ROLE OF VASCULAR OXIDATIVE STRESS IN HYPERTENSION

A Dissertation Presented to

Department of Pharmacological and Pharmaceutical Sciences

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In Partial Fulfillment of the

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Doctor of Philosophy

In

Pharmacology

By

Siddhartha Rajendra Bhatt

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ABSTRACT

Hypertension affects 1 in 3 adult Americans and is a primary risk factor for cardiovascular diseases. Better understanding of hypertension pathogenesis is important for development of effective therapeutic agents. An important underlying factor present during hypertension is oxidative stress (OS). However, causal role of OS in hypertension is unclear.

Increased vascular resistance resulting from enhanced vasoconstriction and impaired vasodilation is a hallmark of hypertension. Enhanced vasoconstriction is associated with increased reactivity to vasoconstrictors such as angiotensin (Ang) II. Ang II-induced vasoconstriction is exaggerated during hypertension and is associated with Ang II type 1 receptors (AT1R) upregulation, the cause of which is unknown. OS modulates redox sensitive transcription factors including nuclear factor kappa B (NFκB), which has been associated with AT1R upregulation. Thus, OS via NFκB can transcriptionally upregulate AT1R. The impaired vasodilation in hypertension is attributed to endothelial dysfunction resulting from attenuated nitric oxide (NO) availability. OS can also contribute to endothelial dysfunction by reducing NO production and increasing NO scavenging.

Our objective was to study the role of OS in hypertension development. The first part of the study investigates whether OS is a cause or consequence of hypertension. Studies in 3-4 week old spontaneously hypertensive rats (SHR) revealed that OS precedes

hypertension development and is associated with NFκB activation and AT1R upregulation. Treatment of young SHR with pyrrolidine dithiocarbamate, an antioxidant with NFκB inhibitory action, attenuated hypertension development and normalized NFκB and AT1R expression. Experiments in human aortic smooth muscle cells also exhibited OS-induced AT1R upregulation through mechanisms involving NFκB. The second part of the study investigates the role of early oxidative stress in endothelial dysfunction with focus on elucidating role of resveratrol, an antioxidant polyphenol. Our results demonstrate, early resveratrol treatment lowers oxidative stress and reduces NO scavenging and eNOS uncoupling thereby preventing endothelial dysfunction and attenuating hypertension development.

In conclusion, early vascular OS in SHR could contribute to hypertension by modulating AT1 receptor upregulation, possibly via NFκB. Additionally, vascular OS could also contribute to endothelial dysfunction by increasing NO scavenging and eNOS uncoupling. Resveratrol treatment lowered oxidative stress, prevented endothelial dysfunction and attenuated hypertension development in SHR.

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
Ang II	Angiotensin II
AT1	Angiotensin II type 1
BH4	Tetrahydrobiopterin
DAG	Diacylglycerol
eNOS	Endothelial nitric oxide synthase
H_2O_2	Hydrogen peroxide
HASMC	Human aortic smooth muscle cells
IP3	1,4,5-Inositol triphosphate
KHB	Krebs-Henseleit Buffer
MAP	Mean arterial pressure
MDA	Malondialdehyde
mRNA	Messenger RNA
NFκB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
OS	Oxidative stress
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction

PDTC	Pyrrolidine dithiocarbamate
PKG	Protein kinase G
RAS	Renin angiotensin aldosterone system
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
sGC	Soluble guanylyl cyclase
SHR	Spontaneously hypertensive rats
SOD	Superoxide dismutase
WKY	Wistar Kyoto

1. INTRODUCTION AND STATEMENT OF PROBLEM

Hypertension is a major risk factor for several cardiovascular diseases such as stroke, atherosclerosis, heart failure and end organ damage. Furthermore, hypertension afflicts almost 1 in 3 adult Americans (Cutler et al., 2008). Despite its wide prevalence, our understanding of the mechanisms involved in hypertension pathogenesis is considerably limited (Romero and Reckelhoff, 1999). This limited knowledge significantly stymies the development of novel therapeutic interventions. Thus, we are in need of better understanding the pathways involved in hypertension pathogenesis.

Oxidative stress has been identified as an important factor associated with hypertension (Ceriello, 2008; Grossman, 2008; Nickenig and Harrison, 2002). An imbalance caused due to increased reactive oxygen species (ROS) production and/or reduced antioxidant systems results in oxidative stress (Wassmann et al., 2004). Oxidative stress has been reported in vascular tissue of several models of experimental hypertension such as spontaneously hypertensive rats (SHR), stroke prone SHR and Dahl salt sensitive rats (Fukui et al., 1997; Kerr et al., 1999; Tanito et al., 2004; Touyz, 2004). However, the mechanisms by which vascular oxidative stress contributes to hypertension are not completely understood. Also, whether oxidative stress is a cause or a consequence of hypertension remains to be established.

A hallmark of hypertension is increased vascular resistance resulting from enhanced vasoconstriction (Jackson et al., 1999; Tahvanainen et al., 2006) and/or

impaired endothelium dependant vasodilation (Lockette et al., 1986; Morawietz et al., 2001; Puddu et al., 2000). The enhanced vasoconstriction is mediated by increased response to vasoconstrictor substances such as Angiotensin II (Ang II) and catecholamines. Ang II is a potent vasoconstrictor and a powerful modulator of vascular tone. Primary vascular effects of Ang II contributing to hypertension are vasoconstriction, vascular remodeling and resultant increase in vascular tone. Ang II elicits most of its vascular effects via AngII type 1 (AT1) receptor which is predominant in the large conduit and small resistance vessels (Levy, 1998). AT1 receptor expression and signaling is upregulated in human and experimental hypertension (Nickenig et al., 1998; Reja et al., 2006; Touyz et al., 2003). However, the cause of AT1 receptor upregulation and the mechanism involved therein are not completely understood.

Several reports identify oxidative stress as an important regulator of gene transcription via modulation of several redox sensitive transcription factors such as nuclear factor kappa B (NFκB), activator protein-1, and Sp1 (Banday et al., 2007a; Lavrovsky et al., 2000; Toledano and Leonard, 1991). Redox modulation of these transcription factors has been associated with pathophysiological conditions such as atherosclerosis, diabetes, hypertension and cancer (Sen and Packer, 1996). Of particular interest is NFκB which has been implicated in several diseases. We and others have previously showed that oxidative stress activates NFκB via nuclear translocation (Banday et al., 2007a; Barchowsky et al., 1995; Hayden and Ghosh, 2004; Toledano and Leonard, 1991). Cowling and colleagues have shown that NFκB is required for AT1 receptor

upregulation in response to tumor necrosis factor α and Interleukin 1β in cardiac fibroblasts (Cowling et al., 2002). Data from our lab also shows that oxidative stress causes activation of NFκB and is associated with AT1 receptor upregulation in rat renal proximal tubules (Banday and Lokhandwala, 2008). Furthermore, the AT1 receptor gene promoter has consensus binding site for NFκB (Heinemeyer et al., 1998). Hence we hypothesized that oxidative stress can upregulate AT1 receptors via NFκB and contribute to hypertension.

As mentioned previously, the increased vascular resistance contributing to hypertension results from enhanced vasoconstriction and impaired vasodilation. The impaired vasodilation seen during hypertension has been attributed primarily to endothelial dysfunction (Lockette et al., 1986; Morawietz et al., 2001; Puddu et al., 2000). Endothelial dysfunction is a consequence of impairment in nitric oxide (NO) synthesis and/or bioavailability. The mechanisms involved in causing endothelial dysfunction during hypertension also remain to be completely established. Oxidative stress has been hypothesized to play a role in modulating endothelial dysfunction. Increased ROS, particularly superoxides, scavenge NO by forming highly reactive peroxynitrite radicals (Escobales and Crespo, 2005; Pryor and Squadrito, 1995). Oxidative stress has also been shown to uncouple endothelial nitric oxide synthase (eNOS) resulting in impaired endothelium dependant relaxations (Landmesser et al., 2003). The uncoupled eNOS generates reactive oxygen species instead of NO thereby reducing NO production and further increasing oxidative stress (Munzel et al., 2005).

Therefore, oxidative stress can contribute to endothelial dysfunction by scavenging NO and uncoupling eNOS.

The present studies were designed to test the involvement of oxidative stress in hypertension pathogenesis and to elucidate the mechanisms involved at the level of the vasculature. The project was essentially divided in to two parts, the first part investigates whether oxidative stress precedes hypertension development. Furthermore, we also studied the role of oxidative stress in AT1 receptor upregulation and the involvement of NFκB. In these sets of studies we used spontaneously hypertensive rats (SHR) as our in vivo animal model, we also used human aortic smooth muscle cells (HASMCs) to further clarify our results from animal studies. The second part of the study was designed to study the role of oxidative stress in mediating endothelial dysfunction and to study the mechanisms involved. We treated 3 – 4 week old SHR and WKY rats with resveratrol, an antioxidant polyphenol, for 10 weeks. At the end of the treatment, development of blood pressure and endothelial dysfunction were studied. Biochemical assays testing nitric oxide production and scavenging as well as eNOS protein expression and uncoupling were performed to elucidate the mechanisms of resveratrol mediated effects on blood pressure and endothelial function.

The present research attempts to link vascular oxidative stress, especially during the early stages, to hypertension pathogenesis. The results from the study might help

better understand the role of oxidative stress in hypertension pathogenesis and aid in development of more effective anti-oxidant therapies and/or regimens.

2. REVIEW OF LITERATURE

2.1 Hypertension

2.1.1 Clinical definition and prevalence

Hypertension is a multifactorial disorder which is phenotypically manifested as a sustained elevation in arterial pressure. According to the guidelines provided by the seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure (JNC7), blood pressure < 120/80 mmHg (Systolic/Diastolic) is optimal whereas a systolic pressure of 140 mmHg or higher and/or a diastolic pressure of 90 mmHg or higher is classified as hypertension (Chobanian et al., 2003). The JNC7 report also introduced a new classification termed as 'Prehypertension' which incorporates individuals who are at higher risk of developing hypertension as indicated by a systolic pressure of 120 - 139 mmHg and/or a diastolic pressure of 80 - 89 mmHg (Chobanian et al., 2003). National Health and Nutrition Examination Survey (NHANES) data suggests that approximately 1 in 3 adult Americans suffer from hypertension requiring some form of treatment (Burt et al., 1995; Hajjar and Kotchen, 2003). Furthermore, approximately 95% to 98% of the hypertension cases fall into the category termed as 'Primary' or 'Essential' hypertension i.e. no clear single identifiable cause is found for hypertension development in these patients (Beevers et al., 2001). Despite significant advances in hypertension research, blood pressure is effectively controlled in only about 31% of the hypertensive patients (Hajjar and Kotchen, 2003).

These alarming statistics underline a considerable gap in our current understanding of development and maintenance of hypertension. It is well recognized that the risk for fatal and non fatal cardiovascular diseases is lowest in adults with blood pressure lower than 120/80 mmHg. These risks increase significantly with elevation in blood pressure above 120/80 mmHg. Sustained elevation in arterial pressure causes pathological changes in vasculature, kidney and heart (Hoffman, 2006). Consequently, hypertension is the primary cause of stroke and is a significant contributor to coronary artery disease, heart failure, renal insufficiency and dissecting aortic aneurysm (Hoffman, 2006). Thus, high blood pressure poses a serious health care challenge and prevention or effective management of hypertension can significantly lower the prevalence of stroke and other serious cardiovascular and renal diseases.

2.1.2 Pathophysiology of hypertension

Hypertension is a polygenic disorder wherein complex interactions involving multiple organ systems and signaling pathways have been implicated (Beevers et al., 2001). These mechanisms include, but are not limited to, increased sympathetic nervous system activity, defect in renal natriuretic and diuretic ability, structural and functional changes in the vasculature and over activity of the renin-angiotensin-aldosterone system (Beevers et al., 2001). The arterial blood pressure is primarily the function of two physiological parameters namely the cardiac output and the total peripheral resistance. The cardiac output is essentially governed by the heart rate and the stroke volume

whereas the total peripheral resistance is influenced by vasoactive mechanisms controlled by local as well as systemic neural, humoral and renal factors (Singh et al., 2010). In cases of primary hypertension either an increase in cardiac output and/or increased total peripheral resistance contributes to elevated arterial pressure (Singh et al., 2010). However, most patients with essential hypertension exhibit normal cardiac output with sustained elevation in peripheral resistance (Beevers et al., 2001). Thus, the hallmark hemodynamic change in hypertension is an increase in vascular resistance (Fink, 2009). The increase in vascular resistance is a consequence of altered vascular reactivity, particularly in the small resistance vessels. The altered vascular reactivity occurs due to structural or functional changes in the arteries resulting in enhanced vasoconstriction and/or impaired vasodilation and plays a crucial role in hypertension development (Lee and Griendling, 2008).

Significant research has also pointed towards an obligatory role of the kidneys in triggering hypertension development. In particular, seminal studies by Guyton and colleagues demonstrated the importance of kidneys in long term blood pressure regulation and in hypertension pathogenesis (Guyton, 1991; Guyton et al., 1972). Furthermore, Dahl and Heine have also shown that hypertension was transferable when kidneys from hypertensive rats were transplanted in to normotensive rats (Dahl and Heine, 1975). The primary mechanism via which kidney contributes to pathogenesis of hypertension is by defects in its sodium excretion capability. However, young patients with hypertension excrete sodium normally and a majority of younger patients do not

show a change in blood pressure with salt loading (Singh et al., 2010). Meta-analyses also suggest that salt restriction may not be important in young patients with hypertension (Singh et al., 2010). Hence, whether the trigger for hypertension pathogenesis occurs in kidneys or vasculature remains to be elucidated. A unifying hypothesis for hypertension development has been proposed by Johnson and colleagues wherein a combined role of vasculature and kidneys has been suggested. The group suggests that although the genesis of hypertension lies in the kidneys, the kidneys function normally in the early prehypertensive state. However, in hypertensive individuals renal injury is induced by enhanced renal vasoconstriction which may be a consequence of hyperactive sympathetic nervous system or renin angiotensin system. This renal injury then contributes to impairment in natriuretic capability of the kidneys affecting salt and water homeostasis thereby contributing to development and maintenance of high blood pressure (Johnson et al., 2005). Hence, the events occurring in the vasculature could play a pivotal role in the pathogenesis of hypertension.

2.2 Vascular pathophysiology during hypertension

The arterial system which comprises of large conduit arteries and small resistance arteries has crucial function in regulation of vascular resistance and consequently in long term control of blood pressure. Mathematically, the vascular resistance is an inverse function of the fourth power of the artery radius. Hence, small reduction in the arterial lumen causes significant increases in vascular resistance and blood pressure (Rehman and

Schiffrin, 2010). Vascular alterations contributing to increased vascular resistance and hypertension are 1) structural changes and/or 2) functional changes in the arteries.

2.2.1 Structural changes

Changes in the vascular structure, also known as vascular remodeling, is a major contributor to general narrowing of the arteries and increase in peripheral resistance. Vascular remodeling is classified based on the nature of changes i.e. outward or inward and change in the media mass i.e. hypertrophic, eutrophic or atrophic (Touyz, 2003). Landmark studies by Folkow established that there was increase in vessel wall thickness during hypertension and also that this structural change contributes to chronic hypertension (Folkow, 1990). Vessels from patients with mild hypertension and animal models such as SHR, 2K1C Goldbaltt rats exhibit vascular remodeling as indicated by reduced lumen diameter and increased meda:lumen ratio (Touyz, 2003). Vascular remodeling involves vascular smooth muscle cell proliferation, inflammation and cell migration. Several stimuli have been implicated in triggering vascular growth and hypertrophy during hypertension including Ang II. Ang II induced activation of MAP kinases, particularly ERK 1\2, influence protein synthesis and cell growth (Touyz, 2003). Vascular remodeling is a chronic process and may also represent adaptive changes caused due to high blood pressure and mechanical stretch. Hence it can be speculated that

structural changes contribute less to development of hypertension and significantly more to maintenance of hypertension.

2.2.2 Functional changes

The functional vascular tone is determined by the action of vasoconstrictor and vasodilatory stimuli on the arteries. The vasoactive substances influencing the vascular tone include vasoconstrictors such as angiotensin II, norepinephrine, endothelin etc and vasodilators such as nitric oxide, prostacyclin and endothelium dependent hyperpolarization factor. Normal arterial pressure results from a dynamic equilibrium of counterregulatory constrictor and dilator stimuli. The functional changes observed during hypertension shift the equillibrium towards enhanced vasoconstriction thereby increasing vascular resistance. These changes in the vascular function primarily result from exaggerated response to vasoconstrictor stimuli and/or an impaired response to vasodilatory stimuli. Furthermore, it has been suggested that these functional changes may be primary events contributing to the development of hypertension and vascular remodeling (Schiffrin, 2007). Several studies in hypertensive humans and rodent models of hypertension have shown exaggerated response to contractile stimuli (Jackson et al., 1999; Tahvanainen et al., 2006). In particular, vascular reactivity to angiotensin (Ang) II, a potent vasoconstrictor, is reportedly enhanced in human and experimental hypertension (Schiffrin et al., 1993; Touyz et al., 1994). However, studies testing the exaggerated

responses to other vasoconstrictors such as endothelin and norepinephrine have produced equivocal results (Touyz, 2003). Additionally, Ang II is an important modulator of blood pressure and blocking Ang II actions has strong antihypertensive effect. Thus, Ang II appears to play an important role in mediating the increased vessel reactivity during hypertension. Vascular effects of Ang II and its role in hypertension has been discussed in detail in the following sections. The second important functional change in hypertensive vasculature is impaired vasodilation. Endothelium is the primary modulator of vascular relaxation in the arteries. Attenuation of endothelium dependent vasodilation resulting from endothelial dysfunction is strongly associated with hypertension (Lockette et al., 1986; Morawietz et al., 2001; Puddu et al., 2000). Endothelial dysfunction has been reported in several models of experimental hypertension and in human hypertensive patients (Touyz, 2003). The endothelium releases several vasoactive substances to influence the vascular tone of which nitric oxide (NO) is the most prominent. NO is a potent vasodilator and aberrations in NO synthesis and signaling is well studied during hypertension (Discussed later).

2.3 Vascular Renin angiotensin system and hypertension

The renin-angiotensin-aldosterone system (RAS) plays a pivotal role in regulation of several cardiovascular processes such as regulation of vascular resistance, salt and water homeostasis and tissue remodeling. RAS functions via endocrine as well as

paracrine and autocrine mechanisms to increase blood pressure. The major physiological effector of the RAS is the multifunctional octapeptide angiotensin II (Ang II). Acute effects of Ang II which contribute towards blood pressure modulation include potent vasoconstriction and regulation of sodium and water homeostasis, whereas chronic effects include vessel remodeling, renal fibrosis and cardiac hypertrophy (Mehta and Griendling, 2007).

2.3.1 Synthesis of angiotensin II

Ang II is primarily synthesized via the classical or renal RAS and released in to the circulation. In the classical pathway, renin which is an aspartyl protease synthesized from the kidney cleaves hepatic derived decapeptide angiotensinogen to angiotensin I. Angiotensin I is further cleaved to active Ang II by angiotensin converting enzyme (ACE), a dipeptidyl carboxypeptidase, in the lungs. angiotensin I can also be cleaved to form a heptapeptide Ang 1-7 by neutral endopeptidases (NEP) in the tissue (Touyz and Schiffrin, 2000). Additionally, presence of several RAS components required for Ang II synthesis has been demonstrated in tissues such as vasculature, kidneys and brain. Thus, Ang II can also be produced locally in various tissues by the tissue RAS. The local production of Ang II has been reported to play a role in regulating local hemodynamics (Danser, 1996). The vascular smooth muscle cells also contain major RAS components except renin (Touyz and Schiffrin, 2000). Hence, local production of Ang II in the

vasculature may be dependent on the circulating renin. Nontraditional Ang II formation can also occur via non-ACE dependent pathways primarily mediated by chymase.

