal pressure was increased incrementally from 3 to 140 mm Hg (10 increments) (Laurant et al., 1997). Lumen and media dimensions were measured at 3 points along the length of the vessel for each pressure level.

5.3.6.3 Vascular geometry

Lumen and media dimensions were measured at a constant intraluminal pressure of 45 mm Hg (Falloon et al., 1993).

5.3.6.4 Formulas

Media stress, which reflects wall tension in the vessel wall, is calculated as $\sigma=(PD)/(2WT)$, where P is the intraluminal pressure, and D and WT are the lumen diameter and media thickness, respectively. Pressure is converted as 1 mm Hg = $1.334x10^3$ dyn/cm^2.

Media strain, which reflects pressure-induced relative change in lumen diameter, is calculated as $\varepsilon=(D–D_o)/D_o$, where D is the observed lumen diameter for a given intraluminal pressure and D_o is the baseline diameter measured at 3 mm Hg.

Elastic modulus describes the intrinsic elastic properties of the wall material. It is obtained by fitting the stress-strain data from each vessel to an exponential curve ($y=ae^{bx}$): $\sigma=\sigma_o e^{\beta\varepsilon}$ where σ_o is the stress at the baseline diameter and β is a constant related to the rate of increase of the stress-strain curve. Tangential elastic modulus (ET) is calculated at several values of stress from the derivative of the exponential curve: $ET=d\sigma/d\varepsilon=\beta\sigma_o e^{\beta\varepsilon}$. Intrinsic stiffness of wall components is represented as the slope of the elastic modulus vs. stress curve.

5.3.7 Tissue collection: At the end of the study (28 weeks of age), all rats were sacrificed; the heart tissues and other organs were isolated and flash frozen in liquid nitrogen.

5.3.8 Histology: Ventricular tissue was fixed in formalin and embedded in paraffin. Paraffin-embedded tissue was cut into 7 μm sections on a Microm HM 550 cryostat. The levels of collagen deposition were determined by Masson trichrome staining. Photographs were taken using a Zeiss LSM 5 Pascal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA).

5.3.9 Oxidative stress measurement: Lipid peroxidation levels in blood plasma, collected at the time of sacrifice, were measured by estimating the amount of malondialdehyde using the Oxiselect TBARS Assay Kit (Cell Biolabs, San Diego, CA) as described by us earlier (Thandapilly et al., 2010; Wojciechowski et al., 2010) by following the manufacturers instructions. Thiobarbituric acid reactive substances (TBARS) values were expressed as nmol/mL of plasma.

5.3.10 Inflammatory marker measurements: Serum samples collected at sacrifice were used for biochemical analysis. Rat interleukin 6 (IL-6) Elisa kit (Thermo Scientific, IL, USA) was used to measure the serum IL6 levels. The assay was performed as described earlier (Louis et al., 2012) by following the manufacturer instructions, and the values were expressed as pg/mL of serum.

5.3.11 Statistics

Results are presented as means ± SEM. Data were analyzed by ANOVA or 2-way ANOVA for repeated measures, followed as appropriate by Student-Newman-Keuls or Bonferroni post-tests to detect between-group differences. $p<0.05$ was considered significant.

5.4 RESULTS

5.4.1 General characteristics of the animal model: All rats had comparable increases in the body weight throughout course of the study. Resveratrol treatment had no effect on the body weight in any of the animals. However, Heart to body weight ratio was significantly higher in 28 weeks SHR when compared to WKY control rats; the treatment with hydralazine alone or in combination with resveratrol significantly regressed the increase in heart to body weight ratio in SHR (Figure: 1A). However, we did not observe any reduction in heart to body weight ratio with 8 weeks of resveratrol treatment (Figure: 1A).

5.4.2 Blood pressure: 20 weeks old SHR had significantly elevated blood pressure compared to normotensive WKY rats before the treatment started (Supplementary figure: 1). Treatment with hydralazine but not resveratrol, significantly reduced the elevated blood pressure (systolic as well as diastolic) in SHR when compared to untreated group (Figure: 2 B, C). Moreover, hydralazine in conjunction with resveratrol further reduced the systolic and diastolic blood pressure in SHR when compared to untreated or hydralazine treated SHR (Figure: 2 B, C).

5.4.3 Cardiac structure:

M-mode echocardiography showed a significant increase in IVSd and LVPWd in 20 weeks old SHR when compared to age matched controls prior to the treatment (Supplementary figure: 2). However, hydralazine but not resveratrol significantly reduced the increase in IVSd and LVPWd in 30-week SHR compared to untreated group (Figure 2A, B). Treatment using hydralazine in combination with resveratrol also significantly reduced this increase in SHR (Figure 2A, B). SHR did not exhibit any change in LV internal dimension when compared to WKY controls at any time point (supplementary figure: 3).

