Effects of Resveratrol on Hypertension and Resistance Arteries in the Spontaneously Hypertensive Rat

by

John Behbahani

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Faculty of Pharmacy

University of Manitoba

Winnipeg

ABSTRACT

Hypertension is accompanied by structural and mechanical abnormalities in resistance arteries. The effects of resveratrol, a phenolic phytoalexin found naturally in various foods, on systolic blood pressure and resistance artery structure and stiffness were assessed in spontaneously hypertensive rats (SHRs). Vascular geometry and mechanical properties of pressurized mesenteric resistance arteries were calculated from media and lumen dimensions measured using pressure myography. Compared to normotensive Wistar-Kyoto (WKY) rats, resistance arteries from SHRs displayed remodeling with narrowed lumen diameters (246.2±21.0 vs. 308.1±14.3 μm, $p<0.05$), thickened media widths (39.8±4.6 vs. 28.5±2.7 μm, $p<0.05$) and augmented media-to-lumen ratios (17.7±2.6 vs. 9.3±1.0, $p<0.05$). Calculations of remodeling and growth indices revealed that SHR vessels underwent mostly eutrophic remodeling. Systolic blood pressure was elevated in 20-week-old SHR versus WKY rats (219±6 vs. 155±6 mmHg, $p<0.01$) and was unaffected by resveratrol (2.5 mg/Kg/d).

In SHRs, resveratrol treatment attenuated eutrophic remodeling and normalized increased vessel compliance ($p<0.01$) as determined by a restorative leftward shift in the stress-strain curve of SHR arteries ($p<0.01$). Resveratrol treatment restored stiffness in SHRs (4.2±0.4 vs. 6.6±0.5, $p<0.05$) through the normalization of vessel geometry. Immunoblotting revealed that resveratrol negated typical pronounced ERK1/2 signaling in SHR arteries. Thus, the results of this study suggest that resveratrol restores vascular mechanical properties in SHRs and attenuates remodeling. Furthermore the attenuation of remodeling in SHR arteries with resveratrol treatment is associated with the inhibition of ERK1/2 activity.

ACKNOWLEDGEMENTS

Thanks first to my supervisor, Dr. Hope Anderson, for her valuable advice, assistance, encouragement and frankness. Through her governance she enabled be to see areas where I could improve the most, something that even my dearest friends have often failed to do. Furthermore, she enabled me to get a solid footing in the research field by allowing me to network from an early stage in my career.

I'd like to extend a sincere thanks to my committee member, and project collaborator Dr. Thomas Netticadan and his graduate students, especially his senior student Sijo Joseph Thandapilly. Dr. Netticadan provided me with great perspective throughout my Masters. His door was always open and welcoming, even after everyone had left for the night. Most importantly, he was always around to help and encourage me when I needed it.

A very special thanks to Dr. Frank Burczynski, who was always willing to support my work endeavours. His approachability was a rare trait and most appreciated.

I'd like to give a heartfelt thanks to all my friends and colleagues who have not been mentioned but have made my academic life an enjoyable and productive one. It is often the company of good friends that makes the long trips worthwhile.

Finally, I'd like to give my deepest thanks to my wonderful and loving family back home in Toronto, Ontario. My parents have taught me that it is more important for a human being to be genuine than to be perfect; a lesson most fitting for a budding researcher.

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Messerli, F. H. (2000) *The ABCs of antihypertensive therapy*, 2nd ed., Authors' Publishing House ; Lippincott Williams & Wilkins, New York, Philadelphia. Permission granted May, 2010.

http://www.lab.anhb.uwa.edu.au/mb140/CorePages/Vascular/Vascular.htm Accessed: March 2009. Special thanks to Professor John McGeachie PhD, University of Western Australia, for permission to use artwork.

CHAPTER I

INTRODUCTION

1. Cardiovascular Disease in Canada

1.1 Cardiovascular disease and mortality

When the first national mortality statistics were published in 1921, cardiovascular disease ranked as the leading cause of mortality for Canadians (*1*). The most current data from Statistics Canada indicate that as of 2005, cardiovascular disease accounted for 31% of all deaths in Canada; 31% and 30% of all female and male deaths were related to cardiovascular disease, respectively (*2*). According to the World Health Organization, the majority of cardiovascular disease-related mortalities in North America are due to hypertension (*3*).

1.2 Hypertension

Hypertension is a condition most prevalent in aging populations. It is commonly diagnosed in individuals as they approach their third, fourth and fifth decades of life (*4*). Due to the asymptomatic nature of the disease, it is often referred to as the "silent killer." An estimated 50% of Canadians over 65 years of age are hypertensive (*5*). Furthermore, 90% of Canadians between 55 and 65 years of age with normal blood pressure will develop hypertension within an average lifespan (*5*).

Hypertension is a heterogeneous condition that falls into two major categories. The first form of hypertension is called essential hypertension (i.e. *primary* hypertension). Essential hypertension is chronic high blood pressure with an unknown cause (*6*). Approximately 85% of diagnosed cases are classified as essential hypertension.

The second form of hypertension is termed non-essential hypertension (i.e. *secondary* hypertension). Here, increased blood pressure is linked to a diagnosed condition. The diagnosed condition can be the cause of dysregulation in various organs or organ systems that regulate

blood pressure, cardiac output and fluid retention. Treatment of the primary ailment often normalizes the compensatory systemic imbalances and reduces blood pressure. Examples of conditions that can lead to secondary hypertension include obstructive sleep apnea, pheochromocytoma, pregnancy, coarctation of the aorta, hyperparathyroidism, Cushings syndrome, chronic renal disease, acromegaly, and hyperthyroidism (*7-15*).

By treating hypertension, the risks of developing heart failure and stroke might be reduced drastically by an estimated 50 and 38% respectively (*16*); this is key since heart disease and stroke cost the Canadian economy more than 22.2 billion dollars annually (*17*). Research and awareness of cardiovascular diseases have proved effective in reducing mortality rates to levels below that of cancer as of 2005. In fact, Statistics Canada shows that since 2000, the rate of death in Canada due to cardiovascular related diseases has continued to decrease annually (*2*). In general, treatment and overall control over hypertension has improved globally since 1988, but only from 6% to a mere 6.4% based on reports arising from Japan, Greece, Germany, England, Spain, the Unites States and Canada (*18*).

1.3 Assessing arterial pressure

Arterial pressure represents the notional pressure exerted by blood flow along the walls of the arteries. Blood pressure is at its maximum value during cardiac contraction (systolic pressure) and lowest at the end of a cardiac contraction (diastolic pressure). The units of measurement for arterial pressure are millimetres of mercury (mm Hg) and are usually expressed in notation as a fraction of systolic blood pressure over diastolic blood pressure. Arterial pressure can be expressed mathematically (Table 1) as a function of cardiac output and total peripheral resistance.

Arterial Pressure = Cardiac Output **X** Total Peripheral Resistance

where Cardiac Output = Heart Rate **X** Stroke Volume

Table 1. Arterial pressure expressed as an equation. Total peripheral resistance and cardiac output are related through arterial pressure.

Cardiac output is defined as the volume of blood ejected by the heart per minute. It is the product of heart rate (number of heart beats per minute) and stroke volume (volume of blood ejected by the left ventricle of the heart per beat). In contrast, total peripheral resistance is defined as the total resistance to ejected blood flow as generated by the arteries in the periphery. For instance, contraction and dilation of arterioles regulate total peripheral resistance.

In terms of total peripheral resistance, arteries with narrow diameters generate more resistance to blood flow than large diameters (Figure 1), affecting diastolic pressure (*19*). As a result, smaller arteries are termed *resistance arteries* as they contribute to the majority of total peripheral resistance.

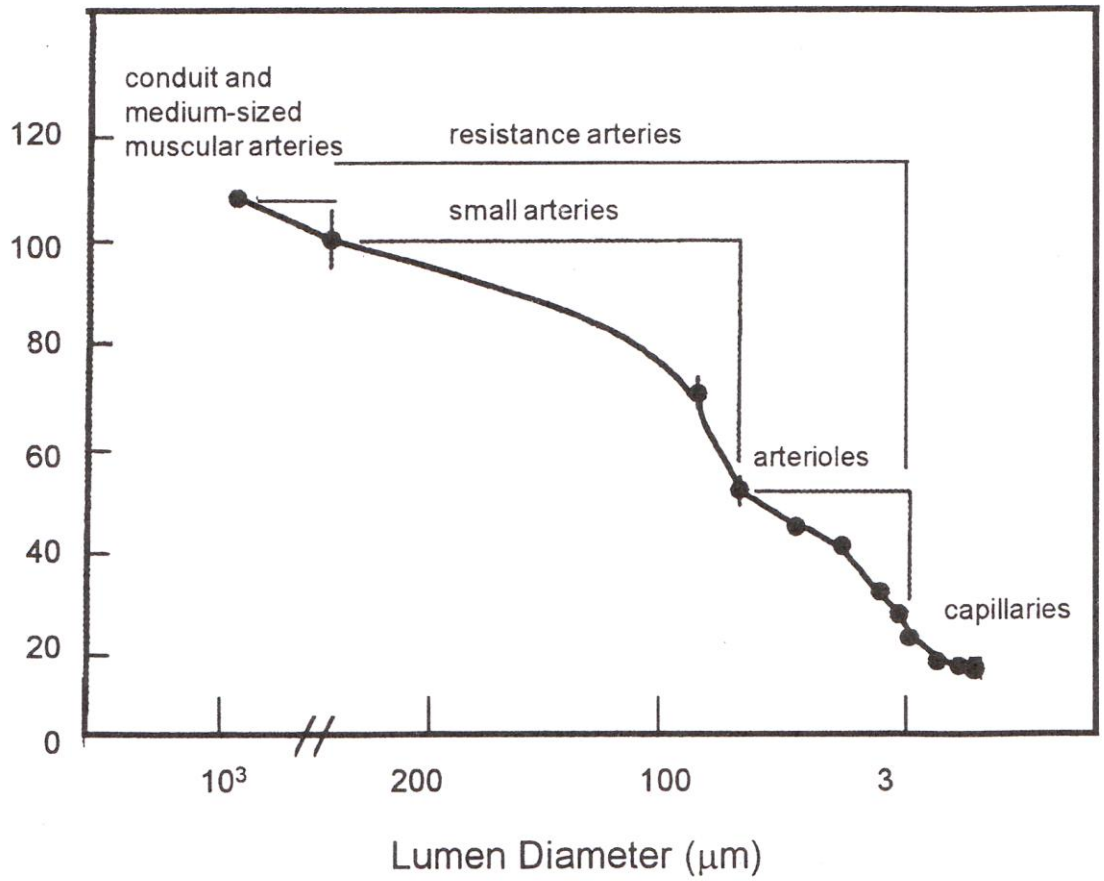

Figure 1. The vascular tree. As blood flow passes from large vessels into the smaller vessels, pressure gradually decreases as the force generated from cardiac output dissipates into the walls of smaller vessels. The majority of resistance to blood flow is generated at the small arteries and arterioles (resistance arteries). By the time blood flow reaches end organs and capillaries, blood pressure (y-axis) is virtually negligible. This figure is modified from Messerli *et al*. 2000 (*20*).

1.4 Resistance arteries

By definition, resistance arteries have lumen diameters between 100 and 500 μm in diameter (*21*). Arterial wall tension is indicative of pulse pressure, which is the difference between systolic and diastolic pressures. Cardiac output largely determines systolic blood pressure, whereas total peripheral resistance largely determines the level of diastolic blood pressure. Microcirculation (i.e. blood flow through the arterioles, capillaries and resistance arteries) is dependent on a number of factors including blood viscosity, elasticity of aortic and arterial walls as well as lumen diameter.

1.5 Classifications of hypertension

For treatment and management of hypertension, the Seventh Joint National Committee on the Detection, Evaluation, and Treatment of High Blood Pressure (JNC-VII) was formed. The JNC-VII has classified hypertension based on measured blood pressures at various stages. Currently, systolic blood pressure is used as a very strong marker to gauge prognosis in cardiovascular disease (*22*). At very high blood pressure levels, acute target-organ injury may exist. The target organ effects of hypertension are particularly manifest in the brain, eyes, heart, kidneys, and arteries in the periphery. Examples of target-organ injury include encephalopathy, intracranial haemorrhage, acute left ventricular failure with pulmonary edema, dissecting aortic aneurysm, unstable angina and eclampsia or severe pregnancy-associated hypertension. Hypertension severity is classified accordingly (Table 2); adults with a diastolic blood pressure of less than 90 mm Hg and systolic blood pressure value of 140 mm Hg or over are classified as having *isolated systolic hypertension*. This condition is believed to be caused by age-related pathophysiological changes in arterial vasculature (*23*); *prehypertension* is the classification

given when blood pressure is elevated above the optimal range, but is not considered hypertensive. Systolic blood pressure levels for normal to high normal individuals are between levels of 120 to 139 mm Hg, with a diastolic pressure between 80 to 89 mm Hg. Once blood pressure levels exceed 140/90, an individual is classified as *hypertensive*. Beyond these levels, a hypertensive individual is grouped into one of three categories or stages depending on resting blood pressure. Incidents of prolonged, untreated hypertension with signs of established target-organ injury are termed *chronic hypertension*.

Category	**Systolic Blood Pressure (mm Hg)**	**Diastolic Blood Pressure (mm Hg)**	**Percent of US Population (1995)**
Optimal	<120	<80	47%
Normal	<130	<85	21%
High normal	130-139	85-89	13%
Stage 1 Hypertension	140-159	90-99	14%
Stage 2 Hypertension	160-179	100-109	4%
Stage 3 Hypertension	≥180	≥110	1%

Table 2. Classification of blood pressure for adults (*24*). Stage four of hypertension may be categorized as systolic blood pressure levels in excess of 210 mm Hg and diastolic pressures above 120 mm Hg.

2. Properties of an Artery

2.1 Vessel geometry and mechanics

Lumen diameter moderates resistance generated by a vessel to blood flow, thus the importance of vessel morphology. In the context of hypertension, a small decrease in lumen diameter of resistance arteries from one arterial bed can increase total peripheral resistance, thereby increasing blood pressure. Conversely, increases in lumen diameter lower total peripheral resistance and might reduce blood pressure. Though there are many factors that might influence lumen diameter, vascular structure and stiffness are the foci of this thesis.

Vascular structure or geometry refers to the physical dimensions of an artery (Figure 2). Important parameters for assessment include lumen diameter, media thickness, media cross-sectional area and media-to-lumen ratio.

Stiffness refers to the ability of a vessel to stretch or distend (Figure 2). Geometry-dependent stiffness describes vessel adaptation affecting distension capacity or stretch caused by alterations in vessel geometry. Conversely, geometry-independent stiffness relates to stiffness caused by the composition of the vessel wall such connective tissue, endothelial cell layering, collagen and elastin content and smooth muscle cell hypertrophy or hypotrophy.

Important parameters affecting lumen diameter can be studied in a controlled setting using specific laboratory techniques. For instance, stiffness can be measured by analysis of mechanical properties such as elasticity. Elasticity is the ability of an artery to stretch in response to pulse and blood volume, this impacts arterial compliance.