2.3.2 Angiotensin receptors and signaling

Ang II is the primary effector of RAS and is a pleiotropic peptide that induces multiple effects on several different cell types. The physiological effects of AngII are mediated primarily by two G-protein receptors namely Ang II type 1 (AT1) and Ang II type 2 (AT2) receptors which share approximately ~30% sequence homology. Binding of Ang II to AT1 receptors elicits effects such as vasoconstriction, remodeling, sodium retention, inflammation and oxidative stress. The AT2 receptor mediates effects such as apoptosis, inhibition of proliferation and hypertrophy and NO production which appear to be counter regulatory to AT1 effects. However, the expression of AT1 is significantly higher in tissues such as vasculature, lung, liver and kidneys whereas the expression AT2 is highest in fetal tissues and decreases in adult tissues (Touyz and Schiffrin, 2000). Thus, AT1 receptors are the primary mediators of the physiological and pathophysiological effects of Ang II. In rodents, AT1 receptors are of two subtypes the AT1a and AT1b which share about 95% sequence homology despite separate chromosomal gene locations. The human AT1 receptor is mapped to chromosome 3 whereas the rodent AT1a and AT1b are mapped to chromosomes 17 and 2 respectively. The AT1 receptor is

a 359 amino acids long protein structurally comprised of seven transmembrane domains and is primarily coupled to Gq and Gi proteins for signal transduction.

2.3.3 Signal transduction of AT1- induced vasoconstriction

Ang II exerts a wide array of functions in the vasculature primarily via the AT1 receptors which are predominant in both the large conduit and small resistance vessels (Levy, 1998). Acutely Ang II causes potent vasoconstriction and an immediate increase in blood pressure. Chronically, it causes inflammation, oxidative stress, cell proliferation and remodeling which contributes to sustained hypertension. The potent vasoconstriction is initiated by Ang II binding to AT1 receptors resulting in coupling of G proteins, particularly Gq, with AT1 receptors. The G protein coupling activates phospholiapse C (PLC) causing hydrolysis of phosphatidylinositol,4-5,biphosphate to inositol, 1-4-5, triphosphate (IP3) and diacylglycerol (DAG). IP3 induces release of Ca^{2+} from the sarcoplasmic reticulum stores thereby increasing intracellular Ca^{2+} concentration which is the primary trigger for vasoconstriction. Increase in intracellular Ca^{2+} causes Ca^{2+}-calmodulin binding which results in activation of myosin light chain kinase by means of phosphorylation, promoting interaction of the contractile proteins actin and myosin and eventually causing vasoconstriction. A secondary increase in Ca^{2+} also occurs via transmembrane calcium channels which are activated by Ang II and this may be responsible for the sustained vasoconstriction seen with Ang II. Furthermore, DAG

along with Ca^{2+} causes activation of the serine threonine kinase protein kinase C (PKC). PKC further contributes to sustained vasoconstriction by increasing intracellular pH most likely by activation of sodium hydrogen exchanger (Touyz and Schiffrin, 2000). Recently, a small gunaosine triphosphatase (GTPase) RhoA and its downstream target Rho kinase have also been shown to play a role in Ang II mediated vasoconstriction (Nguyen Dinh Cat and Touyz, 2011). RhoA is abundantly expressed in arterial cells and contributes to vasoconstriction via phosphorylation of myosin light chain and sensitization of actin and myosin to calcium. AT1 receptor activates RhoA via phosphorylation mediated by activation of G12/13 and Gq proteins. The AT1-RhoA signaling pathway has also been implicated in AngII induced hypertension (Nguyen Dinh Cat and Touyz, 2011).

2.3.4 Angiotensin receptors and hypertension

Given the important role of AT1 receptors in regulation of blood pressure it logically follows that AT1 receptors play a pivotal role in hypertension. Increased Ang II induced vasoconstriction has been demonstrated in vasculature of hypertensive humans as well as in animal models of hypertension (Touyz, 2003). Also, treatment with antisense oligonucleotide directed against AT1 receptors normalizes blood pressure in spontaneously hypertensive rats (SHR) (Iyer et al., 1996). It is interesting to note that in SHR, although there is increased Ang II induced vasoconstriction as compared to the

normotensive control WKY rats, the plasma renin levels are similar in SHR and WKY rats (Haddad and Garcia, 1996). These observations suggest that the increased vasoconstriction observed in SHR could be a result of AT1 receptor upregulation. This conjecture is further supported by reports from Schiffrin and colleagues demonstrating vascular AT1 receptor upregulation in young prehypertensive SHR (Schiffrin et al., 1984). Similar AT1 receptor upregulation is also reported in the renal proximal tubules of young prehypertensive SHR (Cheng et al., 1998) . Furthermore, the AT1 receptor upregulation seen in young prehypertensive SHR may be of functional relevance as suggested by results showing enhanced Ang II-induced vasoconstriction in resistance vessels of these animals (Endemann et al., 1999). Treatment with an AT1 receptor antagonist losartan in young SHR from 3 weeks of age to 8 weeks of age causes a decrease in blood pressure which is maintained until 25 weeks of age (Bergstrom et al., 2002). Thus, based on these reports we hypothesized that vascular AT1 receptor upregulation might precede the development of hypertension and contribute to its pathogenesis.

2.4 Endothelial function and hypertension

2.4.1 Endothelium as a modulator of vascular tone

As mentioned previously the vascular resistance is governed by a dynamic equilibrium between vasoconstrictor and vasodilatory stimuli. This section deals with the vasodilatory stimuli. Endothelium, the single layer of cells forming the inner lining of the blood vessels, plays a critical role in modulation of vascular tone. The importance of endothelium was discovered in landmark studies by Furchgott and Zawadski using a sandwiched preparation of arterial strips with and without endothelium showing the obligatory role of endothelium in acetylcholine induced vasodilation (Furchgott and Zawadzki, 1980). These studies also suggested that the endothelium dependent relaxations were mediated by a diffusible relaxation factor released by the endothelium, which was later identified as nitric oxide (NO) (Vanhoutte, 2009). Since the discovery of NO, endothelium has also been shown to release several vasoactive substances such as endothelium dependent hyperpolarization factor, prostacyclin, thromboxanes etc. However, NO is still the best characterized and prominent vasoactive factor released by the endothelium that plays a major role in regulation of vasomotor tone.

2.4.2 Nitric oxide synthesis and signaling

NO is one of the simplest biological molecules which modulates several vascular protective effects such as vasodilation, inhibition of platelet aggregation and prevention of coronary vasospasm. NO is enzymatically synthesized by a family of three nitric oxide synthase (NOS) enzymes 1) neuronal NOS (nNOS), 2) inducible NOS (iNOS) and endothelial NOS (eNOS). In the endothelial cells NO is primarily produced by the Ca^{2+} - calmodulin dependent endothelial NOS (eNOS) or NOSIII. eNOS requires molecular oxygen and catalyzes oxidation of the guanidine group of L-arginine to produce L-citrulline and NO (Michel and Vanhoutte, 2010). Interestingly, the active catalytic form of eNOS is a homodimer consisting of two NOS monomers and requires several co-factors including tetrahydrobiopterin (BH4) for its catalytic activity. The binding sites of BH4 are on the interface of the two interacting monomers and appear to play a crucial role in promoting dimerization and NOS catalytic activity (Dudzinski et al., 2006). eNOS produces NO constitutively as well as in response to physiological stimuli. NO is a highly lipophillic and easily diffusible molecule with a very short half life of about 5 seconds (Dudzinski et al., 2006). Once released NO diffuses through cell membranes reaching the vascular smooth muscle cells wherein it activates the enzyme soluble guanylyl cyclase (sGC). sGC activation leads to cGMP accumulation and activation of protein kinase G. These events eventually trigger relaxation of smooth muscle cells and powerful vasodilation.

2.4.3 Endothelial dysfunction and hypertension

Impairment in endothelium dependent vasodilation is termed as endothelial dysfunction. Endothelial dysfunction is reported in almost 60% of the patients with mild to moderate uncomplicated hypertension and in almost all the patients with additional cardiovascular risk factors such as obesity and diabetes (Touyz and Schiffrin, 2008). Impaired endothelium dependent vasodilation in response to acetylcholine has been reported in forearm and coronary vascular beds of hypertensive patients (Spieker et al., 2000). Endothelial dysfunction has also been reported in animal models of experimental hypertension including SHR (Feletou and Vanhoutte, 2006). Mice in which NO production is attenuated by genetic deletion of eNOS exhibit elevated arterial pressure (Huang et al., 1995). The fundamental abnormality in endothelial dysfunction appears to be reduction in bioavailability of NO which may be related to reduced NO production and/or increased scavenging of NO. However, the mechanisms involved in impaired endothelial function remain to be elucidated.

2.5 Oxidative stress and hypertension: A complicated relationship

Oxidative stress has been identified as a strong underlying factor in hypertension (Ceriello, 2008; Grossman, 2008; Nickenig and Harrison, 2002). There is a strict balance between reactive oxygen species (ROS) production and ROS neutralization by

antioxidant systems. An imbalance in this system by increased ROS production and/or reduced antioxidant mechanisms results in oxidative stress (Wassmann et al., 2004). ROS such as superoxides ($O2^{\cdot-}$) and hydrogen peroxide (H_2O_2) can react with biological molecules such as lipids, proteins and DNA resulting in structural and functional abnormalities. Also, ROS can directly act as signaling molecules and contribute to various pathophysiological conditions including hypertension. Oxidative stress is also reportedly increased in several models of experimental hypertension and in hypertensive humans. In patients with hypertension biomarkers of oxidative stress such as 8-isoprostane and plasma malondialdehyde are elevated (Touyz and Schiffrin, 2008). This increased oxidative stress is associated with decreased activity of antioxidant enzymes such as SOD, catalase and glutathione transferase and with increased activity of ROS generating enzymes (Touyz and Schiffrin, 2008).

Antioxidant treatment, by reducing oxidative stress, lowers blood pressure in human and experimental hypertension (Akpaffiong and Taylor, 1998; Banday et al., 2007b; Ceriello, 2008; Ceriello et al., 1991; Rodriguez-Iturbe et al., 2003). However, in large scale clinical trials testing the effects of antioxidants such as vitamin E and ascorbic acid on cardiovascular diseases has either shown no beneficial effect or in some cases exhibited detrimental effect (Cook et al., 2007; Steinhubl, 2008). A meta- analysis of randomized clinical trials using vitamin E and β-carotene in prevention of cardiovascular diseases suggests no beneficial effects of vitamin E on all cause mortality, risk of cardiovascular death or cerebrovascular accident when compared to placebo

(Vivekananthan et al., 2003). Furthermore, β-carotene caused a small increase in all cause mortality (Vivekananthan et al., 2003). This paradoxical failure of antioxidants in large scale clinical trials despite strong experimental evidence has been attributed to several factors including choice of antioxidant and duration of trial (Steinhubl, 2008). Nonetheless, these trials suggest that our understanding of the role of oxidative stress in cardiovascular diseases and hypertension is considerably limited.

An important question that remains unanswered is whether oxidative stress is a cause or a consequence of hypertension? Increased oxidative stress has also been demonstrated in prehypertensive humans suggesting that an early increase in oxidative stress could play a causal role in development of hypertension (Nambiar et al., 2009). We have also reported that treatment of Sprague Dawley rats with pro-oxidant L-buthionine sulfoximine (BSO) induces oxidative stress and increases arterial pressure. Furthermore the increase in blood pressure can be prevented by concomitant treatment with anti-oxidant tempol (Banday and Lokhandwala, 2008). Increased renal oxidative stress has also been reported in young SHR prior to blood pressure development. Thus, we speculate that increased oxidative stress prior to blood pressure development may contribute to hypertension pathogenesis. Hence, we performed studies in vessels of 3-4 week old SHR. SHR is a genetic model of hypertension wherein hypertension starts to develop around 5-6 weeks of age. At 3-4 weeks of age hypertension has not developed in SHR and is an ideal age to determine the presence of early vascular oxidative stress. We

also investigated the mechanisms by which vascular oxidative stress can contribute to hypertension development.

2.6 Role of vascular oxidative stress in hypertension

Increased vascular resistance resulting from enhanced vasoconstriction and impaired vasodilation is a hallmark of hypertension. Both vasoconstriction and dilation can be modulated by oxidative stress. Interestingly, vasculature is an important source of oxidative stress as all the cells in the vascular tissue have the ability to generate ROS. The predominant enzymes involved in ROS generation in the vasculature are NADPH oxidase, uncoupled eNOS, xanthine oxidase and the mitochondrial electron transport system (Lee and Griendling, 2008). Oxidative stress has been reported in vascular tissue of several models of experimental hypertension such as spontaneously hypertensive rats (SHR), stroke prone SHR and Dahl salt sensitive rats. (Fukui et al., 1997; Kerr et al., 1999; Tanito et al., 2004; Touyz, 2004). Our goal here was to study the role of oxidative stress in modulation of enhanced vasoconstriction and impaired vasodilation.

2.6.1 Vascular oxidative stress and AT1 receptor upregulation

Recent evidences suggest oxidative stress as being an important regulator of gene transcription via modulation of several redox sensitive transcription factors like nuclear

factor kappa B (NFκB), AP-1, and SP1 (Banday et al., 2007a; Lavrovsky et al., 2000; Toledano and Leonard, 1991). Redox modulation of these transcription factors, particularly NFκB, has been associated with pathophysiological conditions like cancer, diabetes, AIDS and atherosclerosis (Sen and Packer, 1996). NFκB consists of homo or heterodimers of proteins belonging to the Rel family which is activated by nuclear translocation from the cytosol (Hayden and Ghosh, 2008). We and others have previously showed that oxidative stress causes NFκB nuclear translocation in animals and cells in culture (Banday et al., 2007a; Barchowsky et al., 1995; Hayden and Ghosh, 2004; Toledano and Leonard, 1991). Most of our understanding of NFκB and its physiological signaling has been derived from the fields of immunology and oncology. However, novel findings have suggested important role of NFκB in cardiovascular diseases including hypertension (Elks et al., 2009; Rodriguez-Iturbe et al., 2005). Interestingly, AT1 receptor gene promoter has a consensus binding site for NFκB (Heinemeyer et al., 1998). Cowling and colleagues have shown that NFκB is required for the AT1 receptor upregulation in response to tumor necrosis factor α and Interleukin 1β in cardiac fibroblasts (Cowling et al., 2002). Data from our lab also shows that oxidative stress causes activation of NFκB and upregulates AT1 receptor in rat proximal tubules (Banday and Lokhandwala, 2008). Thus, we hypothesized that vascular oxidative stress can contribute to AT1 receptor upregulation and contributing to increased vascular resistance and development of hypertension in SHR.

2.6.2 Vascular oxidative stress and endothelial dysfunction

Endothelial dysfunction is a hallmark of hypertension (Lockette et al., 1986; Morawietz et al., 2001; Puddu et al., 2000). Mechanisms involved in mediating endothelial dysfunction are not completely established. Impairment of NO synthesis and/or bioavailability causes endothelial dysfunction and oxidative stress can contribute to endothelial dysfunction. ROS, particularly superoxides, react rapidly with NO resulting in formation of peroxynitrite, thereby reducing NO availablility and consequently reducing its biological activity (Escobales and Crespo, 2005; Pryor and Squadrito, 1995). The bimolecular reaction of NO and superoxides is ~3-4 times faster than dismutation of superoxides by SOD (Schulz et al., 2008). Thus, increased superoxide production can result in greater scavenging of NO and elevated levels of peroxynitrite (Schulz et al., 2008). Lastly, peroxynitrite itself is also a reactive species and can damage lipids, proteins and DNA in similar fashion as ROS.

The primary source of NO in the endothelium is eNOS. Functional eNOS enzyme is homodimeric and studies have established that eNOS can become ‘uncoupled’ in the absence of substrate L-arginine or co-factor tetrahydrobioterin (BH4). Oxidative stress has also been shown to uncouple endothelial nitric oxide synthase (eNOS) resulting in impaired endothelium dependant relaxations (Landmesser et al., 2003). ROS including peroxynitrites can deplete intracellular BH4 by oxidative modification of BH4 to BH2 resulting in eNOS uncoupling (Schulz et al., 2008). When eNOS is uncoupled, electrons

are directed towards molecular oxygen instead of L-arginine, generating superoxides instead of NO (Munzel et al., 2005). Uncoupled eNOS has been identified as a major source of superoxides and has been reported in blood vessels of SHR, stroke prone SHR, and in rats with Ang II induced hypertension (Lee and Griendling, 2008). Therefore, oxidative stress can contribute to endothelial dysfunction by scavenging NO and uncoupling eNOS. Both these processes further lead to oxidative stress by production of peroxynitrites and superoxides respectively resulting in a vicious cycle of increased oxidative stress and lower NO availability and production.

2.7 General hypothesis

Based on the literature reviewed and findings from our lab we formed the following hypothesis (Diagram 1) to study the role of vascular oxidative stress in hypertension. We hypothesize that vascular oxidative stress precedes the development of hypertension. This early vascular oxidative stress can contribute to increased vascular resistance by enhancing vasoconstriction by AT1 receptor upregulation via NFκB. In addition, the early vascular oxidative stress can also contribute to endothelial dysfunction by uncoupling eNOS and increasing NO scavenging.

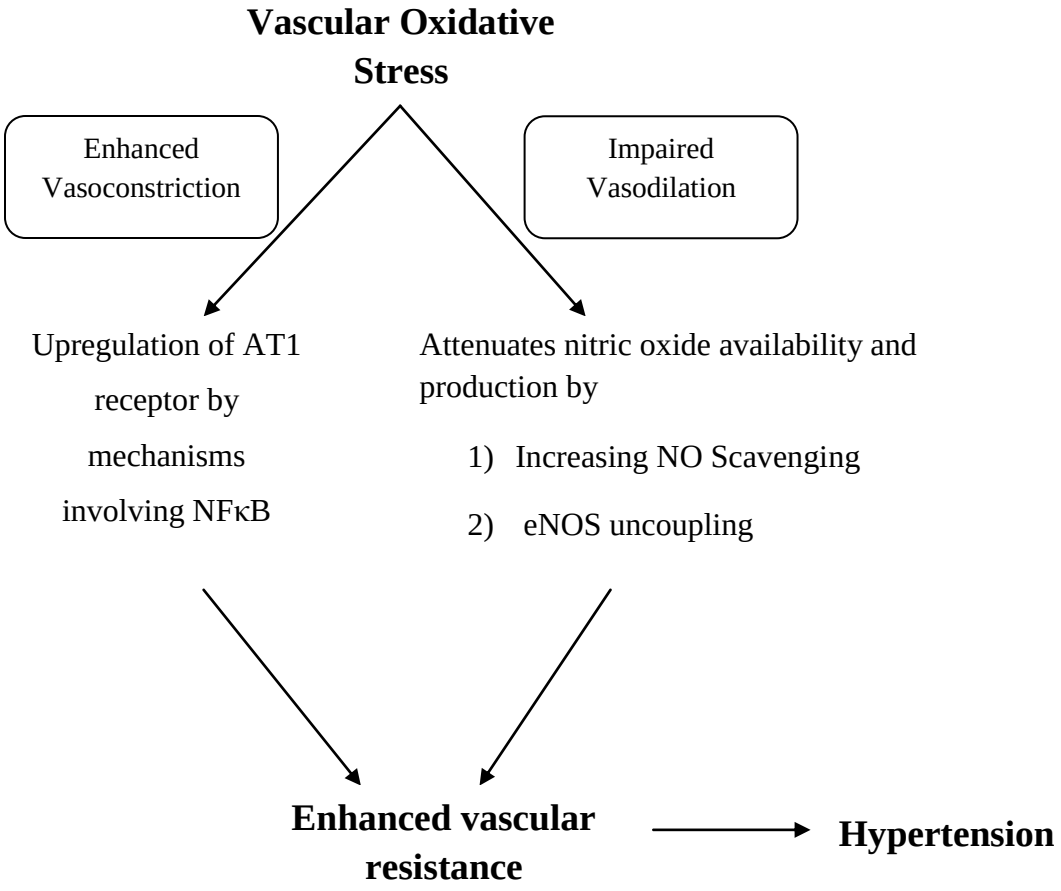


Diagram 1: General hypothesis for the present study as discussed in section 2.7

3. MATERIALS AND METHODS

3.1 Studies in 3 – 4 week old animals and PDTC treatment protocol

Studies were carried out in spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) rats served as controls. SHR and WKY rats (Harlan, Indianapolis, IN) were maintained in the animal care facility with a 12 hour light and 12 hour dark cycle and were given free access to standard rodent chow and drinking water. The initial studies for the first part of the project were performed in 3 - 4 week old SHR and WKY rats. For pyrrolidine dithiocarbamate (PDTC) treatment, 5 week old SHR and WKY rats were used. The rats were divided in to 3 groups 1) Untreated WKY rats (WKY), 2) Untreated SHR (SHR) and 3) SHR treated with PDTC (SHR-PDTC). SHR were treated with PDTC (Sigma Aldrich, St. Louis, MO) in drinking water at a dose of 105 mg/kg/day. Age matched untreated SHR and WKY rats served as controls. The PDTC treatment was carried out for a period of 6 to 7 weeks at the end of which the animals were used for blood pressure measurement and biochemical studies. All experiments were performed in compliance with University of Houston guidelines and protocols for care and use of laboratory animals. The animal protocol was approved by the IACUC.

