OXIDATIVE STRESS AND RENAL AT1R FUNCTION DURING HYPERTENSION

A Dissertation Presented to

Department of Pharmacological and Pharmaceutical Sciences

University of Houston

In Partial Fulfillment of the

Requirement for Degree

Doctor of Philosophy

In

Pharmacology

By

Apurva Javkhedkar

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Abstract

Angiotensin (Ang) II via renal Ang II type 1 receptors (AT1R) modulates proximal tubular Na/K-ATPase such that, low concentrations of Ang II stimulates Na/K-ATPase whereas at high concentrations the stimulatory effect is lost. Studies show that oxidative stress can impair Ang II-induced Na/K-ATPase regulation and can contribute to hypertension.

In the present study we first determined whether renal oxidative stress precedes development of hypertension in SHR. We found that at 3-4 weeks of age, SHR are normotensive and do not exhibit proximal tubular oxidative stress. Also, renal AT1R protein expression and Ang II-induced Na/K-ATPase stimulation was similar in SHR and WKY rats.

Next we determined if antioxidant treatment at an early age can prevent renal oxidative stress and alleviate increase in blood pressure in SHR. We found that in adult SHR, there was high blood pressure, increased renal oxidative stress and decreased proximal tubular Nrf-2 activation as well as antioxidant activity compared to WKY rats. In WKY rats Ang II exerted a biphasic effect on Na/K-ATPase whereas in SHR, there was loss of biphasic effect such that Ang II produced stimulation at both high and low concentrations. In presence of nitric oxide (NO) synthase (NOS) inhibitor, L-NAME, Ang II (high concentration) produced stimulation of Na/K-ATPase in WKY rats. Ang II via AT1R caused NO

production in WKY rats but not in SHR. Ang II caused enhanced activation of NADPH oxidase and L-arginine dependent NOS mediated superoxide production in proximal tubules of SHR. NADPH oxidase inhibitor DPI prevented Ang II-induced L-arginine dependent superoxide production and normalized NO production in SHR. There was increased renal NF kappaB activation, Giα protein expression and AT1R-G protein-coupling in SHR compared to WKY rats. Antioxidant resveratrol decreased blood pressure, increased Nrf-2 mediated antioxidant enzymes, reduced oxidative stress, normalized NOS coupling and restored Ang II-induced NO production as well as biphasic effect on Na/K-ATPase. Resveratrol treatment in SHR decreased Giα protein expression and normalized AT1R-G protein-coupling.

Together these results demonstrate mechanism by which renal oxidative stress contributes to hypertension in SHR.

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Ang II	Angiotensin II
AT1R	Angiotensin II type 1 receptor
Na/K-ATPase	Sodium/potassium adenosine triphosphatase
NO	Nitric oxide
L-NAME	L-Nitro-Arginine Methyl Ester
cAMP	cyclic adenosine monophosphate
DAG	Diacylglycerol
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H_2O_2	Hydrogen peroxide
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
EDTA	Ethylenediaminetetraacetic acid

KHB	Krebs-Henseleit Buffer
NFkappa B	Nuclear factor kappa B
NHE	Sodium/hydrogen Exchanger
Nrf-2	Nuclear factor E2-related factor-2
ROS	Reactive oxygen species
SDS-PAGE electrophoresis	Sodium dodecyl sulfate-polyacrylamide gel
SOD	Superoxide dismutase
NOS	Nitric oxide synthase
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase

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1. INTRODUCTION AND STATEMENT OF PROBLEM

Regulation of sodium and water balance by kidneys is important for maintenance of blood pressure. Renal proximal tubules reabsorb about 70% of the filtered sodium and Na/K-ATPase, a plasma membrane sodium transporter, plays a major role in sodium reabsorption (1). Both natriuretic and antinatriuretic hormones such as dopamine, angiotensin (Ang) II, and norepinephrine have been shown to regulate renal Na/K-ATPase activity (2-4).

It is recognized that Ang II via AT1R can modulate sodium reabsorption in a manner such that low concentrations (pM) of Ang II promotes sodium reabsorption by stimulating Na/K-ATPase whereas; at high concentrations (μM) of Ang II the stimulatory effect on Na/K-ATPase is lost (5). At circulating concentrations, Ang II maintains positive sodium balance by directly stimulating sodium transporters or indirectly via aldosterone release. However, the renal proximal tubular concentrations of Ang II are reported to be high during pathological conditions such as hypertension (6) and thus regulation of renal Na/K-ATPase at high concentration of Ang II is physiologically relevant.

The mechanism by which low concentration (pM) of Ang II causes stimulation of Na/K-ATPase is widely studied and is shown to be mediated via activation of various kinases including protein kinase C (PKC), Mitogen-activated protein (MAP) Kinase or decrease in protein kinase A (7-9). However, the mechanism by which high concentration of Ang

II (μM) exerts its effect on Na/K-ATPase is not completely understood. It is reported that high concentrations of Ang II via AT1R can activate nitric oxide (NO)-cGMP signaling in rat proximal tubules (6, 10). Additionally, there are reports suggesting that NO-cGMP signaling may be involved in mediating inhibition of Na/K-ATPase at high concentrations of Ang II in cultured opossum kidney cells and rat renal proximal tubules (11, 12).

Evidences suggest that oxidative stress can modulate renal AT1 R signaling and affect Na/K-ATPase regulation (6, 8, 9, 13, 14). Ang II via AT1R can activate NADPH oxidase and increase superoxide production which can reduce NO bioavailability by either formation of peroxynitrite or by uncoupling nitric oxide synthase. Therefore, hypothesis for our studies was oxidative stress via defective NO signaling impairs Ang II-induced Na/K-ATPase regulation and contributes to hypertension. To test this hypothesis, we determined the involvement of NO in AT1R mediated Na/K-ATPase regulation at high concentrations of Ang II under conditions of high oxidative stress in spontaneously hypertensive rats (SHR), genetic model of essential hypertension, and normotensive control Wistar-Kyoto (WKY) rats which do not exhibit oxidative stress.

Oxidative stress is known as one of factors contributing to hypertension in SHR (15) whereas antioxidant treatment has been reported to decrease blood pressure (16, 17). However, the mechanism through which antioxidants decrease blood pressure is not yet known. Studies show that polyphenols e.g. resveratrol can activate transcription factor

Nuclear Factor-E2-related Factor-2 (Nrf-2), which in turn increases transcription of phase 2 antioxidant enzymes. Phase 2 antioxidant enzymes e.g. glutathione-S-transferase and NAD(P)H:quinone reductase play a critical role in eliminating oxidative stress. Under normal conditions, redox sensitive transcription factor Nrf-2 is located in the cytoplasm, bound to Keap-1 protein which is a repressor of Nrf-2. Upon receiving a stimulus (e.g. antioxidants such as polyphenols), Keap-1 is degraded and Nrf-2 is translocated to the nucleus where it binds to stress or antioxidant response elements [StRE/ ARE] in the promoter regions of antioxidant genes (18). Induction of these antioxidant enzymes reduces cellular oxidative stress which in turn is beneficial in ameliorating oxidative stress. Thus, next part of the project was designed to determine the potential beneficial effects of antioxidant resveratrol, a polyphenol, on blood pressure in SHR and WKY rats. We started the resveratrol treatment in 3-4 weeks old SHR and WKY rats. At 3-4 weeks of age, SHR are normotensive and do not exhibit oxidative stress. The studies were carried out to determine if antioxidant treatment at early age can reduce renal oxidative stress and alleviate increase in blood pressure in SHR. The blood pressure was measured using radiotelemetry and the treatment was continued till the difference between blood pressure of vehicle and resveratrol treated SHR remained constant.

The research presented here aims to establish understanding of intermediate molecules involved between renal oxidative stress and hypertension with particular emphasis on Na/K-ATPase regulation by Ang II. Also targeting Nrf-2 for activation of proteins which

provide protection from oxidative stress-induced cell damage might be important for developing novel therapeutic strategies for treating hypertension.

2. REVIEW OF LITERATURE

2.1 Role of kidneys in the maintenance of sodium homeostasis:

Fine control of sodium and water balance is required to maintain blood pressure and kidneys play an important role in maintaining sodium homeostasis in the body. To accomplish this function, kidneys have various transporter systems that are controlled by specific hormones. Na/K-ATPase, expressed in basolateral membranes throughout the nephron, and various apical ion transport proteins (exchangers and co-transporters) maintain the function of the kidneys (19). Various transporters e.g. Na/H-exchanger, located in proximal tubules is an important apical sodium transporter. Other co-transporters such as Na-phosphate co-transporter reabsorbs phosphate, Na-glucose and Na-amino acid co-transporters reabsorbs sodium whereas water channels reabsorb fluid volume (1). Na/K/2Cl co-transporter is the primary mediator of sodium transport in thick ascending limb of loop of Henle. In the distal convoluted tubule thiazide sensitive Na-Cl transporter reabsorbs sodium whereas in collecting ducts amiloride sensitive Na channels regulate sodium reabsorption (20).

Proximal tubules reabsorb about two-thirds of the filtrate that flows through the kidneys and thus plays an important site for sodium reabsorption. Antinatriuretic hormones such as Ang II synthesized locally, and nor-epinephrine released from sympathetic nerves promote sodium reabsorption (21). On the other hand, natriuretic hormones such as dopamine inhibit sodium reabsorption (22). Under normal physiological conditions,

antinatriuretic hormones e.g. Ang II predominates to conserve sodium, whereas during conditions of increased sodium intake natriuretic hormones such as dopamine dominate to maintain sodium balance in the body. These hormones act in counter regulatory manner to maintain sodium homeostasis.