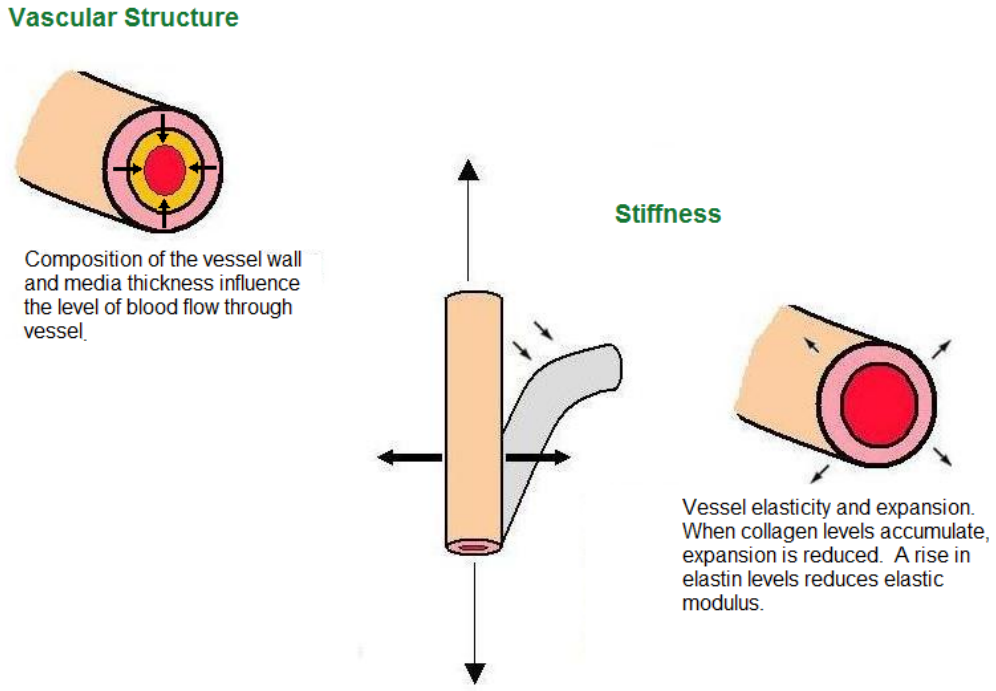

Figure 2. Vessel geometry and expansion. Morphological adaptations to flow within a vessel result in remodeling over time, with changes to both the vessel geometry and composition. As collagen and elastin ratios change within the vessel, the stiffness parameters are affected, resulting in altered blood flow through vessel lumen.

Compliance is the ability of an artery to withstand changes in blood volume and added pressure. The elastic lamina and the smooth muscle layers of an artery contribute to the lowered compliance seen in arteries as compared to veins.

Chronic hypertension can lead to abnormal compliance in the arteries under basal conditions (*25-26*). Adaptive responses vary across different arterial beds. For instance, mesenteric resistance vessels in spontaneously hypertensive rats (SHRs) initially display reduced stiffness due to increased elastin content of the vessel walls (*27*); over time, there is a steady increase in stiffness while the composition of the vessel wall adapts, increasing collagen and decreasing elastin (*28*).

2.2 Tunica layering

The arterial wall is layered with three tunica: tunica intima, tunica media and the tunica adventitia (Figure 3). These layers refer to the innermost layer (exposed to blood circulation), the middle and the outermost layers, respectively. The tunica intima consists of the endothelium and the internal elastic lamina. The internal elastic lamina plays a major role in determining vessel elasticity. In conditions such as atherosclerosis, calcification of the internal elastic lamina contributes to hardening of the artery (*29*). The tunica media consists of a smooth muscle layer, which functions to moderate arterial tone. Relaxation and constriction of the smooth muscle layer is influenced by crosstalk between the endothelial layer and local factors. Adjacent to the smooth muscle layer, there is an external layering of elastic lamina (the external elastic lamina). Similar to the internal elastic lamina, the external elastic lamina also contributes to the elastic properties of the artery. Finally, the tunica adventitia consists primarily of connective tissues (including for example collagen, fibronectin and fibroblasts etc.) which are involved in artery insulation, tissue repair and physical support.

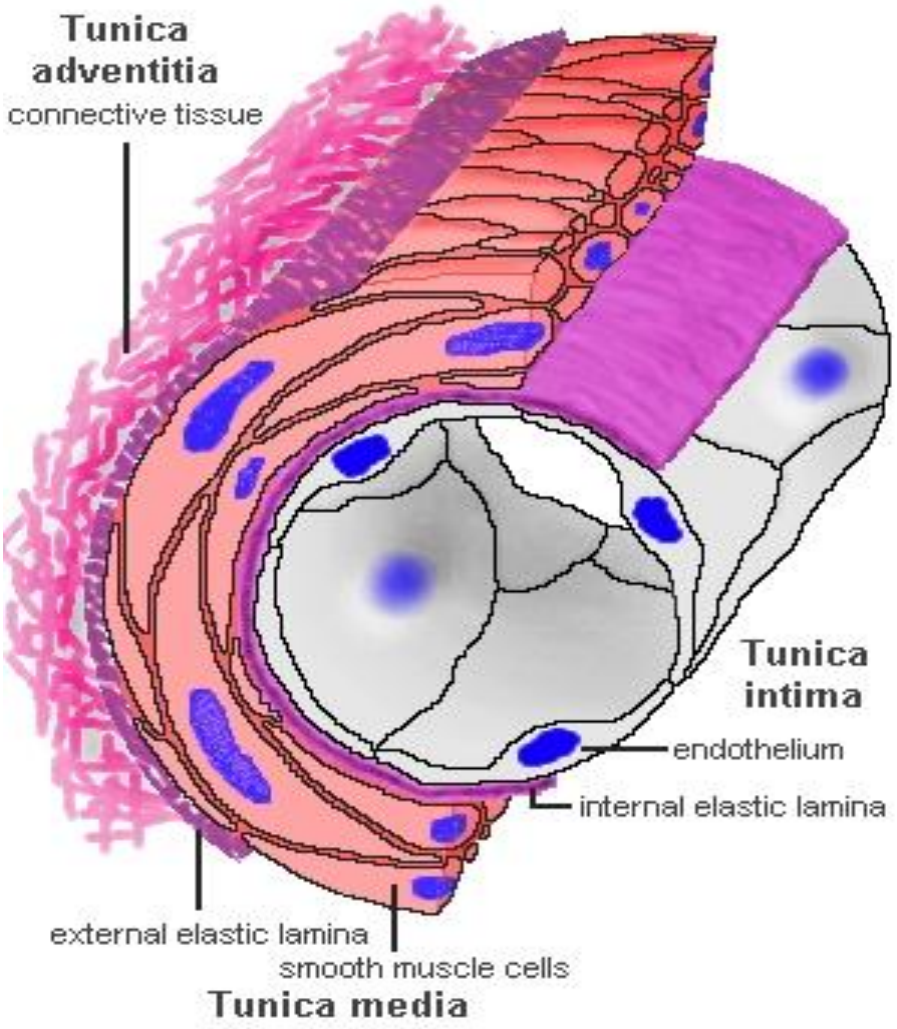

Figure 3. The layers of an artery.

(http://www.lab.anhb.uwa.edu.au/mb140/CorePages/Vascular/Vascular.htm Accessed: March 2009)

2.3 Arterial remodeling

Arterial remodeling is a multidirectional process. For example, *outward* remodeling refers to an increase in lumen diameter whereas *inward* remodeling is a decrease in lumen diameter (*30*). Other terminology utilizes the media cross-sectional area as the point of reference. Remodeling that involves an increase in the area occupied by the vessel media is called *hypertrophic* remodeling, whereas remodeling that yields a decreases in media cross-sectional area is termed *hypotrophic* remodeling. Finally, remodeling in which there is no change in media cross-sectional area is called *eutrophic* remodeling. The type of remodeling greatly affects the resistance generated to flow. For instance, compared to a normal arteriole, a vessel with eutrophic remodeling generates more resistance to blood flow upon smooth muscle contraction than a vessel that has undergone hypertrophic remodeling (Figure 4).

Some terms that describe remodeling refer to a combination of processes resulting from various effectors. For instance *hypertrophic inward remodeling* is a term used to describe a decrease in lumen diameter associated with change and growth of vessel wall material (Figure 5), a response more specific to increased pulse pressure than mean pressure. *Eutrophic inward remodeling* describes a decrease in lumen diameter without an accompanying growth in the area of the vessel wall (*31*). Human essential hypertension is characterized by eutrophic remodeling of small arteries with little evidence of hypertrophic remodeling (*32*).

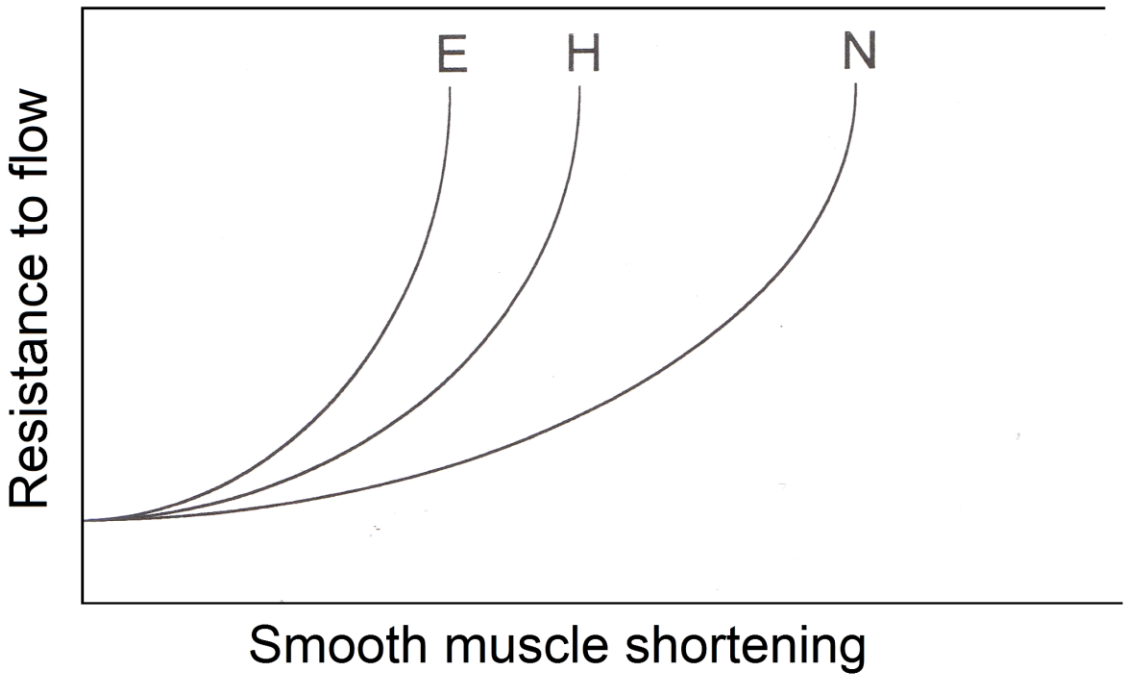

Figure 4. Remodeling and resistance. As remodeling occurs, the resistance generated to blood flow is increased in proportion to the media-to-lumen ratio of a vessel. The above diagram illustrates the effect of media-to-lumen ratio during contraction of smooth muscle in the vascular wall. Represented are vessels under eutrophic (E) and hypertrophic (H) remodeling and normotensive (N) vessels. This figure is modified from Messerli *et al*. 2000 (*20*).

The top row indicates the types of inward remodeling where lumen diameter is decreased. In the top left, there is a decrease in media cross-section as well as a decrease in lumen diameter. In the top right, there is an increase in media cross-sectional area as well as a decrease in lumen diameter.

The bottom row indicates the types of outward remodeling (lumen diameter is increased). In the bottom left, there is a decrease in media cross - sectional area with an increase in lumen diameter. In the bottom right, the artery undergoes an increase in media cross-sectional area and the lumen diameter is increased.

Remodeling can also occur without any change in lumen diameter, this is called *compensated remodeling.*

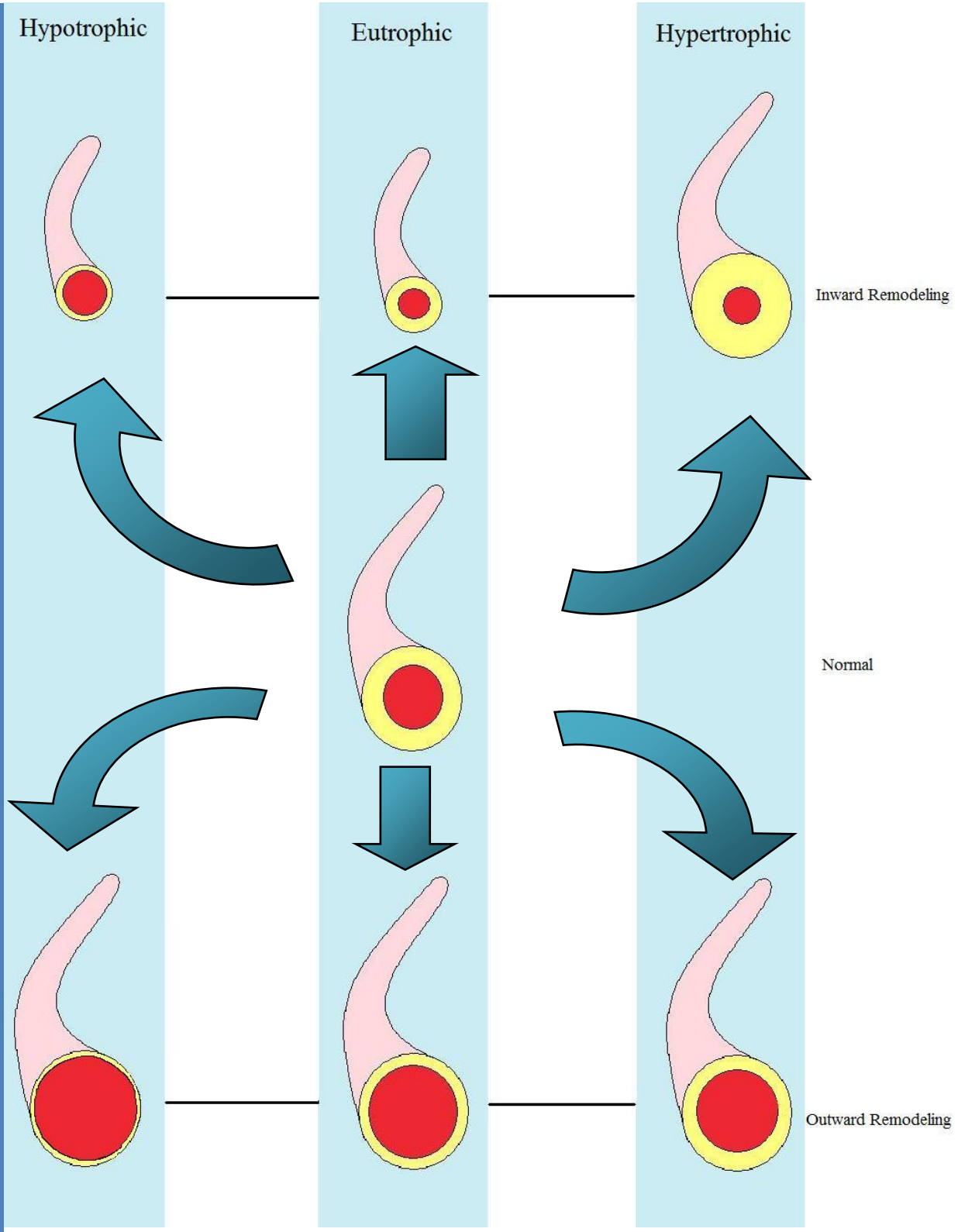

Figure 5. The various forms of arterial remodeling.

2.4 Determinants of resistance artery remodeling

Determinants of vessel remodeling include artery size, as well as type or location of the artery within the circulatory system. It is widely believed that arteriolar flow and hormonal levels largely influence the degree, mechanism and type of remodeling. Prolonged exposure to increased intraluminal pressures cause sustained wall stress, stimulating hypertrophic processes which cause increased media thickness over time (*33*). Some studies have indicated that increased arteriolar flow will also lead to increased media thickness (*34*). The specific mechanism for this process can be explained (to an extent) by endothelium-mediated vasodilation of the vessel in response to the increased flow, which causes increased wall stress (and in turn, thickening) (*33*).

Laplace's law (or Laplace relation) describes the relationship that exists between increased transmural pressure through the lumen of an artery to the tension, thickness and radius of the vessel wall. Tension is the result of the pressure difference that exists between intraluminal pressure and the surrounding pressure that exists outside the vessel. Increased intraluminal pressures will cause an increase in pressure difference resulting in added tension. Thickening the vessel wall will reduce tension, while increasing vessel radius further augments tension. Hence, Laplace relation is consistent with the remodeling that occurs within a vessel to reduce tension within the vessel wall in response to increased intramural pressure. As part of the Laplace relation, growth factors are believed to contribute to the hypertrophic processes within a vessel (*35*).