3.2 Blood pressure determination

Blood pressure was measured as previously reported (Marwaha et al., 2004). Briefly, rats were anesthetized with Inactin® (100 mg/kg ip) and tracheotomy was performed to facilitate breathing. To measure blood pressure, the left carotid artery was catheterized with PE-50 tubing, connected to a Grass pressure transducer PT300, and blood pressure was recorded using a data acquisition system (PolyView, Grass Ins). After 30 to 45 minutes of stabilization period, the systolic and diastolic blood pressures were recorded. At the end of the blood pressure measurement, blood was collected from the carotid artery in vials coated with sodium EDTA for plasma preparation. Femoral artery and aorta were excised and flash frozen using liquid nitrogen and stored at -80°C for further analysis. For functional studies mesenteric artery was also excised as discussed later.

3.3 Markers of systemic oxidative stress

3.3.1 Lipid peroxidation in plasma

Lipid peroxidation products in plasma of SHR and WKY rats were assessed by measuring thiobarbituric acid reactive substances namely malondialdehyde (MDA) using the method previously described by Buege and Aust (1978) and routinely used in our lab. Frozen plasma was thawed on ice, diluted using 1.15% potassium chloride. The diluted

sample and boiled with 15% trichloroacetic acid, 0.375% thiobarbituric acid and 0.25N hydrochloric acid for 15 min. The reaction mixture was allowed to cool down and centrifuged at 1000g for 10 min to remove flocculent precipitate. The color was read at 535 nm using a spectrophotometer. The MDA levels were determined using the molar extinction co-efficient ($1.56 \times 10^5\ M^{-1}\ cm^{-1}$) and expressed as nmoles/ml of plasma

3.3.2 Urinary 8-isoporstane levels

Urinary 8-isoprostane was measured by a commercially available EIA kit (516351; Cayman chemical, Ann Arbor, MI). Essentially the assay is based on competition between 8-isoprostane in the urine sample and 8-isoprostane-Acetylcholinesterase conjugate (tracer) for the limited 8-isoprostane antiserum binding sites. Since the concentration of the tracer is constant the amount of tracer bound to antiserum binding sites is determined by concentration of 8-isoprostane in the urine. Diluted urine samples or standards were mixed with EIA buffer, tracer, 8-isoprostane antiserum in a 96 well plate precoated with rabbit IgG and incubated for 18 hours at 4º C. At the end of the incubation period the wells were washed with wash buffer and bound tracer was detected by addition of Ellman's reagent. Incubation with Ellman's reaction was carried out for 90 minutes at the end of which absorbance was detected at 405-420 nm using a spectrophotometer. The 8-isoprostane levels in the urine were calculated as per manufacturer's instructions and represented as ng/ml of urine.

3.3.3 Superoxide dismutase (SOD) activity in plasma

SOD activity was measured in the plasma using the superoxide dismutase assay kit from Cayman Chemical. The SOD activity in the biological sample is determined by detecting the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine. The kit quantifies the activities of all SOD isoforms. Briefly, the plasma samples were diluted using sample dilution buffer (50 mM Tris-Hcl, pH 8.0). Assay solution to detect radicals was prepared by diluting tetrazolium salt in assay buffer [50 mM Tris-Hcl. pH 8.0, containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA)]. Samples or standards and radical detector solution were mixed in a 96 well plate by gentle shaking. Bovine erythrocyte SOD was used to generate a standard curve. The reaction was initiated by addition of xanthine oxidase and incubated for 20 minutes at room temperature. The absorbance was read at 440-460 nm and SOD activity was calculated as per kit manufacturer's instructions. Results are represented as units/ml of plasma wherein one unit of activity indicates amount of enzyme needed to induce 50% dismutation of superoxide radicals

3.4 Markers of vascular oxidative stress

3.4.1 Protein carbonylation levels

Protein carbonyl is a marker of oxidative stress (Shacter et al., 1994) and has been used in our previous studies (Asghar et al., 2008). Protein carbonyls (addition of aldehydes and ketones) were determined using a protein oxidation detection assay kit (Millipore, Temecula, CA) according to the manufacturer's protocol. Briefly, artery homogenates were denatured by treatment with 6% SDS. The denatured protein samples are derivatized by addition of equal volume of 2,4 dinitrophenylhydrazine (DNPH). DNPH treatment derivatizes the carbonyl groups on the protein chains to 2,4 dinitrophenylhydrazone (DNPhydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH). The DNP-derivatized protein samples were resolved by polyacrylamide gel electrophoresis (PAGE) and transferred on to a Polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA). The membranes were incubated with primary antibody, specific to the DNP moiety of the proteins. This step was followed by incubation with a horseradish peroxidase-antibody conjugate directed against the primary antibody (secondary antibody: goat anti-rabbit IgG). After washing, the PVDF membranes were incubated with enhanced chemiluminescence reagent (Alpha Diagnostics, San Antonio, TX) and protein carbonyl bands were visualized on X-Ray film. The bands were quantified by densitometric analysis using Kodak Imaging software.

3.4.2 Superoxide dismutase activity in vascular homogenate

Superoxide dismutase (SOD) activity was measured in the femoral artery homogenate using the SOD assay kit as described previously in section 3.3.3.

3.4.3 Hydrogen peroxide levels in vascular homogenate

H_2O_2 levels were determined in femoral artery homogenates (30 microgram protein) using amplex red H_2O_2 kit (Molecular Probes, OR) as detailed by Rush and colleagues (Rush et al., 2007). Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) reacts with H2O2 in a 1:1 stoichiometry in the presence of peroxidase to produce a highly fluorescent oxidation product resorufin, which can be measured spectrophotometrically. Briefly, artery homogenates were incubated with 50 µl Amplex Red reagent (100 µM Amplex Red stock and 0.2 U/ml horse-radish peroxidase in 50 mM sodium phosphate buffer, pH 7.4) and incubated in the dark at room temperature for 60 minutes. At the end of the incubation absorbance was determined spectrophotometrically at 560 nm using a Synergy 2 plate reader (Biotek, Winooski, VT). The H_2O_2 levels are expressed as arbitrary units of absorbance relative to 30 micrograms of tissue protein.

3.5 Immunoblotting for NFκB p65 levels

Nuclear and cytosolic fractions from freshly collected aortic tissue were isolated as per the manufacturer's instructions using NE-PER nuclear and cytosolic extraction reagents (Thermo Scientific, Rockford, IL). Proteins were resolved by sodium dodecyl sulfate (SDS) PAGE. The resolved proteins were transferred electrophoretically onto a PVDF membrane. The PVDF membrane was blocked with 5% milk overnight at 4ºC followed by incubation with rabbit NFκB p65 antibody (catalog number-4764s, cell signaling technology, Danvers, MA) for 60 min. After washing the membranes with phosphate buffer saline (PBS) with Tween-20 (PBST), incubation with secondary horseradish peroxidase-conjugated goat anti-rabbit antibody was carried out for 60 minutes. The PVDF membranes were washed with PBST and incubated with enhanced chemiluminescence reagent (Alpha Diagnostics, San Antonio, TX) and protein bands were visualized on X-Ray film. The bands were quantified by densitometric analysis using Kodak Imaging software.

3.6 Immunoblotting for AT1 receptor

Portions of isolated femoral artery samples were homogenized in lysis buffer containing 0.25 mol/L of sucrose, 50 mmol/L of dithiothreitol, 3 mmol/L of HEPES, 0.5 mmol/L of EGTA, 0.4 mmol/L of PMSF, protease inhibitor mixture, and 1% Triton X-

100. Samples were centrifuged, and supernatants were solubilized in Laemmli buffer (62.5 mM Tris-HCL, 10% glycerol, 1% SDS and 1% β-mercaptoethanol and 0.8% bromophenol blue). Equal protein was loaded and western blotting was performed using primary antibody directed against the AT1 receptor (Sc-1173, Santa Cruz Biotechnology, Santa Cruz, CA). Alpha actin was used as loading control (Sc-58669, Santa Cruz Biotechnology). The density (arbitrary units) of the bands was quantified by Kodak Imaging software.

3.7 Vascular function studies to determine angiotensin II- induced contraction

The vascular function studies were performed in mesenteric artery rings. For mesenteric preparations, a midline abdominal incision was made, and the mesenteric artery was removed and immediately placed in ice-cold Krebs–Henseleit buffer (mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, and glucose 10.0 pH 7.4). The arteries were cleaned of adherent tissue and cut into rings under a dissecting microscope. Each ring was fixed under a resting tension of 0.6 g in a 10 mL organ bath filled with Krebs–Henseleit buffer (37°C) and continuously aerated with a 95% O_2/5% CO_2 gas mixture, and the rings were allowed to equilibrate for 90 minutes before the start of the experiments. Isometric tension change in response to angiotensin II (10^{-7}M) was measured with a digital force isometric transducer (Harvard Apparatus, Holliston, MA) connected to a data acquisition system (AD instruments)

3.8 Methods used in cell culture studies

The following methods were used to study the effects of oxidative stress-induced AT1 receptor upregulation in cell cultures

3.8.1 Human aortic smooth muscle cell cultures

Human aortic smooth muscle cells (HASMCs) were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in manufacturer recommended modified F12K medium (Gibco, Carlsbad, CA) supplemented with 0.05 mg/ml ascorbic acid, 0.01 mg/ml bovine insulin, 0.01 mg/ml human transferrin, 10 ng/ml sodium selenite, 0.03 mg/ml endothelial cell growth supplement, 10 mM HEPES, 10mM TES, 10% fetal bovine serum and cocktail of antibiotics and antimycotics (penicillin, streptomycin and ampotericin B) (Invitrogen, Carlsbad, CA). The cells were maintained in a humidified cell culture incubator maintained at 37ºC under 6% carbon dioxide. 80-85% confluent cells were used to perform further experiments. For experiments involving combination of oxidants (BSO and H_2O_2), serum starved cells were treated with BSO (400 µM) for 24 hours and in the final 3 hours of the BSO treatment the media was supplemented with 50 µM H_2O_2). In experiments involving antioxidant treatment with catalase, catalase (100 U/ ml) was added 15 minutes prior to the oxidant treatment and maintained throughout the duration of the treatment. At the end of the treatments the cells were used for the experiments described below.

3.9 Quantitative PCR for AT1 receptor

3.9.1 RNA extraction

HASMCs were grown in 6 well plates and treated with oxidants or oxidants + catalase as indicated in results. After the end of treatment period media was aspirated and the cells were washed with sterile PBS. Total RNA was prepared from cells using the RNeasy mini kit (Qiagen, Valencia, CA) as per the manufacturer's recommendations. Briefly, cells were disrupted by addition of 600 µL of buffer RLT and collected by scraping using a sterile cell scraper. The disrupted cells were mixed with equal volume of of 70% ethanol. The sample (700µL) was transferred to an RNeasy spin column using DNAse, RNAse free pipette tips and centrifuged at 8000g for 15 sec. The flow through was discarded and the column was washed once with RW1 buffer (700 µL) and twice with RPE buffer (500 µL) by centrifugation at 8000g for 15 sec and 2 minutes respectively. RNA bound to the spin column was eluted with 40 µL RNase-free water by centrigugation at 8000g for 1 min and collected in to sterile microcentrifuge tube. The purity of the extracted RNA was determined as a ratio of absorbance at 260 and 280 nm and yield was calculated using absorbance at 260 nm (U-2910 Spectrophotometer, Hitachi, Kernersville, NC).

3.9.2 cDNA synthesis

Total RNA (1 µg) was reverse transcribed to cDNA using commertically available Advantage RT for PCR kit (Clonetech, Mountain View, CA) using manufacturer recommended protocol. Briefly, 1 µg RNA diluted in 12.5 µL DEPC-treated H_2O was mixed 1 µL of random hexamer primer, heated at 70ºC for 2 minutes and cooled rapidly by placing on ice. cDNA synthesis was carried out by mixing the RNA-primer mixture with 4 µL 5X reaction buffer, 1 µL dNTP mix (10mM each), 0.5 µL recombinant RNase inhibitor, 1 µL MMLV reverse transcriptase and incubating the sample at 42ºC for 1 hour. Therafter, the reaction was terminated by heating the sample at 94ºC for 5 minutes. The cDNA yield was diluted by adding 60 µL of DEPC-treated H_2O and stored at -80ºC until the quantitative PCR was carried out.

3.9.3 Quantitative PCR (qRT-PCR)

cDNA synthesized in the previous section was used to perform qRT-PCR with specific primer probe assay mix directed towards human AT1 receptor (HS00258937_m1, Applied biosystems) using 18s rRNA as internal control using RT-PCR machine (Applied Biosystem 7300). Briefly, 5 µL of cDNA was mixed with 25 µL of Taqman Gene expression master mix and 2.5 µL of AT1 assay mix or 18s rRNA assay mix and the final volume made up to 50 µL with DEPC-treated water. The PCR reaction

was performed on the Applied Biosystems 7300 Real-Time PCR System. The reaction was initiated by heating at 50ºC for 2 minutes and 95ºC for 10 minutes. The samples were amplified for 40 thermal cycles (95ºC for 15 seconds and 60ºC for 1 minute). The relative fold expression to the control cells was calculated using the delta/delta Ct method.

3.10 Detection of intracellular oxidative stress

Intracellular oxidative stress was determined by measuring the levels of reactive oxygen species (ROS). Intracellular ROS were determined using cell permeable fluorescent probe, dichlorofluorescein diacetate (CM-H_2DCFDA) (Invitrogen, Carlsbad, CA). CM-H_2DCFDA is non fluorescent until it is taken by the cell and its acetate groups cleaved by intracellular esterase. Thereafter, oxidation by ROS produces fluorescent product which can be detected. The cells were grown in 12 well plates and after treatment were loaded with 5 µM CM-H_2DCFDA in DMSO at 37ºC for 30 min. The excess dye was removed by washing the cells with PBS 2-3 times. The cells were incubated in PBS in dark for 15 additional minutes. The fluorescence intensity as a result of CM-H_2DCFDA oxidation by intracellular ROS was recorded using a Synergy 2 microplate reader (Excitation: 492-495 nm/ Emission: 517-527 nm). The cells were collected using a cell scraper and the protein concentration was calculated using bicinchoninic acid assay. The results were normalized using protein concentration.

3.11 siRNA transfection protocol

HASMC were seeded in 6 well plates at 30-50% confluence and used for siRNA transfection. The siRNA against p65 subunit of NFκB (cell signaling technology, Danvers, MA) was used to knock down p65 expression in HASMC. siRNA was diluted to achieve final concentration (200 nM) in the serum free media without growth factors and antibiotics/antimycotics. For each well, the siRNA solution was mixed with the transfecting reagent oligofectamine (4 µL) and the volume was adjusted to 200 µL. The siRNA-oligofectamine mixture was incubated for 20 minutes at room temperature to allow complex formation. The siRNA-oligofectamine mixture was further diluted to 500 µL with serum free media. The cells in each well were washed with sterile PBS. The siRNA-oligofectamine mixture was added to each well and the cells were incubated for 4 hours in a humidified cell culture incubator maintained at 37ºC under 6% carbon dioxide. After 4 hours, 1.5ml of 1.5X complete growth media was added and cells were allowed to grow for 48 hrs. At the end of which treatment with oxidants was performed.

3.12 Detection of NFκB p65 expression

The effect of siRNA transfection on the levels of NFκB p65 protein expression was determined by western blotting. Whole cell lysates were prepared by disrupting cells using cell lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA,

1% Triton X-100, 2.5 mM $Na_4P_2O_7.10\ H_2O$, 1 mM β-glycerophosphate, 1 mM sodium orthovandate) containing PMSF and protease inhibitor cocktail and collected using a cell scraper. The lysates were homogenized and used for western blotting using anti NFκB antibody as described previously in section 3.5.

3.13 Studies performed in Resveratrol treated animals

3.13.1 Resveratrol treatment of SHR and WKY rats

Treatment was carried out in male, 3-4 week old spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) rats. The animals were fed standard rat chow diet, and had free access to water. The rats were divided in to 4 groups: 1) untreated WKY rats, 2) resveratrol treated WKY rats (WKY-R) 3) untreated SHR and 4) SHR treated with resveratrol (SHR-R). The SHR-R and WKY-R groups were given resveratrol (Sigma-Aldrich, St. Louis, MO) dissolved in drinking water ad libitum at a concentration of 50mg/L. At this concentration the dose of resveratrol per day calculated using the daily water intake and body weight was approximately 5mg/kg/day. The resveratrol treatment was started at 3-4 weeks of age and carried out for 10 weeks. At the end of the treatment animals were used for blood pressure measurement and biochemical studies.

3.14 Blood pressure determination and sample preparation

Blood pressure was determined and samples were prepared as described previously in section 3.2.

3.15 Vascular function studies to determine endothelial function

The vascular function studies were performed in mesenteric artery rings. For mesenteric preparations, a midline abdominal incision was made, and the mesenteric artery was removed and immediately placed in ice-cold Krebs–Henseleit buffer (mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, and glucose 10.0 [pH 7.4]). The arteries were cleaned of adherent tissue and cut into rings under a dissecting microscope ensuring minimal damage to the endothelium. Each ring was fixed under a resting tension of 0.6 g in a 10-mL organ bath filled with Krebs–Henseleit buffer (37°C) and continuously aerated with a 95% O_2/5% CO_2 gas mixture, and the rings were allowed to equilibrate for 90 minutes before the start of the experiments. Isometric tension change was measured with a digital force isometric transducer (Harvard Apparatus, Holliston, MA) connected to a data acquisition system (AD instruments). Acetylcholine (Ach) induced cumulative dose response was measured in mesenteric artery rings preconstricted with phenylephrine (0.2μmol/L) to determine the endothelium dependant relaxation. The relaxation responses are represented as percentage. Complete relaxation of the phenylephrine induced constriction was considered as 100%. To determine endothelium independent relaxation, responses to sodium nitroprusside (SNP) were measured in phenylephrine preconstricted mesenteric artery rings.

3.16 Markers of oxidative stress

3.16.1 Superoxide dismutase activity in aortic homogenate

Superoxide dismutase (SOD) activity was measured in the aortic homogenate using the SOD Assay kit from Cayman Chemical (Ann Arbor, MI).

3.16.2 Hydrogen peroxide levels in the aorta

Aortic H_2O_2 levels were determined in aortic homogenates (30 microgram protein) using amplex red H_2O_2 kit (Molecular Probes, OR) as detailed by Rush and colleagues (Rush et al., 2007).

3.16.3 Nitrotyrosine levels in the aorta

3-nitrotyrosine levels were measured in aortic homogenate using a competitive ELISA kit which detects tyrosine nitration as per protocol recommended by the manufacturer (Millipore, Billerica, MA). Briefly, the assay was performed in a 96 well plate with wells coated with nitrated BSA. Tissue samples (30µg) were added to the wells followed by addition of rabbit anti-nitrotyrosine antibody and incubated at 37ºC for 60 minutes. The wells were emptied by aspiration and washed with tris buffered saline (TBS) and incubated with secondary antibody (Anti rabbit IgG, HRP-conjugate) at 37ºC

for 60 minutes. The wells were aspirated again after incubation and washed once again with TBS. The nitrotyrosine levels were detected by incubation with LumiGLO® Chemiluminescent Substrate for 10 minutes at room temperature and detecting the relative light units (RLU) using Synergy 2 microplate reader. The results are expressed as RLU per 30 µg of aortic protein.

3.17 Nitrite/Nitrate levels in the aorta

The nitrite/nitrate levels were determined by a nitrite/nitrate assay kit from Cayman Chemicals in 30 micrograms of tissue protein for each sample as per the manufacturer recommended protocol. The assay is primarily a two step process. In the first step all the nitrate is converted to nitrite by enzyme nitrate reductase. In the second step, nitrite levels are detected by reaction with Griess reagent which yields a purple azo compound that can be detected spectrophotometrically. Briefly, aortic homogenates were incubated with nitrate reductase cofactors and the nitrate reductase enzyme for 180 minutes at room temperature. Following the incubation, Griess reagent was added and the total nitrite and nitrate levels were determined by measuring absorbance at 540 nm wavelength using a Synergy 2 microplate reader.

3.18 Endothelial nitric oxide synthase (eNOS) and soluble guanylyl cyclase (sGC) protein expression

The protein expression of eNOS and soluble guanylyl cyclase (sGC) β1 subunit was determined by immunoblotting using anti-eNOS and anti-sGC antibodies from BD transduction laboratories and Santa Cruz Biotechnology Inc. respectively as previously described (Banday et al., 2007b).