5.4.4 Cardiac function:

A significant increase in the diastolic functional parameter, IVRt, was observed in 20-week SHR prior to the treatment in comparison with their respective WKY controls (Supplementary figure: 4). Hydralazine but not resveratrol treatment moderately but significantly improved the prolonged IVRt in SHR when compared to untreated SHR (Figure: 3A). More interestingly, combination treatment of hydralazine with resveratrol completely normalized the diastolic functional parameter IVRt in SHR (Figure: 3A). There was no significant reduction in the systolic functional parameter fractional shortening in 20-week SHR prior to the treatment (Supplementary figure: 4); however, at 28 weeks, we observed significant reduction in fractional shortening in untreated SHR compared to normotensive WKY rats (Figure: 3B). Both resveratrol as well as hydralazine significantly improved the fractional shortening in SHR when compared to untreated group at 28 weeks time point (Figure: 3B). Resveratrol in combination with hydralazine normalized the fractional shortening in SHR (Figure: 3B). Cardiac output was unchanged in all groups at the end of the study (Figure: 3C).

5.4.5 ECG measurements:

The ECG recordings did not show any abnormalities in the QT interval in any of the groups at any time points of the study (Figure: 4).

5.4.6 Pressure myography of resistance arteries:

Media-to-lumen ratios were greater in SHR vessels compared to WKY (Table 1; $p<0.01$). Resveratrol alone had no effect on vascular geometry in WKY and SHR rats. However, hydralazine (whether alone or combined with resveratrol) significantly corrected media-to-lumen ratio in SHR vessels ($p<0.05$). Vascular compliance is influenced by the fact that transduction of intraluminal pressure to the vessel wall as stress is modulated by the geometry of the artery. Isobaric elastic

modulus (i.e. elastic modulus vs. pressure) is determined by two factors – wall component stiffness and vessel geometry. However, when elastic modulus is plotted against stress, geometry is mathematically eliminated as a contributor, and therefore provides information regarding solely the stiffness of wall components such as elastin, collagen, and smooth muscle cells. Wall component stiffness, presented as the slope of elastic modulus vs. stress (Table 1), was increased in SHR vs. WKY vessels ($p<0.01$). Here, resveratrol treatment alone attenuated stiffening of wall components of SHR arteries, as did hydralazine and the combination of resveratrol with hydralazine ($p<0.01$).

5.4.7 Histological analysis:

The histological analysis of ventricular tissue showed increased collagen deposition at 28 weeks SHR compared to its normotensive counterparts (Figure: 5). Resveratrol alone or in combination with hydralazine was able to reduce the collagen deposition in the left ventricular tissue from SHR (Figure: 5).

5.4.8 Oxidative stress:

Twenty eight weeks old SHR had a significantly increased levels of plasma TBARS, when compared to their age matched control rats; treatment with resveratrol or hydrlazine significantly reduced elevated TBARS levels in SHR (Figure: 6A). More importantly, resveratrol in combination with hydralazine further reduced the TBARS levels in SHR when compared to hydralazine or resveratrol alone (Figure: 6A).

5.4.9 Inflammatory markers

There was a significant increase in the plasma TNF-α level in 28-week SHR when compared to their controls (Figure: 6C). Resveratrol alone or in combination with hydralazine significantly decreased the serum TNF-α level in SHR (Figure: 6C). Serum IL-6 levels were increased in SHR when compared to age matched WKY, and

were significantly reduced upon resveratrol treatment alone or in combination with hydralazine (Figure: 6B). Hydralazine treatment alone did not have significant effect on both TNF-α and IL-6 levels in SHR (Figure: 6B, C).