Processes surrounding eutrophic remodeling are less clear. However, it has been suggested that neurohumoral activity leading to increased vasoconstriction and blood pressure will, through the Laplace relation, lead to increased media thickness and reduced lumen diameters. This will insure that the wall stress remains normal, thereby eliminating hypertrophic

processes. Over time, it is suggested that the active vasoconstriction will turn into passive remodeling, as has been demonstrated *in vitro* (*36*).

In hypertension, increases in both cell size (hypertrophy) and cell number (hyperplasia) have been observed within the tunica media. However, the relative contribution of hyperplasia and hypertrophy in increasing muscle mass appears to vary depending on the location and nature of the hypertensive state (*37*). In the mesenteric vasculature of SHRs, increased media-to-lumen ratio is commonly associated with hyperplasia (*38-40*).

Flow-induced remodeling of mesenteric vasculature in SHRs has also been characterized. Mesenteric arteries from hypertensive and normotensive rats respond differently to increased blood flow. In healthy Wistar Kyoto rats, response to increased blood flow typically results in lumen enlargement with reduced smooth muscle cell apoptosis. In SHRs, the same stimulus results in an increase in media-to-lumen ratio with enhanced smooth muscle cell growth (*41*). It remains to be determined whether flow alteration is the initiating factor in the development of vascular remodeling in hypertension.

3. Resveratrol

3.1 Known cardiovascular effects of antioxidants

Trans-resveratrol is a potent polyphenol antioxidant. Antioxidants including vitamins (B6, C, E) thiols (lipoic acid and cystine) and the quinine enzyme Q10 have been shown to lower blood pressure in animal models and humans with essential hypertension (*42-43*). The antihypertensive effects of such antioxidants are possibly achieved by the reduction of conjugate/advanced glycation end product (AGE) formation as well as oxidative stress. Studies have shown that plasma AGEs levels are significantly higher in hypertensive individuals and that

these products greatly influence pulse pressure (and vascular stiffness) (*44-45*). In addition, a number of antioxidants have been shown to improve endothelial function, and normalize peripheral vascular resistance (*43*). Vitamins C and E have also been shown to prevent the development of hypertension in stroke prone SHRs, improve acetylcholine-induced vasodilation, and reduce media-to-lumen ratios of mesenteric vessels (*42*). These effects were achieved through modulation of enzyme systems that generate free radicals such as decreasing activation of vascular NADPH oxidase and increasing activation of superoxide dismutase (*42*). In studies of human LDL oxidation and ferric reducing/antioxidant power assays, resveratrol has been shown to have greater oxidative potency compared to vitamin E but less than vitamin C (*46-50*). While investigating the *in-vivo* and *in-vitro* antioxidant actions of resveratrol on endothelial health, Ungvari *et al*. showed that resveratrol treatment attenuated glucose-induced mitochondrial and cellular oxidative damage, inhibited vascular oxidative stress associated with impaired endothelial function in mice fed high-fat diet, improved acetylcholine-induced vasodilation, and inhibited apoptosis of femoral arterial branches in mice (*51*). In addition, inhibition of lipid oxidation, reduction of hydroperoxide formation, and scavenging of free radicals have also been identified (*52-53*).

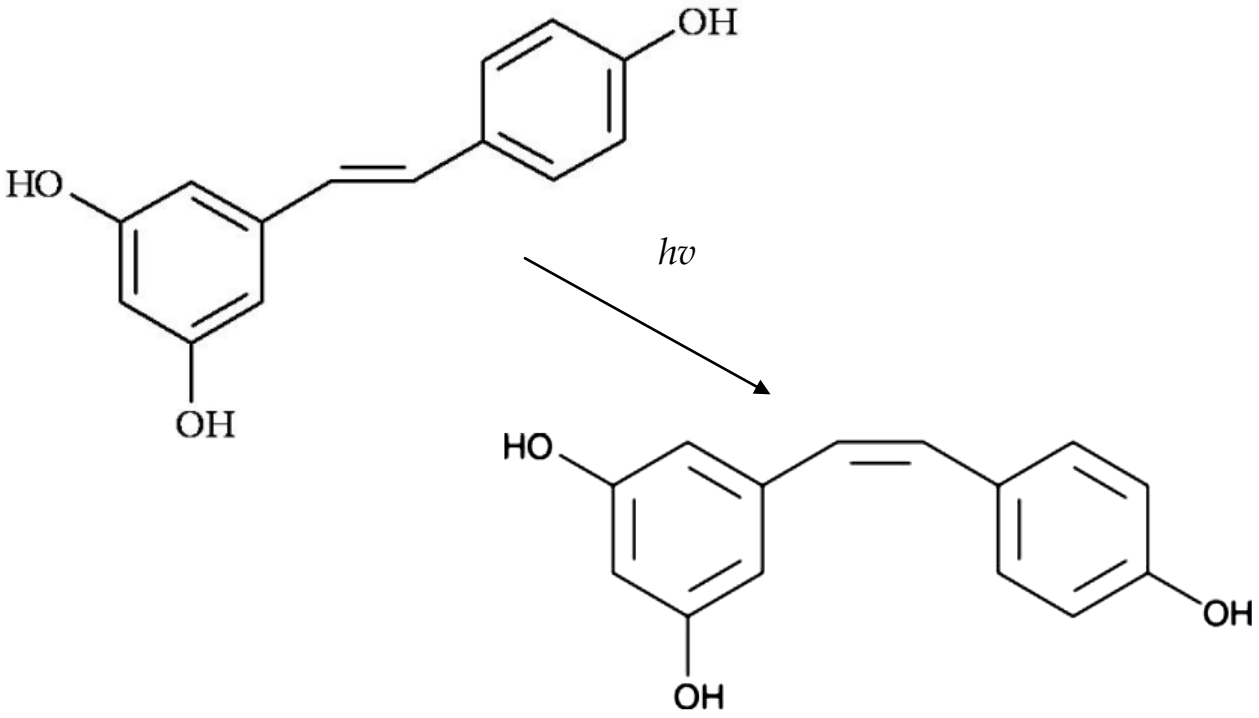

Figure 6. Resveratrol isomers. The chemical structure of resveratrol, a stilbenoid. *Trans*-resveratrol is made into the *cis* isomer through simple photoisomerization (with UV light). The *trans* isomer of resveratrol is the most bioavailable and abundant form of the compound.

3.2 Classification of resveratrol

Resveratrol is a naturally occurring phytoalexin, consisting of two hydroxylated benzene groups connected by a double bond (Figure 6). The structure of resveratrol is that of a stilbenoid, produced in plants though an enzyme called stilbene synthase. As a phytoalexin, resveratrol is produced as an antimicrobial substance by plants to defend against pathogen infection. The amount of resveratrol present in foods varies greatly. Non-muscadine red wines contain roughly 0.2 to 5.8 mg/L of resveratrol (*54*), whereas wines from muscadine grapes typically contain up to nine times higher concentrations of resveratrol (*55*).

Functional foods are classified as foods with health benefits beyond basic nutrition due to specific physiologically active components, which may or may not be manipulated or modified for enhancement of bioactivity (*56*). According to Canada's Ministry of Health, resveratrol falls into the category of a natural health product (*57*). Natural health products, by definition are over-the-counter products which fall into two components: The first component is that of function, which relates to its use in preventing, restoring or promoting physical health and function. The second component is that of substance, indicating the medicinal component of the natural health product (*58*). Resveratrol is also sold as a nutraceutical. Nutraceuticals are, by definition:

"...any substance that is a food or a part of a food and provides medical or health benefits, including the prevention and treatment of disease. Such products may range from isolated nutrients, dietary supplements and specific diets to genetically engineered designer foods, herbal products, and processed foods such as cereals, soups and beverages (*58*) *."*

Resveratrol is found in lesser quantities in at least 72 plant species (*59-60*) and foods including peanuts, Itadori tea, legumes, mulberries, ACAI berries, wild Indian gooseberries,

raspberries, cranberries, blueberries, bilberries (*61-62*). Cooking foods significantly reduces active resveratrol content by up to 50% (*63*).

3.3 Isomers of resveratrol

Resveratrol exists in *cis* [(Z)-RESV] and *trans* isomers [(E)-RESV], differentiated by the double bond that exists in between the two phenolic groups (*64-66*). The *trans* isomer is most abundant in the skin of grapes, especially red grapes and is also present in the seed and leaf epidermis. It undergoes isomerisation into the *cis* form when exposed to ultraviolet radiation.

The *trans* isomer was identified by Michio Takaoka in 1940 in the resin of *Veratrum grandiflorum* (false hellebore) (*67*). In 1963, Nonomura *et al*. detected resveratrol in dried Japanese Knotweed root (*Polygonum cuspidatum*), a common remedy used in Japanese and Chinese folk medicine (called Ko-jo-kon or Itadori) to treat numerous ailments including, suppurative dermatitis, gonorrhoea favus, hyperlipemia, athlete's foot (tinea pedis), atherosclerosis, allergic and inflammatory diseases (*59, 64, 66, 68*). The first investigation of resveratrol in wine was conducted by Langcake and Pryce in 1976, in which resveratrol was detected in grapevines and found to be synthesized in the leaf tissues in response to fungal infection or exposure to ultraviolet light (*69*).

3.4 Non-cardiovascular effects of resveratrol

Through *in vitro*, *in vivo*, and *ex vivo* experiments, the *trans* isomer has been shown to have a wide variety of beneficial biological effects which include anti-carcinogenic properties (*69-70*), anti-inflammatory properties in respiratory diseases such as chronic obstructive pulmonary disease (*71*) and protective effects against neurodegenerative diseases such as

Alzheimer's disease (*64*). Recent findings show that resveratrol increases the life span of *Saccharomyces cerevisiae* (yeast) (*72*), *Caenorhabditis elegans* (roundworm) (*73*), *Drosophila melanogaster* (fruit fly) (*74*), *Northobranchious furzeri* (fish) (*75*) and high fat-fed C57BL/6NIA "black 6" mice (*76*). However, resveratrol was found only to improve health, not longevity, in aging mice fed a standard diet (*77*).

3.5 Cardiovascular effects of resveratrol – The French Paradox

In the early 1990's a large epidemiological study investigated the low incidence of cardiovascular disease in the areas of southern France and the Mediterranean. The term "French Paradox" was coined to describe the low incidence of coronary heart disease in the area despite the population trend for increased dietary saturated fats, increased consumption of alcohol and wines (especially red wines), widespread smoking and sedentary lifestyle (*78*). Independently, Siemann and Creasy were studying the presence and effects of resveratrol in red wines (*78-79*). Around this time, reports began to emerge suggesting that the protective effects of red wine consumption are independent of alcohol content (*79*). A number of studies attempted to identify the cardioprotective constituents of red wine. Focus turned to resveratrol as one such candidate responsible for the beneficial effects.

Resveratrol has been linked to a number of cardiovascular-related health benefits across numerous models. Some of the most recent findings in SHR include protection against atherosclerosis and coronary heart disease (*80*), inhibition of collagen synthesis and smooth muscle cell proliferation by AGEs in stroke-prone SHRs (*81*), improvement in endothelial cell function (*82*), prevention of hypertrophy and diastolic impairment (*83*), and protection from pathological cardiac hypertrophy and contractile dysfunction (*84*). In addition, a number of studies have previously shown that resveratrol exerts inhibitory effects on platelet aggregation

(*85-87*), anti-inflammatory effects (*88*), as well as vasorelaxatory effects associated with upregulation of NO synthase (*89*). Furthermore, resveratrol has shown protective effects on reactive oxygen species-induced cardiomyocyte cell death *in vitro* (*90*).

CHAPTER II

STUDY PHASE ONE:

VASCULAR MORPHOLOGY

AND

MECHANICAL PROPERTIES

1. Essential Hypertension – A Rat Model

1.1 General information

Increased peripheral resistance to blood flow is a prominent hallmark of essential hypertension (*91*). Resistance is generated mostly by the transfer of force from cardiac output to the smaller arteries and arterioles in the periphery. As a result of this transfer, the energy generated by a single cardiac cycle is greatly dissipated by the time blood reaches the endpoints of the arterial bed (*92*). According to Poiseuille's law, resistance to flow is inversely related to the fourth power of the vessel radius. Accordingly, a small decrease in the lumen diameter of arterioles and small arteries will increase total resistance. Resistance arteries therefore play a key role in the regulation of blood pressure. Changes that result in decreased lumen diameter may be due to structural, functional or mechanical changes in the arteries. The general size range of an artery greatly affects the type of adaptive changes that will occur (*93*).

1.2 Spontaneously Hypertensive Rats

In 1963, Okamoto and Aoki developed a rat model of genetic hypertension by breeding a hypertensive, male Wistar Kyoto rat with a female Wistar Kyoto rat with higher than average blood pressure. Selective brother and sister mating of the offspring with elevated blood pressure were conducted until a full strain of hypertensive Wistar Kyoto rats was created. This strain of hypertensive rats was named the Spontaneously Hypertensive Rat (SHR) (*94-95*). The SHR is the closest known animal model to human essential hypertension (*96*). The SHR model has been shown to display most of the characteristics of human essential hypertension, as well as the various pathogenic mechanisms linked to the establishment of increased blood pressure (*96-100*).

SHRs are commonly used as a model of essential hypertension to study antihypertensive drugs to further understand the aetiology of hypertension (*101*).

1.3 SHR- Pathogenesis of hypertension

SHRs begin to show early signs of hypertension with pathological signs of disease such as increased heart weight, starting at 10 weeks of age (*101*). Blood pressure levels begin to rise as early as 4 weeks, eventually reaching levels greater than 200 mm Hg in mature males and 175 mm Hg in mature females (*101*). Female SHRs live significantly longer than males (*102*). SHRs develop spontaneous hypertension by their 7-15th week without exception (*98*). As well, SHRs develop increased peripheral vascular resistance, which plays a central role in the maintenance of SHRs hypertension (*103-104*). The genetic basis of hypertension in the SHRs is polygenic, meaning many genes are associated with the condition. Early studies identified at least three genes contributing to the condition (*105-106*), but over the years many new genes have been implicated.

2. Rationale and Hypothesis

Resveratrol has been shown to induce vasorelaxation of mesenteric resistance arteries in guinea-pigs (*107*) and in male Wistar rats (*108*). Moreover, resveratrol inhibits vascular smooth muscle cell proliferation, DNA synthesis, and collagen synthesis in stroke-prone SHRs, *in vitro* (*81*). Despite these protective vascular actions, resveratrol failed to reverse established hypertension in SHR (*82*). To our knowledge, no study has investigated the protective nature of resveratrol on resistance arteries prior to the onset of chronic hypertension.

The specific aim of this study was to investigate the preventative potential of a pharmacological dose of resveratrol in SHR. Since structural and mechanical aberrations typically follow prolonged periods of high blood pressure (*109*), the query was posed whether resveratrol might exert protective effects on resistance arteries and thwart the development of elevated blood pressure.

To achieve this specific aim, an *in vivo* study was designed to test the following hypothesis: Chronic treatment with resveratrol would prevent:

a. the development of elevated blood pressure in SHR.
b. the development of abnormal vascular geometry and wall stiffness in resistance arteries from SHR.

3. Experimental Design and Methodology

3.1 Animals

This study was conducted in accordance to recommendations from the Animal Care Committee of the University of Manitoba and the Canadian Council of Animal Care. Eight-week old male SHR and Wistar-Kyoto (WKY) rats were obtained from Charles River, housed under a 12-hour light/dark cycle at 22°C and 60% humidity and offered standard rat chow and water *ad libitum* (Figure 7). After a 7-day acclimatization period, rats were then trained for 5 days using a tail plethysmography apparatus (CODA Non-Invasive Blood Pressure System for Mice and Rats, Kent Scientific) for motionless restraint in a heated chamber (26°C) and blood pressure measurement. Systolic blood pressure was measured biweekly.

3.2 Treatments

As previously discussed, the aim of this study was to investigate the preventative potential of a pharmacological dose of resveratrol. Several pharmacological interventions such as L-ascorbic acid, alpha-tocopherol, coenzyme Q10, alpha-lipoic acid, stannous chloride, lovastatin and N-acetylcysteine attenuate the onset of or reduce high blood pressure and resistance artery abnormalities in SHR (*110-116*). Therefore, we determined whether resveratrol would be similarly effective.