3.19 Determination of eNOS uncoupling

eNOS uncoupling was determined by measuring L-arginine dependent superoxide production using fluorescence spectrometric assay as described previously(Satoh et al., 2005). Superoxide production was detected by dihydroethidium (DHE) conversion to ethidium. Briefly, the aortic homogenates were incubated with NOS substrate L-arginine (1 mmol/L), dihydroethidium (DHE) dye (0.02 mmol/L) and $CaCl_2$ (1 mmol/liter) at 37°C for 40 min away from light. The oxidation of DHE was assessed by measuring fluorescence at an excitation wavelength of 480 nm and an emission wavelength of 610 nm using a fluorescence plate reader. To ensure that the superoxide production was eNOS specific the above procedure was carried out in presence or absence of NOS inhibitor NG-nitro-L-Arginine (L-NNA, 1 mmol/L). To investigate the role of cofactor

tetrahydrobiopterin (BH_4) the assay was also performed in presence or absence of BH_4 (3 µmol/L).

3.20 Studies in Sprague Dawley rats

Acute studies investigating the effects of resveratrol on Ang II-induced pressor response were performed in adult Sprague Dawley (SD) rats. SD rats weighing 200-250g were purchased from Harlan (Indianapolis, IN) and maintained in the animal care facility with a 12 hour light and 12 hour dark cycle and were given free access to standard rodent chow and drinking water. Rats were fasted overnight prior to performing experiments. SD rats were anesthetized by intraperitoneal administration of Inactin® (100mg/kg). In order to facilitate breathing, a tracheotomy was performed. To measure blood pressure, the left carotid artery was catheterized with PE-50 tubing, connected to a Grass pressure transducer PT300, and blood pressure was recorded using a data acquisition system (PolyView, Grass Ins). The left jugular vein was isolated and catheterized with PE-50 tubing for saline and drug administration. Following the catheterizations, normal saline (1% body weight, ml/hr) was continuously infused throughout the experimental period to prevent dehydration and associated hemodynamic changes.

The drug treatment protocol has been exhibited in the following schematic. Briefly, Adult SD rats were first given a bolus dose of vehicle followed by a pressor dose

of AngII (300ng/Kg). The rise in blood pressure was recorded. After 60–90 min the same animal was given a bolus dose of resveratrol 12mg/Kg followed by a pressor dose of AngII (300ng/Kg). To test the involvement of nitric oxide the rats were infused with 11micromol/Kg/min of NOS inhibitor L-NAME for 25 min and the above treatment protocol was repeated

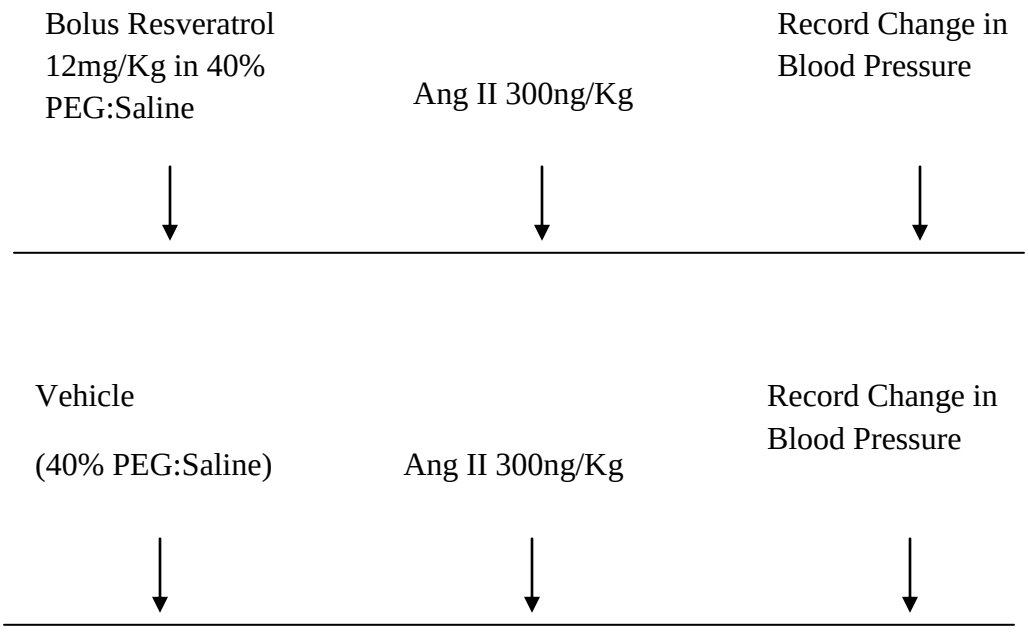


Diagram 2: Schematic representing drug treatment protocol in SD rats to study acute effects of resveratrol on Ang II induced pressor response

3.21 Data analysis

Results are presented as mean ± SEM. Difference between means were analyzed using paired, unpaired student t test or one way analysis of variance. Post hoc (Newman-Keuls) test was performed with one way analysis of variance to make comparisons between groups. Statistical significance was set at P values less than 0.05. Statistical analysis was performed using Graph Pad Prism software (GraphPad Software, San Diego, CA)

4. RESULTS

4.1 Studies performed in 3 - 4 week old SHR and WKY rats

4.1.1 Blood pressure

The mean arterial pressure (MAP) of SHR at 3 - 4 weeks of age was similar to that of WKY rats (Fig. 1). The hypertension in SHR starts to develop around 5 - 6 weeks of age and blood pressure rises consistently with age until it plateaus at 13 - 14 weeks of age. Hence, 3 - 4 week old SHR represent an appropriate age for studying the factors contributing to the pathogenesis of hypertension.

4.1.2 Markers of systemic oxidative stress

The plasma malondialdehyde levels were similar in SHR and WKY rats (Fig. 2A) indicating no significant systemic lipid peroxidation. We also determined 8-isoprostane levels in urine samples of SHR and WKY rats. The urinary 8-isoprostane levels were actually lower in SHR as compared to the WKY controls (Fig. 2B). Furthermore, superoxide dismutase (SOD) activity was higher in SHR in comparison to WKY (Fig. 2C). Thus, our results do not indicate any systemic increase in oxidative stress in SHR at 3 - 4 weeks of age.

4.1.3 Markers of oxidative stress in the vasculature

The protein carbonylation levels were higher in SHR femoral artery homogenates in comparison to WKY rats (Fig. 3A). The nitrotyrosine content was also significantly elevated in SHR artery homogenates as compared to WKY rats, suggesting increased oxidative stress in the cell milieu of the vasculature (Fig 3B). To test whether the increase in oxidative stress was associated with attenuated endogenous antioxidant defenses we measured SOD activity. The SOD activity in SHR artery homogenate was 50% lower in comparison to that of WKY rats (Fig. 3C) suggesting significant decrease in the endogenous ability of the SHR to neutralize the large amounts of reactive oxygen species that are generated in the vasculature.

4.1.4 Nuclear factor kappa B (NFκB) expression and activation in the vasculature

The levels of the p65 subunit of NFκB were determined in nuclear and cytosolic fractions of SHR and WKY aortas using western blotting. We found significantly higher nuclear levels of p65 in SHR as compared to WKY (Fig. 4A) suggesting increased NFκB activation in SHR vascular tissue. Furthermore, p65 expression in the cytosolic fraction was also significantly elevated in SHR as compared to WKY rats (Fig. 4B). Thus, there appears to be an increase in total expression of NFκB p65 subunit in the SHR.

4.1.5 Vascular Angiotensin II Type I (AT1) receptor expression in SHR

To test whether increased oxidative stress and NFκB activation seen in SHR were associated with vascular AT1 receptor upregulation, we measured AT1 receptor protein levels in femoral artery homogenates of SHR and WKY rats. The expression of AT1 receptors was significantly higher in SHR as compared to WKY rats (Fig. 5).

4.2 Studies performed in 11-12 week old animals after Pyrrolidine dithiocarbamate (PDTC) treatment

SHR exhibited increased vascular oxidative stress associated with increased NFκB activation and AT1 receptor upregulation prior to development of hypertension. Hence, to investigate the hypothesis that this early vascular oxidative stress contributes to AT1 receptor upregulation and hypertension SHR were treated with an antioxidant pyrrolidine dithiocarbamate (PDTC), which has well recognized NFκB inhibitory activity

4.2.1 Effect of PDTC treatment on blood pressure of SHR

SHR at 5 weeks of age were treated with PDTC for a period of 5-6 weeks. Age matched untreated SHR and WKY rats served as control. As expected, at 11-12 weeks of age untreated SHR displayed significantly higher mean arterial pressure as compared to

age matched WKY rats. However, the mean arterial pressure of PDTC treated SHR (SHR-PDTC) was 31 mmHg lower in comparison to untreated SHR (Fig. 6). Significantly lower diastolic as well as systolic pressures (Data not shown) contributed to the lower MAP in SHR-PDTC.

4.2.2 Effect of PDTC treatment on vascular oxidative stress

We determined vascular levels of hydrogen peroxide as a marker for oxidative stress. Untreated SHR exhibited enhanced oxidative stress in comparison to WKY rats as evidenced by elevated levels of vascular H_2O_2. PDTC treatment significantly lowered the hydrogen peroxide levels in SHR-PDTC (Fig. 7).

4.2.3 Effect of PDTC treatment on NFκB expression

Similar to the 3 - 4 week old animals, the adult untreated SHR also exhibited increased p65 subunit expression in the vascular homogenate as compared to WKY rats, as determined by western blotting. In addition, PDTC treatment caused a marked reduction of the elevated p65 expression in SHR-PDTC (Fig. 8).

4.2.4 Effect of PDTC treatment on AT1 receptor expression and function

Untreated SHR showed increased vascular AT1 receptor expression as compared to WKY rats. PDTC treatment normalized the AT1 receptor expression in SHR-PDTC (Fig. 9). To test whether the upregulation in AT1 receptor has any functional consequences we measured angiotensin II induced constriction in mesenteric artery rings. Untreated SHR exhibited enhanced angiotensin II induced vasoconstriction in comparison to WKY rats. Interestingly, PDTC treatment normalized the enhanced angiotensin II induced vasoconstriction in SHR-PDTC (Fig. 10).

4.3 Studies performed in Human Aortic Smooth Muscle Cells

To further probe a direct link between oxidative stress, NFκB activation and AT1 receptor upregulation and to validate our results from the animal studies in human cells, we performed studies in human aortic smooth muscle cells (HASMCs)

4.3.1 Effect of oxidative stress on AT1 receptor messenger RNA (mRNA) levels

The HASMCs were exposed to two different oxidants either alone or in combination and the AT1 receptor mRNA levels were determined using quantitative RT-PCR (qRT-PCR). Treatment with L-buthionine sulfoximine, a glutathione synthesis

inhibitor, at a concentration of 100uM or 400uM for 24h caused a small but insignificant increase in AT1 mRNA (Fig. 11). Further, H_2O_2 (50uM for 3h) failed to increase AT1 receptor mRNA in HASMCs. However, a robust increase of more than 2 fold was seen when an oxidative insult was provided by combining these two oxidants (BSO (400uM, 24h) + H_2O_2 (50uM, 3h)) (Fig. 11). Hence, for all further experiments this combination of oxidants was used to expose the cells to oxidative insult.

4.3.2 Effect of antioxidant enzyme catalase on oxidative stress-mediated AT1 receptor upregulation

To confirm that the effects on AT1 receptor mRNA were induced by oxidative stress we concomitantly treated the HASMCs with the oxidants and anti-oxidant enzyme catalase.

4.3.2.1 Effect of catalase on oxidative stress

The intracellular oxidative stress was determined using a ROS sensitive dye CM-DCFDA. The oxidant treatment markedly increased the oxidative stress in HASMCs as evidenced by significantly high CM-DCFDA fluorescence (Fig. 12). Concomitant treatment with catalase (100 U/ml) prevented the increase in oxidative stress whereas catalase by itself had no effect (Fig. 12).

4.3.2.2 Effect of catalase on AT1 receptor upregulation

Similar to our previous results, treatment of HASMCs with oxidants exhibited a significant increase in AT1 receptor mRNA levels. However, concomitant treatment with antioxidant catalase prevented the oxidant induced increase in AT1 receptor mRNA. These results suggest an important role of oxidative stress in upregulating AT1 receptors (Fig. 13).

4.3.3 Role of NFκB in oxidative stress-mediated AT1 receptor upregulation

To study the role of NFκB in oxidative stress mediated AT1 receptor upregulation we knocked down the p65 subunit of NFκB using a siRNA approach. The p65 siRNA significantly knocked down the expression of p65 as determined using western blotting (Fig. 14). The effect of oxidant treatment on AT1 receptor in presence or absence of p65 siRNA was also determined using qRT-PCR (Fig. 15). Oxidant treatment once again caused a robust increase in AT1 receptor mRNA in the absence of p65 siRNA. However, in the cells where p65 expression was knocked down using siRNA oxidant treatment failed to increase the AT1 receptor mRNA (Fig 15).

4.4 Studies performed at the end of resveratrol treatment in SHR and WKY rats

As mentioned previously endothelial dysfunction is a hallmark of hypertension. Furthermore, oxidative stress has been reported to play an important role in endothelial dysfunction by attenuating synthesis of nitric oxide and also by increasing NO scavenging. This section of our project was directed towards studying the effects of oxidative stress on endothelial dysfunction, using resveratrol as an antioxidant. Resveratrol is an antioxidant polyphenol that has been shown to have beneficial effects on endothelial function. Role of resveratrol in hypertension has not been established. Thus, we were also interested in elucidating the mechanisms of resveratrol-induced effects on endothelial dysfunction and hypertension development

4.4.1 Effects of resveratrol treatment on blood pressure

The food/water intake and body weights were similar in all the groups suggesting that resveratrol did not have any effect on eating and drinking behaviors of the rats (Table 1). As expected the mean, systolic and diastolic blood pressure of SHR were significantly higher in comparison to WKY rats (Table 1). Resveratrol treatment for 10 weeks significantly attenuated the development of hypertension in SHR as reflected by lower values of systolic, diastolic and mean arterial pressure (Table 1). SHR also had significantly elevated heart weight/body weight ratio which was reduced by resveratrol

treatment (Table 1). Resveratrol had no effect on blood pressure and heart weight/body weight ratio in WKY rats (Table 1).

4.4.2 Effects of resveratrol on vascular oxidative stress

SHR exhibited vascular oxidative stress as evidenced by increased aortic H_2O_2 levels (Fig. 16A). SHR also exhibited attenuated antioxidant capacity as indicated by significantly lower aortic SOD activity in comparison to WKY rats (Fig. 16B). Resveratrol treatment reduced H_2O_2 levels and improved SOD activity in SHR (Fig. 16 A&B). In WKY rats, resveratrol did not affect H_2O_2 levels but caused a modest nonsignificant increase in SOD activity (Fig. 16 A&B).

4.4.3 Effects of resveratrol on endothelial function

Acetylcholine (Ach) induced endothelium dependant relaxation in the mesenteric artery rings of SHR was attenuated as indicated by marked rightward shift of the Ach dose response curve in SHR compared to that of WKY rats (Fig. 17). There was a significant decrease in pD2 (-LogEC50) value of SHR dose response curve in comparison to that of WKY rats (7.82 ± 0.12 in SHR vs. 8.72 ± 0.26 in WKY rats, $P < 0.05$) (Fig. 17). Resveratrol treatment restored the endothelium dependant relaxation as indicated by marked leftward shift of the Ach dose response curve of SHR-R in comparison to the

untreated SHR. Resveratrol also increased the pD2 values of SHR-R (7.82 ± 0.12 in SHR vs. 8.2 ± 0.1 in SHR-R) (Fig. 17). Resveratrol treatment had no effect on Ach induced vasorelaxation in WKY rats (8.72 ± 0.26 in WKY rats vs. 8.5 ± 0.23 in WKY-R). The endothelium independent relaxation in response to SNP, a direct NO donor, was similar in SHR and WKY rats. However, resveratrol treatment increased the relaxation in response to higher doses of SNP in SHR but not in WKY rats (110 ± 7.16% in SHR vs. 131.8 ± 5.18% in SHR-R, $P < 0.05$) (Fig. 18).

4.4.4 Effects of resveratrol on NO availability and production

4.4.4.1 Effects of resveratrol on NO availability

Reactive oxygen species such as superoxides reduce NO availability by scavenging it via conversion of NO to peroxynitrite. To test the effects on resveratrol on NO scavenging we determined the levels of 3-nitrotyrosine in the aortic homogenates. Aortic 3-nitrotyrosine content, which is an indicator of protein modification mediated primarily by peroxynitrite, was significantly higher in SHR compared to WKY rats (Fig. 19). Resveratrol treatment normalized the nitrotyrosine levels in SHR with no change in WKY rats (Fig. 19).

4.4.4.2 Effects of resveratrol on NO production

The NO production was determined by measuring the aortic nitrite/nitrate levels. Aortic nitrite nitrate levels were significantly reduced in SHR compared to WKY rats, suggesting attenuated NO production (Fig. 20). Resveratrol treatment restored the NO levels in the aortic homogenate of SHR while having no effect in WKY rats (Fig. 20).

4.4.4.3 Effects of resveratrol on expression of eNOS and soluble guanylyl cyclase

To determine if resveratrol mediated improvement of endothelial function was associated with changes in protein expression of NO synthesizing enzyme eNOS and its proximal effector soluble guanylyl cyclase, we determined the expression of eNOS and β1 subunit of soluble guanylyl cyclase by western blotting. Protein expression of eNOS was slightly but insignificantly higher in SHR compared to WKY rats (Fig. 21). Resveratrol treatment significantly increased the expression of eNOS in both SHR and WKY rats (Fig. 21). The basal expression of aortic sGC (β1 subunit) was higher in SHR as compared to WKY rats, resveratrol treatment further increased the expression of sGC in both SHR and WKY rats (Fig. 22).

4.4.5 Effects of resveratrol on eNOS coupling

To determine eNOS uncoupling we measured eNOS induced superoxide production in the presence and absence of NOS substrate L-arginine in aortic homogenates. The basal superoxide levels were similar in all experimental groups (42174 ± 5716 in WKY, 44384 ± 6363 in WKY-R, 45263 ± 6473 in SHR, and 49250 ± 4334 in SHR-R, arbitrary units). Incubation of aortic homogenates with L-arginine did not increase superoxide generation in WKY rats (Fig. 23). However we found a significant increase in superoxide production in SHR. This superoxide generation was inhibited by L-NNA suggesting that the superoxides production was eNOS dependent (Fig. 23). The superoxide production in response to L-arginine was abolished in resveratrol treated SHR (Fig. 23). Furthermore, incubation of aortic homogenates with NOS cofactor tetrahydrobiopterin (BH4) also abolished the increased superoxide production in response to L-arginine seen in SHR (Fig. 24).

4.5 Studies performed in Sprague Dawley (SD) rats to test the acute effects of resveratrol on angiotensin II induced pressor response

To test acute effects of resveratrol in vivo, pressor response to a bolus dose of Ang II was measured either in presence or absence resveratrol pretreatment. Resveratrol pretreatment significantly attenuated the increase in systolic, diastolic and mean pressure

in response to ang II (Fig. 25). Furthermore, the ability of resveratrol to block ang II induced increase in systolic, diastolic and mean pressure was prevented by eNOS inhibitor L-NAME, suggesting involvement of NO pathway in mediating resveratrol's effects (Fig. 26).