Figure: 1 Effect of treatment on cardiac hypertrophy and blood pressure

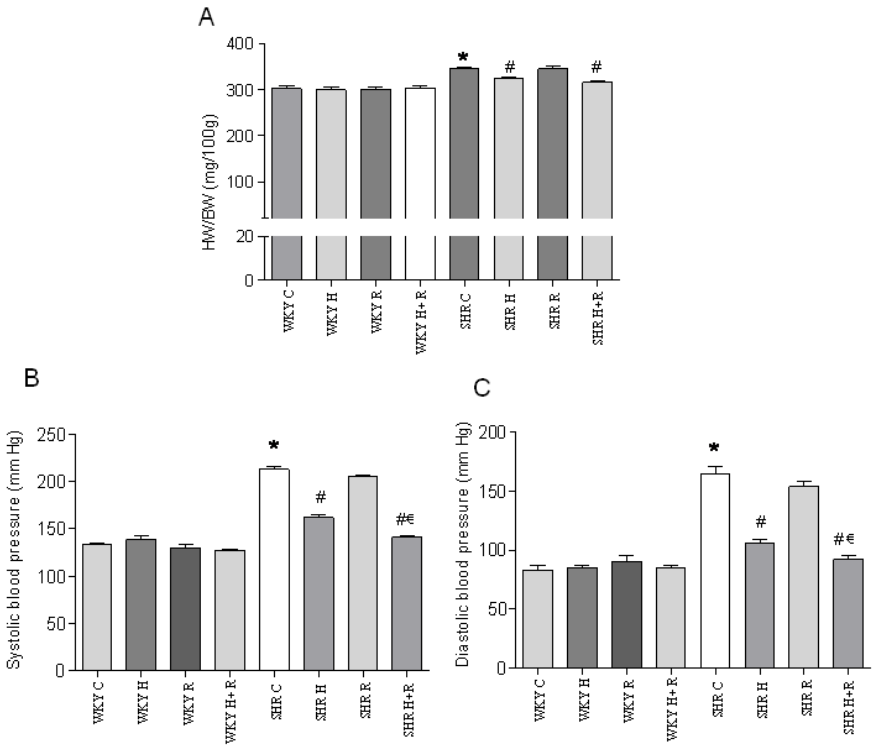


Figure 1. Effect of treatment on cardiac hypertrophy and blood pressure in 28 week WKY and SHR treated with resveratrol and or hydralazine. (A), Heart weight/body weight ratio. (B), Analysis of systolic blood pressure. (C), Analysis of diastolic blood pressure. C: control; H: hydralazine; R: resveratrol. Data are mean ± SE. n = 8-10. * $P < 0.05$ Vs WKY C; $^{\#}$ $P < 0.05$ Vs. SHR C; $^{€}$ $P < 0.05$ Vs. SHR H.

Figure: 2 Effect of treatment on cardiac structure

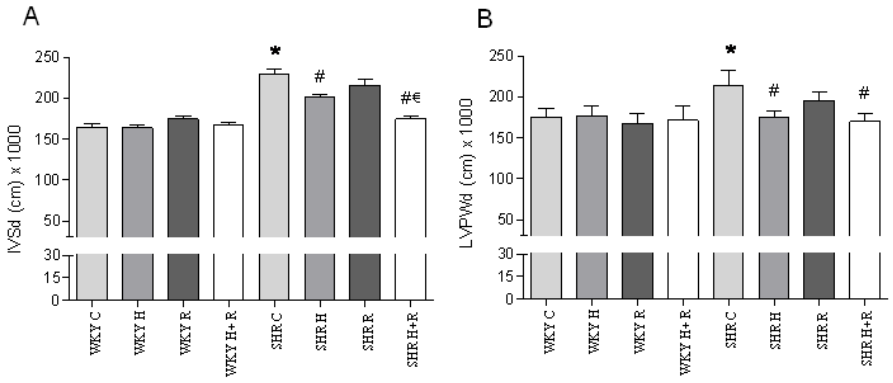


Figure 2. Effect of treatment on cardiac structure in 28 week WKY and SHR treated with resveratrol and or hydralazine. (A), Interventricular septal wall thickness at diastole (IVSd). (B), left ventricular posterior wall thickness at diastole (LVPWd). C: control; H: hydralazine; R: resveratrol. Data are mean ± SE. n = 8-10. * $P < 0.05$ Vs WKY C; $^{\#}$ $P < 0.05$ Vs. SHR C; $^{€}$ $P < 0.05$ Vs. SHR H.

Figure: 3 Effect of treatment on cardiac function

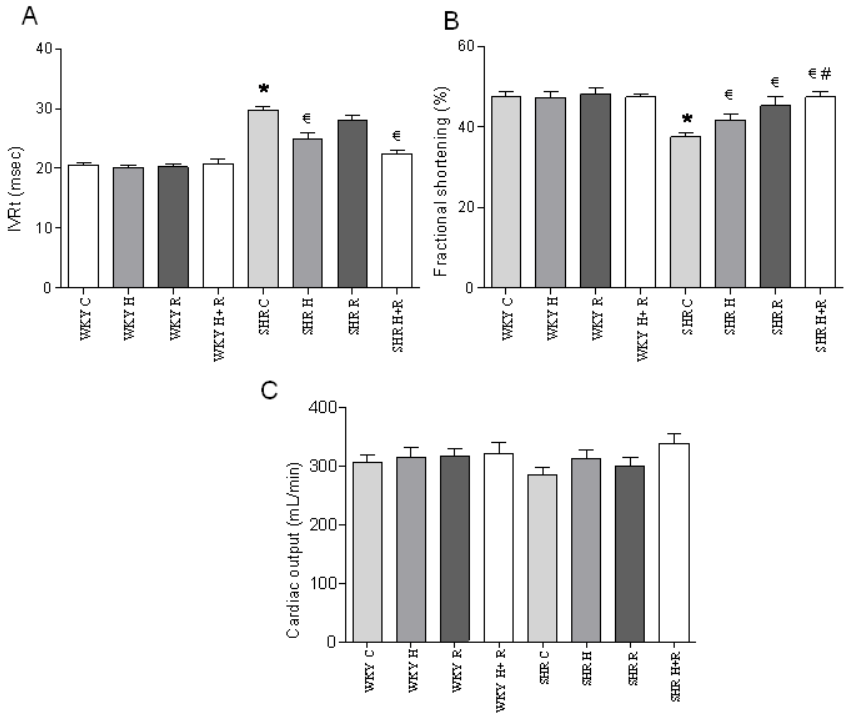


Figure 3. Effect of treatment on cardiac function in 28 week WKY and SHR treated with resveratrol and or hydralazine. (A), isovolumetric relaxation time (IVRt). (B), Fractional shortening. (C), Cardiac output. C: control; H: hydralazine; R: resveratrol. Data are mean ± SE. n = 8-10. * $P < 0.05$ Vs WKY C; $^{€}$ $P < 0.05$ Vs. SHR C; $^{\#}$ $P < 0.05$ Vs. SHR H.