As opposed to the dose of resveratrol one might receive through foods such as muscadine grapes or red wine, we focused on a pharmacological dose of 2.5 mg/Kg/d (Table 3). Admittedly, this dose is lower than that used in a number of *in vivo* studies (*117-118*). However, based on speculation of cardiac toxicity associated with such higher doses (T. Netticadan, oral communication) and the efficacy of a low dose of resveratrol to suppress cardiac hypertrophy (*83*), 2.5 mg/Kg/d was selected as the dose for this study.

Group	**Control (vehicle of 50% ethanol)**	**Resveratrol Treated (2.5 mg/Kg/d)**
Normotensive	WKY (n=8)	WKY (n=8)
Hypertensive	SHR (n=8)	SHR (n=8)

Table 3. Treatment paradigms. Each group consisted of sixteen male rats, subdivided into control and treated subgroups.

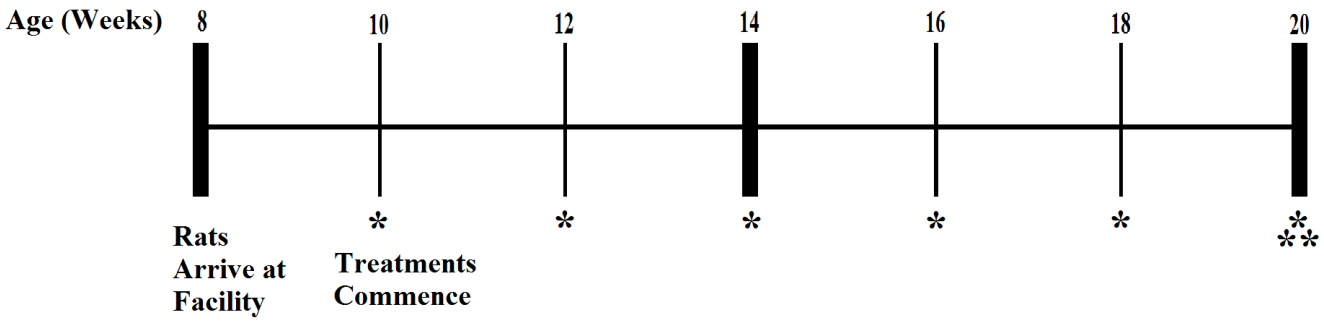

Figure 7. Study timeline. Treatments given from 10 to 20 weeks of age. At the 20th week, rats were sacrificed; mesenteric vasculature was extracted and studied.

3.3 Mesenteric arteries

At 20 weeks of age, rats were given an i.p. injection of 110 mg/Kg sodium pentobarbital then sacrificed by decapitation, and third-order mesenteric arteries (<500 µm) were extracted for study using pressure myography as previously described (*119*). The use of mesenteric resistance arteries was based on several factors. First, several studies have shown that essential hypertension is associated with resistance artery remodeling and dysfunction within the mesenteric arterial bed (*120-123*). Second, mesenteric arteries are first to receive blood flow following ejection from the left ventricle through the descending aorta (Figure 8). As a result, the calibre of resistance arteries from this region contributes to the degree of total peripheral

resistance (*40, 124*). Third, resistance arteries from the mesentery are easily accessible for study, with minimal branching. Resistance arteries must be quickly isolated with minimal time spent between animal sacrifice and equilibration in the Krebs solution to keep the vessel viable.

Successful pressure myograph experimentation requires the maintenance of constant luminal pressure which is confounded when there is branching within the mounted artery. Unsealed branches cause pressure leakage, which dissipates the constant intraluminal pressure required for accurate measurements using pressure myography.

In addition to minimal branching, mesenteric arteries are also lengthy and plentiful. In this study, third-order branches of arteries were used to ensure unbiased sampling for arteries used in pressure myography (Figure 9). The abundance of arteries in the mesenteric region also provided sufficient tissue for pressure myography and biochemical analysis for phase two of the study.

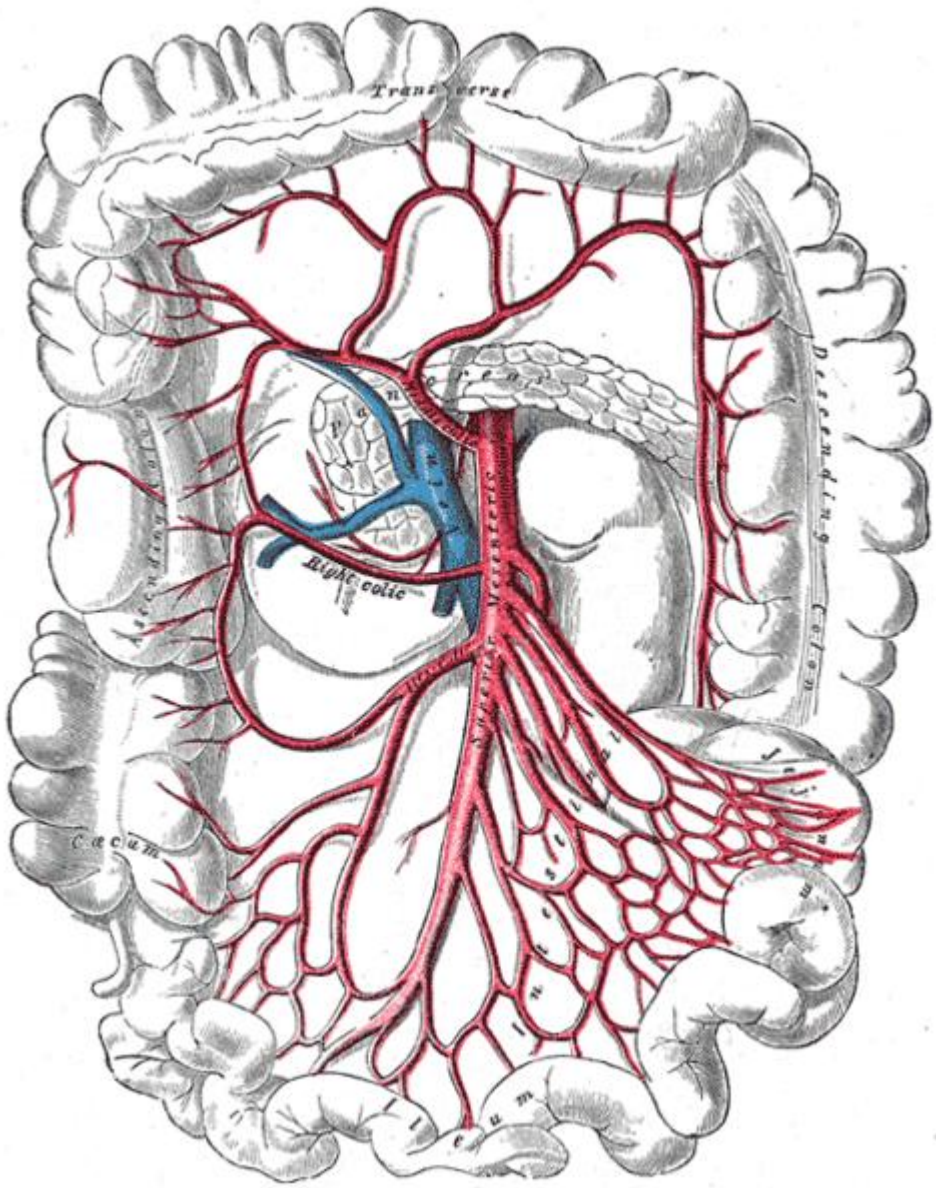

Figure 8. The mesenteric arterial bed. The superior mesenteric artery branches directly from the abdominal aorta to the intestinum. Figure illustrates branching of the mesenteric arterial bed.

(http://education.yahoo.com/reference/gray/subjects/subject/154#i534 Accessed February 2009). Copyright expired.

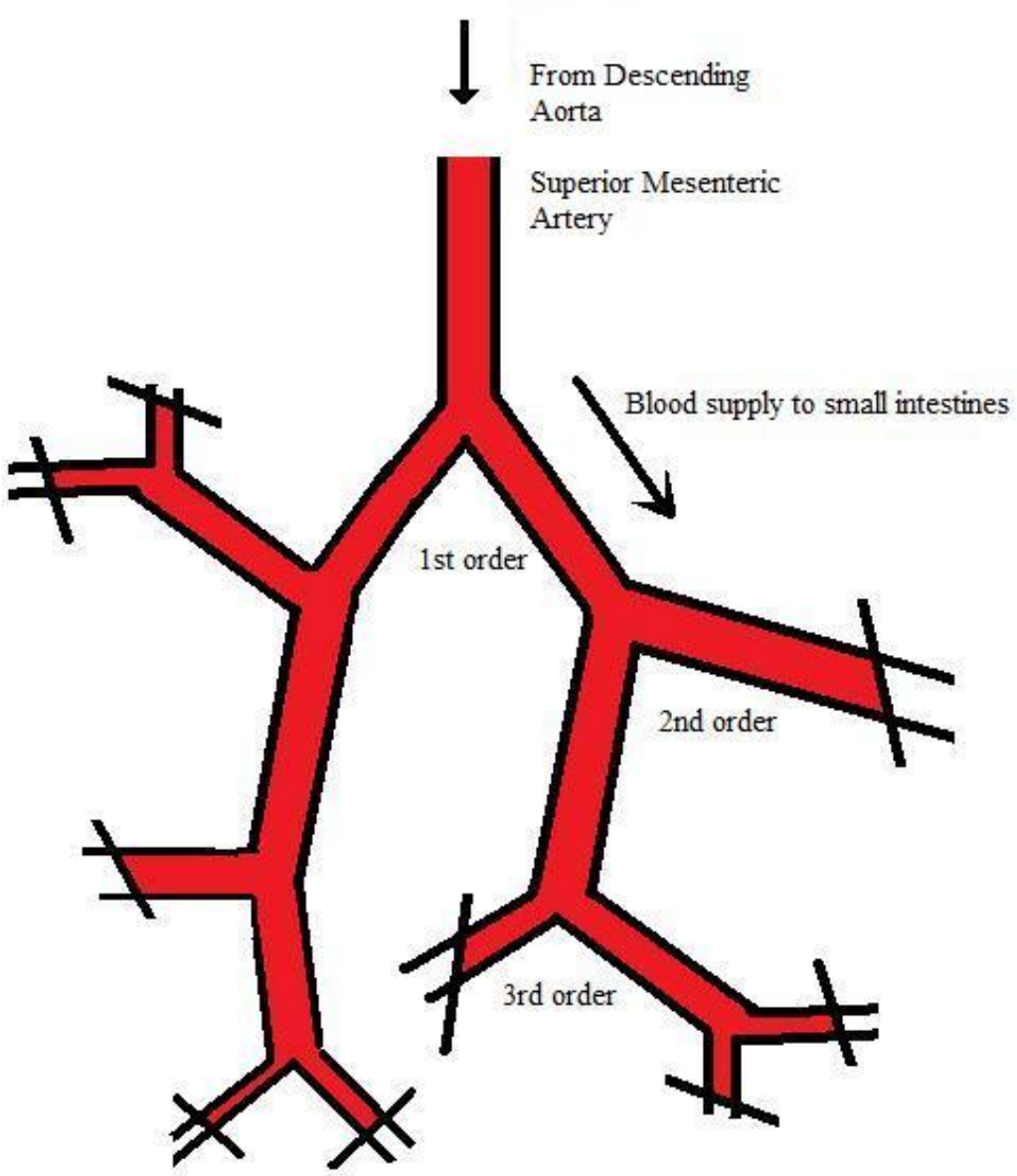

Figure 9. Identification of 3rd-order mesenteric arteries. Branching order ensures unbiased sampling of vessels used for experimentation.

3.4 *Pressure myography - Living Systems instrumentation*

Upon excision, the third-order resistance artery was mounted between two glass microcannulae in a pressure myograph chamber that was perfused with Krebs solution (Figure 10). The cannulae were then adjusted so that the vessel were parallel without stretch (*125*), magnified and projected onto a monitor to facilitate measurements of lumen diameter and media thickness. A constant intraluminal pressure of 45 mm Hg was established using a servo-controlled pump. The Krebs solution was maintained at a constant temperature of 37°C, and bubbled with 95% air and 5% CO_2 to achieve a pH of 7.4 to 7.45. As widely used in studies with resistance arteries from the mesentery, an optimal pressure of 45 mm Hg was selected as this pressure provides maximal resting lumen diameter while also allowing for maximal contraction (*126*).

After equilibration for 60 minutes with a constant flow of Krebs solution, the vessel was tested to ensure it was viable for study; a minimum contraction of 50% in lumen diameter to KCl (125 mmol/l) was necessary to determine viability of mounted vessel for study. Afterwards, the vessel was equilibrated again with constant flow of fresh Ca^{2+}-free Krebs solution containing 10 mmol/L EGTA for 30 minutes (prepared daily). This ensured that all myogenic tone was eliminated from the vessel prior to making dimension measurements. During this time an intraluminal pressure of 45 mm Hg was maintained within the mounted vessel.

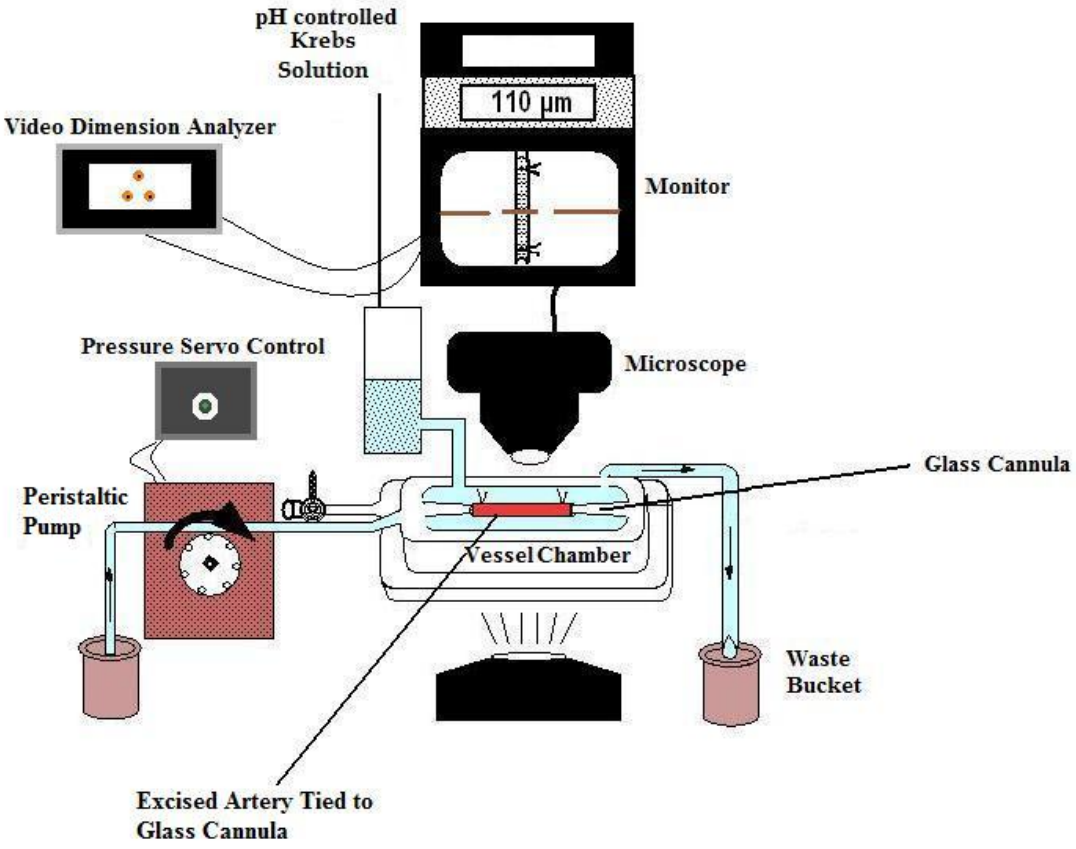

Figure 10. Pressure myograph instrumentation.

4. Vascular Measurements and Calculations

4.1 Vascular geometry

Measurements of the vessel dimensions included that of lumen diameter, external diameter, and media thickness for three different locations along the length of the artery (Figure 11). Averages of the three measurements were recorded for each pressure.