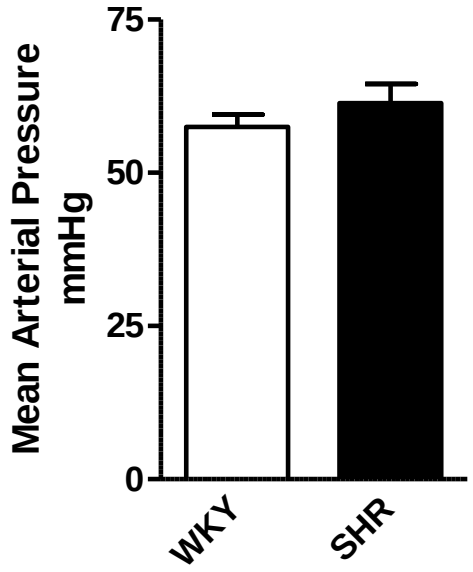


Figure 1: Similar mean arterial pressure in SHR and WKY rats at 3 - 4 weeks of age as measured by intra-aortic catheterization. Bars represent mean ± SE of 6 rats in each group

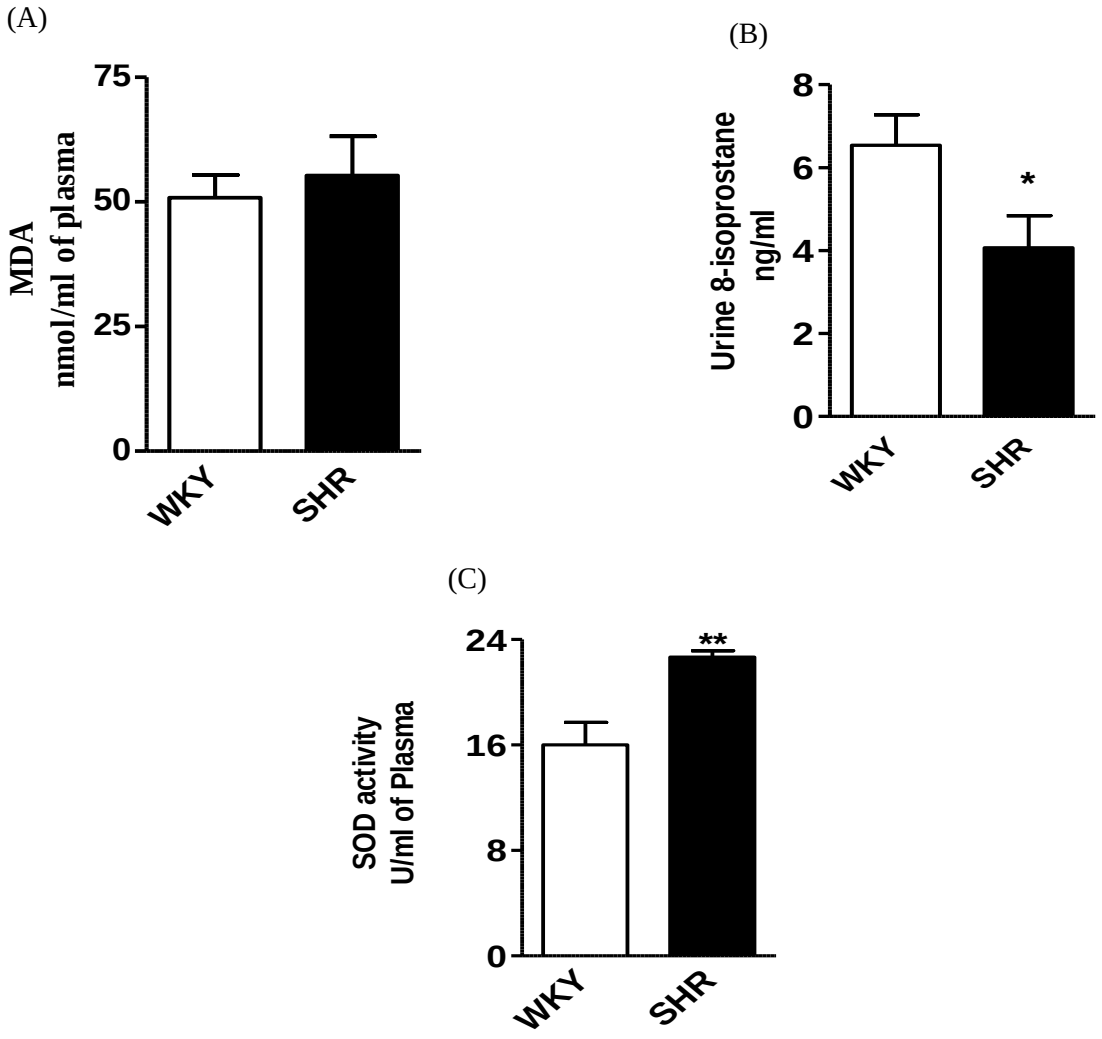


Figure 2: Markers of systemic oxidative stress in 3 - 4 week old SHR and WKY. (A) Similar levels of plasma malondialdehyde in SHR and WKY. (B) Lower levels of urinary 8-isoprostane in SHR. (C) Higher activity of antioxidant enzyme SOD in SHR plasma in comparison to WKY. Bars represent mean ± SEM and data (n = 6-7 rats in each group) were analyzed using Student's t test. **P<0.05 vs. WKY* and ***P<0.01 vs. WKY*

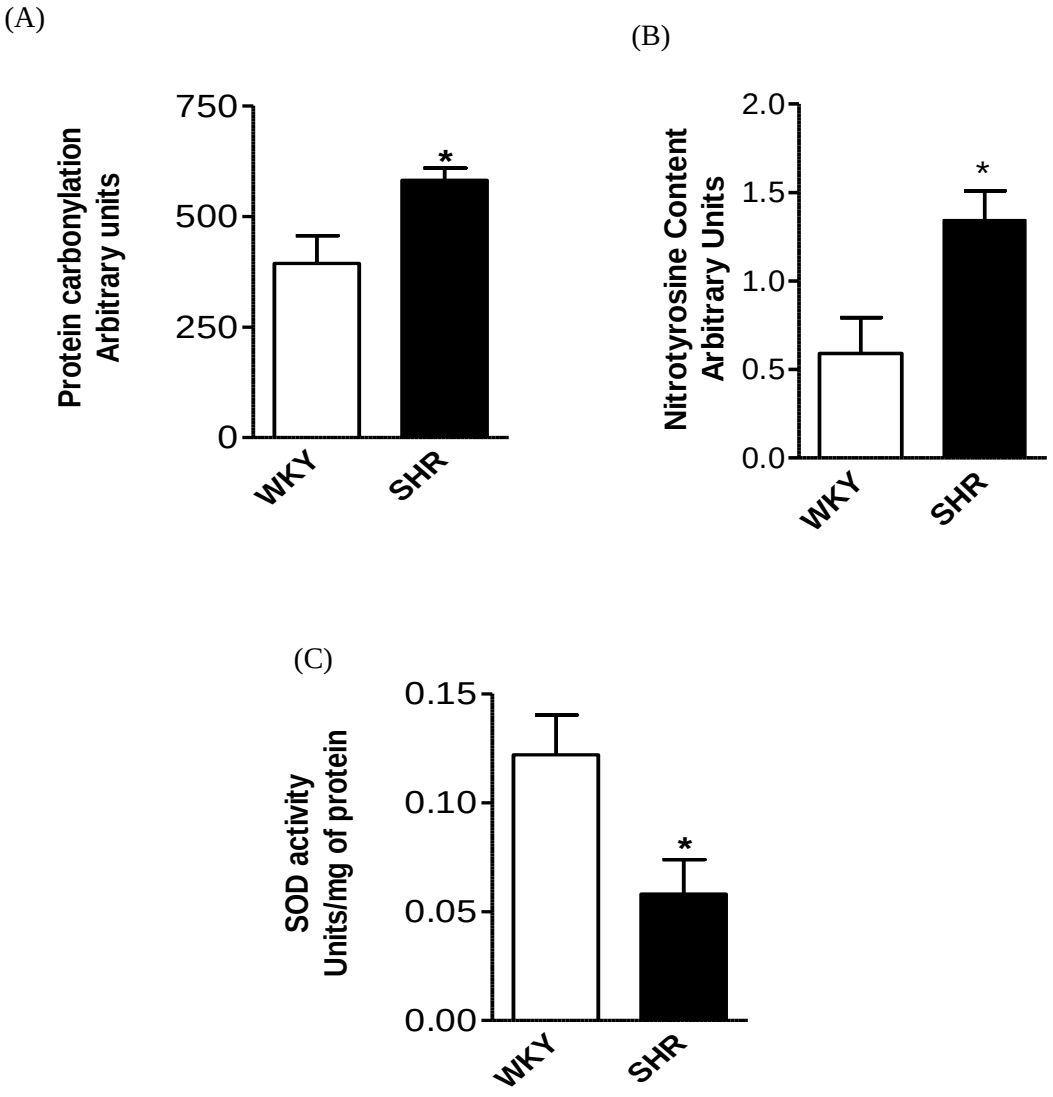
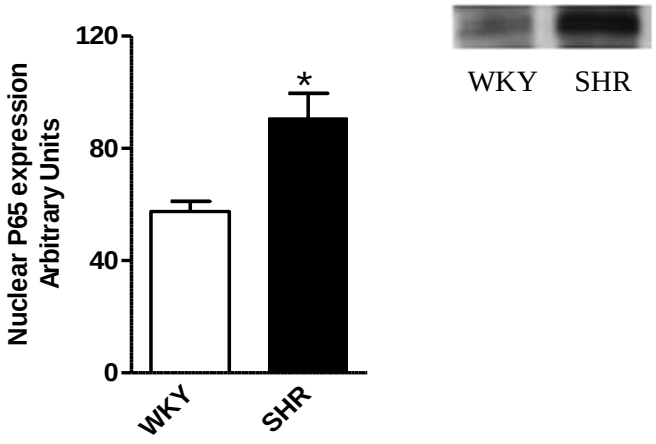


Figure 3: Markers of oxidative stress in artery homogenates of 3-4 weeks old SHR and WKY rats. (A) Protein carbonylation levels (B) Nitrotyrosine content and (C) Superoxide dismutase (SOD) activity in the vascular homogenate. Bars represent mean ± SEM. Data (n = 5 - 6 rats per group) were analyzed using Student's t test. **P < 0.05 vs. WKY*.

(A)



(B)

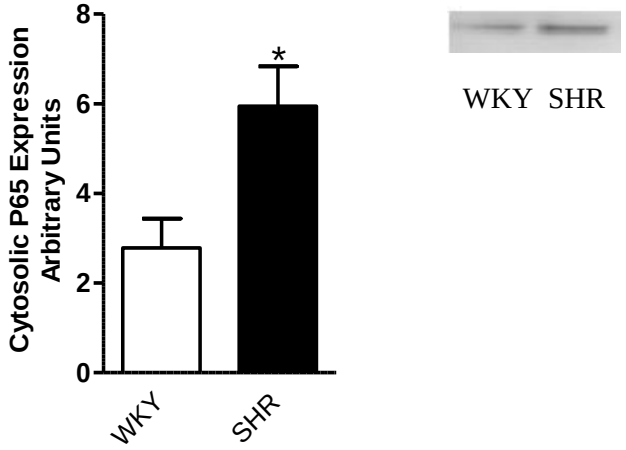


Figure 4: Vascular NFκB activation in 3 - 4 week old SHR and WKY rats. (A) Aortic NFκB p65 subunit expression in nuclear fraction of 3 - 4 week old SHR and WKY rats. (B) NFκB p65 subunit expression in cytosolic fraction 3 - 4 week old SHR and WKY rats' aortas. Bands are representative western blots. Bars represent mean ± SEM. Data (n = 5 rats) were analyzed using Student's t test. **P < 0.05 vs. WKY.*

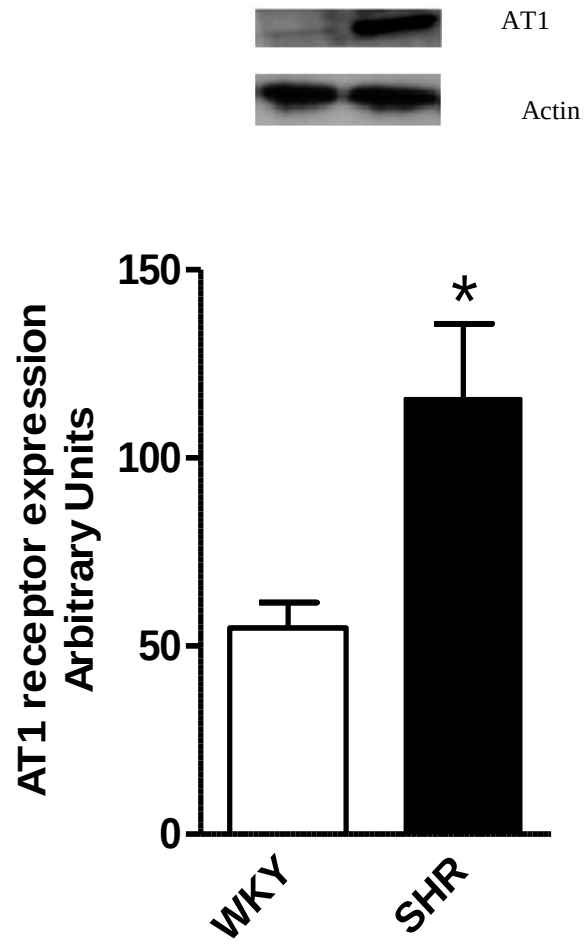


Figure 5: Increased AT1 receptor protein in 3 - 4 weeks old SHR and WKY rats' femoral artery homogenate. Bands are representative western blots. Bars represent Mean ± SEM. Data (n = 5 - 6 rats in each group) were analyzed using Student's t test. **P<0.05 Vs WKY*.

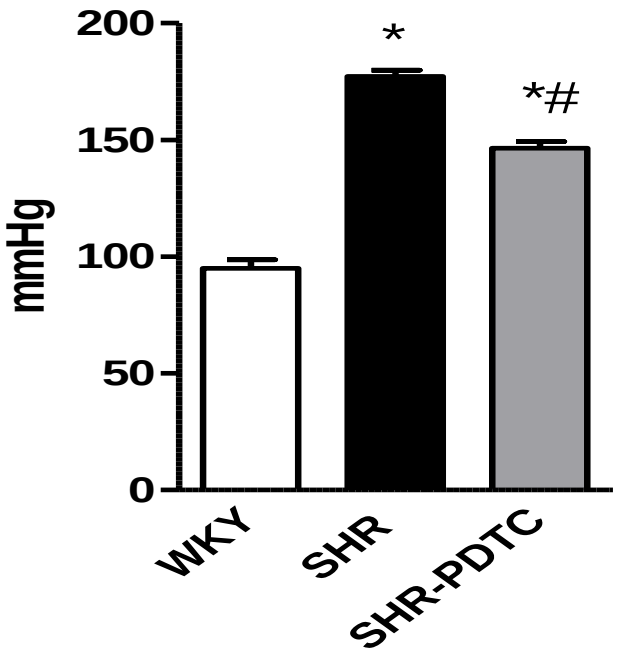


Figure 6: Mean arterial pressure in adult untreated WKY rats (WKY), untreated SHR (SHR) and PDTC treated SHR (SHR-PDTC). Bars represent mean ± SEM. Data (n = 5 - 6 rats per group) were analyzed using one way ANOVA followed by posthoc Newman-Keuls test. *$*P < 0.05$ vs. WKY. $^{\#}P < 0.05$ vs. SHR.*

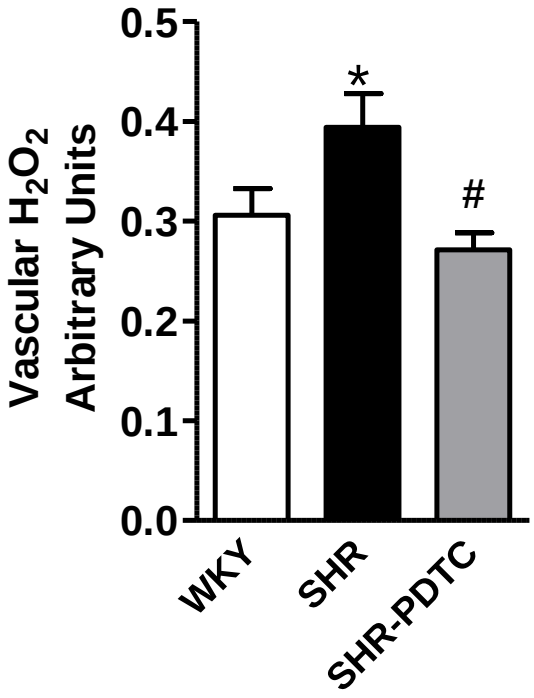


Figure 7: Vascular hydrogen peroxide levels after PDTC treatment in adult untreated WKY rats (WKY), untreated SHR (SHR) and PDTC treated SHR (SHR-PDTC). Bars represent mean ± SEM. Data (n = 5 - 6 rats per group) were analyzed using one way ANOVA followed by posthoc Newman-Keuls test. $^{*}P < 0.05$ vs. WKY. $^{\#}P < 0.05$ vs. SHR.

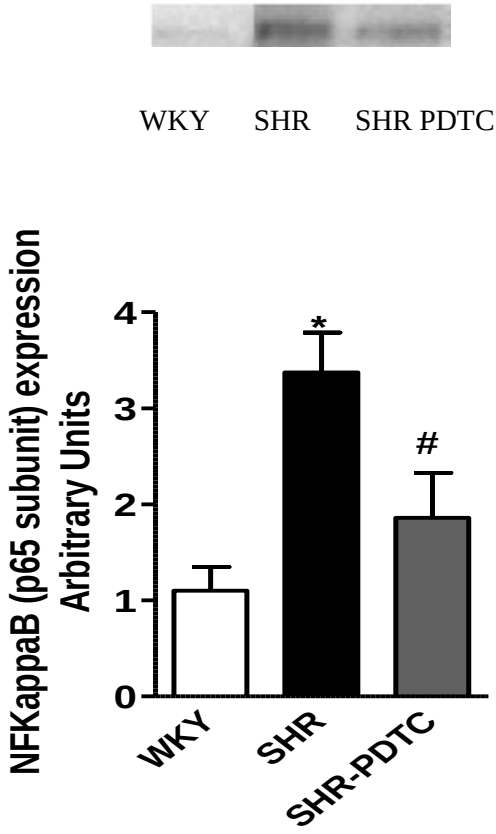


Figure 8: NFκB p65 subunit expression in adult untreated WKY rats (WKY), untreated SHR (SHR) and PDTC treated SHR (SHR-PDTC). Bars represent mean ± SEM. Data (n = 5-6 rats) were analyzed using one way ANOVA followed by posthoc Newman-Keuls test. $^{*}P < 0.05$ vs. WKY. $^{\#}P < 0.05$ vs. SHR.

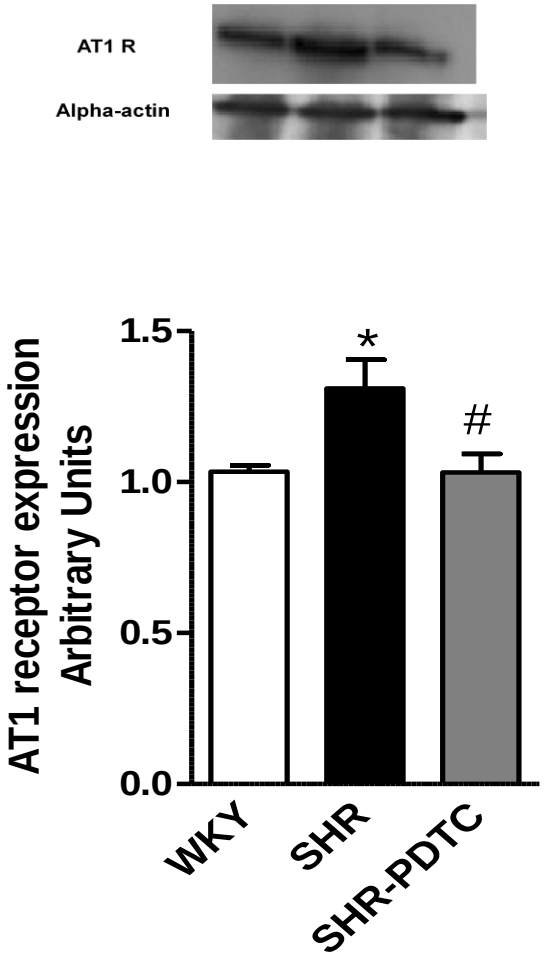


Figure 9: AT1 receptor expression in vascular homogenate of adult untreated WKY rats (WKY), untreated SHR (SHR) and PDTC treated SHR (SHR-PDTC). Bars represent mean ± SEM. Data (n = 5 - 6 rats) were analyzed using one way ANOVA followed by posthoc Newman-Keuls test. $^{*}P < 0.05$ vs. WKY. $^{\#}P < 0.05$ vs. SHR.