Figure 4. Effect of treatment on electrical activity

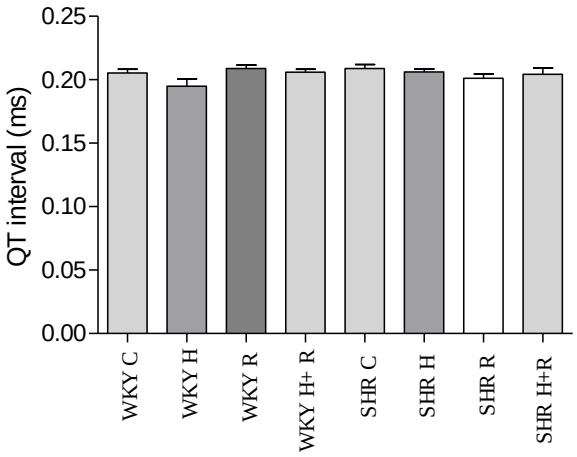


Figure 4. Effect of treatment on QT interval in 28 week WKY and SHR treated with resveratrol and or hydralazine. C: control; H: hydralazine; R: resveratrol. Data are mean ± SE. n = 8-10.

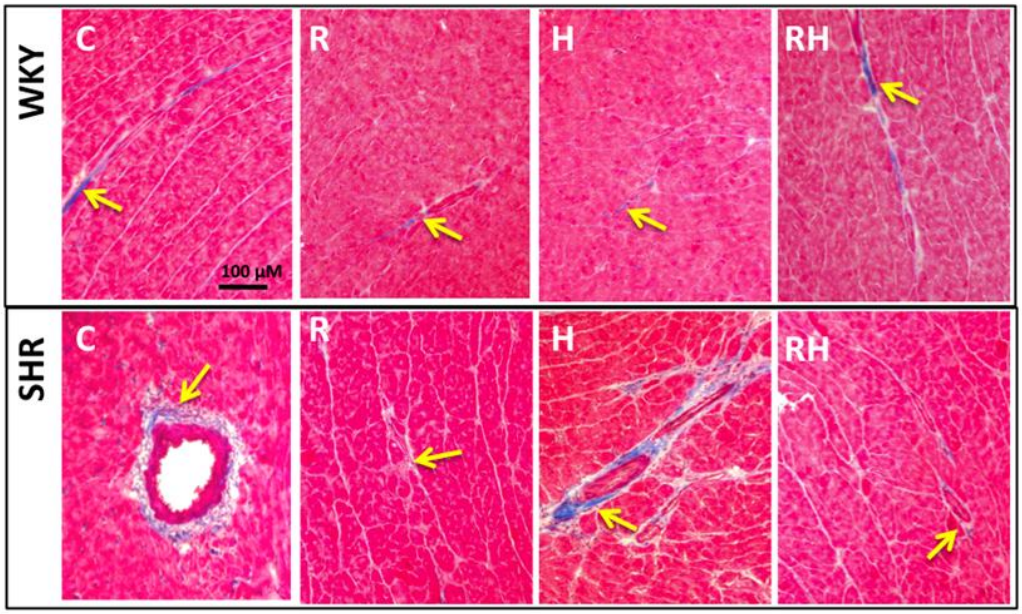


Figure: 5 **Effect of treatment on myocardial fibrosis**

Figure 5. Effect of treatment on myocardial fibrosis in 28 week WKY and SHR treated with resveratrol and or hydralazine. Representative images of WKY or SHR transverse heart ventricular sections from different treatment groups, as indicated, and stained for collagen deposition (Masons trichrome). Fibrotic areas (blue) are indicated by yellow arrows. Muscle stains reddish-purple. C, control;R, resveratrol; H, hydralazine; RH, resveratrol plus hydralazine.