4.2 Growth and remodeling indices

Remodeling index is defined as the percentage of observed difference in lumen diameter between hypertensive and normotensive vessels which can be accounted for by remodeling of the normotensive vessel. It is calculated as $100 \times [(D_i)_n - (D_i)_{remodel}]/[(D_i)_n - (D_i)_h]$, where $(D_i)_n$ and $(D_i)_h$ are mean lumen diameters of normotensive and hypertensive vessels, respectively; and $(D_i)_{remodel}$ is $[(D_e)_h{}^2 - 4xCSA_n/\pi)]^{0.5}$, where $(D_e)_h$ is the mean external diameter of hypertensive vessels and CSA_n is the mean cross-sectional area of normotensive vessels (*127*).

Growth index is defined as the percentage of observed difference in lumen diameter in between hypertensive and normotensive vessels which can be accounted for by hypertrophy of the normotensive vessel. It is calculated as $(CSA_h - CSA_n)/CSA_n$ where CSA_n and CSA_h are mean media cross-sectional areas of normotensive and hypertensive vessels, respectively (*128*).

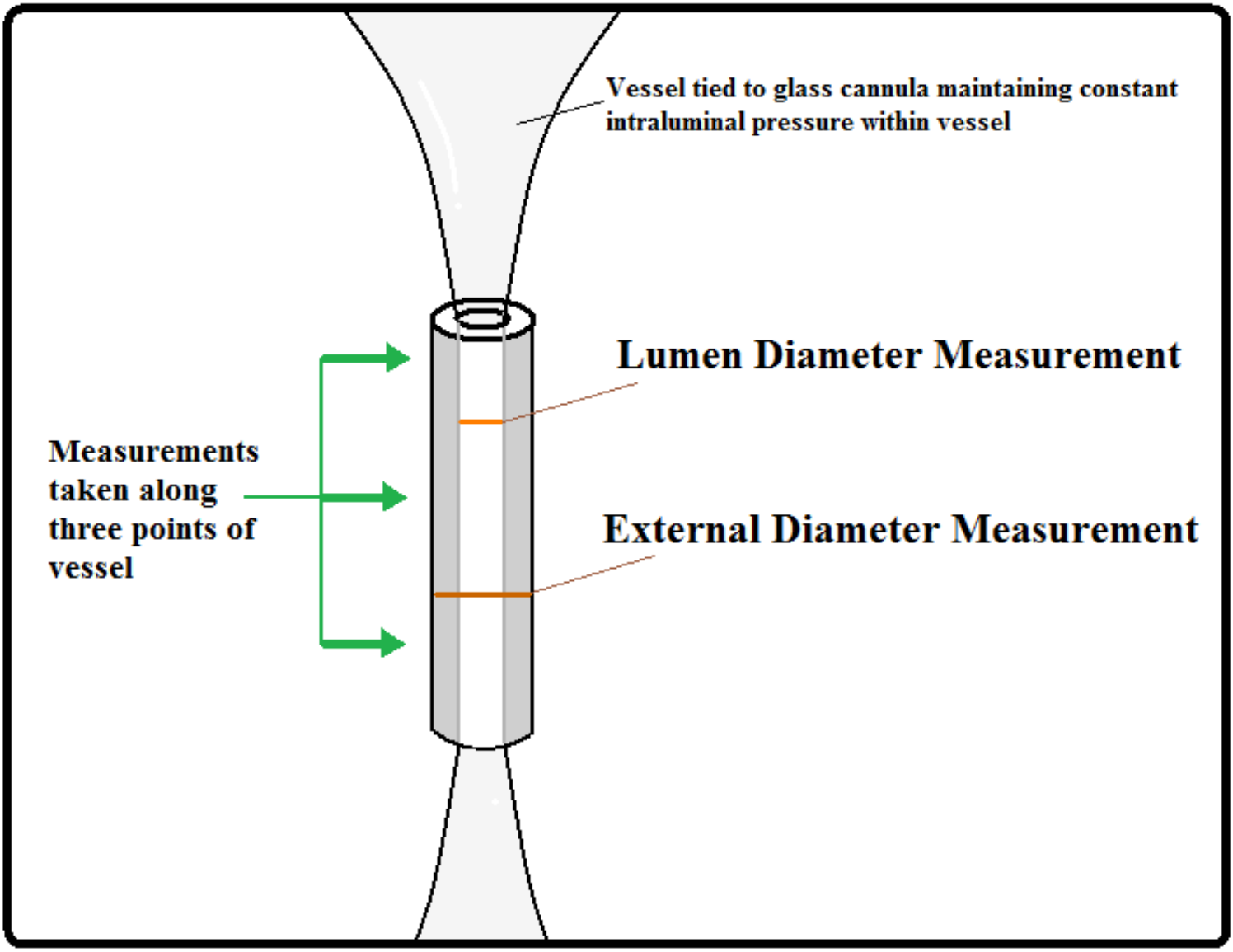

Figure 11. Vascular measurements made using the pressure myograph.

4.3 Mechanical properties

Mechanical properties were calculated from the vessel dimensions measured of 10 different intraluminal pressures (3, 10, 20, 30, 40, 60, 80, 100, 120 and 140 mmHg). Before the first measurement was made at 3 mm Hg, the intraluminal pressure within the vessel is gradually increased to 140 mm Hg. This is done three times and adjustments are made to the positioning of the cannula to ensure that vessels does not buckle during measurements (*27*). The precision of the system as determined with a micrometer was 0.7 μm. Once measurements are completed for all 10 pressure readings, calculations are made of the following parameters (Table 4) using these formulas:

Media cross-sectional area (CSA) is calculated by subtraction of the internal CSA from external CSA: media CSA = $\pi(D_e^2-D_i^2)/4$, where D_e is the external diameter, and D_i is the internal (lumen) diameter.

Circumferential stress, which reflects wall tension in the vessel wall, is calculated as $\sigma = (PD)/(2WT)$ where P is the intraluminal pressure, D and WT are the lumen diameter and media thickness, respectively. Pressure is converted from mm Hg to dynes per centimeter (1 mm Hg = 1.334×10^3 dyn/cm^2).

Circumferential 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 represents stress at the original vessel diameter and β is a constant related to the rate of increase of the stress-strain curve. The 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.

4.4 Statistical analysis:

Results are presented as means ± SEM. Statistical analysis was performed using ANOVA or 2-way ANOVA, followed by Bonferroni and Student-Newman Keuls post-tests to determine differences between groups, where appropriate. Statistical significance was considered as $p<0.05$.

Name	Formula	Symbols	Description
Cross-Sectional Area	$CSA = (\pi /4)*(D_e^2 - D_i^2)$	D_e = External Diameter D_i = Internal Diameter	Surface area perpendicular to the longitudinal length of the vessel.
Circumferential Stress	$\sigma = (PD)/(2M)$	P = Pressure D = Internal Diameter M = Average Wall Thickness	Resistance to pressure exerted along the circumference of an artery.
Incremental Distensibility	$ID = (1/\Delta P) * (\Delta D/D)*100$	ΔP = Change in Pressure ΔD = Change in Diameter D = Diameter	Ability to expand due to incremental increase in pressure exerted within artery.
Circumferential Strain	$\varepsilon = (D - D_0) /D_0$	D = Given Lumen Diameter D_0 = Lumen Diameter at 3 mmHg	Degree of stretch along the circumference of an artery due to pressure, normalized for baseline diameter.
Tangential Elastic Modulus	$ET = d\sigma/d\varepsilon = \beta\sigma_0 e^{\beta\varepsilon}$	β = Stress Strain Rate σ_0 = Initial Circumferential Stress ε = Circumferential Strain	Ability to resist deformation caused in response to pressure exerted along the circumference.
Media-to-lumen Ratio	M:L = (M/D)*100	M = Average Media D = Lumen Diameter	Relation comparison between arterial wall thickness and lumen diameter.
Stress-Strain Rate Constant	$\beta = \ln (\sigma/\sigma_0) * (1/\varepsilon)$	σ = Circumferential Stress σ_0 = Initial Circumferential Stress ε = Circumferential Strain	Slope constant of the natural logarithm taken from the stress-strain comparison generated at given pressure.

Table 4. Equations and abbreviations for mechanical properties.

5. Phase One Results

5.1 Systolic blood pressure

Over the duration of the study, systolic blood pressure rose in SHR to a significantly greater degree than in WKY rats (Table 5 and Figure 12; $p<0.05$). Resveratrol treatment did not affect blood pressure in either WKY rats or SHR. The baseline blood pressure measured at 10 weeks suggests a trend where the SHR group might have already developed elevated blood pressure compared to that of the normotensive WKY control group (Figure 12).

5.2 Vascular geometry

Remodeling and growth indices are indicative of eutrophic and hypertrophic forms of remodeling respectively. The untreated SHR arteries had a remodeling index of 78%, and a growth index of 20% (Figure 13). This suggests a greater degree of eutrophic remodeling in the artery with a lesser degree of hypertrophic remodeling. Resveratrol treatment reduced SHR remodeling index from 78% to 35%, suggesting that resveratrol affects processes leading to eutrophic remodeling despite not having significant effects on systolic blood pressure.

Table 5 indicates that the untreated SHR arteries exhibited reduced lumen diameters and increased media thickness. These parameters were improved with resveratrol treatment towards WKY levels. Figures 14 and 15 illustrate that under isobaric conditions, the untreated SHRs exhibited reduced lumen and external diameters (Figure 14 and 15; $p<0.05$). Compared to WKYs, SHR arteries had a reduced ability to expand and comply with increased intraluminal pressure. Again, resveratrol treatment restored isobaric lumen and external dimensions in the SHR to near WKY levels. No significant changes detected with treatment in Figures 16 and 17.

5.3 Stiffness

Compared to normotensive WKY, the SHR arteries had a much greater isobaric strain curve, indicating greater compliance in response to increased intraluminal pressures compared to that of the WKY arteries. Resveratrol treatment partially corrected the compliance of SHR vessels back towards normotensive control levels (Figure 18; $p<0.05$).

Circumferential stress corresponds to wall tension or distending force on the vessel wall. Figure 19 indicates that the untreated SHR arteries had the lowest circumferential stress levels in response to increases in intraluminal pressures. Resveratrol treatment normalized isobaric circumferential stress in the SHR arteries back toward control or untreated WKY group.

In Figure 20 circumferential stress is plotted against circumferential strain. This plot shows that the stress strain curve of the SHR has an extreme rightward shift compared to that of the normotensive control. This increased compliance in the SHR vessels is partially corrected with resveratrol treatment as noted by the leftward shift.

Figure 21 indicates that SHR vessels exhibited the lowest range of elastic modulus when exposed to increased intraluminal pressures. Resveratrol treatment caused a partial restorative shift in the slope of elastic modulus versus pressure of the SHR back towards the normotensive WKY arteries.

In Figure 22, elastic modulus is plotted against circumferential stress. Here, the slope of the SHR arteries is the lowest and most deviated compared to the normotensive WKY arteries and resveratrol treatment had almost no effect on the slope of the SHR. Thus, intrinsic stiffness of wall components was lower in SHR arteries compared to WKY vessels, and resveratrol treatment had virtually no affect on wall component stiffness.

6. Discussion of Phase One Results

Systolic blood pressure in SHRs begins to rise from 5 weeks of age until approximately 12 weeks (*98*). Throughout the 10 week period of the study, resveratrol had no effect on blood pressure in either strain. There are a few of possible explanations for this result. The selected dose of 2.5 mg/Kg/d of resveratrol may have not been sufficient to evoke a systemic response in the SHR to result in lowered blood pressures. Also, at the start of the study, the SHR group appears to have already developed a trend towards increased levels of systolic blood pressure as compared to the normotensive WKY group. Therefore, the age at which the resveratrol treatment was initiated may not have been early enough to produce any cardioprotective-effects which would prevent the onset of hypertension in the SHR. It is possible that adjusting a combination of dose, duration of treatment, and starting age (perhaps around 5 weeks?) might lead to a scenario where resveratrol can affect systolic blood pressure in the SHR group. There is also a possibility of a technical limitation in the use of tail-cuff plethysmography in assessing changes in blood pressure. This can to be investigated with future studies.

In explaining the results obtained from measurements of systolic blood pressure, the technical limitations in using tail-cuff plethysmography might also be explored. In some studies the use of tail-cuff plethysmography has yielded conflicting results in comparison to other more sensitive techniques such as telemetry (*129*). The difference in measurement sensitivity between the blood pressure measuring techniques was described by the Subcommittee of Professional and Public Education of the American Heart Association Council of High Blood Pressure Research (*130*). In addition, the use of tail-cuff plethysmography is a short time frame measurement of

blood pressure, yet this measure is generally taken to reflect the overall duration of experimentation. As well, the short time frame measurement of blood pressure includes restraint and thermal stressors for the animals being tested. Finally, using tail-cuff has been suggested as eliciting a pressor response (*131*). These factors suggest that tail-cuff plethysmography may not have detected some subtle but significant differences that may have existed between the treatment groups.

Figures 14 and 15 indicate that the mesenteric vasculatures of the SHR have compromised passive dilation as noted by reduced lumen and external diameters at increased intraluminal pressures. Resveratrol treatment restored lumen and external diameters in SHR to near WKY levels, thereby restoring passive dilation capacity in SHR. The restoration of passive dilation capacity can be mostly attributed to correction in vessel morphology. Figure 16 suggests that treatment had no significant effect on media-to-lumen ratio of SHR at high intraluminal pressures. As well, Figure 17 indicates no significant changes with treatment noted in media cross sectional area at increased pressures. The flattened nature of the curves in Figure 17 indicates incompressibility of material composition in the vessel walls.

Strain corresponds to the relative change in lumen diameter in response to increased intraluminal pressure, normalized to baseline lumen diameter. An upwards shift in the isobaric strain curve (Figure 18) is indicative of increased compliance. Resveratrol treatment corrected isobaric strain in the SHR back towards normotensive control levels (Figure 18; $p<0.05$). Thus, resveratrol led to alterations in vessel morphology and stiffness that potentially decrease the capacity of SHR vessels to withstand tension from increased intraluminal pressures, while attenuating changes that reduce tension at the cost of increased total peripheral resistance.

Circumferential stress corresponds to wall tension or distending force on the vessel wall. Figure 19 indicates that the SHR group had the lowest circumferential stress at all measured

intraluminal pressures. In chronic hypertension, such vessel adaptation would accommodate increased pressures in hypertensive rats. The untreated SHR vessels had adapted and remodelled in such a manner to alleviate the increased wall tension or distending force exerted along the vessel walls. Resveratrol treatment caused a normalizing shift in the isobaric circumferential stress curve of SHR toward the WKY control arteries, almost completely.

In Figure 20 circumferential stress is plotted against circumferential strain. Since circumferential strain represents relative change in lumen diameter normalized to baseline lumen dimensions, a plot of stress versus strain would indicate stiffness parameters of a vessel made independent of baseline lumen diameter. This plot removes the influence of vessel geometry, at least in terms of lumen diameter, on the ability of a vessel to react to added pressure. An extreme rightward shift in a stress-strain plot indicates decreased vessel stiffness and increased compliance, as seen in young SHR mesenteric vasculature (*27*). Figure 19 shows that in this study, the stress-strain curve of the SHR arteries displayed an extreme rightward shift, compared to that of the normotensive control. Increased compliance coupled with increased media thickness (Table 5) most certainly serves as protective adaptation of the SHR arteries, such that damage to the vessel wall that might be caused by increased intraluminal pressures might be minimized. Increased compliance in SHR vessels is partially reversed with resveratrol treatment as noted by the leftward shift. The partial reversal of the stress-strain curve of the SHR versus the almost complete normalization seen in the isobaric stress slope in SHRs suggests that the correction in stiffness is due mostly to the changes seen in vessel geometry (Table 5).

Elastic modulus is a measure of arterial elasticity and stiffness and is indicative first, of wall component stiffness from various wall components (elastin, fibronectin, collagen, etc.) and second, the influence of vessel geometry on stiffness. The plot of isobaric elastic modulus is affected by any adaptive mechanisms which protect the vessel at high intraluminal pressures

through rearrangement of vessel composition. Figure 21 indicates that SHR vessels exhibited the lowest levels of elastic modulus when exposed to increased intraluminal pressures. Resveratrol treatment caused a partial upward shift in the curve of elastic modulus versus pressure of the SHR back towards the normotensive WKY control group. A partial correction in the curve of elastic modulus suggests a limited effect of resveratrol on wall component stiffness.