2.2 Effect of Angiotensin II on Sodium transport

Ang II exerts a biphasic effect on sodium reabsorption. Studies done by Harries et al and others have demonstrated that low concentration (pM-nM normal physiological concentration) of Ang II stimulates sodium and fluid reabsorption, whereas high concentrations of Ang II (µM-pathological concentrations) inhibits sodium and fluid transport (5, 23, 24). Likewise, the regulation of Na/K-ATPase in response to Ang II is such that picomolar- nanomolar concentrations of Ang II stimulate Na/K-ATPase whereas at micromolar concentration, Ang II either inhibits or causes loss of stimulation of Na/K-ATPase (7, 23). Navar et al (25, 26) have reported that proximal tubular concentration of Ang II is much higher than plasma concentration because there are two sources of Ang II in proximal tubules, 1) local synthesis and 2) glomerular filtration. During pathological conditions such as hypertension, Ang II levels further increase in the kidneys(27). Thus Ang II (high concentration) mediated regulation of Na/K-ATPase is physiologically important because increase in intrarenal Ang II levels may contribute to sodium retention and hypertension if sodium transporter stimulation is maintained at high

concentration. Thus biphasic regulation of sodium reabsorption by Ang II is important for maintaining normal blood pressure during pathological conditions.

2.3 Signaling pathways for Ang II-induced Na/K-ATPase stimulation

Studies in rat proximal tubules suggests that the stimulatory effect of low concentration of Ang II on Na/K-ATPase is mediated by decrease in cAMP accumulation (7). Microperfusion studies show that Ang II decreased cAMP levels in luminal fluid and effect of Ang II on fluid transport was prevented through inhibition of Gi by pertussis toxin (28). Alternatively, studies using PKC inhibitors or activators suggested that Ang II (low concentration)-mediated PKC activation contributes to stimulation of sodium transport (29, 30). Previous studies done by Banday et al suggest a role of MAPK activation by low concentrations of Ang II in mediating stimulation of Na/K-ATPase and treatment of rat proximal tubules with MAPK inhibitor U0126 prevented Ang II-induced Na/K-ATPase stimulation at low concentration of Ang II (9).

2.4 Signaling pathways for effect of high concentration of Angiotensin II on Na/K-ATPase

High concentrations of Ang II either inhibits or causes loss of stimulation of Na/K-ATPase, however the mechanism by which high concentration of Ang II modulates Na/K-ATPase is still being investigated. Studies in rat renal proximal tubules suggest a role of PLA2 in mediating inhibitory effect of Ang II on Na/K-ATPase (7) . There are

reports that show Ang II via AT1R activation can activate NO formation which through activation of downstream messengers such as PKG, inhibits Na/K-ATPase in rat kidneys (6, 10). NO mediated inhibition of Na/K-ATPase decreased sodium reabsorption resulting in diuresis and natriuresis in rats (6, 10). Acetylcholine and bradykinin via activation of NO are shown to mediate salt and water loss in rat kidneys (31, 32). Studies in rat proximal tubules and cultured opossum kidney cells also show that NO decrease Na/K-ATPase activity (9, 12).

2.5 Formation of Angiotensin II

Classical renin angiotensin system (RAS) is an enzymatic cascade system initiated by renin release by kidneys (from juxtaglomerular apparatus). Renin further cleaves circulating angiotensinogen, generated from liver, to form decapeptide Ang I. Angiotensin converting enzyme (ACE), expressed on surface of endothelial cells further cleaves Ang I to form octapeptide Ang II. Ang II is then delivered systemically to peripheral tissues such as vasculature, heart, brain, gonads or kidneys where it exerts its effects through Ang II receptors namely AT1R and AT2R (33).

Although not all components of classical RAS are synthesized locally, as shown in Fig. 1, alternative enzymatic nonclassical pathways permit the formation of Ang II. Chymase, stored in secretory granules of mast cells, and cathepsin G can enzymatically generate Ang II from Ang I (34). Furthermore, Ang II can also be directly generated from angiotensinogen by the action of tonin and cathepsin D.

Kidneys, particularly proximal tubules possesses all components of RAS and thus regulates its own local RAS activity independent of systemic RAS (35). Immunohistochemical studies show angiotensinogen mRNA localizes in proximal tubules suggesting a primary source of intrarenally produced Ang II (36, 37). Micropuncture studies show that higher concentrations of Ang II are present in proximal tubules compared to plasma (38). Studies done in adult SHR show high concentrations of Ang II in proximal tubules (27). Thus regulation of sodium transporters at high concentrations of Ang II is important for maintaining sodium homeostasis.

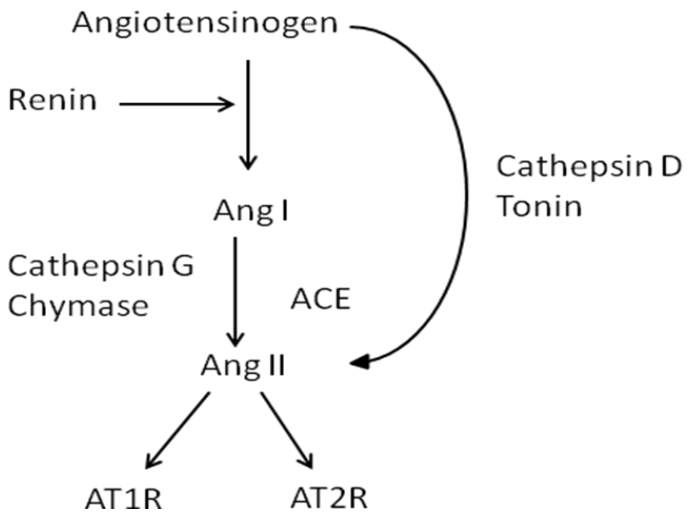


Figure 1: Classical and non classical pathways for formation of Ang II.

Ang II can be formed by classical pathway which involves formation of Ang I from angiotensinogen via renin and further formation of Ang II from Ang I via ACE. In non-classical pathway, Ang II can be formed from Ang I via chymase or cathepsin G.

2.6 Ang II receptor classification

Ang II receptors are classified into AT1 and AT2 receptors based on pharmacological and radioligand studies (39). Both these receptors bind Ang II with high affinity. AT1R displays a high affinity for AT1R antagonist Losartan and Dup753, but not for AT2R antagonist CGP42442A, whereas AT2R bind with CGP42112A with high affinity but not

Losartan or Dup753 (39). Studies in rat mesangial cells showed that binding of ^{125}I (Sar-Ile) Ang II to renal cortex was significantly displaced by AT1 R antagonist Losartan, whereas AT2 receptor antagonist CGP42112 showed no effect on binding (39). These studies suggest that 95% of Ang II receptors are AT1R subtype, whereas AT2R are only 5% of the total receptor pool.

In rodents, 2 different subtypes of AT1R have been identified as AT1a and AT1b (40). AT1a receptor subtype is classified as PD123319 insensitive and Losartan sensitive, whereas AT1b receptor subtype is sensitive to PD123319 as well as to Losartan.

Fig. 2 summarizes the Ang II receptor subtypes, transduction mechanism and actions.