Figure:6 Effect of treatment on oxidative stress and inflammation

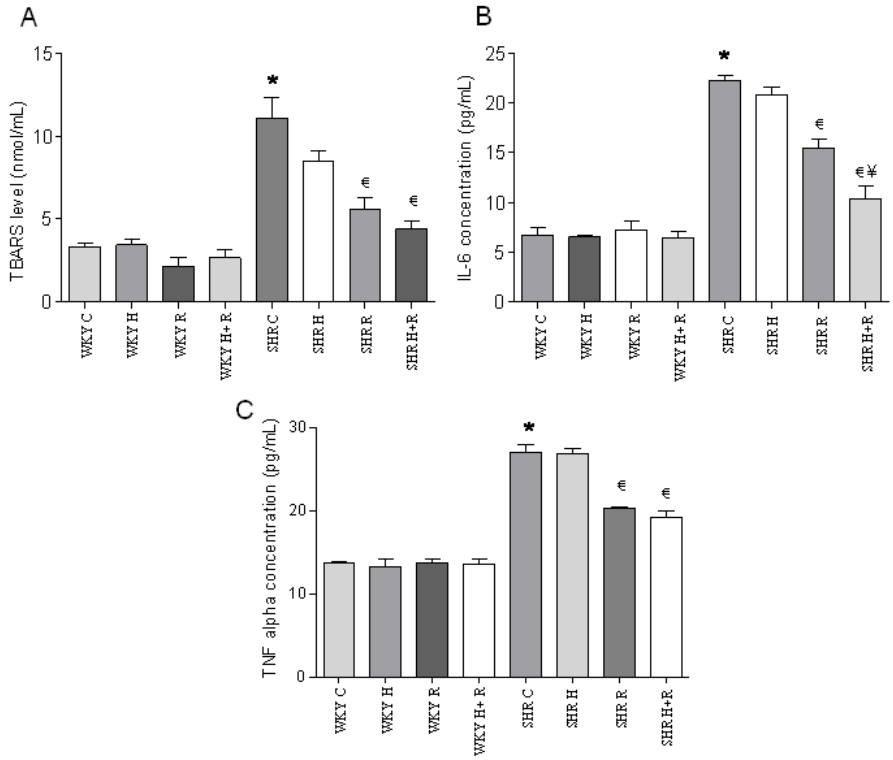
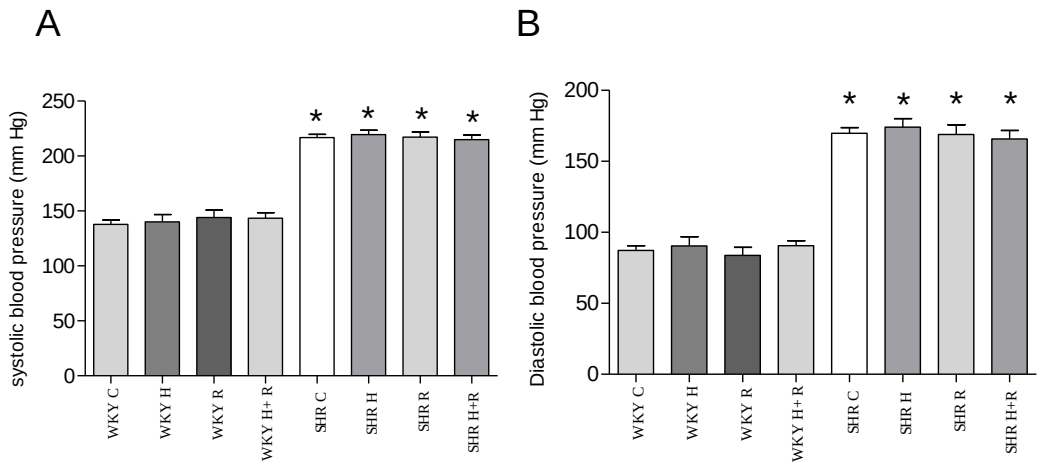


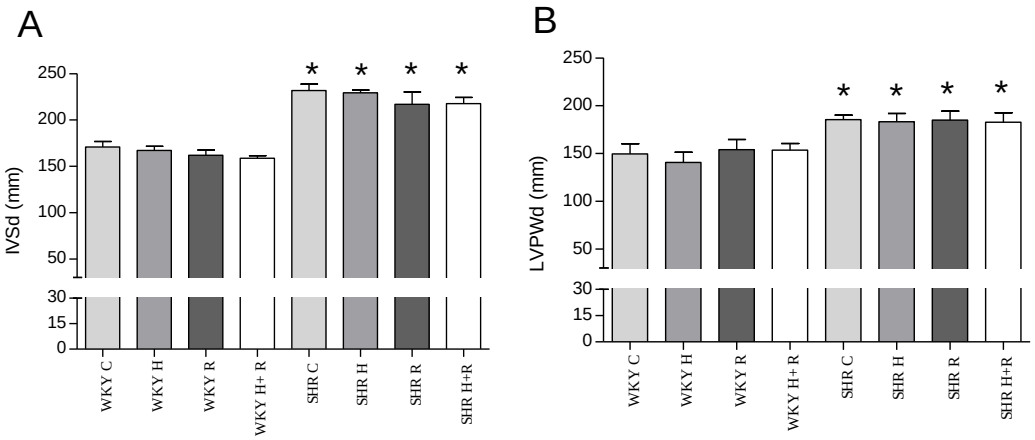
Figure 6. Effect of treatment on oxidative stress and inflammation in 28 week WKY and SHR treated with resveratrol and or hydralazine. (A), Thiobarbituric Acid Reactive Substances (TBARS) (B), Interleukin-6 (IL-6). (C), Tumor necrosis factor (TNF) alpha. C: control; H: hydralazine; R: resveratrol. Data are mean ± SE. n = 4-6. * $P < 0.05$ Vs WKY C; $^{€}$ $P < 0.05$ Vs. SHR H; $^{¥}P < 0.05$ Vs SHR R.