In Figure 22, elastic modulus is plotted against circumferential stress. Plotted in this manner, vessel compliance is determined almost entirely by stiffness of wall components and not vessel geometry. This graph allows visualization of the relationship of vessel compliance with the tension placed along the vessel wall. As the tension along the vessel wall (along the x-axis) is increased, the stiffness of the wall components through its elastic modulus can be assessed. The plot of elastic modulus versus stress shows that the slope of the SHR is the lowest and most deviated compared to the normotensive WKY; and resveratrol treatment had almost no effect on the slope of the SHR curve. Thus, intrinsic stiffness of wall components was lower in SHRs compared to WKY vessels and resveratrol treatment had almost no affect on wall component stiffness. Figure 22 indicates that the restorative effect of resveratrol on vessel stiffness is due mostly to correction of vessel geometry.

Adaptive remodeling of resistance vessels is hypothesized as being one of the first sites of target organ damage due to increased intraluminal pressures from hypertension (*132-134*). In SHRs, early remodeling may be independent of high blood pressure (*20, 135-139*). In this study, resistance artery remodeling was mostly attenuated with resveratrol treatment despite no significant change in the systolic blood pressure of SHRs (Figure 12). These findings suggest that resveratrol acts directly on vessel walls to attenuate remodeling. They also led to speculation that the process of remodeling in SHRs may be independent of blood pressure, at least in early stages. A possible cause of early remodeling in SHRs, rather than elevated blood

pressure, may be the presence of oxidative damage along the vessel walls (*140*). As a potent antioxidant, resveratrol may protect against oxidative and inflammatory damage within the vessel walls and/or invoke signaling mechanisms that attenuate smooth muscle cell proliferation (*141*).

It is important to note that the results obtained from this study are not intended to support the validity of the purported contribution of resveratrol to the French Paradox phenomenon. This can be verified by comparing the resveratrol content of red wine with the dose used in this study. Red wines normally have an average resveratrol content of 1.14-1.98 mg/L (*142-143*). The current study uses 2.5 mg/Kg/d of resveratrol in the rat. As an average 90 Kg adult male would need to consume in excess of 100 litres of red wine per day in order to reach an equivalent dose of the resveratrol used in this study, these findings are not consistent with consumption of red wine as the source of resveratrol.

Parameter	WKY	WKY + Resveratrol	SHR	SHR + Resveratrol
systolic blood pressure, mm Hg	155 ± 6	153 ± 7	219 ± 6 **	211 ± 15 **
baseline lumen diameter, μm (measured at 3 mm Hg)	196.1 ± 33.8	186.6 ± 20.0	144.9 ± 46.2 *	174.4 ± 30.0
lumen diameter, μm	308.1 ± 14.2	335.5 ± 18.8	246.2 ± 21.0 *	292.5 ± 11.5
media thickness, μm	28.5 ± 2.7	25.6 ± 0.9	39.9 ± 4.6*	34.1 ± 1.6
media-to-lumen ratio, %	9.3 ± 1.0	8.8 ± 1.3	17.7 ± 2.6 **	11.8 ± 0.62 ‡
media cross-sectional area, μm^2	30262 ± 3497	32901 ± 3584	36447 ± 5929	35125 ± 2375
slope of elastic modulus vs. media stress	6.6 ± 0.5	5.3 ± 0.2 *	4.2 ± 0.4 **	4.5 ± 0.2 **

Table 5. Blood pressure & mesenteric resistance artery morphology and stiffness from SHR and WKY rats at 20 weeks of age. Values are mean ± SEM. Arterial parameters were measured in relaxed arteries at an intraluminal pressure of 45 mm Hg unless otherwise indicated. *$p<0.05$ and **$p<0.01$ vs. untreated WKY rats. ‡$p<0.01$ vs. untreated SHR.

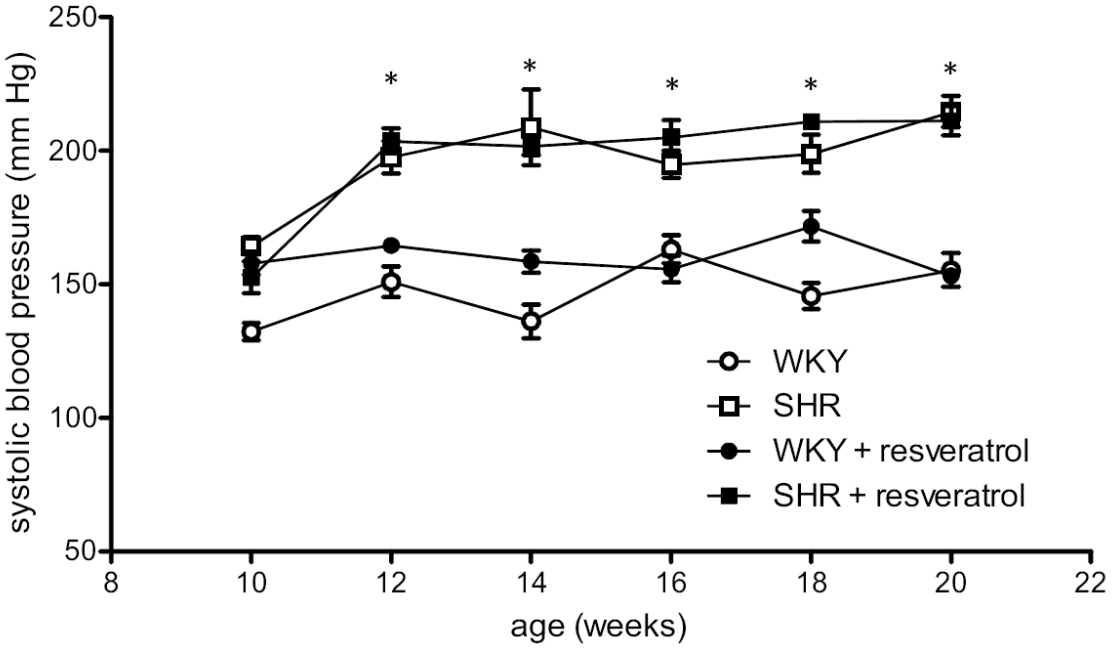

Figure 12. Systolic blood pressure levels. The above graph illustrates the effects of resveratrol on systolic blood pressure of rats throughout the 10 week study period treated with vehicle or resveratrol (2.5 mg/Kg/d). Measurements were taken biweekly by tail-cuff plethysmography. Data represent mean ± SEM; n = 5-8. *$p<0.05$.

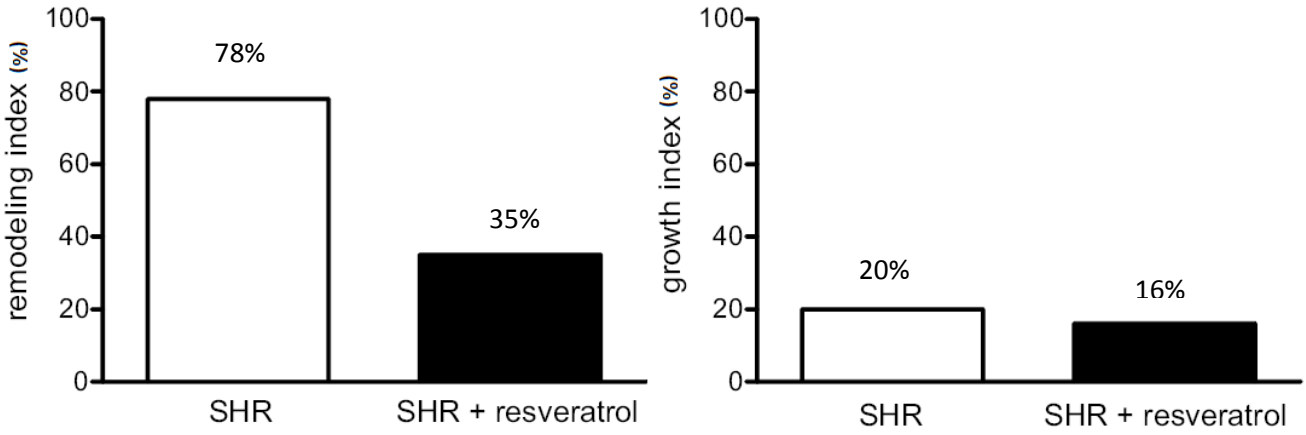

Figure 13. Effect of resveratrol on vascular remodeling indices in SHR. The above graph illustrates the remodeling and growth indices of mesenteric arteries from SHR, showing untreated SHR (open bars) and resveratrol-treated SHR (solid bars), compared to WKY at 20 weeks.

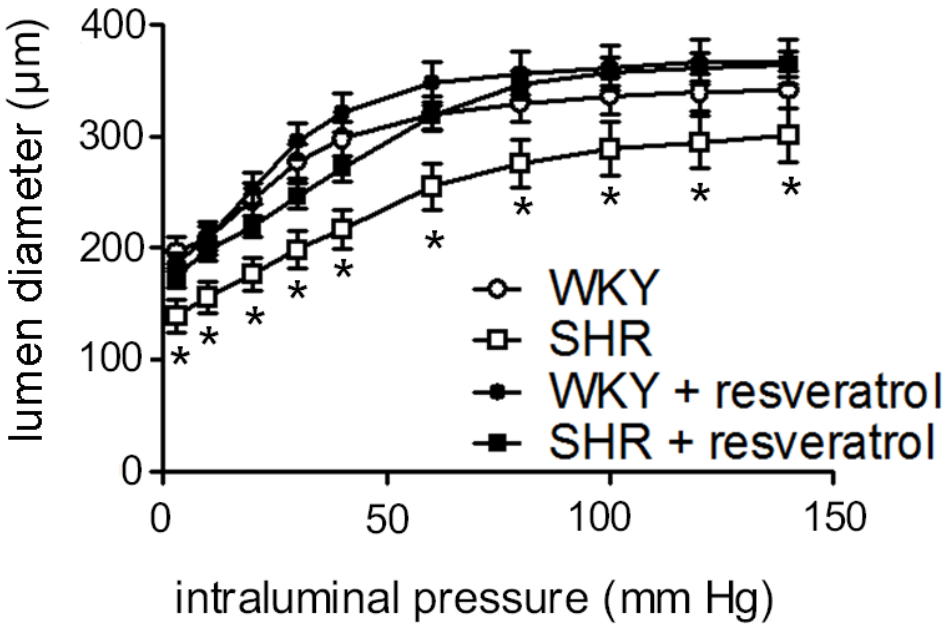

Figure 14. Effect of resveratrol on lumen diameter of relaxed mesenteric resistance arteries in 20-week old SHR. The above graph illustrates the effects of resveratrol treatment on lumen diameter in SHR and WKY, plotted against intraluminal pressure. Data represent mean ± SEM; n = 5-8. *$p<0.05$ versus untreated WKY.

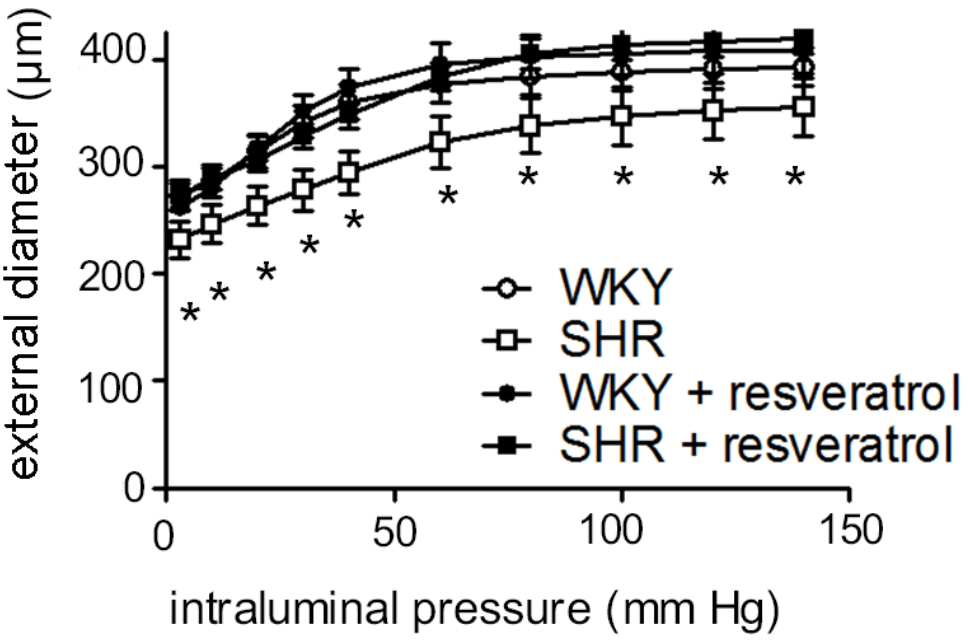

Figure 15. Effect of resveratrol on external diameter of relaxed mesenteric resistance arteries in 20-week old SHR. The above graph illustrates the effects of resveratrol treatment on external vessel diameter in SHR and WKY, plotted against intraluminal pressure. Data represent mean ± SEM; n = 5-8. *p<0.05 versus untreated WKY.

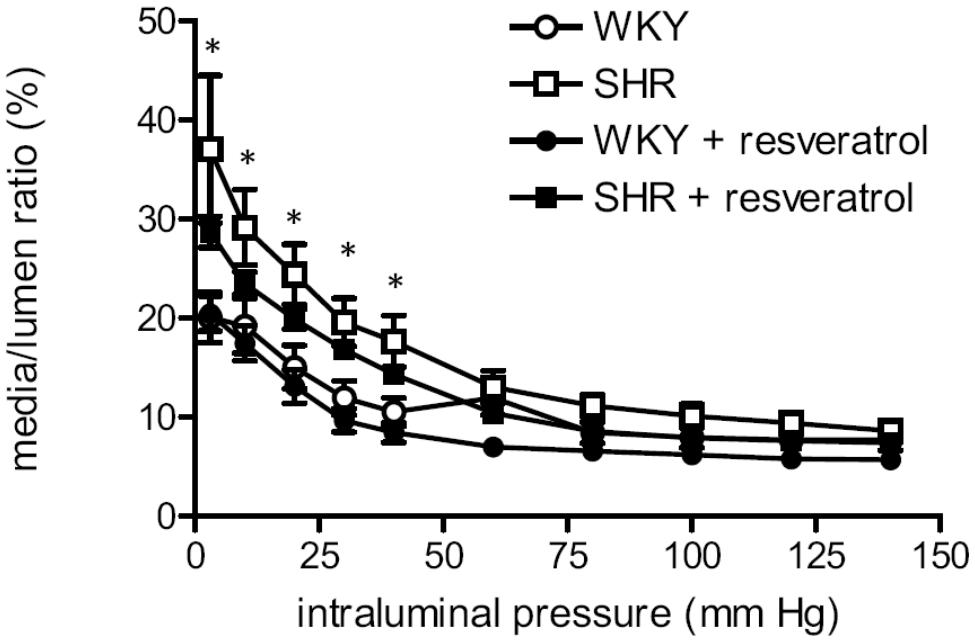

Figure 16. Effect of resveratrol on media-to-lumen ratio of relaxed mesenteric resistance arteries in 20-week old SHR. The above graph illustrates the effects of resveratrol treatment on the media-to-lumen ratio of SHR and WKY, plotted against intraluminal pressure. Data represent mean ± SEM; n = 5-8. *$p<0.05$ versus untreated WKY.