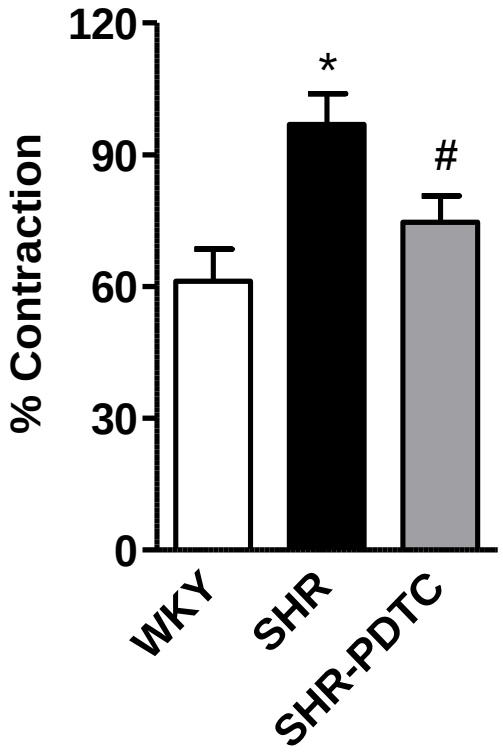


Figure 10: Angiotensin II induced contractions in isolated mesenteric artery rings of adult untreated WKY rats (WKY), untreated SHR (SHR) and PDTC treated SHR (SHR-PDTC). Bars represent mean ± SEM. Data (n = 5-6 rats) were analyzed using one way ANOVA followed by posthoc Newman-Keuls test. $^{*}P < 0.05$ vs. WKY. $^{\#}P < 0.05$ vs. SHR.

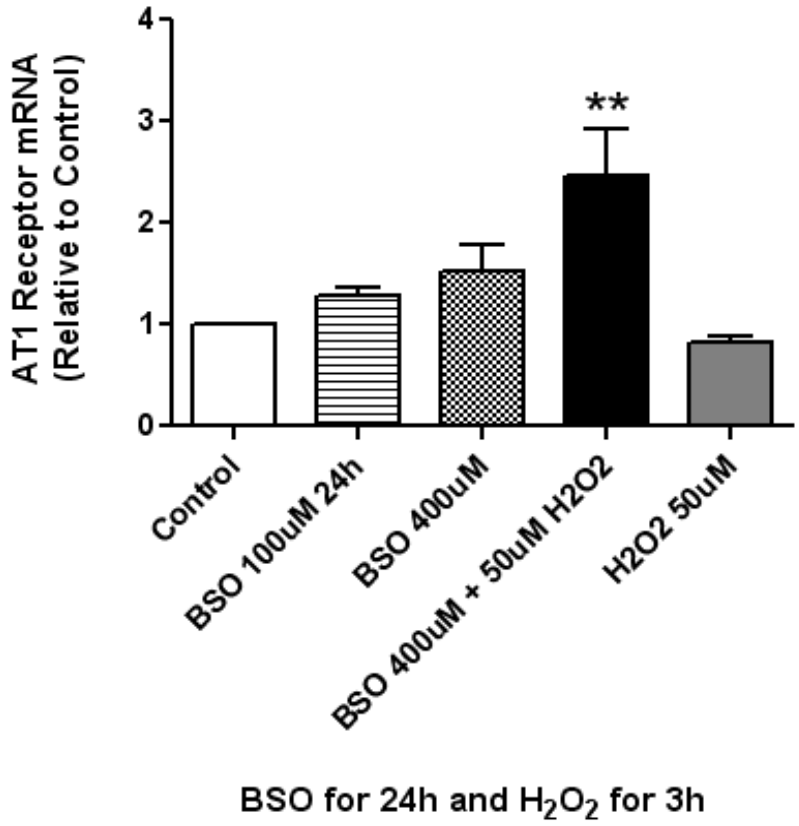


Figure 11: AT1 receptor mRNA levels in HASMC determined using qRT-PCR following treatment with oxidants BSO and H_2O_2 either alone or in combination as indicated. Bars represent mean ± SEM. Data (n = 3-5) was analyzed using one way ANOVA followed by posthoc Newman-Keuls test. *$P < 0.05$ vs. Control

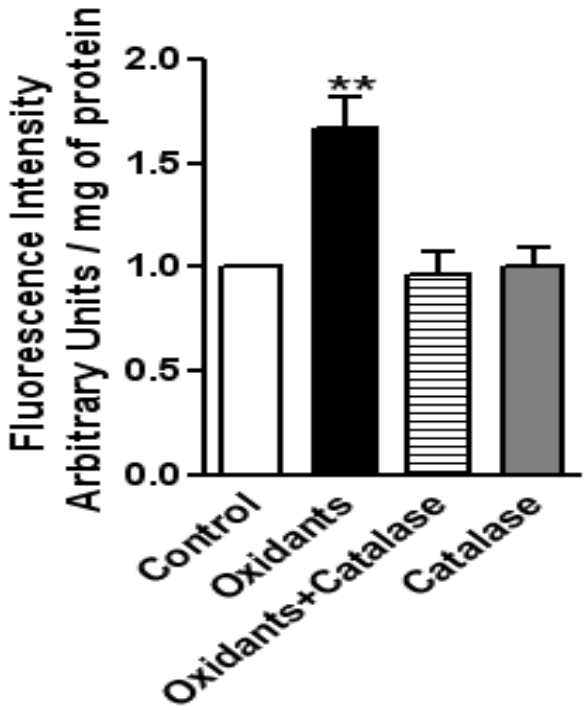


Figure 12: Intracellular oxidative stress determined using cell permeable fluorescent probe, CM-H_2DCFDA, in control HASMCs, HASMCs treated with oxidants (BSO+H_2O_2), Catalase (100U/mL) + Oxidants or Catalase (100U/mL) alone. Bars represent mean ± SEM. Data (n = 3) was analyzed using one way ANOVA followed by posthoc Newman-Keuls test. *$P < 0.05$ vs. Control

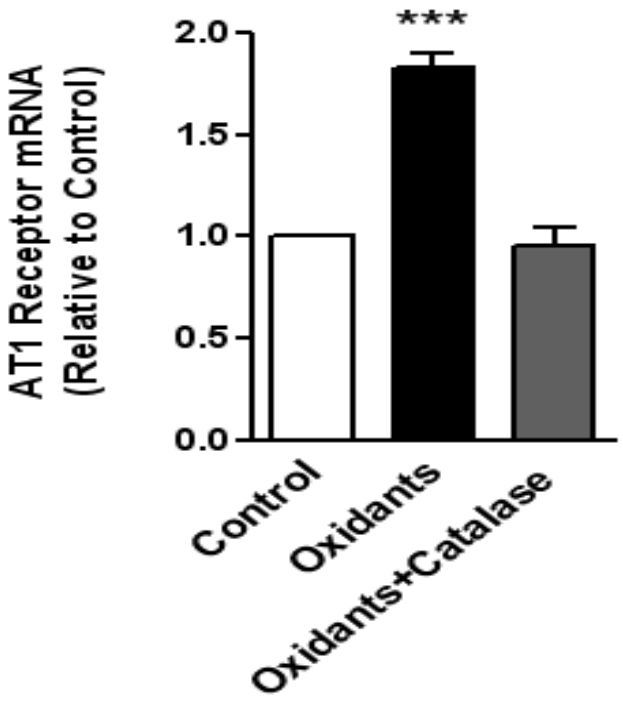


Figure 13: AT1 receptor mRNA levels determined using qRT-PCR in control HASMCs, HASMCs treated with oxidants (BSO + H_2O_2) or oxidants + Catalase (100 U/ml) indicated. Bars represent mean ± SEM. Data (n = 3) was analyzed using one way ANOVA followed by posthoc Newman-Keuls test. *$P < 0.05$ vs. Control

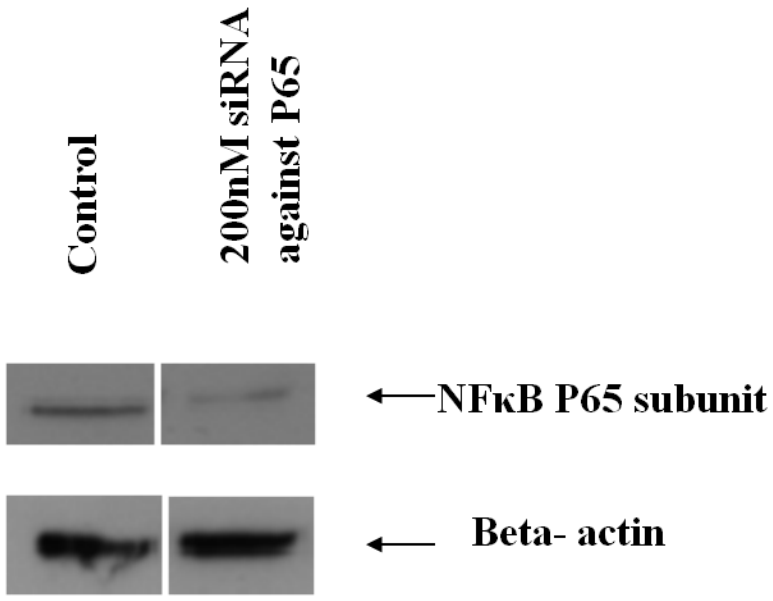


Figure 14: Representative blot showing knockdown of NFκB p65 subunit expression following treatment with siRNA against NFκB p65.

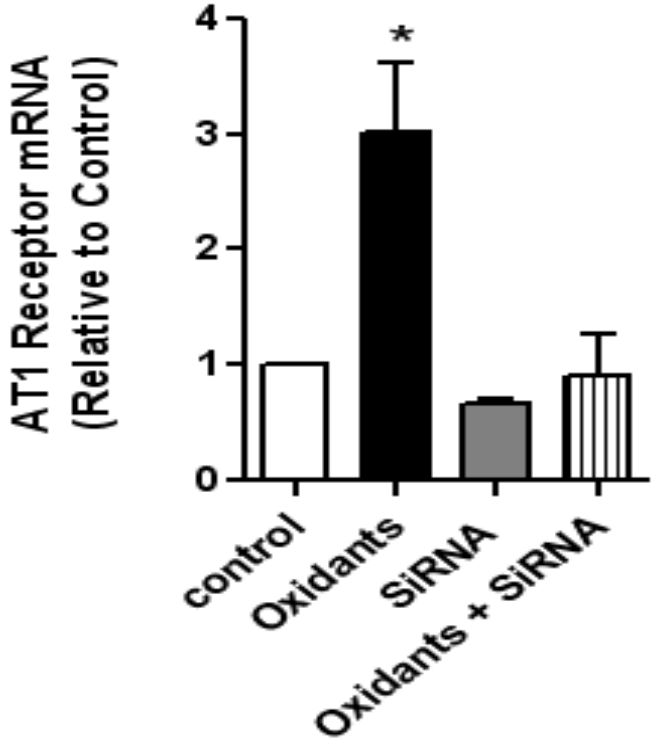


Figure 15: AT1 receptor mRNA levels determined using qRT-PCR in control HASMCs and HASMCs treated with oxidants (BSO + H_2O_2) either in presence or absence of siRNA against NFκB p65 subunit. . Bars represent mean ± SEM. Data (n = 3 - 5) was analyzed using one way ANOVA followed by posthoc Newman-Keuls test. *$P < 0.05$ vs. Control

Table 1: General parameters and blood pressure with resveratrol treatment.

PARAMETERS	WKY	WKY-R	SHR	SHR-R
Body Weight (g)	313.4 ± 9.4	320.6 ± 6.4	321.1 ± 10.6	321.7 ± 6.3
Water Intake (ml/day)	27.5 ± 0.5	25.6 ± 0.8	28.2 ± 0.7	29.7 ± 0.7
Food Intake (g/day)	22.0 ± 0.4	21.3 ± 0.5	21.5 ± 0.42	22.1 ± 0.5
Heart wt/Body wt	3.16 ± 0.17	3.06 ± 0.08	3.78 ± 0.11*	3.45 ± 0.05$^{\#}$
SBP (mmHg)	116.0 ± 1.4	114.7 ± 3.0	221 ± 1.9*	**195.6 ± 3.2*$^{\#}$**
DBP (mmHg)	82.1 ± 2	81.4 ± 2.8	160.0 ± 1.4*	**142.0 ± 2.7*$^{\#}$**
MAP (mmHg)	93.6 ± 1.8	92.5 ± 2.7	180.7 ± 1.3*	**160.6 ± 1.9*$^{\#}$**

Food and water intake, body weight, heart weight/body weight (ratio x 10^3), systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP) in WKY rats, resveratrol treated WKY rats (WKY-R), SHR and resveratrol treated SHR (SHR-R). Data are represented as mean ± SEM (n = 5–6 rats) and were analyzed using one way ANOVA followed by posthoc Newman-Keuls test.* $P < 0.05$ vs. WKY, $^{\#}P < 0.05$ vs. SHR

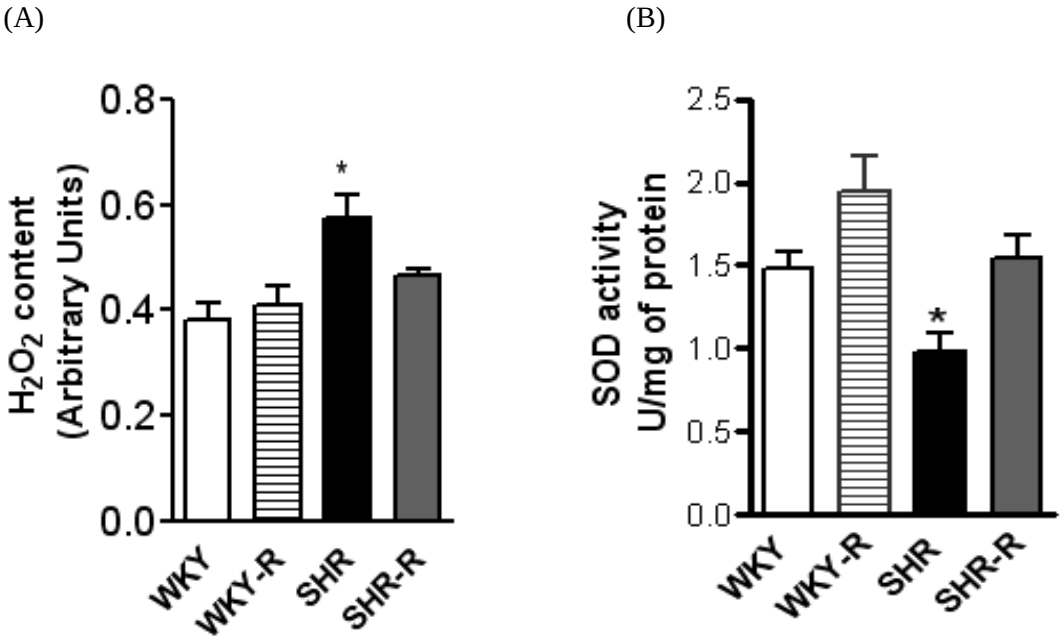


Figure 16: Markers of oxidative stress in aortic homogenates of WKY rats, resveratrol treated WKY rats (WKY-R), SHR and resveratrol treated SHR (SHR-R). A) H2O2 levels in the aortic tissue. B) Superoxide dismutase (SOD) activity in the aortic homogenate. Bars represent mean ± SEM and data (n = 5 - 6 rats) were analyzed using one way ANOVA followed by posthoc Newman-Keuls test. *$P < 0.05$ vs. WKY

pD2	WKY	WKY-R	SHR-C	SHR-R
(Mean±SEM)	8.72±0.26	8.53±0.23	7.82±0.12*	8.2±0.10

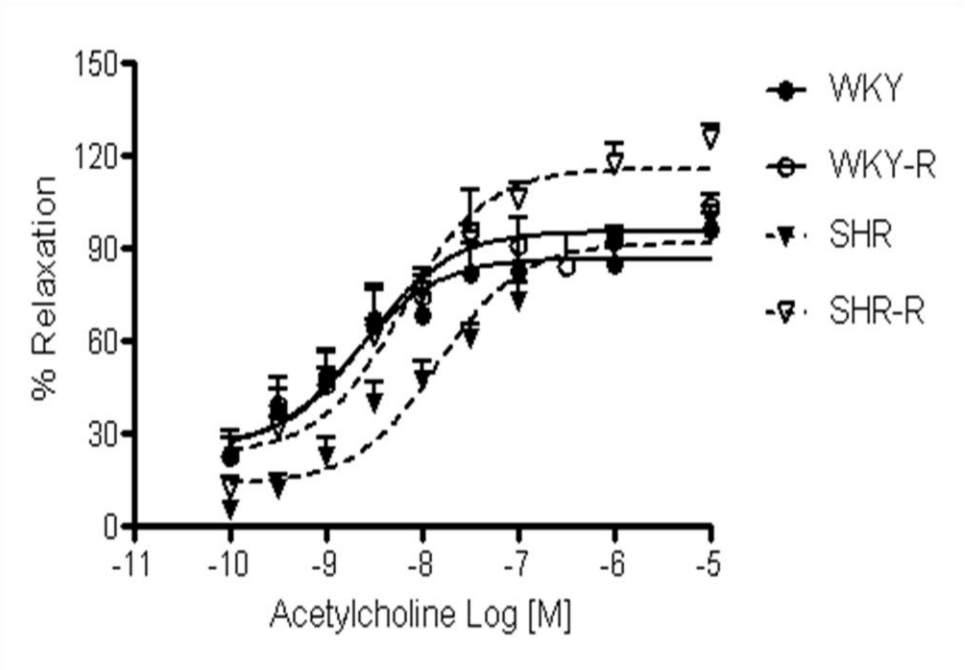


Figure 17: Endothelium dependant relaxations in response to acetylcholine (Ach) in mesenteric artery rings of WKY rats, resveratrol treated WKY rats (WKY-R), SHR and resveratrol treated SHR (SHR-R) A). pD_2 (-Log EC50) values represented in the inset. Data represented as mean ± SEM, data (n = 5 – 6 rats) were analyzed using one way ANOVA followed by posthoc Newman-Keuls test. *$P < 0.05$ vs. WKY

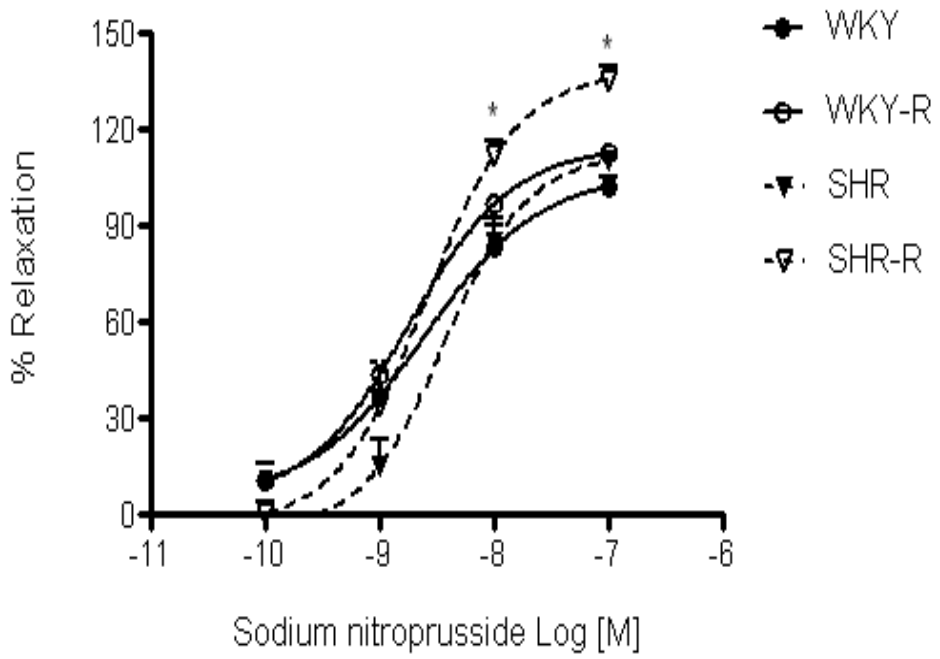


Figure 18: Endothelium independent relaxation in response to sodium nitroprusside (SNP) in mesenteric artery rings of WKY rats, resveratrol treated WKY rats (WKY-R), SHR and resveratrol treated SHR (SHR-R). Data represented as mean ± SEM, data (n = 5 – 6 rats) were analyzed using one way ANOVA followed by posthoc Newman-Keuls test. *$P < 0.05$ vs. WKY

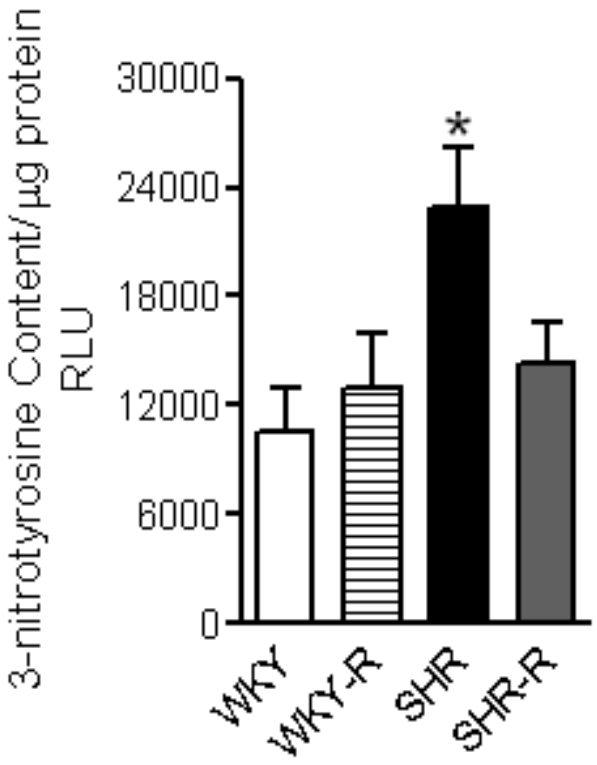


Figure 19: Vascular 3-nitrotyosine levels in WKY rats, resveratrol treated WKY rats (WKY-R), SHR and resveratrol treated SHR (SHR-R). Bars represent mean ± SEM and data (n = 5 - 6 rats) were analyzed using one way ANOVA followed by posthoc Newman-Keuls test. *$P < 0.05$ vs. WKY.