	WKY C	WKY H	WKY R	WKY H+R	SHR C	SHR H	SHR R	SHR H+R
Media:lumen at 45 mm Hg	10.57±0.6	7.79±0.3	11.16±0.9	7.59±0.5	16.56±1.4**	13.15±1.2†	18.29±1.3**	13.52±1.1†
Slope of elastic modulus vs. stress	5.34±0.3	4.19±0.2	5.57±0.4	5.32±0.2	8.16±1.2**	4.57±0.2‡	5.88±0.7‡	4.37±0.3‡

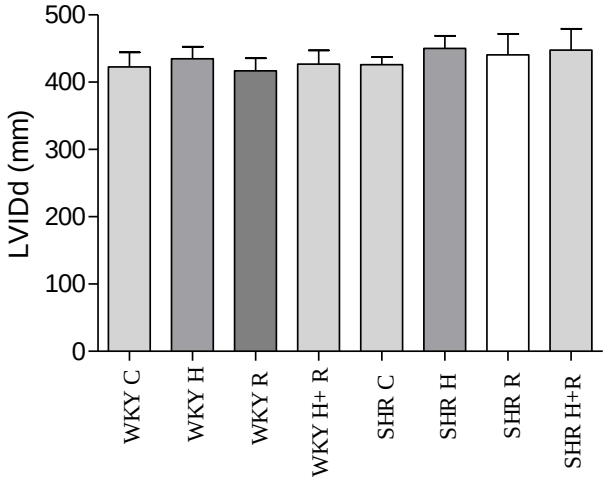
Table 1. Effect of treatment on arterial structure in 28 week WKY and SHR treated with resveratrol and or hydralazine. C: control; H: hydralazine; R: resveratrol. Data are mean ± SE. n = 6-7. **P<0.01 *vs.* untreated WKY. †P<0.05 and ‡P<0.01 vs. untreated SHR.



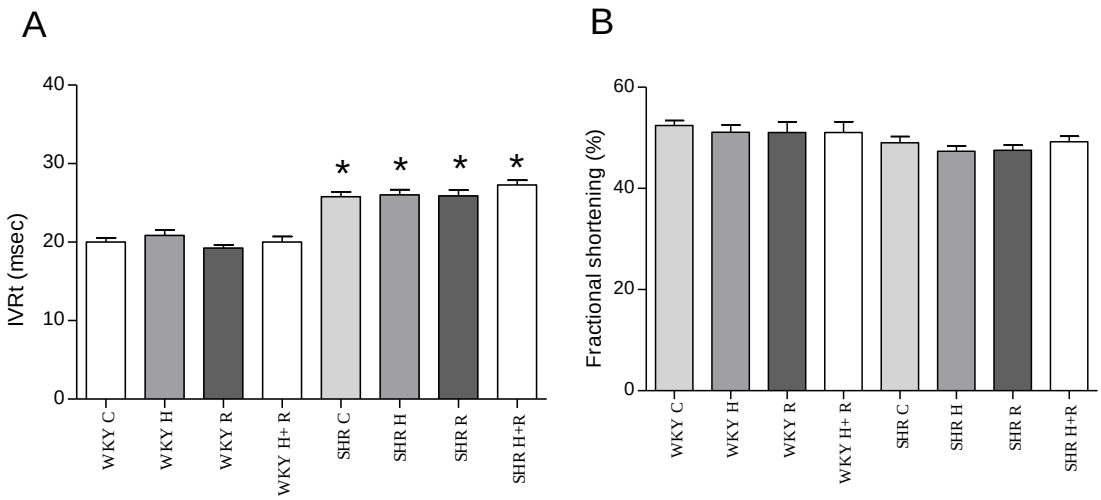
Supplementary figure 1. Blood pressure measurement in 20 week WKY and SHR before starting the treatment. (A), Analysis of systolic blood pressure. (B), Analysis of diastolic blood pressure. C: control; H: hydralazine; R: resveratrol. Data are mean ± SE. n = 8-10. * $P < 0.05$ Vs WKY groups.



Supplementary Figure 2. Analysis of cardiac structure in 20 week WKY and SHR before starting the treatment (A), Interventricular septal wall thickness at diastole (IVSd). (B), left ventricular posterior wall thickness at diastole (LVPWd). C: control; H: hydralazine; R: resveratrol. Data are mean ± SE. n = 8-10. * $P < 0.05$ Vs WKY groups



Supplementary Figure 3. Effect of treatment on left ventricular internal dimension at diastole (LVIDd) in 28 week WKY and SHR treated with resveratrol and or hydralazine. C: control; H: hydralazine; R: resveratrol. Data are mean ± SE. n = 8-10.



Supplementary Figure 4. Assessment of cardiac function in 20 week WKY and SHR before starting the treatment. (A), Isovolumetric relaxation time (IVRt). (B), Fractional shortening. C: control; H: hydralazine; R: resveratrol. Data are mean ± SE. n = 8-10. * P < 0.05 Vs WKY groups.

5.5 DISCUSSION

Although hemodynamic load is a major determinant of cardiac hypertrophy in hypertension, there are also blood pressure independent factors involved in the pathogenesis and its progression (Pachori et al., 2002). Accordingly, prevention or regression of cardiac hypertrophy with different pharmacological agents has had an enormous impact on the prognosis in hypertensive patients (Katholi and Couri, 2011). In this context, we had recently reported the prevention of pressure overload–induced cardiac hypertrophy and its deleterious consequences on heart function in resveratrol-treated abdominal aortic-banded rats as well as young SHR rats (Juric et al., 2007; Thandapilly et al., 2010; Wojciechowski et al., 2010). Li *et al*. (Li et al., 2005) also reported prevention of cardiac structural and functional alterations by resveratrol treatment in another experimental model of pressure overload created by transverse aortic constriction. However, in most of the studies, the anti-hypertrophic effect observed with resveratrol was independent of the blood pressure lowering effects (Dolinsky et al., 2009; Thandapilly et al., 2010) suggesting the direct action of this polyphenol acts directly on the cardiac tissue. This is consistent with our in vitro study (Thandapilly et al., 2011), where we observed anti-hypertrophic effect of resveratrol on isolated adult cardiomyocytes exposed to norepinephrine. Moreover, all these studies examined the preventive efficacy of resveratrol in the early stages of hypertension rather than its effectiveness in reversing the cardiac abnormalities. However, in the present study we investigated whether resveratrol alone or in a combination with a pure blood pressure lowering agent will **reverse** the cardiac impairment due to hypertension once it is developed.