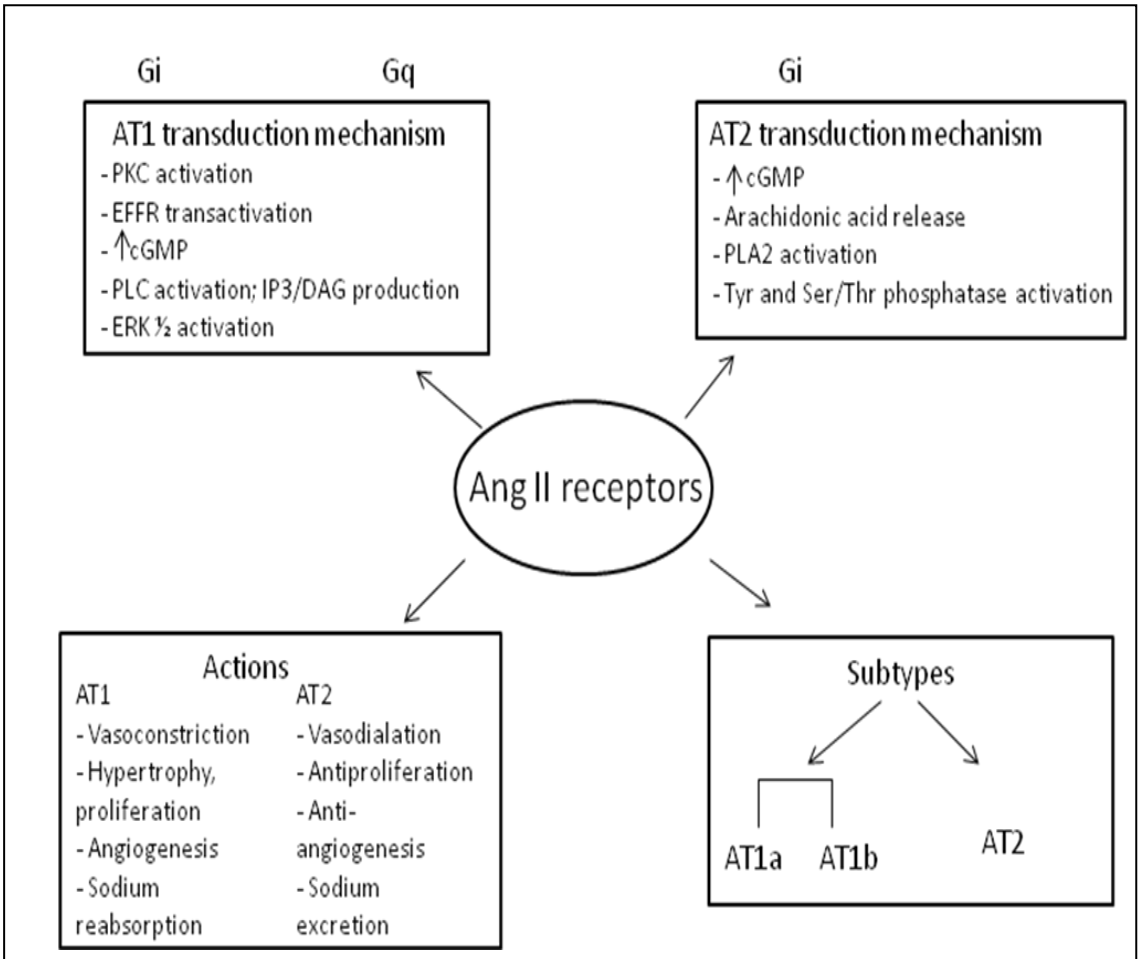


Figure 2: Ang II receptor subtypes, transduction mechanisms and actions in the body: Ang II receptors are classified into AT1 (AT1a and AT1b) and AT2 receptors. The actions of both the receptors are mostly opposite of each other. AT1R are coupled to Gi protein (decrease cAMP levels) and also to Gq (increase calcium levels and activate PKC, PLC, cGMP, ERK1/2). AT2 receptors are coupled to Gi proteins to decrease cAMP levels and activate cGMP, arachidonic acid and PLA2 activation.

2.7 Oxidative stress and hypertension

Over the past decade, role of reactive oxygen species (ROS) in cardiovascular system has been a subject of interest. ROS encompasses various molecules having divergent effects on cellular function such as regulation of cell growth and differentiation, inactivation of nitric oxide (NO) and stimulation of various kinases. Many of these effects of ROS are deleterious and are associated with cardiovascular disorders such as hypertension (13).

Oxidative stress is defined as a condition involving chronically elevated ROS levels and/or decreased activity of antioxidant enzymes. Studies show that hypertensive patients have increased levels of oxidative stress byproducts, increased oxidative DNA damage and also decreased activity of antioxidant enzymes when compared to normotensive patients (41). Enhanced ROS production and NO inactivation are seen in various experimental models of hypertension (42-44). Patients with renovascular hypertension show enhanced oxidative stress along with impaired endothelium dependent vasodialatation (44). These evidences suggest a role of ROS in pathogenesis of hypertension.

Different chemical properties of ROS have important role in cellular signaling. Superoxides ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$) have short biological half life and require ion channels to cross the membranes. Hydrogen peroxide (H_2O_2) has longer biological

half life and is able to cross lipid bilayers. Thus all these ROS are capable of activating signaling pathways to initiate the deleterious effects of oxidative stress.

2.8 Production of ROS

The most common enzymatic sources of ROS are xanthine oxidase, uncoupled NOS and NADPH oxidase. Xanthine oxidase is a metalloenzyme that catalyses oxidation of hypoxanthine and xanthine to form superoxides ($O_2^{\bullet -}$). Nitric oxide synthase (NOS) can also contribute to ROS formation under certain conditions. Uncoupling of NOS under the conditions of absence of cofactors such as L-arginine or tetrathydrobiopterin (BH_4) can lead to $O_2^{\bullet -}$ production instead of NO. Superoxides can further reduce the available NO by formation of peroxynitrite. Figure 3 describes the byproducts of NOS during normal coupled and uncoupled state. In various rat models of hypertension, oxidative stress can cause NOS uncoupling which contributes to hypertension (45). Supplementation of antioxidant such as tempol has shown to recover NOS coupling and ameliorating blood pressure in these rats.

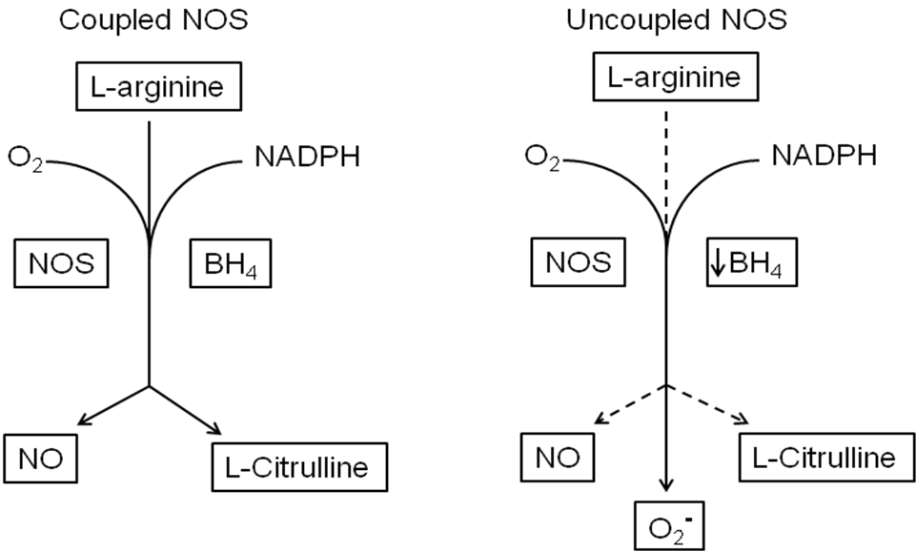


Figure 3: NOS byproducts during coupled and uncoupled state

Coupled NOS utilizes L-arginine, O_2 and NADPH to produce NO and L-citrulline. Under the conditions of BH4 or L-arginine deficiency, NOS can get uncoupled and produce superoxide instead of NO, which can further reduce available NO by converting it to peroxynitrite.

Another major source of ROS is NADPH oxidase which utilizes NADH/NADPH as electron donor to reduce molecular oxygen and produce $O_2^{\bullet -}$. Assembly of cytosolic (p47phox and p67phox) and membrane bound (gp91phox/Nox1/NOX4 and p22) subunits are required to form a functional enzyme complex of NADPH oxidase. Activation of NADPH oxidase is regulated by Ang II, growth factors like TGFβ and mechanical stimuli. Studies show that Ang II activates NADPH oxidase via activation of protein kinase C, phospholipase D and receptor tyrosine kinases (46). It is reported that inhibition of NADPH oxidase improves endothelial function and lowers blood pressure in SHR (47).

Many evidences suggest a role of enhanced ROS in pathophysiology of renal diseases such as glomerulopathy, tubulointerstitial damage and chronic kidney disease (CKD) (48). Studies show that superoxide production via NADPH oxidase plays an important role in tubulointerstitial damage. Ang II via AT1R plays an important role in nephropathy by activation of NADPH oxidase (49, 50). Thus enhanced AT1R signaling can exaggerate renal damage via superoxide production. Studies show that oxidative stress mediated endothelial dysfunction is involved in CKD (51, 52). In two kidney one clip rat model, oxidative stress in ischemic kidney has an important role in maintenance of hypertension (52). ROS can decrease NO bioavailability and cause enhanced renal vasoconstriction thereby affecting tubuloglomerular feedback. Vascular mediators such as Ang II and endothelin are affected by oxidative stress. Ang II-induced influx of

phagocytes, cytokines and activation of NFkappaB in turn upregulates proinflammatory proteins (53-55). Thus ROS modulate renal hemodynamics by causing constriction and inducing renal inflammation.

2.9 Cellular antioxidants

Some of the major cellular antioxidant system in the body is composed of Vitamin A, C and E, reduced glutathione (GSH), superoxide dismutase (SOD), catalase, heme oxygenase (HO) and glutathione peroxidase. Antioxidant vitamins and GSH donate electron to unstable molecules such as ROS and stabilize them. In this process, reduced GSH reacts with another GSH to form GSSG. Glutathione reductase can regenerate GSH from GSSG.

Depending on the cellular location where it is present, SOD is classified into 3 isoforms Cu/Zn SOD (cytosolic), Mn SOD (mitochondria) and EC SOD (extracellular). SOD catalyses the conversion of superoxides to H_2O_2 in presence of metal cofactors such as zinc, copper and manganese. H_2O_2 can further –a) be eliminated by catalase and glutathione peroxidase antioxidant enzymes which catalyze the conversion of H_2O_2 to H_2O and O_2 or, b) undergo Fenton reaction and get converted to hydroxyl radical.

Glutathione system comprises of a) glutathione reductase (catalyses the conversion GSSG to GST), b) glutathione S-transferase (catalyzes transfer of SH groups to oxidized lipids) and c) glutathione peroxidase (catalyses the conversion of H_2O_2 to H_2O and O_2).

Heme oxygenase (HO) is an antioxidant enzyme that lowers the cellular levels of heme and converts it to bilirubin, iron and carbon monoxide. Excess heme acts as an oxidant and has been shown to cause vascular dysfunction whereas, bilirubin acts as an antioxidant. HO exists in 2 different isoforms, HO-1 which is inducible by stress and HO-2 which is constitutive isoform activated by corticosteroids (56).