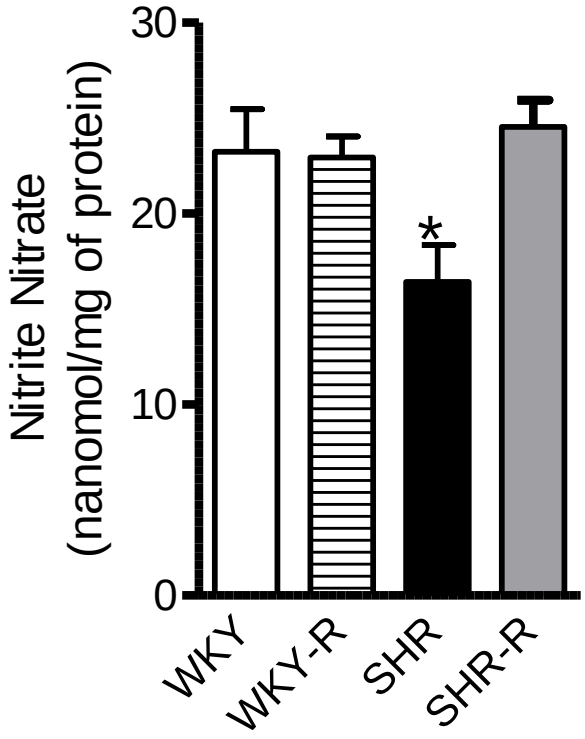


Figure 20: Vascular nitrite/nitrate levels in WKY rats, resveratrol treated WKY rats (WKY-R), SHR and resveratrol treated SHR (SHR-R). Bars represent mean ± SEM and data (n = 5 - 6 rats) were analyzed using one way ANOVA followed by posthoc Newman-Keuls test. *$P < 0.05$ vs. WKY.

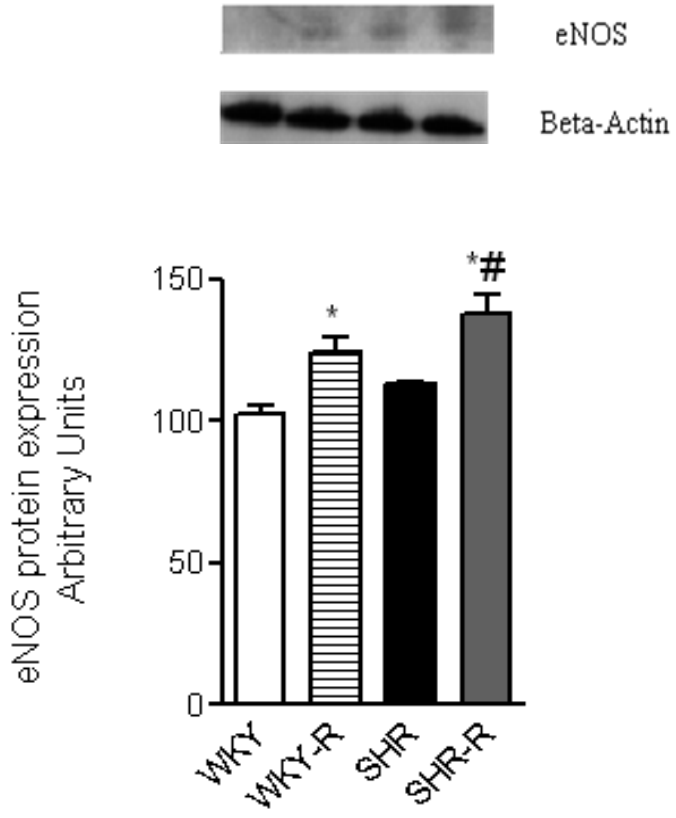


Figure 21: Vascular eNOS protein expression in WKY rats, resveratrol treated WKY rats (WKY-R), SHR and resveratrol treated SHR (SHR-R). Bands are representative western blots. Bars represent mean ± SEM and data (n = 5 - 6 rats) and were analyzed using one way ANOVA followed by posthoc Newman-Keuls test. $^{*}P < 0.05$ vs. WKY, $^{\#}P < 0.05$ vs. SHR

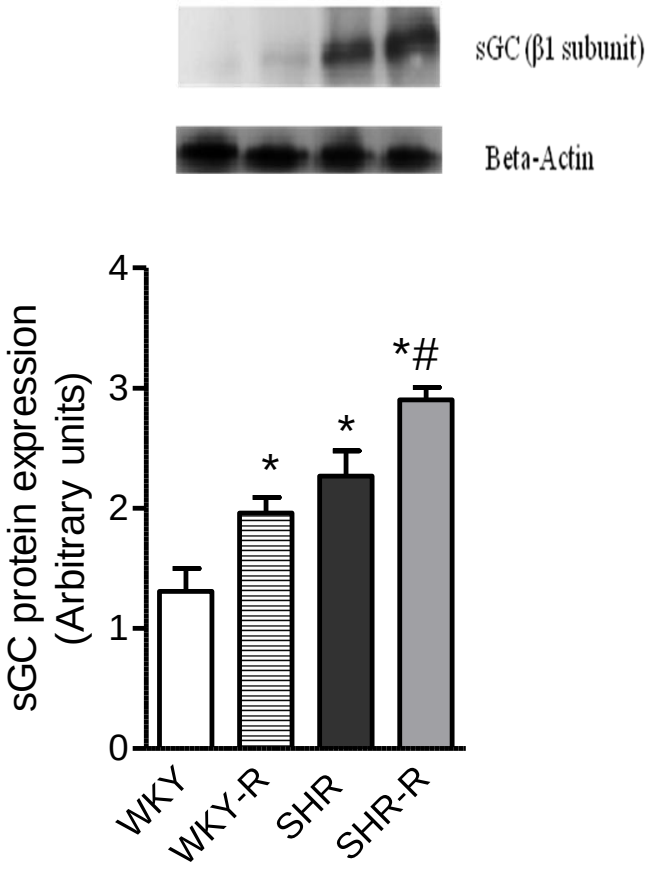


Figure 22: Vascular soluble guanylyl cyclase protein expression in WKY rats, resveratrol treated WKY rats (WKY-R), SHR and resveratrol treated SHR (SHR-R). Bands are representative western blots. Bars represent mean ± SEM and data (n = 5 - 6 rats) and were analyzed using one way ANOVA followed by posthoc Newman-Keuls test. *$P < 0.05$ vs. WKY, $^{\#}P < 0.05$ vs. SHR

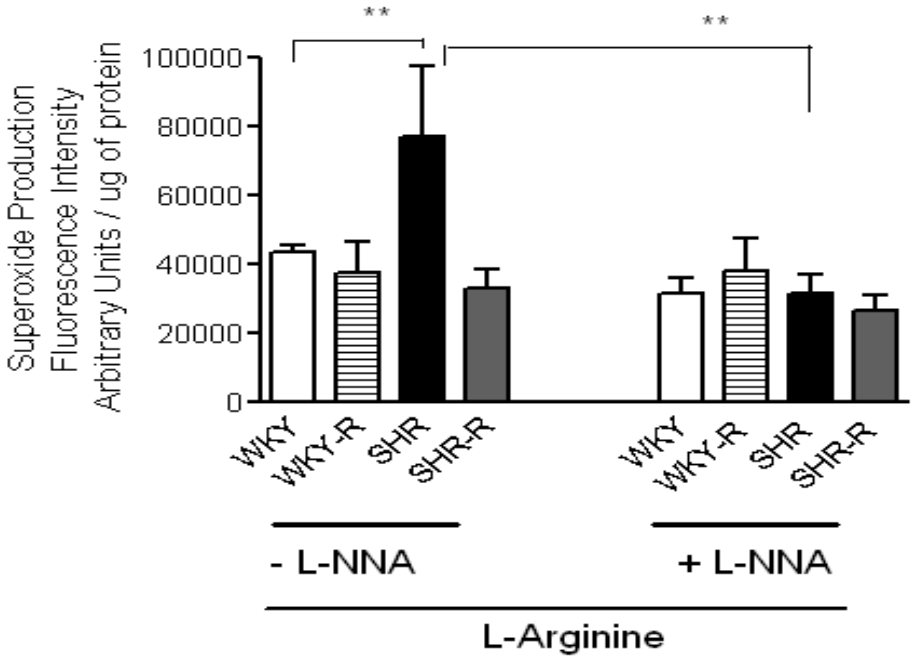


Figure 23: Superoxide production in presence or absence of eNOS inhibitor L-NNA in aortic homogenates of WKY rats, resveratrol treated WKY rats (WKY-R), SHR and resveratrol treated SHR (SHR-R) in response to eNOS substrate L-arginine. Bars represent Mean ± SEM. Data (n = 5 rats) were analyzed using one way ANOVA followed by posthoc Newman-Keuls test. ***P < 0.01*.

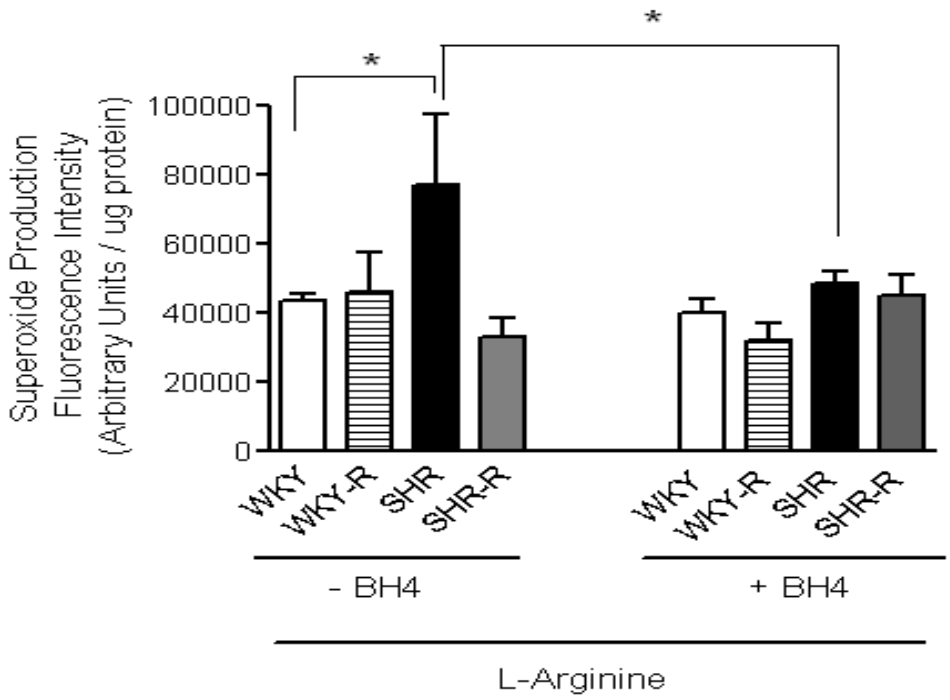


Figure 24: Superoxide production in presence or absence of eNOS cofactor tetrahydrobiopterin (BH4) in aortic homogenates of WKY rats, resveratrol treated WKY rats (WKY-R), SHR and resveratrol treated SHR (SHR-R) in response to eNOS substrate L-arginine. Bars represent Mean ± SEM. Data (n = 5 rats) were analyzed using one way ANOVA followed by posthoc Newman-Keuls test. **P <0.05.*

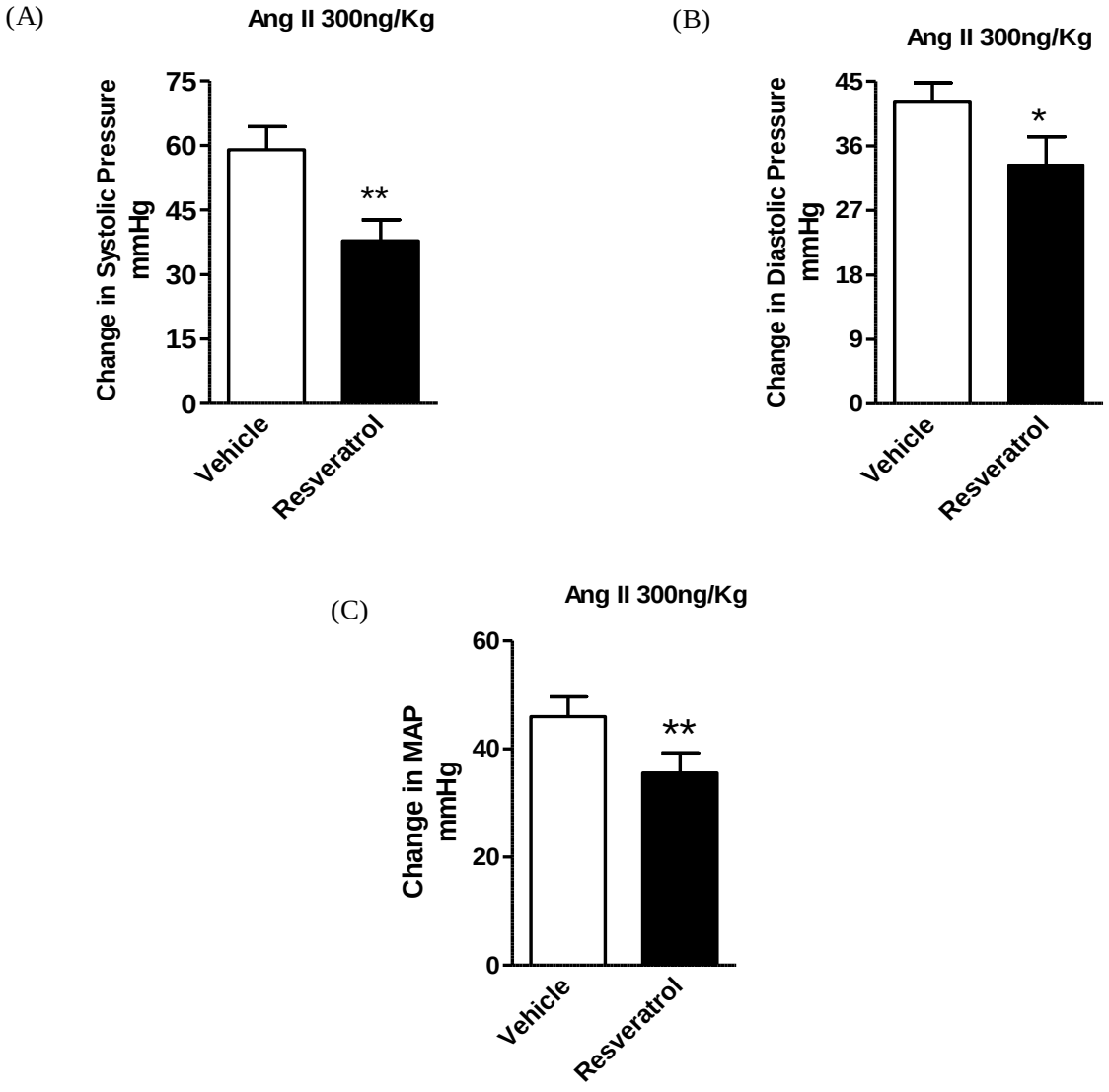


Figure 25: Change in blood pressure with a bolus administration of 300 ng/kg angiotensin II in SD rats pretreated with vehicle (white bars) or resveratrol (black bars). (A) Systolic pressure, (B) Diastolic pressure and (C) Mean arterial pressure. Bars represent Mean ± SEM. Data (n = 5 rats) were analyzed using paired Student's t test. **P <0.05 vs.* vehicle *and **P<0.01 vs.* vehicle.

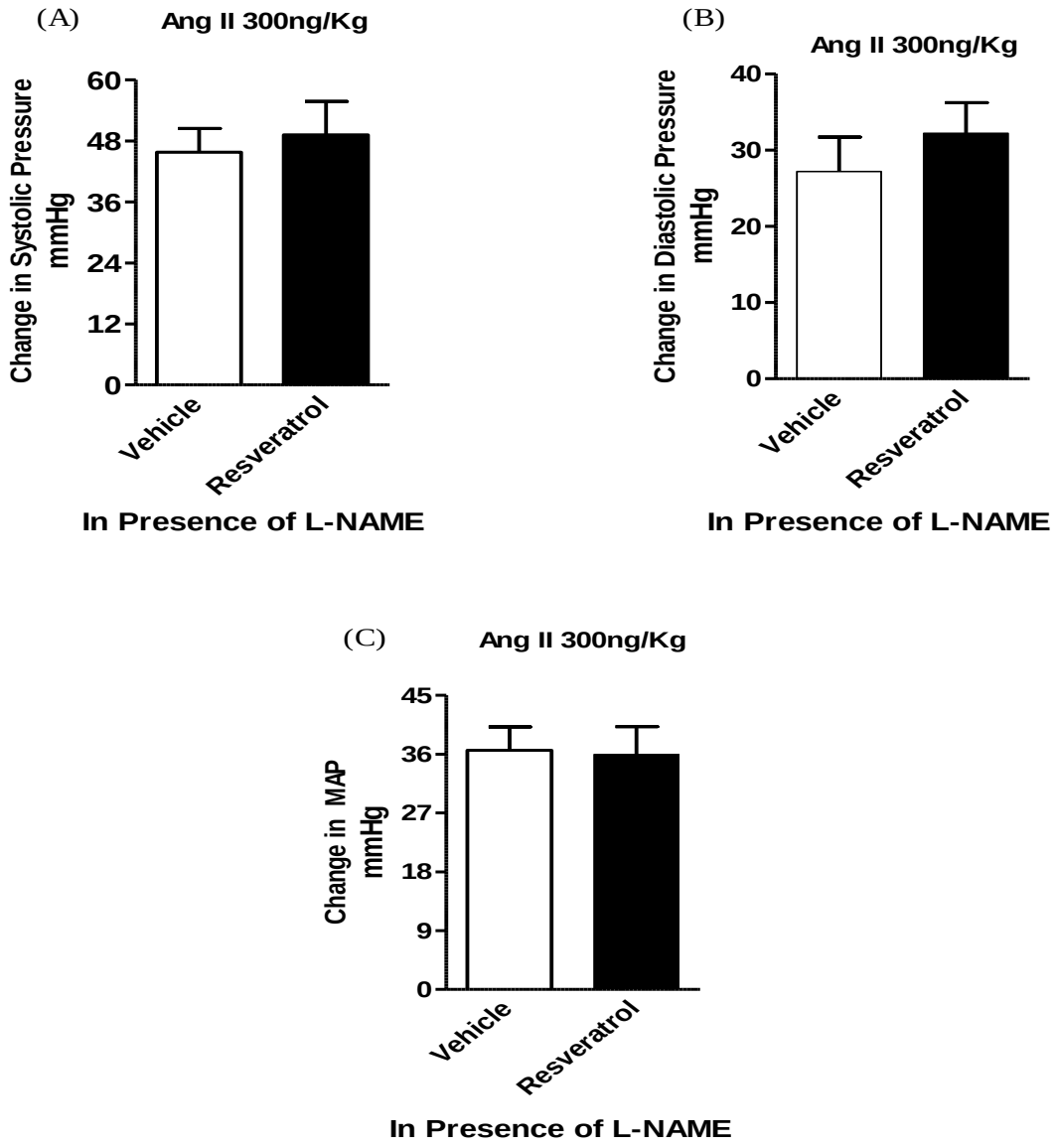


Figure 26: Change in blood pressure with a bolus administration of 300 ng/kg angiotensin II in SD rats pretreated with NOS inhibitor L-NAME and either vehicle (white bars) or resveratrol (black bars) . (A) Systolic pressure, (B) Diastolic pressure and (C) Mean arterial pressure. Bars represent Mean ± SEM.