In the current study, 20 week old SHR had established hypertension and moderate systolic and diastolic dysfunction before starting the treatment. Consistent with our

previous study, we did not observe a decrease in blood pressure with 8 weeks of resveratrol treatment alone in SHR; but hydralazine treatment was able to significantly reduce blood pressure in SHR. However, when resveratrol was administered in conjunction with hydralazine, it enhanced the anti-hypertensive action of hydralazine by further decreasing it to a normal level. This added effect of resveratrol might be attributed to its anti-oxidant and anti inflammatory activities.

Although, we have reported the prevention of both systolic and diastolic dysfunction in SHR rats (Thandapilly et al., 2010), in the present study 8 weeks of resveratrol treatment was effective in reversing systolic dysfunction and not diastolic dysfunction in 30 week old SHR. However, resveratrol in combination with a vasodilator, hydralazine, was able to restore both systolic and diastolic dysfunction in SHR. Contrary to previous reports, hydralazine alone was able to reduce hypertrophy and some of the functional abnormalities in 30 week old SHR.

The increased deposition of collagen is a hallmark of the hypertrophic remodeling process and which can predispose to increased risk of adverse cardiac events (Grobe et al., 2006). Resveratrol has been reported to inhibit collagen deposition and fibroblast proliferation, two key events in the development of cardiac fibrosis both in *in vivo* and *in vitro* settings (Olson et al., 2005; Wang et al., 2007). The reduction in collagen deposition observed in the present study with resveratrol, and resveratrol/hydralazine treatment might have contributed to the reduction in the myocardial stiffness thereby improved the cardiovascular function in hypertensive rats.

It is also well known fact that ventricular hypertrophy significantly increases the risk of ventricular arrhythmias in hypertensive patients (Akdeniz et al., 2002; Bikkina et al., 1993), and it seem to result from prolonged repolarization such as long QT

intervals represented in the ECG measurements. SHR rats have been reported to exhibit long QT syndrome by the age of 20 weeks (Rousseau-Ralliard et al., 2009). However in the present study, we did not observe any change in the QT intervals of SHR rats when compared to normotensive rats. Neither resveratrol nor hydralazine affected the QT interval of the rats at any time points during the treatment.

Abnormalities of small arteries are also major contributors to the pathogenesis and maintenance of hypertension (Mulvany, 2002). Changes in the structural and functional properties have been also detected in small arteries from the spontaneously hypertensive rat. In this regard, vascular compliance is determined by passive geometry as well as intrinsic stiffness of arterial wall components. (Laurant et al., 1997). Thus, the effects of resveratrol on these parameters were considered. The elastic modulus vs. stress plot provides information regarding the stiffness of wall components (i.e. connective tissue, elastin and collagen fibers, smooth muscle cells, and endothelial cells) which is independent of arterial geometry (Intengan et al., 1999). We previously reported that, in the context of developing hypertension (i.e. 10-20 weeks of age) in the SHR, the beneficial effects of lie in correction of vascular geometry (Behbahani et al., 2010). In contrast, we observed here in SHR with established hypertension (i.e. 20-30 weeks of age) that resveratrol fails to improve vascular geometry. Instead, resveratrol improved the intrinsic stiffness of the arterial wall. Although we did not measure levels of extracellular matrix proteins such as collagen, these findings in the microvasculature are consistent with alleviation of fibrosis that we observed here in other organs such as heart.

In order to understand the mechanism underlying the protective effect of resveratrol/hydralazine treatment in reversing the cardiovascular abnormalities in SHR, we examined the status of inflammation and oxidative stress, two major

contributors in the pathogenesis of hypertensive heart disease (Pashkow, 2011; Vaziri and Rodriguez-Iturbe, 2006). It is well established that oxidative stress and inflammation are inextricably linked and form a circuit in the progression of cardiovascular events, and if not blocked, culminates in progressive target organ injury and dysfunction (Vaziri, 2008). Moreover, previous studies from our laboratory and others have reported that the cardioprotective effect of resveratrol might be attributable to its anti-oxidant enhancing activity and or anti-inflammatory activity (Li et al., 2005; Louis et al., 2012; Thandapilly et al., 2010). Consistent with the previous reports (Li et al., 2005; Louis et al., 2012; Thandapilly et al., 2010) we found that resveratrol alone or in combination with hydralazine was able to reduce the oxidative stress and inflammation in hypertensive rats which was evident from the TBARS, IL-6 and TNF-alpha measurements. However, hydralazine treatment alone was able reduce oxidative stress (TBARS) only but not inflammation (IL-6 and TNF-alpha) in hypertensive rats. We speculate that the cardiac structural and functional improvement observed with resveratrol/hydralazine combination therapy might be attributed to **a,** reduction in the oxidative stress -inflammation axis; **b,** reduction in the hemodynamic load.