2.10 Role of transcription factor Nrf-2 in regulation of oxidative stress

Nuclear factor-erythroid-2-related factor 2 (Nrf-2), a member of basic leucine zipper transcription factors, plays a crucial role in induction of stress responsive and cytoprotective genes that encode for phase 2 antioxidant enzymes such as NADPH quinine oxidoreductase-1 (NQO-1), heme oxygenase-1(HO-1), superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione peroxidase (GPx), thioredoxin etc.

In the resting cells, Nrf-2 is located in the cytoplasm forming an inactive complex with Kelch like ECH-associated protein 1 (Keap1). Upon receiving a stimulus (e.g. polyphenols), Nrf-2 is released and translocated to the nucleus. After the translocation, Nrf-2 forms a heterodimer with other transcription factors such as Maf, and binds to antioxidant responsive elements (ARE) or electrophile response elements (EpRE) located in promoter region of genes encoding phase 2 detoxifying enzymes or various antioxidant enzymes (57). Fig. 4 shows the pathway for activation of Nrf-2.

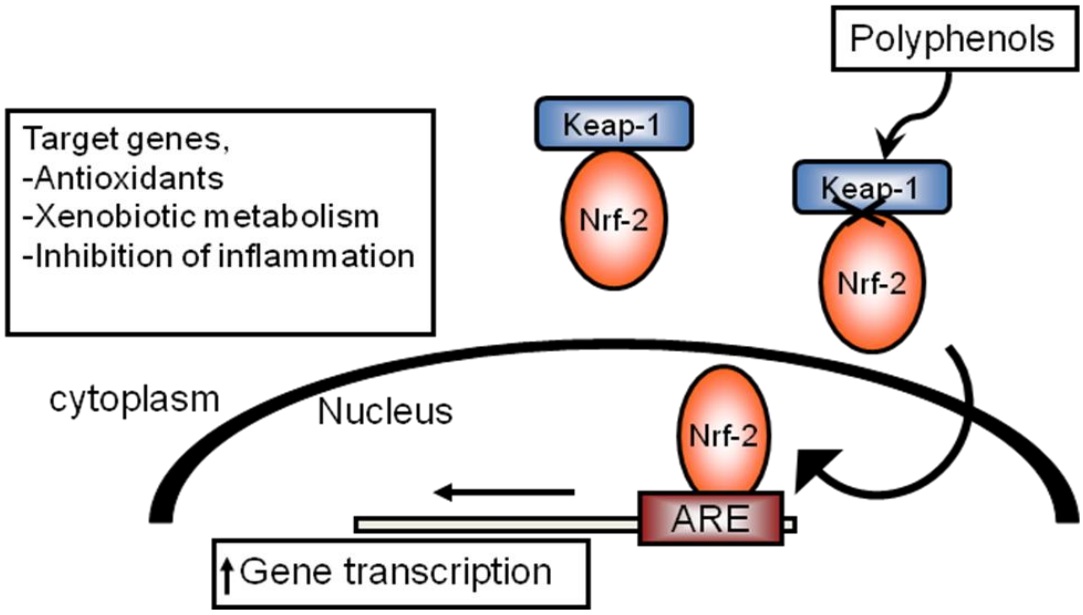


Figure 4: Activation of Nrf-2

Under the resting cell conditions Nrf-2 is located in the cytosol, forming a complex with Keap-1 protein. Upon receiving a stimulus e.g. polyphenols, the complex is broken and Nrf-2 is translocated to the nucleus where it increases transcription of target genes.

2.11 Resveratrol mediated Nrf-2 activation

Resveratrol is a natural antioxidant, phytochemical present in grapes, nuts, berries and other plant species. Resveratrol exerts its antioxidant and chemoprotective activities by

modulating various events in cellular signaling such as activation of Nrf-2. Studies in K562 cells treated with resveratrol showed 2.5 fold increase in NQO-1 protein levels and 3-5 fold increase in its enzymatic activity. The stimulation of NQO-1 expression was associated with increased state of Nrf-2 phosphorylation and its translocation to nucleus (57).

Treatment of human alveolar epithelial cells (A549) with cigarette smoke extract was associated with enhanced ROS production and decreased GSH activity (58, 59). Treatment with resveratrol restored GSH levels by activation of Nrf-2 and also quenched ROS.

2.12 Beneficial effects of antioxidant supplementation in treating hypertension

Evidences in human and various experimental models of hypertension suggest that enhanced ROS plays an important role in pathogenesis of hypertension. In human hypertension, markers of systemic oxidative stress are elevated and treatment with SOD mimetics have been shown to improve vascular, renal function and reduce blood pressure (41, 60). Mouse models deficient in ROS producing enzymes have lower blood pressure than the wild-type. Ang II infusion fails to increase blood pressure in these mice model suggesting an important role of Ang II-mediated ROS production in elevating blood pressure (60). Various experimental models of hypertension such as spontaneously hypertensive rats have elevated ROS generating enzymes along with compromised antioxidant capacity (9, 61). Treatment with SOD mimetic, BH_4 and AT1R blockers

have shown to decrease superoxide generation and attenuate the development of hypertension in these models. It has been suggested that antihypertensive agents such as AT1R blockers, ACE inhibitors and adrenergic blockers mediate their effects at least in part by decreasing oxidative stress (62-64).

Studies from our lab show that treatment of normotensive rats with pro-oxidants results in development of hypertension and SOD mimetic Tempol normalizes the elevated blood pressure (9). Recent studies in SHR show that treatment of SHR with red wine, which has resveratrol as one of the major components, along with physical exercise showed beneficial effects on HDL levels and also on blood pressure (65). Melatonin treatment in young SHR showed decreased oxidative stress and restoration of NO signaling which contributed to decrease in blood pressure (66).

Although the studies in animals suggests beneficial effects of antioxidant treatment on hypertension, clinical trials using antioxidant supplementation for treating hypertension in humans have been quiet disappointing. Currently antioxidants are not considered for prevention or treatment of hypertension in humans. Clinical trial data fails to demonstrate any beneficial cardiovascular effects of antioxidant treatment. Following are few of the reason for disappointing clinical trial outcomes with antioxidant treatment.

1) Antioxidants used in the trial: The antioxidants used in the trials are mostly vitamins such as vitamin C and E. It is possible that these agents were ineffective

due to insufficient dosing. It is also possible that orally administered antioxidants may be inaccessible to source of free radical.

2) Patients included in the trial: The patients included in such trials had significant cardiovascular disease and thus damaging effects of oxidative stress may be irreversible.

Thus targeting Nrf-2 to increase expression of antioxidant enzymes might be important for developing novel therapeutic strategies for treating hypertension.

Taken together, main objective of our project was to understand the mechanisms by which oxidative stress contributes to hypertension with particular emphasis on alteration of sodium transporter activity, particularly Na/K-ATPase. Since SHR have enhanced Ang II levels, our project was designed to determine the mechanisms by which Ang II (μM) regulates Na/K-ATPase activity under the conditions of enhanced oxidative stress in SHR and under the conditions of normal oxidative stress in WKY rats. We also determined the molecular mechanisms by which antioxidant resveratrol improves oxidative stress and ameliorates hypertension in SHR.

3. MATERIAL AND METHODS

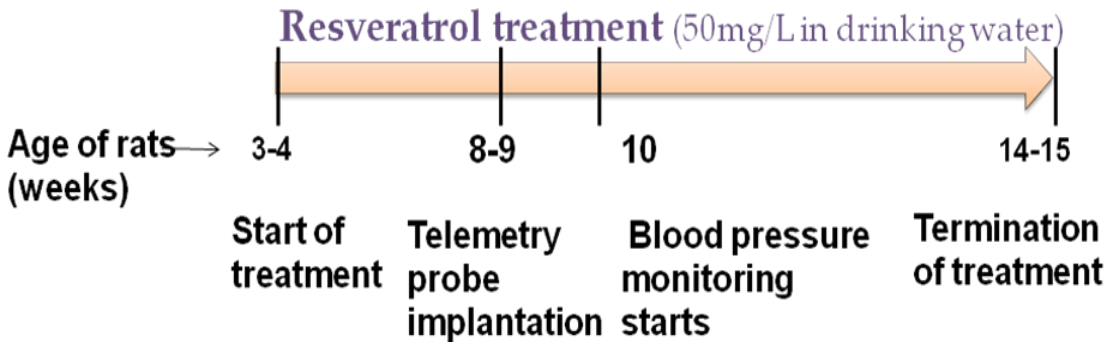
3.1 Animals

Male 3-4 weeks old spontaneously hypertensive rats (SHR) and Wistar Kyoto (WKY) rats were purchased from Charles River Laboratories (Wilmington, MA). The rats were placed in plastic cages and provided with free access to tap water and standard rat chow (Purina Mills, St. Louis, MO). All experiments were performed according to University of Houston guidelines and protocols were approved by Institutional Animal Care and Use Committee.

3.2 Treatment protocol

SHR and WKY rats were randomly divided into groups receiving either resveratrol (50mg/L) or tap water. Resveratrol was prepared by first dissolving 50mg resveratrol in 1mL ethanol. This solution was then diluted with tap water. Rats were supplied with either fresh solution of resveratrol or tap water everyday.