5. DISCUSSION

This project was designed to study the pathophysiological role of vascular oxidative stress in development of hypertension. The project was essentially divided in to two parts. The first part investigates whether oxidative stress precedes the development of hypertension in SHR. Our results demonstrate that oxidative stress is detected prior to development of hypertension in SHR. Furthermore, this increased oxidative stress was localized to the vasculature and it was not a systemic phenomenon. Our results also show for the first time that this early vascular oxidative stress is associated with NFκB activation and AT1 receptor upregulation. We also investigated the causal effect of oxidative stress on vascular AT1 receptor upregulation in SHR and the role of NFκB in this phenomenon, by treatment of SHR with antioxidant PDTC throughout the developmental phase of hypertension. PDTC treatment attenuated oxidative stress and significantly lowered NFκB expression. Inhibition of NFκB with PDTC normalized AT1 receptor expression and attenuated the development of hypertension in SHR. Additionally, we also validated our results regarding oxidative stress and AT1 receptor upregulation in HASMCs. HASMCs exposed to oxidative stress exhibited increased AT1 receptor mRNA levels. The role of oxidative stress and NFκB were confirmed by blocking the AT1 receptor upregulation using antioxidants and siRNA against NFκB respectively.

The second part of the project was designed to study the role of oxidative stress on endothelial dysfunction with specific focus on investigating the effect of resveratrol,

an antioxidant polyphenol, on endothelial dysfunction and hypertension development in SHR. The novel findings of this section of the study are that early chronic resveratrol treatment prevents endothelial dysfunction and significantly attenuates development of hypertension in SHR. The beneficial effects of resveratrol appear to be mediated by enhanced synthesis and availability of nitric oxide due to attenuation of eNOS uncoupling and lower NO scavenging in SHR.

5.1 Early vascular oxidative stress and AT1 receptor upregulation

Oxidative stress has been associated with experimental hypertension as well as human hypertension and antioxidant supplementation has been shown to lower blood pressure (Akpaffiong and Taylor, 1998; Banday et al., 2007b; Ceriello, 2008; Ceriello et al., 1991; Rodriguez-Iturbe et al., 2003). To test whether oxidative stress precedes development of hypertension, 3 - 4 wk old SHR and WKY rats were used for our initial studies. Hypertension in SHR starts to develop by the age of 5 - 6 weeks, blood pressure continues to rise and plateaus around 13 - 14 weeks of age (Adams et al., 1989). SHR at 3 - 4 weeks of age are prehypertensive i.e. the hypertension has not manifested at this stage and is in the developmental phase. Hence, at this age we can identify factors which may play a role in the development of hypertension. In our study we confirmed that blood pressure was similar in 3 - 4 weeks old SHR and WKY rats. However, we found that oxidative stress in the vasculature was elevated as evidenced by elevated protein

oxidation and elevated nitrotyrosine levels in the femoral artery homogenate. Furthermore, SHR artery homogenates also exhibited significantly reduced SOD activity. These results suggest that vascular oxidative stress precedes development of hypertension and may be contributing towards pathogenesis of hypertension in SHR. This is supported by report showing a 14% and 26% decrease in blood pressure of male and female SHRs respectively that were treated with antioxidant tempol from birth (Fortepiani and Reckelhoff, 2005). Nabha and colleagues have reported increased vascular oxidative stress as evidenced by increased superoxide levels in prehypertensive 6 weeks old SHR, which is consistent to our findings. They also report that chronic antioxidant treatment with tempol in prehypertensive 6 week old SHR for a period of 5 weeks completely inhibits hypertension development (Nabha et al., 2005). However, the role of oxidative stress in pathogenesis of hypertension and the mechanisms involved have not been elucidated.

The AT1 receptor plays a major role in regulation of high blood pressure by various central and peripheral mechanisms. Several studies have shown that AT1 receptor expression and signaling is upregulated in human and experimental hypertension (Nickenig et al., 1998; Reja et al., 2006; Touyz et al., 2003). We also found significantly increased vascular AT1 receptor expression in 3-4 wks old SHR as compared to WKY rats in the femoral artery homogenate. This finding is consistent with those of Schiffrin and colleagues showing increased density of vascular angiotensin receptors in the mesenteric arteries of 4 and 6 week old SHR (Schiffrin et al., 1984). Furthermore,

Endemann and colleagues have reported increased angiotensin II induced vasoconstriction in small arteries of 6 week old SHR as compared to WKY rats (Endemann et al., 1999). Lastly, treatment with an AT1 receptor antagonist losartan in young SHR from 3 weeks of age to 8 weeks of age causes a decrease in blood pressure which is maintained until 25 weeks of age (Bergstrom et al., 2002). Thus, our results alongwith these reports suggest that vascular AT1 receptor upregulation exists early in the life of SHR and could contribute to the development of high blood pressure. However, the mechanisms leading to this upregulation have not been established.

In several models of hypertension, oxidative stress and AT1 receptor upregulation co-exist. Oxidative stress can act as an important regulator of gene transcription via modulation of several redox sensitive transcription factors like AP1 and nuclear factor kappa B (NFκB). The transcriptional activity of NFκB is mainly dependent on the nuclear translocation of the P65 subunit (Schmitz and Baeuerle, 1991). Our results indicate that the nuclear as well as cytosolic p65 levels were significantly increased in the vasculature of SHR as compared to WKY at 3 - 4 weeks of age. The elevated nuclear p65 levels are indicative of enhanced vascular NFκB activation in young SHR. Thus, Studies from 3-4 weeks old rats demonstrated that vascular oxidative stress, NFκB activation and AT1 receptor upregulation precedes the development of hypertension in SHR.

Based on our results from 3 – 4 week old animal studies, we hypothesized that early oxidative stress could play a causal role in hypertension development by upregulating AT1 receptor via NFκB. To test this hypothesis we treated prehypertensive 5 wk old SHR with an antioxidant pyrrolidine dithiocarbamate (PDTC) which has well recognized NFκB inhibitory activity (Elks et al., 2009). PDTC treatment significantly attenuated the development of hypertension in SHR as indicated by 31 mmHg lower mean arterial pressure in PDTC treated SHR as compared to untreated SHR. It is worth mentioning that Rodrıguez-Iturbe and colleagues have also shown that chronic treatment of SHR from 7 weeks to 25 weeks of age with PDTC attenuates the development of hypertension (Rodriguez-Iturbe et al., 2005). We also found a significant lowering of oxidative stress in SHR with PDTC treatment as evidenced by lower vascular hydrogen peroxide levels. PDTC treatment also reduced the protein expression of p65 subunit which was elevated in SHR as compared to WKY rats. This is in concurrence with previous studies which have used PDTC in this model of hypertension (Elks et al., 2009; Rodriguez-Iturbe et al., 2005).

The AT1 receptor expression was also elevated in adult control SHR as compared to WKY rats. The increase in AT1 receptor expression was functionally relevant as evidenced by increased vasoconstriction in response to Ang II of SHR mesenteric artery rings in comparison to WKY rats. PDTC treatment normalized the AT1 receptor expression seen in vasculature of SHR. PDTC treatment also attenuated the exaggerated vasoconstriction observed in SHR. Interestingly, the AT1 receptor gene promoter has

been demonstrated to have a consensus binding site for NFκB (Heinemeyer et al., 1998). Furthermore, Cowling and colleagues have shown that AT1 receptor upregulation in response to tumor necrosis factor α and Interleukin 1β in cardiac fibroblasts involves NFκB (Cowling et al., 2002). These results, in combination, strongly suggest a role of oxidative stress in upregulation of AT1 receptor via NFκB activation.

To validate our results from the SHR studies and to further investigate the causal role of oxidative stress on AT1 receptor upregulation we used human aortic smooth muscle cells (HASMCs). HASMCs were exposed to oxidative insult by co-incubating cells with BSO and H_2O_2, either alone or in combination. These two oxidants were chosen since they act via two different mechanism of action. BSO is a pro-oxidant which acts by depleting glutathione,a major endogenous antioxidant whereas H_2O_2 is a direct oxidant. Cells exposed to BSO at a concentration of 100μM and 400μM for 24h exhibited moderate increase in AT1 receptor mRNA as detected using quantitative PCR. Cells incubated with H_2O_2, 50μM for 3h showed no increase in AT1 receptor message levels. This, we anticipate, may be due to the ability of the endogenous antioxidant system to neutralize H_2O_2. However, when the cells were treated with a combination of BSO and H_2O_2 we observed a robust increase in AT1 receptor mRNA. Thus, our results suggest that an attenuation of endogenous antioxidant capacity combined with an exogenous oxidative insult results in a robust increase in AT1 receptor mRNA levels. This is interesting because in SHR animals, we had also observed attenuated antioxidant capacity

combined with increased oxidative stress. Furthermore, the oxidative stress and AT1 receptor upregulation seen with oxidant treatment were abolished by concomitant treatment with antioxidant enzyme catalase (100U/ml). These results strongly support a pivotal role of oxidative stress in upregulation of AT1 receptors. Interestingly, oxidative stress has also been shown to play a role in Ang II induced AT1 receptor upregulation in neuronal cell line (Liu et al., 2008). To study the involvement of NFκB in mediating AT1 receptor upregulation we adopted siRNA approach. Oxidant treatment was unable to upregulate AT1 receptors in cells transfected with siRNA against p65 subunit of NFκB. This data indicates that NFκB mediates the oxidative stress induced AT1 receptor upregulation. However, whether NFκB directly upregulates AT1 receptors by increasing its transcription or indirectly via an intermediate molecule remains to be established. As an example, Mitra and colleagues have recently shown that AT1 receptor upregulation in neuronal cell line occurs by sequential activation of NFκB and Elk-1 (Mitra et al., 2010). Further experiments are required to completely elucidate the mechanisms of AT1 receptor upregulation and the involvement of NFκB in this process. However, our results from this section of the study suggest that oxidative stress upregulates AT1 receptors in SHR and HASMCs. Furthermore, NFκB plays a crucial role in oxidative stress mediated AT1 receptor upregulation.

5.2 Early vascular oxidative stress and endothelial dysfunction: Role of resveratrol

This section of the project aims to investigate the effects of the polyphenol resveratrol, an important component of red wine, on mitigation of hypertension in SHR and to study the mechanisms involved. Resveratrol treatment from 3-4 weeks to 11-12 weeks of age significantly attenuated the rise in blood pressure of SHR. Recently, multiple studies have investigated the effects of resveratrol and other red wine polyphenols on endothelial function (Lopez-Sepulveda et al., 2008; Rivera et al., 2009; Rush et al., 2007; Thandapilly et al.). Although all of them report significant improvement in the endothelial function the mechanisms involved and its role in hypertension has not been established. As an example, Rush and colleagues. (Rush et al., 2007) and Thandapilly et.al. (2010) have seen no change in blood pressure with chronic resveratrol treatment in SHR. Interestingly in both these studies resveratrol was administered to adult SHR with established hypertension. In the present study resveratrol treatment was started prior to the onset of hypertension and continued throughout the developmental phase of hypertension. Our results show that resveratrol treatment normalized the endothelial function and significantly lowered blood pressure in SHR. Thus, it appears that beneficial effects of resveratrol treatment on blood pressure may be related to events occurring prior to increase in blood pressure.

Herein, untreated SHR showed significantly higher vascular oxidative stress in comparison to WKY rats as indicated by elevated levels of H2O2 in the vascular homogenate. Also, aortic SOD activity in untreated SHR was lower as compared to WKY rats. Resveratrol treatment normalized the SOD activity and H2O2 levels in SHR. Our results are in concurrence with previous reports demonstrating that resveratrol increases SOD activity and gives protection against oxidative stress in cultured rat aortic smooth muscle cells (Li et al., 2006). Thus, it is possible that prevention of vascular oxidative stress by early resveratrol intervention seen in the present study could be due to up regulation of antioxidant enzymes such as SOD.

Oxidative stress in the vasculature contributes to hypertension by detrimentally affecting the endothelial function. We carried out endothelial function studies in isolated mesenteric arteries since these resistance vessels are significant contributors to the regulation of vascular tone. The endothelium dependant relaxations induced by lower concentrations of Ach were attenuated, with significantly lower pD2 values in untreated SHR as compared to WKY rats. Resveratrol treatment significantly improved Ach induced relaxation in SHR. Enhanced Ach induced relaxation suggests increased NO response which can result from reduced scavenging/deactivation of NO by reactive oxygen species which react rapidly with NO to form peroxynitrite. To test the involvement of NO scavenging we measured vascular 3-nitrotyrosine levels. Protein nitrosylation is an indicator of peroxynitrite formation in the vascular tissue. SHR had significantly elevated nitrotyrosine levels in the aortic homogenate as compared to WKY

rats which were normalized by resveratrol treatment. Thus, resveratrol by lowering oxidative stress prevents NO scavenging and increases its biological availability in SHR.

In addition to NO scavenging, another important mechanism by which oxidative stress can contribute to endothelial dysfunction is by 'uncoupling' of the eNOS enzyme. The physiological consequences of eNOS uncoupling are doubly harmful since it will not only reduce the production of NO but also result in formation of superoxides thereby further increasing oxidative stress (Munzel et al., 2005). We measured superoxide production in vascular homogenates in presence of eNOS substrate L-arginine. The superoxide production in SHR was almost two fold higher than in WKY rats. This superoxide production was sensitive to eNOS inhibitor L-NNA suggesting that it was derived from eNOS which was most likely uncoupled in SHR. Interestingly, in SHR the superoxide production was completely normalized with resveratrol treatment suggesting a novel role of resveratrol in preventing uncoupling of eNOS. It is well recognized that an important contributor to eNOS uncoupling is oxidation of the co-factor tetrahydrobiopterin (BH4) to BH2. Supplementation of BH4 abolished the elevated NOS dependent superoxide production in SHR indicating that vascular oxidative stress contributes to endothelial dysfunction and hypertension by uncoupling eNOS possibly by oxidation of BH4. It has been reported that BH4 supplementation from an early age of 5 weeks to 16 weeks suppressed the development of hypertension in SHR to similar extent as seen in the present study (Hong et al., 2001). Lastly, resveratrol upregulated eNOS protein both in SHR and WKY rats suggesting transcriptional upregulation. However, the

endothelial function was improved only in SHR, which exhibited oxidative stress and eNOS uncoupling. These data suggest that eNOS uncoupling plays a role in endothelial dysfunction and resveratrol prevents eNOS uncoupling and rescues endothelial function in SHR.

We also investigated the effect of resveratrol on endothelium independent vasorelaxation in response to SNP. The SNP induced vasorelaxation was similar in SHR and WKY rats. Surprisingly, resveratrol significantly increased relaxation in response to higher doses of SNP in SHR. Similar results have also been reported in porcine retinal arterioles (Nagaoka et al., 2007). The proximal mediator for NO induced vasorelaxation is soluble guanylyl cyclase (sGC). We measured the expression of the catalytic β1 subunit of sGC in vascular homogenate. The β1 subunit is responsible for the responsiveness of sGC to NO (Lucas et al., 2000). The basal expression of sGC was higher in SHR as compared to WKY rats which could be a compensatory increase in response to reduced NO bioavailability. Resveratrol treated SHR as well as WKY rats showed higher expression of sGC β1 subunit. The observation that sGC upregulation was seen in both SHR and WKY rats suggests that it may be a transcriptional upregulation caused by resveratrol. However, the mechanism by which it improved endothelium independent vasorelaxation in SHR remains to be established.

Our data suggest improvement of NO synthesis and availability as a mechanism of action for resveratrol mediated attenuation of hypertension development. To further support this observation in vivo we studied the effects of a bolus dose of resveratrol on Ang II induced pressor response in Sprague Dawley rats. Administration of a resveratrol bolus injection significantly attenuated the increase in blood pressure caused by Ang II. Interstingly, the effect of resveratrol was abolished by NOS inhibitor L-NAME implying the role of NO in mediated resveratrol's effect.

Taken together, our studies in animals and cell culture suggest a novel role of early vascular oxidative stress in development of hypertension. We propose that early vascular oxidative stress which precedes hypertension development upregulates AT1 receptors contributing to enhanced vasoconstriction. Furthermore, the oxidative stress-induced AT1 receptor upregulation appears to be mediated by the redox sensitive transcription factor NFκB. In addition we also discovered that early oxidative stress also causes eNOS uncoupling consequently contributing to endothelial dysfunction and hypertension development. Finally, resveratrol treatment attenuates development of hypertension in SHR by preventing oxidative stress, eNOS uncoupling and endothelial dysfunction.

6. Summary and Conclusions

1. Vascular oxidative stress precedes the development of hypertension in SHR as evidenced by elevated markers of oxidative stress and attenuated antioxidant activity in the vascular tissue. However, the systemic markers of oxidative stress were not elevated indicating that the early oxidative stress might be tissue specific to blood vessels and not a systemic phenomenon. Furthermore, the early oxidative stress is also associated with increased activation of NFκB and AT1 receptor upregulation in the vasculature of SHR as compared to WKY rats.

2. Treatment of SHR with PDTC, an antioxidant with NFκB inhibitory activity, throughout the developmental phase of hypertension attenuates hypertension pathogenesis. Furthermore, this attenuation is associated with lowering of oxidative stress and normalization of the NFκB p65 subunit and AT1 receptor expression in SHR. Thus, we concluded that the early vascular oxidative stress can contribute to hypertension development by inducing vascular AT1 receptor upregulation. Additionally, the AT1 receptor upregulation by oxidative stress appears to involve NFκB.

3. Experiments performed in human aortic smooth muscle cells also showed increased AT1 receptor mRNA levels in cells exposed to oxidative stress,

confirming the results from our studies in SHR and WKY rats. Our results also suggest that a concomitant increase in ROS and attenuation in antioxidant activity was required for AT1 receptor upregulation. Furthermore, oxidative stress-induced AT1 receptor upregulation was prevented by treatment with antioxidant catalase. Oxidative stress also failed to upregulate AT1 receptors when NFκB was knocked down using siRNA.

4. Early treatment with resveratrol, an antioxidant polyphenol, attenuated development of hypertension and prevented endothelial dysfunction in SHR. The mechanisms involved appear to be three fold 1) Attenuation of vascular oxidative stress resulting in increased NO bioavailability, 2) Prevention of eNOS uncoupling possibly via inhibition of BH4 oxidation by free radicals and 3) Increased expression of important proteins in NO pathway namely eNOS and sGC. Additionally, we also report that acute resveratrol treatment attenuates Ang II-induced pressor response in a NO dependent manner.

7. MANUSCRIPTS AND ABSTRACTS

Manuscripts:

Bhatt, S., Lokhandwala, M.F. and Banday, A.A.: Resveratrol prevents endothelial nitric oxide synthase uncoupling and attenuates development of hypertension in SHR. (*Manuscript under revision*)

Bhatt, S., Lokhandwala, M.F. and Banday, A.A.: Early vascular oxidative stress upregulates AT1 receptor via NFκB and contributes to development of hypertension in SHR (*Submitted Manuscript*)

Bhatt, S., Lokhandwala, M.F. and Banday, A.A.: Oxidative stress upregulates AT1 receptor in human vascular smooth muscle cells (*In preparation*)

Abstracts:

Bhatt, S., Lokhandwala, M.F. and Banday, A.A.: Oxidative Stress via Nuclear Factor Kappa B Causes Vascular Angiotensin II Type 1 Receptor Upregulation and Contributes to Development of Hypertension in SHR. *64th Annual High Blood Pressure Research Conference 2010 (P478)*

Bhatt, S., Banday, A.A. and Lokhandwala M.F.: Resveratrol via Nitric Oxide Attenuates Angiotensin II Induced Acute Increase in Blood Pressure in Sprague Dawley Rats *FASEB J. 2010 24:lb559 (Experimental Biology 2010)*

Bhatt, S., Lokhandwala, M.F. and Banday, A.A.: Early Chronic Resveratrol Treatment Reduces Blood Pressure and Vasoconstriction and Improves Endothelium-Dependent Vasorelaxations by Lowering Oxidative Stress in Spontaneously Hypertensive Rats *Hypertension.2009*; *54: e26-e127 (P268)*

Bhatt, S., Banday, A.A. and Lokhandwala M.F.: Vascular Oxidative Stress, NFκB Activation And AT1 Receptor Upregulation Precede the Development of Hypertension in Spontaneously Hypertensive Rats (SHR) *FASEB J. 2009 23:LB53*

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