Two novel findings of the present study are (1) resveratrol itself was able to reverse cardiac fibrosis and some of the functional abnormalities in 30 week old SHR (2) resveratrol in combination with hydralazine was more effective in reducing blood pressure, improving cardiac structure and function in SHR when compared to resveratrol or hydralazine alone.

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CHAPTER 6

OVERALL SUMMARY

6.1 CONCLUSION

Despite great advances in its treatment, the incidence of heart failure related deaths are on the rise (Alba and Delgado, 2009; Horwich and Fonarow, 2010). This calls for finding improved therapeutic strategies to prevent or reverse this ailment. Hypertension is one of the major independent risk factors for the development of heart failure (Bui et al., 2011). Studies have reported on the cardioprotective effects of resveratrol, a compound derived from grapes, in different *in vitro* and *in vivo* models of heart disease (Li et al., 2012; Yu et al., 2012., Juric et al., 2007; Li et al., 2005). Although our laboratory and others have reported the potential of resveratol in experimental models of pressure overload induced by surgery (Juric et al., 2007; Li et al., 2005), no study has investigated the potential of resveratrol in preventing/reversing cardiovascular abnormalities induced by essential hypertension. In this project, we used a suitable model of essential hypertension, the SHR which develops hypertension in the early stages of life. The SHR develops cardiac hypertrophy gradually in response to the progressive hypertensive disease and not abruptly as a consequence of the surgical procedure. Thus, the nature of cardiac hypertrophy in SHR resembles development of cardiac hypertrophy in humans secondary to systemic hypertension ((Bing et al., 2002; Doggrell and Brown, 1998). In this project, we also used an *in vitro* model of cardiomyocyte hypertrophy induced by norepinephrine, a potent inducer of hypertension and pathological hypertrophy to elucidate the molecular mechanism of resveratrol's action.

The novel findings of the present study can be summarized as follows

1, Resveratrol treatment was beneficial in preventing the development of concentric hypertrophy and cardiac dysfunction in 20 week SHR without lowering
blood pressure. This effect was associated with a reduction in oxidative stress.

2, Resveratrol prevented adult cardiomyocyte hypertrophy induced by norepinephrine by activating the NO-AMPK pathway. Consistent with the *in vitro* findings, the cardioprotective effect of resveratrol in SHR was also associated with increase in NO and AMPK activity.

3, Resveratrol was not able to reverse hypertension, cardiac hypertrophy and diastolic dysfunction by itself in 28 week SHR. However, in combination with the blood pressure lowering agent hydralazine, resveratrol reversed hypertension, cardiac hypertrophy and contractile dysfunction in SHR.

6.2 LIMITATIONS OF THE STUDY

In hypertensive patients, reduction of blood pressure is key factor in preventing the development cardiovascular events. In the present study we did not observe any reduction in blood pressure with resveratrol mono treatment. However, a few studies have found a significant reduction in blood pressure with resveratrol treatment (Bhatt et al., 2011; Liu et al., 2005; Rivera et al., 2009). One of the reasons for the observed discrepancy of the effects of resveratrol in lowering blood pressure may be the lower dosage of resveratrol used by us compared to other studies as well as the nature and the stage of hypertension in these models.

Secondly, the drug used for combination therapy in the third study (hydralazine) is not a clinically recommended drug for essential hypertension because of its' side effects (Magee et al., 2003). However, we used hydralazine as a tool to elucidate the blood pressure-independent mechanism of resveratrol.

6.3 SIGNIFICANCE OF THE STUDY

The studies carried out in this project are pre-clinical in nature and established the efficacy of resveratrol (by itself) in the treatment of cardiac abnormalities secondary to essential hypertension. These results also revealed that resveratrol can complement

and possibly reduce the dose requirement of well established anti-hypertensive/heart failure drugs in treating hypertension induced heart failure. One of the most relevant aspect of the present study is the concentration of resveratrol used (2.5 mg/kg/day in rat, which is approximately a human equivalent dosage of 28 mg in a 70-kg person) is not at pharmacological levels (with hundreds of milligrams or grams) and more likely achievable through regular wine intake (Goncalves and Camara, 2011). Moreover, the results generated from the current study will be useful in designing future clinical trials testing the potential of resveratrol in preventing cardiac complications in patients with hypertension.

6.4 FUTURE DIRECTIONS

The ineffectiveness of resveratrol in reducing blood pressure in SHR is a concern and future studies with a prolonged treatment and or higher dosage of resveratrol will be helpful to resolve this question. It would be also very interesting to investigate the effects of resveratrol on other major target organs of hypertension, the brain and kidney, in SHR. The ultimate aim would be to conduct human clinical trials in the future to examine the effects of resveratrol in preventing/reversing cardiac complications in hypertensive patients. Furthermore, exploring resveratrol analogues with improved pharmacokinetic potential will also enable the resveratrol research to move forward.

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