Treatment protocol:



3.3 Animal surgery

Implantable rat radio transmitters (TA11PA-C40; Data Sciences International, St. Paul, MN) were used to measure conscious blood pressure in individual animals. Animals were anaesthetized with 5% isoflurane and anesthesia was maintained by 2.5-3% isoflurane with a face mask throughout the surgery. After a midline abdominal incision was made, the abdominal aorta was exposed by using sterile cotton swabs. The catheter of the telemetric device was inserted into the abdominal aorta and guided upstream. To secure the catheter to the aorta, tissue adhesive (Vetbond, 3M Animal Care Products, St Paul, MN) was used. The body of the telemetric device was placed in the abdominal cavity and sutured to the abdominal musculature. The abdominal musculature and skin were closed individually. The surgery was performed under sterile conditions on a heating pad (37°C). Analgesia was achieved by the administration of buprenorphine (0.05 mg/kg SQ) every 12 h for 2 days. Animals were allowed 1 week for recovery before blood pressure measurements were recorded.

3.4 Preparation of renal proximal tubules

Proximal tubules were prepared as described previously (67). Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, IP) and after a midline incision the aorta was cannulated with PE50 tubing below the renal artery. Kidneys were perfused with Krebs-Hanseleit Buffer (KHB) (KHB-A in [mM: 118 NaCl, 27.2 NaHCO3, 4 KCl, 1.2 MgCl2, 1 KH2PO4, 1.25 $CaCl_2$, 5 glucose, and 10 HEPES, pH 7.4] at 37°C with flow

rate of 8mL/minute till blood was washed off. *In situ* digestion was accomplished by perfusing an enzyme solution of 230 U/ml collagenase and 250 U/ml hyaluronidase (Sigma, St. Louis, MO) in KHB-A. The kidneys were excised and decapsulated. Outer cortex was removed, chopped and digested with 20 mL of KHB-A solution (Collagenase 460 U/mL and Hyaluronidase 500 U/mL) under 95% O_2 and 5% CO_2 for 20 minutes to obtain uniform suspension. The suspension was filtered through a nylon mesh (Spectrum Medical Industries, Los Angeles, CA, mesh size: 105 µm). The filtrate was centrifuged (Centra-MP4R centrifuge, IEC, Needleham Heights, MA) at 50g for 2 minutes to recover pallet. The pellet was washed thrice with 15 mL of KHB-B buffer. Enrichment of proximal tubules was carried out using 25% Ficoll density gradient in KHB-B (mM: 118 NaCl, 27.2 $NaHCO_3$, 4 KCl, 0.12 $MgCl_2$, 1 KH_2PO_4, 5 Glucose and 10 HEPES, pH 7.4). The band at Ficoll-KHB-B buffer interface was collected and washed by centrifugation at 250g for 5 min in KHB-C (mM: 118 NaCl, 27.2 NaHCO3, 4 KCl, 1.2 MgCl2, 1.25 mM CaCl2, 5 glucose, and 10 HEPES, pH 7.4).

3.4.1 Cell viability studies

For determining proximal tubular viability in the cell suspension, we used trypan blue exclusion test (68). The cell viability was tested by mixing an equal volume of proximal tubules and trypan blue. The dye uptake by proximal tubular cells was observed under the microscope. The proximal tubules were considered viable if more than 95% of the cells excluded trypan blue and were used in the experiments. The proximal tubules that were used in our experiments had more than 95% viablility.

3.4.2 Proximal tubular membrane preparation

The isolated proximal tubular suspension in KHB-C (which included protease inhibitors) was homogenized. The homogenate was centrifuged at 250g for 10 minutes. The supernatant was centrifuged at 20,000g for 35 minutes to obtain a membrane pallet. The fluffy layer of pallet was resuspended into sucrose buffer (10mM Tris, 250mM Sucrose, protease inhibitors, pH 7.4).

3.5 Biochemical markers of oxidative stress

3.5.1 Measurement of 8-Isoprostane

Proximal tubular 8-Isoprostane levels were measured by using a commercially available EIA-based kit (Cayman Chemical Company, Ann Arbor, MI). This assay is based on competition between 8-isoprostane and 8-isoprostane-acetylcholinesterase (AChE) conjugate (8-isoprostane tracer) for limited number of 8-isopostane specific rabbit antiserum binding sites. The amount of tracer bound to rabbit antiserum is inversely proportional to concentration of 8-isoprostane present in the sample and is measured by adding Ellman's reagent. The intensity of the developed yellow color is measured spectrophotometrically (Biotek, Winooski, VT) at 412 nm.

3.5.2 Immunoblotting for protein carbonylation

Proximal tubular protein carbonylation levels were determined by using commercially available Millipore's protein carbonyl assay kit (Millipore, Billerica, MA). The assay is based on the reaction between protein carbonyls and 2,4-dinitrophenylhydrazine

(DNPH). Protein carbonyls react with DNPH to form corresponding hydrazone which can be analyzed spectrophotometrically at an absorbance 360 nm.

3.6 Determination of Nrf-2 activation and antioxidant enzymes

3.6.1Western blotting for Nrf-2 and GST

Proximal tubular nuclear fractions were isolated using commercially available NE-PER nuclear and cytisolic protein extraction kit (Pierce biotechnology, Rockfold, IL). Nuclear proteins (20µg) were resolved by SDS-PAGE and transferred to PVDF membrane (Immunobilon-P, Millipore, Bedford, MA) followed by overnight blocking with 5% bovine serum albumin (BSA, Pierce, IL). The membranes were further incubated with Nrf-2 specific primary antibodies (1:500) for 60 minutes. Secondary horse radish peroxidase conjugated anti-rabbit antibody was incubated for 60 minutes. After washing, the PVDF membranes were incubated with enhanced chemilumunescence reagent (Alpha Diagnostics, San Antonio, TX) and protein bands were visualized on X-ray film. The bands were quantified using Alphainnotech software (Cell biosciences, Santa Clara, CA). For immunoblotting of glutathione-S-transferase (GST), 40 µg of proximal tubular homogenates were used. Anti-GST rabbit polyclonal antibody (1:1000) from Santa Cruz was used in the protocol. The procedure for separation and visualization of proteins was done as described previously.

3.6.2 Hemeoxygenase-1 by enzyme linked immunosorbant assay (ELIZA)

Proximal tubular hemeoxygenase-1 (HO-1) was measured by a using commercially available sandwich ELIZA kit (Assay Designs, Ann Arbor, MI). Briefly, samples were

added to 96 well plates precoated with mouse monoclonal antibody specific against HO-1. HO-1 in the samples was captured by rabbit polyclonal antibody specific against HO-1 and detected with anti-rabbit IgG antibody conjugates to horseradish peroxidase.
The wells were washed after each antibody incubation. The HRP substrate tetramethylbenzidine (TMB) was added and a blue color was developed which was proportional to the amount of captured HO-1. The color development was stopped with an acid stop solution. The endpoint yellow color was read at 450 nm on microplate reader (Biotek, Winooski, VT). The standards for HO-1 were prepared simultaneously and used to quantify the amount of HO-1 present in the samples. The values in proximal tubules were expressed as per microgram protein.

3.6.3 Superoxide dismutase activity

Proximal tubular superoxide dismutase (SOD) activity was measured by using commercially available kit from Cayman Chemical (Ann Arbor, MI). This method detects SOD activity by utilizing tetrazolium salt to detect superoxides from xanthine oxidase and hypoxanthine. The amount of enzyme needed for dismutation of 50% of superoxide radical was considered as one unit of SOD. The radical detector system was prepared by diluting 50µL tetrazolium salt solution in 19.95 mL assay buffer (50mM Tris-HCl, pH 8.0, and containing 0.1 mM diethylenetriaminepentaacetic acid). Samples (10µg/µL) and radical detector solution were added in 96 well plate and the reaction was initiated by addition of xanthine oxidase. The plate was incubated at room temperature, with gentle shaking, for 20 min. SOD standards were prepared simultaneously and used

to quantify SOD activity in the samples. The absorbance was read at 440 nm and SOD activity was calculated using manufacturer's instructions.

3.6.4 Antioxidant assay

The total antioxidant capacity in proximal tubules was determined using a commercially available kit (Cayman Chemical, MI). Briefly, the assay relies on the ability of antioxidants present in the sample to inhibit the oxidation of substrate ABTS® (2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABTS^{®,+}$ by metmyoglobin. The amount of $ABTS^{®,+}$ produced was determined by reading the absorbance at 405 nm. The capacity of the antioxidants in the sample to prevent ABTS® oxidation was compared with that of Trolox and is quantified as millimolar Trolox equivalents.

3.7 Protein kinase C activity

Proximal tubular tubular protein kinase C (PKC) activity was determined using PepTag Protein Kinase C assay kit (Promega, Madison, WI) according to manufacturer's instructions. Renal proximal tubules were homogenized in buffer (mM: 125 Tris, 0.5 EDTA, 0.05 Triton X-100, 10 β-mercaptoethanol and protease inhibitor cocktail) followed by centrifugation at 14000g for 5 minutes at 4°C. The supernatant was used for performing the assay. Reaction mixture contained 5µL each of 5X reaction buffer, PepTag Cl peptide (0.4 µg/µL), 5 X activator solutions and 10 µL sample (2.5µg/ µL). The reaction mixture was incubated at 95°C for 10 minutes to start the reaction. Glycerol (80%, 1 µL) was added to all the samples and were separated on 0.8% agarose gel at 100V for 15 minutes. The phosphorylated C1 peptide band that migrated towards cathode

(+) side was excised under UV light. The agarose containing peptide was melted at 95°C, transferred to 96 well plate and fluorescence was read using excitation (540 nm) and emission (600 nm) wave lengths.