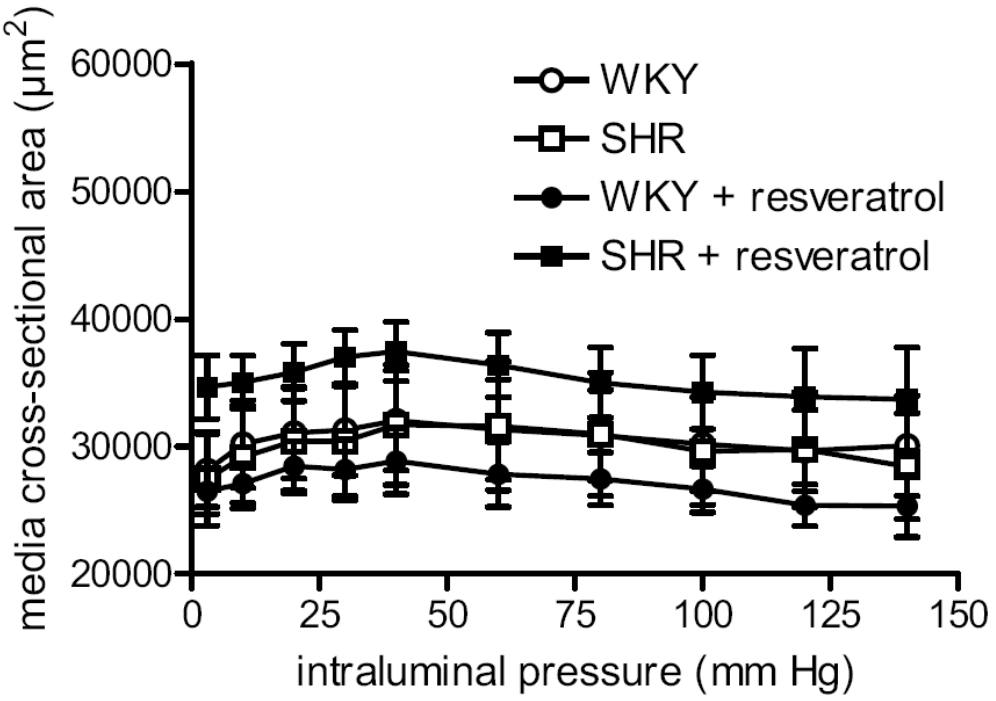

Figure 17. Effect of resveratrol on media cross-sectional area of relaxed mesenteric resistance arteries in 20-week old SHR. The above graph illustrates the effects of resveratrol treatment on the media cross-sectional area of SHR and WKY, plotted against intraluminal pressure. Data represent mean ± SEM; n = 5-8.

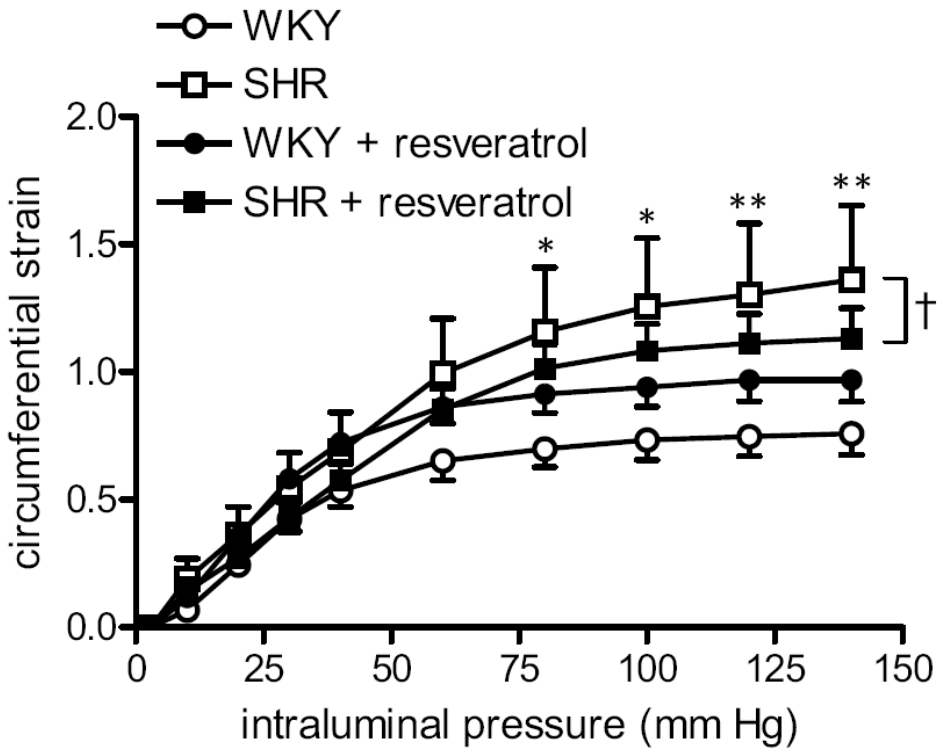

Figure 18. Effect of resveratrol on isobaric strain of relaxed mesenteric resistance arteries in 20-week old SHR. The above graph illustrates the effects of resveratrol treatment on the circumferential strain of SHR and WKY, plotted against intraluminal pressure. Data represent mean ± SEM; n = 5-8. *$p<0.05$ and **$p<0.01$ versus WKY; †$p<0.05$ versus untreated SHR.

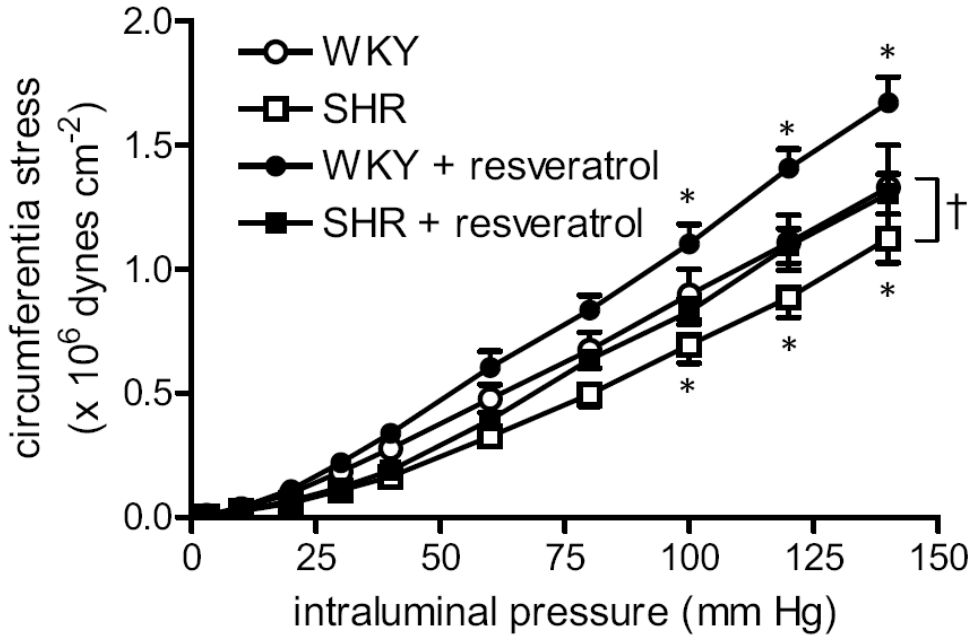

Figure 19. Effect of resveratrol on isobaric stress of relaxed mesenteric resistance arteries in 20-week old SHR. The above graph illustrates the effects of resveratrol treatment on the circumferential stress of SHR and WKY, plotted against intraluminal pressure. Data represent mean ± SEM; n = 5-8. *$p<0.05$ versus WKY; †$p<0.05$ versus untreated SHR.

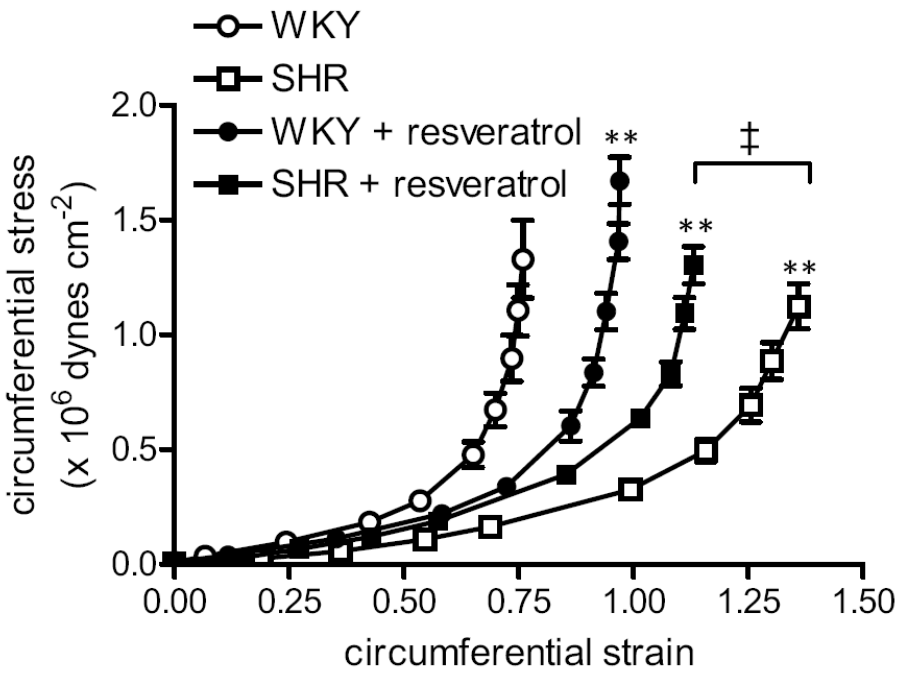

Figure 20. Effect of resveratrol on stress-strain curves of relaxed mesenteric resistance arteries in 20-week old SHR. The above graph illustrates the effects of resveratrol treatment on the circumferential stress of SHR and WKY, plotted against circumferential strain. Data represent mean ± SEM; n = 5-8. **p<0.01 versus WKY; ‡p<0.01 versus untreated SHR.

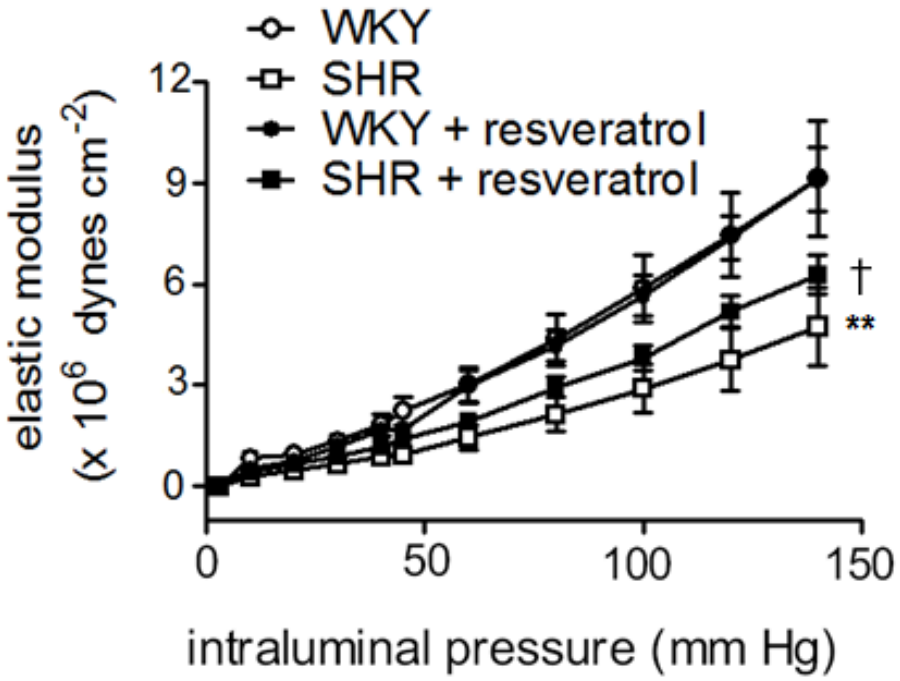

Figure 21. Effect of resveratrol on elastic modulus of resistance arteries in 20-week old SHR. The above graph illustrates the effects of resveratrol treatment on the elastic moduli of SHR and WKY, plotted against intraluminal pressure. Data represent mean ± SEM; n = 5-8. **p<0.01 versus WKY.

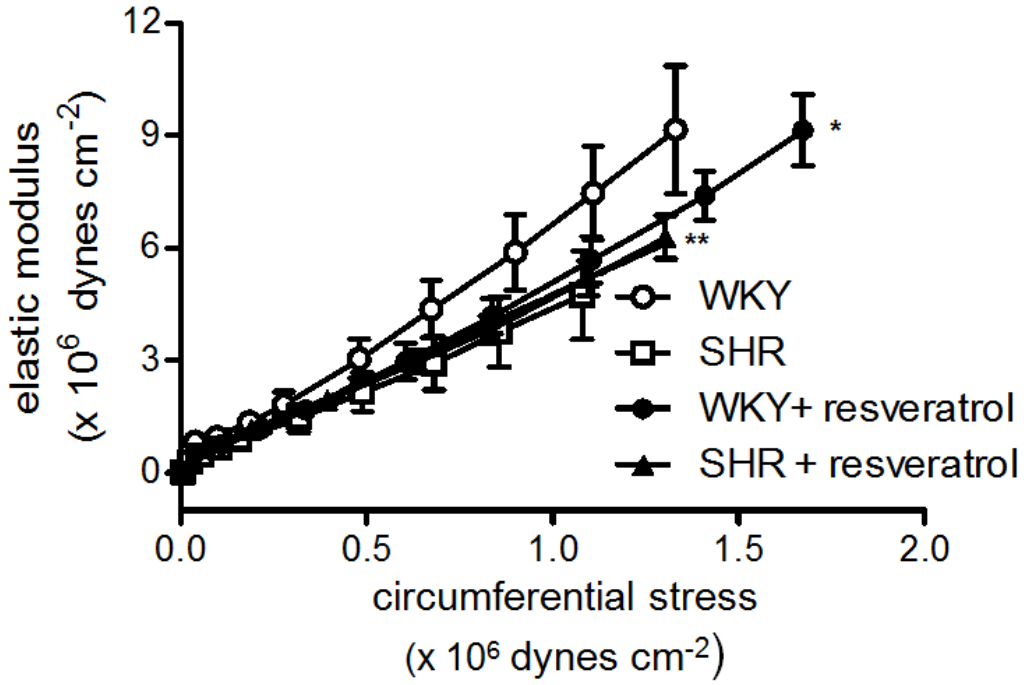

Figure 22. Effect of resveratrol on intrinsic stiffness of vessel wall from resistance arteries in 20-week old SHR. The above graph illustrates the effects of resveratrol treatment on the elastic moduli of SHR and WKY, plotted against circumferential stress. Data represent mean ± SEM; n = 5-8. *$p<0.05$ and **$p<0.01$ versus WKY.

CHAPTER III

STUDY PHASE TWO:

SIGNALING

1. Resveratrol Effect on Hypertension – Signaling

1.1 General information

A number of studies have shown that in the SHR, the renin-angiotensin-aldosterone system contributes to the establishment and maintenance of the hypertensive state. Studies have shown anti-hypertensive effects can be achieved in SHRs by a blockade of the renin-angiotensin system (*28*, *144-145*). In fact, a study by Kost *et al* showed that in the SHR, there seems to be hypersensitivity towards the effects of angiotensin II on renal arterial remodeling from SHRs compared to that of the normotensive WKYs (*146*).

1.2 Signaling pathway

The extracellular signal regulated kinases 1 and 2 (ERK1/2), are mitogen-activated protein kinases that are involved in the signaling pathways leading to cell proliferation and differentiation. Evidence has pointed to the key importance of ERK1/2 in the mediation of growth responses from angiotensin II in both *in-vitro* (*147*) and *in-vivo* studies (*148-149*). Early *in-vitro* studies have indicated that an important non-receptor kinase mediator for the activation of ERK1/2 by intravascular pressure and mechanical strain is cellular-Src or c-Src (*150*). This kinase has been shown to play an important role in cyclic stretch of vascular smooth muscle cells (*151*) and responses of rabbit aorta to raised intravascular pressure (*152*). It is possible that c-Src mediates the interaction between strain responses of integrins and focal adhesion kinases of the endothelial layer to processes leading to ERK1/2 activation, ultimately resulting in arterial remodeling. The relationship between these kinases was later shown in SHRs (in-vivo) (*153*). The activation of c-Src has also been suggested to trigger processes downstream of platelet-

derived growth factor β receptors (PDGFβ) (*154-155*). It has since been speculated that angiotensin II receptor activation causes transactivation of PDGFβ (*154*) and this process mediates the downstream activation of ERK1/2 (*156*).