3.8 Na/K-ATPase activity

Na/K-ATPase activity was determined as described by Quigley and Gotterer (69). Briefly, renal proximal tubules (1 mg protein) were incubated without (basal) and with Ang II (10^{-10} M or 10^{-6} M) at 37°C for 15 min in a water bath. After incubation, the proximal tubules were lysed by snap freezing in liquid nitrogen. The lysed tubules were used to assay ouabain (4 mM) sensitive Na/K-ATPase activity, which was determined by phosphate hydrolysis of ATP (4 mM). The inorganic phosphate released was determined colorimetrically as described by Taussky and Shorr (70).

3.9 Determination of nitric oxide (NO) level

Renal proximal tubules were incubated without (basal) or with Ang II (10^{-6}M) in presence or absence of candesartan (1 µM) and tissues were snap frozen in liquid nitrogen followed by homogenization. The levels of NO were determined colorimetrically using nitrate/nitrite assay kit (Cayman chemical, Ann Arbor, MI) according to manufacturer's protocol.

3.10 Superoxide detection

Fluorescence spectrometric assay of superoxide production was performed as detailed by Satoh et al (71). Briefly, proximal tubules were incubated with and without Ang II (10^{-6}M) for 15 min. Oxidation of dihydroethidium to ethidium in presence of NADH or L-

arginine was used as a measure of superoxide production. Conversion of dihydroethidium to ethidium in absence of 1 µM Ang II was considered as basal superoxide production

3.11 Determination of [^{35}S]GTPγS binding

The assay was performed as described in previous studies (72). Briefly, the reaction mixture containing 100 µl of 0.6 nM [^{35}S]GTPγS, Ang II (10^{-6}M), 5 µg of proximal tubular membrane protein was incubated at 30°C for 60 min. Nonspecific [^{35}S]GTPγS binding was determined in the presence of 100 µM unlabelled GTPγS. The reaction was stopped by rapid filtration on GF/C filters under vacuum and radioactivity was determined by liquid scintillation counter (Beckman Coulter, Brea CA).

3.12 AT1R Saturation binding using [^{125}I]Sar-Ang II

Radiolabelled [^{125}I]Sar-Ang II, an AT1R antagonist, was used to detect Kd (affinity) and Bmax (receptor numbers) of AT1 receptors. The binding of [^{125}I]Sar-Ang II to proximal tubular membranes was performed as describe previously (73, 74). Proximal tubular membranes (50µg) were incubated with varying concentrations of [^{125}I]Sar-Ang II ranging between 0.03 nM to 4 nM. Non-specific binding was determined by unlabelled Ang II (1 µM). The reaction volume was adjusted to 250 µg with assay buffer (50mM Tris HCl, 1mM $MgCl_2$, protease inhibitors). The final reaction volume was incubated at 30^{o}C for 1 hour. The binding was terminated by rapid filtration under vacuum followed by 3-4 washes with assay buffer. The radioactivity on the membranes was determined using Beckman gamma counter.

3.13 Immunoblotting

Immunoblotting was performed as described previously in section 3.6.1.

1) AT1R : Proximal tubular homogenate (20 µg) was separated and immunoblotted against AT1R (1:200, Santa Cruz Biotechnology) and Glyceraldehyde 3-phosphate dehydrogenase (1:1000, Santa Cruz Biotechnology).
2) gp91: Proximal tubular homogenate (30 µg) was separated and immunoblotted against gp 91(1:1000, BD Biosciences, San Jose, CA).
3) Giα and Gq/11: Proximal tubular membrane proteins (40 µg) were separated and immunoblotted against Gq/11 and $Gi\alpha_{1\text{-}2}$ (1:1000, Calbiochem, Gibbstown, NJ)
4) NFkappaB subunit p65: Proximal tubular nuclear fractions (20µg) were separated and immunoblotted against p65 subunit (1: 1000, Cell Signaling, Danvers, MA).

The procedure for separation and visualization of the proteins was performed as mentioned in section 3.6.1.

3. 14 DATA ANALYSIS

Results are presented as Mean ± SEM. Differences between the means were evaluated using unpaired *student's t-test* or one way ANOVA with *post hoc* Newman-Keuls multiple test, as appropriate. $P<0.05$ was considered statistically significant (N=6-8 rats). Statistical analysis was performed using Graph Pad Prism, version 4.03 (GraphPad Software, San Diego, CA).

4 RESULTS

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

4.1.1 Blood pressure and markers of oxidative stress

At 3-4 weeks of age, SHR and WKY rats had similar mean arterial pressure (MAP) (Fig. 1A). There were no differences in proximal tubular protein carbonylation levels (Fig.1B), expression of NADPH oxidase subunit gp91 (Fig. 1C) and superoxide dismutase (SOD) activity (Fig. 1D) in SHR and WKY rats.

4.1.2 AT1R protein expression

To determine if there are any changes in AT1R protein expression we performed western blotting. Densitometry analysis of blots showed equal AT1R protein expression in proximal tubules of SHR and WKY rats (Fig.2).

4.1.3 Ang II-induced Na/K-ATPase regulation

To determine AT1R mediated Na/K-ATPase regulation, we detected Na/K-ATPase activity in presence of Ang II. Incubation of proximal tubules with Ang II (10^{-10}M) activated Na/K-ATPase in SHR and WKY rats to similar extent (Fig. 3).

4.1.4 Protein kinase C activity

There are reports showing involvement of protein kinase C (PKC) in modulating Na/K-ATPase activity (75). Proximal tubular PKC activity was equal in both, SHR and WKY rats (Fig. 4).

4.2 Studies in adult SHR and WKY rats

4.2.1 Effect of chronic resveratrol treatment on blood pressure of SHR and WKY rats

The blood pressure was measured by radiotelemetry. Resveratrol treatment was started in SHR and WKY at 3-4 weeks of age. Telemetry probe transducer was implanted in these rats at 7-8 weeks of age and the blood pressure was measured continuously after 1 week of recovery period. Once the blood pressure difference between resveratrol treated and untreated SHR remained constant, animals were sacrificed to perform biochemical experiments. As expected, there was a significant increase in MAP in SHR compared to WKY rats (Fig. 5). Compared to untreated SHR, resveratrol treated SHR showed a significant decrease in MAP starting at 7 weeks and continued through 11 weeks of treatment (Fig. 5). Untreated and resveratrol treated WKY showed similar MAP (Fig. 5).

4.2.2 Effect of resveratrol on markers of oxidative stress

Proximal tubular levels of 8-isoprostane were significantly higher in SHR compared to WKY rats (Fig. 6). Resveratrol treatment significantly decreased proximal tubular 8-

isoprostane levels in SHR (Fig. 6). The resveratrol treatment did not change 8-isoprostane levels in WKY rats (Fig. 6).

Proximal tubular protein carbonylation levels, as determined by western blotting, were significantly higher in SHR compared to WKY rats (Fig. 7). Resveratrol treatment significantly decreased protein carbonylation levels in SHR (Fig. 7). Resveratrol treatment did not change carbonylation levels in WKY rats (Fig. 7).

4.2.3 Effect of resveratrol on antioxidant capacity

Proximal tubular total antioxidant activity in SHR was significantly lower than WKY rats (Fig. 8). Resveratrol treatment in SHR significantly increased total antioxidant activity (Fig. 8). Resveratrol treated and non treated WKY rats had equal proximal tubular antioxidant activity (Fig. 8).

4.2.4 Effect of resveratrol on Nrf-2 activation and Nrf-2 induced antioxidant enzymes

Protein expression of Nrf-2 in nuclear fraction of proximal tubules was significantly lower in SHR compared to WKY rats (Fig. 9). Resveratrol treatment caused a significant increase in protein expression of Nrf-2 in proximal tubular nuclear fractions of SHR (Fig. 9). However, resveratrol treatment did not change Nrf-2 nuclear expression in WKY rats (Fig. 9).

Expression levels of glutathione-S-transferase (GST) were significantly low in proximal tubules of SHR compared to WKY rats (Fig. 10). Resveratrol treatment caused a significant increase in GST protein expression in SHR (Fig. 10). Both treated and untreated WKY rats showed equal GST protein expression (Fig. 10).

The basal proximal tubular SOD activity was equal in SHR and WKY rats (Fig. 11). Resveratrol treated SHR showed significant increase in SOD activity compared to untreated SHR (Fig. 11). There was no change in SOD activity in WKY rats after resveratrol treatment (Fig.11).

Protein levels of Hemeoxygenase-1 (HO-1), as determined by ELIZA, were similar in proximal tubules of SHR and WKY rats (Fig. 12). Resveratrol treatment caused significant increase in protein levels of HO-1 in SHR (Fig. 12). Protein levels of HO-1 were equal in untreated and resveratrol treated WKY rats (Fig. 12).

4.2.5 Effect of resveratrol on Ang II-induced Na/K-ATPase activation

Incubation of proximal tubules with low concentration of Ang II (10^{-10}M) stimulated Na/K-ATPase activity in both SHR and WKY rats. However, Ang II induced stimulation of Na/K-ATPase was significantly higher in SHR compared to WKY rats (Fig. 13A). Resveratrol treatment in SHR significantly decreased the stimulation of Na/K-ATPase in response to Ang II (Fig. 13A). Ang II-induced Na/K-ATPase stimulation was equal in untreated and resveratrol treated WKY rats (Fig. 13A).

High concentration of Ang II (10^{-6}M) failed to activate Na/K-ATPase in WKY rats; yet at this concentration Ang II continued to stimulate Na/K-ATPase in SHR (Fig. 13A). Ang II induced Na/K-ATPase stimulation was significantly decreased in resveratrol treated SHR compared to untreated SHR (Fig. 13A). Ang II also failed to stimulate Na/K-ATPase in resveratrol treated WKY rats (Fig. 13A).

The basal Na/K-ATPase activity was equal in untreated and resveratrol treated SHR and WKY rats (Fig. 13B).

As shown in Fig. 14, Na/K-ATPase activation by Ang II was sensitive to AT1R antagonist candesartan in untreated SHR and resveratrol treated SHR and WKY rats.

4.2.6 Effect of resveratrol on nitric oxide mediated Na/K-ATPase regulation

Incubation of proximal tubules of WKY rats with NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME, 1mM) caused a significant stimulation of Na/K-ATPase by Ang II (Fig. 15). However, L-NAME treatment did not change Ang II-induced Na/K-ATPase stimulation in SHR (Fig. 15). Furthermore, L-NAME treatment produced stimulation of Na/K-ATPase by Ang II in resveratrol treated SHR and WKY rats (Fig. 15).

4.2.7 Effect of resveratrol on Ang II mediated nitric oxide (NO) production

There are reports showing involvement of NO system in mediating Na/K-ATPase regulation at high concentrations of Ang II (9, 23). Since our results show defective Na/K-ATPase regulation at high concentration of Ang II, we wanted to determine NO

production in presence of Ang II (μM) in proximal tubules of SHR and WKY rats. Incubation of proximal tubules with Ang II (10^{-6}M) caused a significant increase in NO production in WKY rats which was sensitive to AT1R antagonist candesartan (Fig. 16). However, Ang II failed to increase NO levels in proximal tubules of SHR (Fig. 16). Resveratrol treatment in SHR significantly increased Ang II-induced NO levels (Fig. 17A). Ang II induced NO accumulation was similar in untreated and resveratrol treated WKY rats (Fig. 17A).

Basal NO production was equal in all the groups (Fig. 17B).

4.2.8 Effect of resveratrol on Ang II-induced NOS dependent superoxide production

NOS uncoupling is one of the mechanisms which causes decrease in NO levels. Uncoupled NOS can produce superoxides instead of NO. Thus we determined Ang II-induced superoxide production in presence of NOS substrate L-arginine. Preincubation of proximal tubules with Ang II (10^{-6}M) and L-arginine (1mM) caused superoxide production in SHR but not in WKY rats (Fig. 18). Superoxide production in proximal tubules of SHR was sensitive to NOS inhibitor L-NAME (1mM) (Fig. 18). There was no effect of L-NAME treatment on superoxide production in proximal tubules of WKY rats (Fig. 18). Ang II failed to produce NOS dependent superoxides in resveratrol treated SHR (Fig. 19).

4.2.9 Effect of resveratrol on Ang II mediated NADPH oxidase activation

Incubation of proximal tubules with Ang II (10^{-6}M) and NADPH oxidase substrate, NADH (0.1mM), resulted in superoxide production in all the groups (Fig. 20 and 21). However, the generation of superoxides in response to Ang II was significantly higher in SHR compared to WKY rats (Fig. 20). Ang II-induced superoxide production in proximal tubules of SHR and WKY rats was sensitive to NADPH oxidase inhibitor diphenyliodonium (DPI, 0.1mM) (Fig. 20). Ang II-induced NADPH oxidase dependent superoxide production was significantly decreased in proximal tubules of resveratrol treated SHR compared to untreated SHR (Fig. 21). Incubation of proximal tubules with Ang II activated NADPH oxidase to similar extent in untreated and resveratrol treated WKY rats (Fig. 21).

4.2.10 Involvement of NADPH oxidase in NOS uncoupling

To further verify if Ang II-induced superoxide production plays any role in reducing NO levels in SHR, we determined the effect of NADPH oxidase inhibitor DPI on Ang II-induced NO production in proximal tubules of SHR and WKY rats. As shown in Fig. 22, DPI (0.1mM) treatment normalized Ang II-induced NO production in untreated SHR. However, there was no effect of DPI treatment on Ang II-induced NO production in proximal tubules of untreated WKY rats and resveratrol treated SHR and WKY rats (Fig. 22).

In addition, DPI treatment blocked L-arginine dependent NOS mediated superoxide production in Ang II treated proximal tubules of SHR, but had no effect in WKY rats and resveratrol treated SHR and WKY rats (Fig. 23).

4.2.11 AT1R protein expression

Proximal tubular AT1R protein expression was equal in SHR and WKY rats (Fig. 24A). To determine AT1R affinity and numbers we performed saturation binding studies in proximal tubular membranes. Saturation binding experiments using radiolabelled ^{125}I-sar-Ang II showed a modest but significant decrease in Kd in proximal tubular membranes of SHR compared to WKY rats (Fig. 24B). AT1R numbers, Bmax, were equal in SHR and WKY rats (Fig. 24C).

4.2.12 Effect of resveratrol treatment on G protein coupling

To detect if any changes in AT1R-G protein-coupling could possibly enhance AT1R signaling in SHR, we determined Ang II-induced [^{35}S]GTPγS binding in proximal tubular membranes of SHR and WKY rats. Incubation of proximal tubules with high concentration of Ang II increased membrane [^{35}S]GTPγS binding in all the groups (Fig. 25A). However, the membrane [^{35}S]GTPγS binding was significantly higher in SHR compared to WKY rats (Fig. 25A). Resveratrol treatment normalized [^{35}S]GTPγS binding in SHR but did not change [^{35}S]GTPγS binding in WKY rats (Fig. 25A). The basal [^{35}S]GTPγS binding was equal in SHR and WKY rats (Fig. 25B).

4.2.13 Effect of resveratrol on G protein expression and role of G proteins in AT1R-G protein-coupling

To investigate the mechanism for enhanced Ang II-induced G protein coupling in SHR, we determined G protein expression by Western Blot in proximal tubular membranes of SHR and WKY rats. Western blot analysis showed higher levels of Giα protein in proximal tubular membranes of SHR compared to WKY rats (Fig. 26). In resveratrol treated SHR, there was significant decrease in Giα protein expression levels compared to untreated SHR (Fig. 26). There was no effect of resveratrol treatment on protein expression of Giα protein in WKY rats (Fig. 26). Incubation of proximal tubular membranes with pertussis toxin decreased [^{35}S]GTPγS binding in untreated and resveratrol treated SHR and WKY rats (Fig. 27).

Gq/11 protein expression in proximal tubular membranes was equal in proximal tubular membranes of untreated and resveratrol treated SHR and WKY rats (Fig. 28).

4.2.14 Effect of resveratrol on activation of NFkappa B

To determine if NFkappa B activation could be responsible for enhanced Giα protein expression in SHR, we determined NFkappa B subunit p65 protein levels in nuclear fraction of proximal tubules of SHR and WKY rats. Western blot analysis showed higher levels of NFkappa B subunit p65 in proximal tubular nuclear fraction of SHR compared to WKY rats (Fig. 29). Resveratrol treatment in SHR significantly decreased p65 subunit

expression in proximal tubular nuclear fractions (Fig. 29). There was no effect of resveratrol treatment on protein expression of p65 subunit in WKY compared to untreated WKY rats (Fig. 29).