1.3 Effect of resveratrol on signaling

Resveratrol has been shown to suppress angiotensin II receptor type I activity *in-vitro* and *in-vivo* via activation of the Sirtuin 1 gene (*157*). Studies have also found that resveratrol inhibits hypoxia-induced lysophosphatidic acid production of HIF-1alpha and VEGF through the inhibition of a number of kinases including ERK1/2 (*158*). Other effects include inhibition of angiotensin II-induced intracellular ROS levels, and angiotensin II-induced ERK phosphorylation in cultured neonatal rat ventricular myocyte cells (*159*). As well, resveratrol was shown to possess anti-inflammatory properties via inactivation of ERK1/2 in rat hippocampus slices *ex vivo* (*160*), a link believed to be closely associated with Alzheimer's disease. Furthermore, resveratrol suppresses ERK1/2 activity in various other cell types including cardiac fibroblasts (*161*), mouse adipocytes (*162*), human coronary arteries (*163*) and smooth muscle cell from mouse aorta (*164*).

1.4 Rationale for investigating angiotensin II receptors and ERK

Based on the above studies, phase two of the study was designed to identify the possible signaling pathway by which resveratrol attenuated remodeling. Since wall component vessel stiffness was unaffected by resveratrol treatment (Figure 19), we therefore considered attenuation of eutrophic remodeling to be the predominant action by which resveratrol treatment affected vessel compliance. Considering that the renin-angiotensin-aldosterone system largely influences vascular remodeling (*93, 165*) and that this system is implicated in growth signaling of SHR

mesenteric arteries through the AT1 and AT2 receptor subtypes (*166*), we first investigated whether AT1 and AT2 receptor subtypes may have been affected by resveratrol.

The activation of ERK1/2 was also investigated as the signaling effector downstream of angiotensin receptors as it mediates, at least in part, the vascular actions of angiotensin II. Mesenteric arteries from SHR exhibit increased ERK1/2 activation, and angiotensin II-promoted growth in SHR vessels is decreased by inhibition of ERK1/2 (*166*).

2. Methodology

2.1 Immunoblotting

As previously described (*167*), frozen mesenteric arteries were sonicated on ice in Buffer A (10% glycerol, 20 mM Tris–HCl pH 7.3, 100 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 2 mM EGTA, 10 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), then centrifuged at 15000 rpm for 30 minutes. The pellet was extracted and resuspended in Buffer B [1% Igepal CA 630 in Buffer A] and the subsequent lysate was clarified by centrifugation. Antibodies against AT1 (Santa Cruz Biotechnology), AT2 (Santa Cruz Biotechnology), phospho-ERK1/2 (Cell Signaling Technology) and native ERK1/2 (Cell Signaling Technology) were used in conventional Western blotting experiments. Membranes were stripped and re-probed with β-actin antibody (Sigma-Aldrich Canada) to account for loading variations among lanes.

3. Phase Two Results

3.1 AT1, AT2 and ERK1/2 signaling

Findings show that resveratrol had no detectable effect on AT1 or AT2 receptor expression in mesenteric arteries from WKY or SHR (Figure 23). Immunoblotting of phosphorylated ERK1/2 revealed that the SHR arteries had significantly greater levels of activated ERK1/2 activity within the mesenteric vasculature as compared to the normotensive WKY group (Figure 24). Resveratrol treatment abolished exaggerated ERK1/2 signaling in SHRs.

4. Discussion of Phase Two Results

4.1 Effect of resveratrol on AT1, AT2 and ERK1/2 signaling

These findings indicate augmented signaling of ERK1/2 in SHR mesenteric arteries, which is completely normalized to WKY levels with resveratrol treatment (Figure 24; $p<0.05$). Therefore this study shows that the ability of resveratrol to attenuate resistance artery remodeling in SHRs is associated with suppression of ERK1/2 activation, with no detectable effect on AT1 or AT2 receptor activity. As such, the signaling pathway triggered by resveratrol to decrease ERK 1/2 activity needs to be investigated.

4.2 Recap of findings and implications

As previously noted, SHRs are genetically predisposed to renal dysfunction, promoting

the hypertensive state. This has been confirmed in renal graft studies where transplantation of healthy Wistar kidneys in SHRs caused correction of the hypertensive state (*168-169*). SHRs also develop initial signs of vessel remodeling prior to the onset of hypertension (*137*). This study further suggests that resistance artery remodeling and the hypertensive state may play independent roles in the initial development of hypertension in SHRs as noted by the maintenance of increased systolic pressures in resveratrol-treated SHRs (Figure 12), despite correction of resistance artery morphology (Table 5). As such, it is possible that the direct restorative effects of resveratrol on resistance artery morphology (Figures 13, 14, 15, Table 5) may be due to the potent antioxidant properties of the compound which include anti-proliferative and anti-inflammatory effects (*81, 141*). The full mechanistic pathway in which the noted vascular effects were achieved with resveratrol treatment have yet to be fully established; however this study suggests that resveratrol attenuated mesenteric vascular remodeling via downstream ERK1/2 signaling (Figure 24).

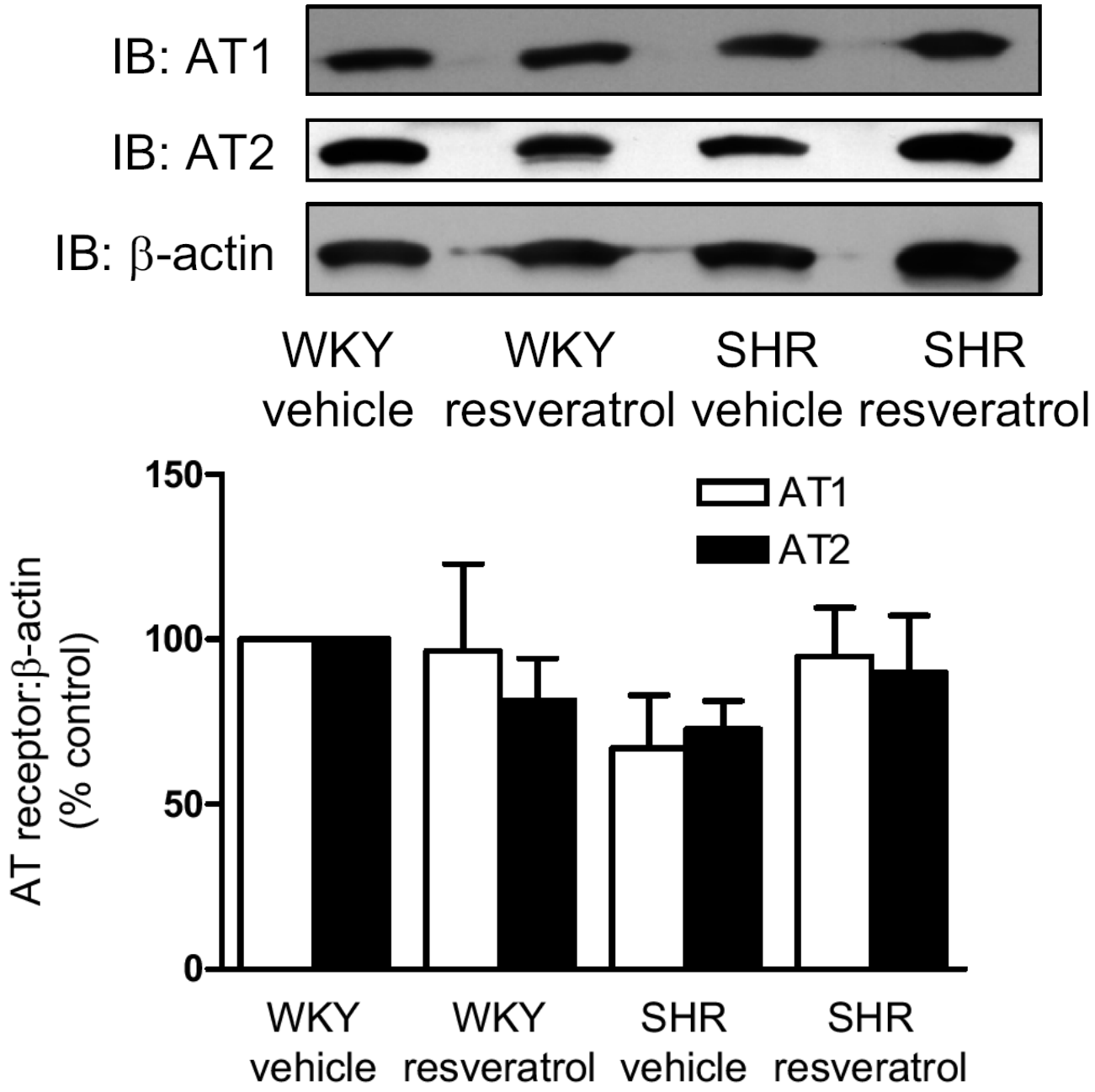

Figure 23. Effect of resveratrol on AT1 and AT2 in mesenteric arteries from SHR. *Top:* Representative blots *Bottom:* Quantification data demonstrating lack of effect of resveratrol on AT1 and AT2 levels in mesenteric arteries from WKY or SHR. n≥4. Error bars indicated SEM.

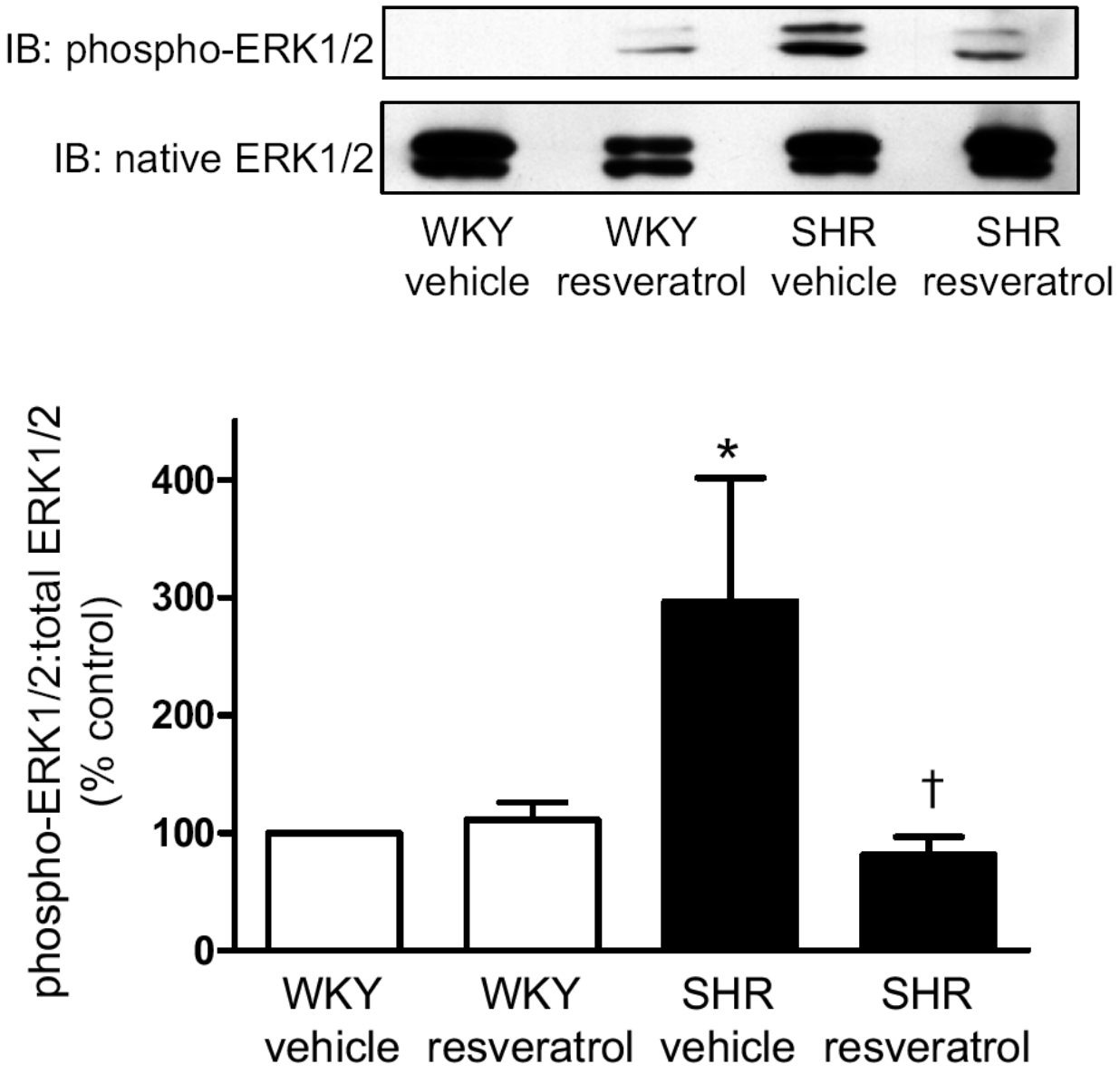

Figure 24. Effect of resveratrol on ERK1/2 activation in mesenteric arteries from SHR. *Top:* Representative blot *Bottom:* Quantification data demonstrating the ability of resveratrol to abolish exaggerated ERK1/2 signaling in mesenteric arteries from SHR. n≥4. Error bars indicated SEM. *p<0.05 vs. untreated WKY. †p<0.05 vs. untreated SHR.

CHAPTER IV

CONCLUSIONS

1. Conclusion and Clinical Implications

1.1 Conclusion

Resveratrol has been widely studied since the early 1990's because of the numerous purported beneficial properties. Recently, attention has turned to the various cardioprotective properties of this compound. The focus of this study was to investigate the protective nature of resveratrol on the establishment of hypertension and resistance artery health.

Our study showed that resveratrol attenuated eutrophic remodeling in SHRs, improved passive dilation capacity, elastic modulus, and isobaric stress and strain. Through its normalizing effects on vessel geometry, it reversed aberrant compliance seen in SHRs. In addition, the attenuation of remodeling in SHRs by resveratrol was possibly related to the ability of resveratrol to restore ERK1/2 activity in mesenteric arteries to normotensive WKY levels. Immunoblotting revealed that the effects of resveratrol on mesenteric vasculature were likely unrelated to AT1 and AT2 receptor activity.

The effects noted by resveratrol in this study were counteractive to innate protective mechanisms within the physiology of SHRs that null the effects of increased blood pressure on microvasculature. Treatment had no apparent effect on the establishment and maintenance of high blood pressure within the SHRs. As a result, despite the normalizing effects of resveratrol on vessel geometry and mechanical properties, in a continued hypertensive state, these changes may be highly undesirable and damaging. Therefore, it is important understand how adaptive mechanisms that protect SHR arteries in the hypertensive state were bypassed with resveratrol treatment and how this can be used to treat chronic cases where remodeling of resistance vessels is undesirable.

1.2 Resveratrol - A clinical perspective

The results of this study give new insight into the potential use of resveratrol in future clinical settings. Based on the results of this study, it would seem that if resveratrol treatment is to be used to attenuate arterial remodeling in hypertension, it would have to be accompanied by medication that would keep blood pressure under control.

However, despite evidence of benefits from resveratrol, there are some potential caveats when considering use of high doses in humans. Use of resveratrol supplementation by children, nursing, pregnant or conceiving women should be strictly controlled due to potential anti-growth effects of the compound (*170-171*). Due to the blood-thinning effects of resveratrol, all supplementation with resveratrol would likely need to be taken with severe caution in situations where it is taken in combination with other blood-thinning medications (*172-173*). Finally, anecdotal reports of short termed, high dose use of the compound (500-1000 mg/day) in humans caused reversible side-effects including: headaches, diarrhea, Achilles heel tendinitis, slight peripheral numbness, blood thinning and anxiety (*174*). Most of the symptoms linked with high doses are speculated as being the result of the chelation of copper by resveratrol, as copper is needed for collagen generation and nerve regeneration.

1.3 Final remarks

The results of this study suggest that resveratrol treatment was not effective at preventing increased systolic blood pressures in SHRs. Nevertheless, the treatment did attenuate remodeling within the mesenteric vasculature. Establishing a full mechanistic pathway in which remodeling was attenuated can provide insight into future studies to develop therapeutic agents for hypertension.

CHAPTER V

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