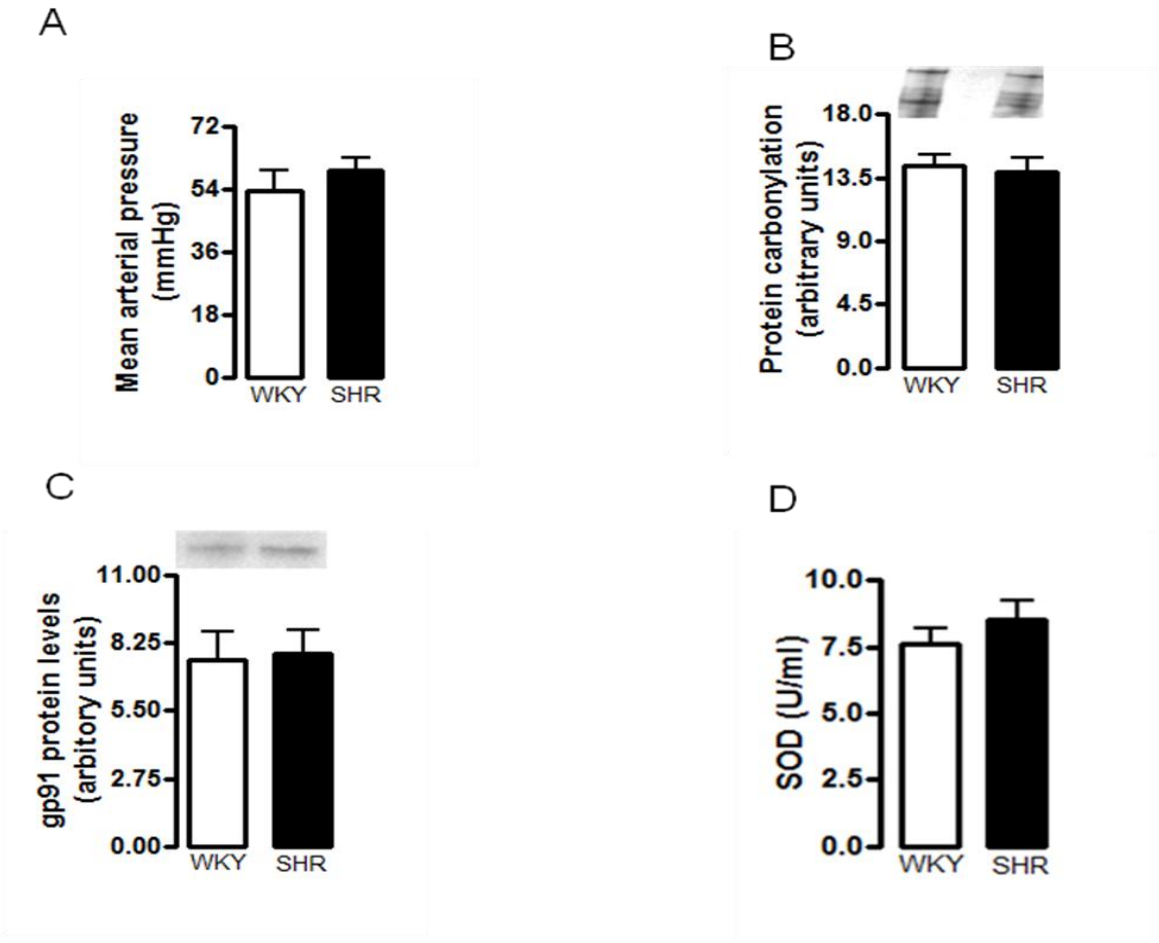


Figure 1: Blood pressure and oxidative stress markers in 3-4 weeks old SHR and WKY rats: A: Mean aterial pressure (MAP) of SHR and WKY rats. B) Proximal tubular protein carbonylation levels, measured by immunoblotting, in SHR and WKY rats. C) gp91 protein expression, measured by immunoblotting, in proximal tubules of SHR and WKY rats. Bands are representative Western Blots. D) Antioxidant superoxide dismutase activity in proximal tubules of SHR and WKY rats. Data represents Mean ± SEM from 6-8 rats.

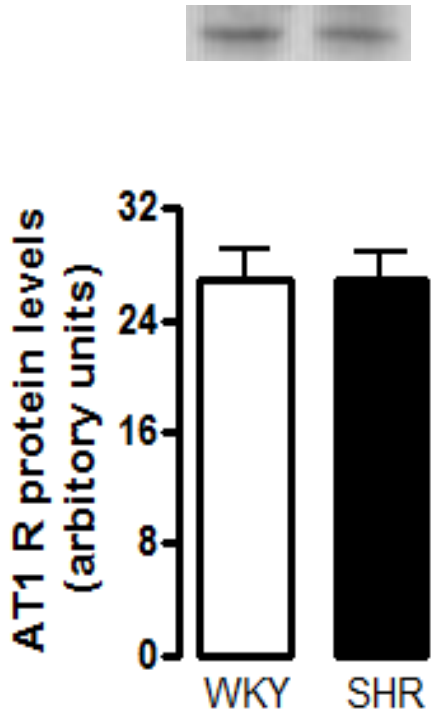


Figure 2: AT1R protein expression: Proximal tubular proteins were resolved by SDS-PAGE and immunoblotted for AT1R specific antibody. Bands are representative Western Blots. Data represents Mean ± SEM from 6-8 rats.

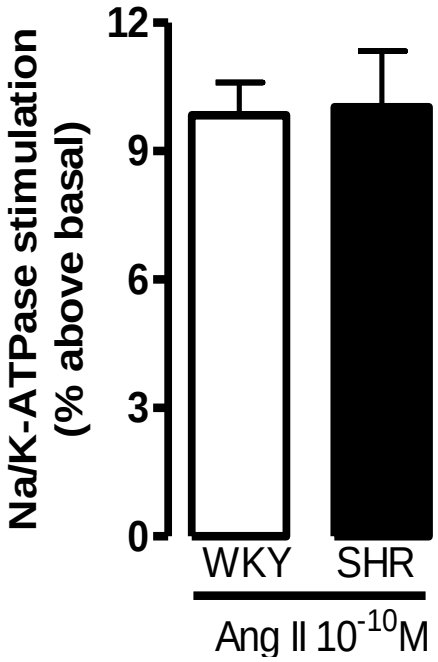


Figure 3: Ang II-induced Na/K-ATPase activity. Proximal tubules of SHR and WKY were treated with Ang II (10^{-10}M) for 15 minutes and Na/K-ATPase activity was determined as described in methods section. Data is expressed as % stimulation over basal. Bars represent Mean ± SEM from 6-8 rats.

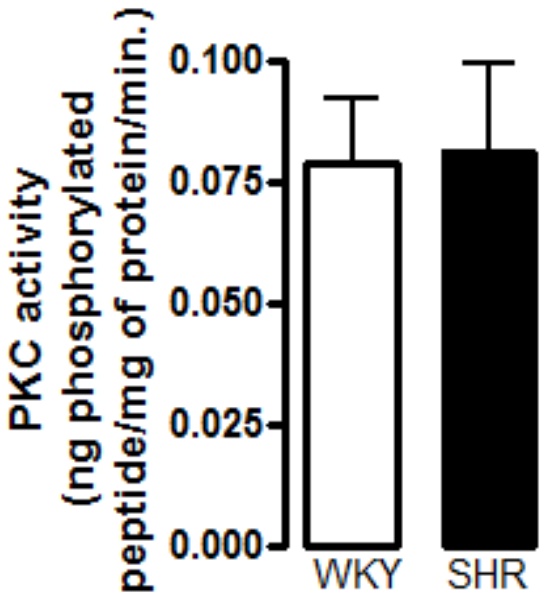


Figure 4: Protein kinase C activity: PKC activity was measured by using PepTag assay kit from Promega. Bars represent basal proximal tubular PKC in SHR and WKY rats. . Data represents Mean ± SEM from 6-8 rats.

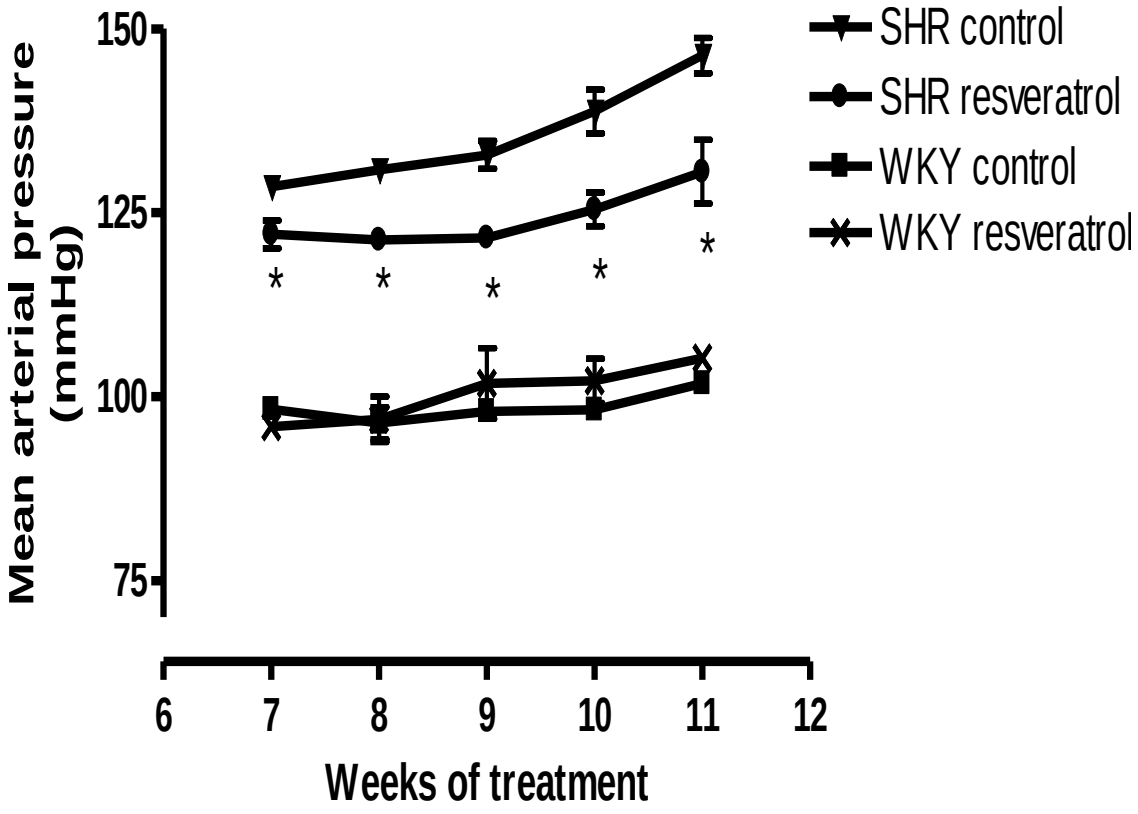


Figure 5: Mean arterial pressure (MAP): Blood pressure was measured by telemetry as described in methods section. Blue bars = WKY rats, red bars = SHR, grey bars=WKY treated with resveratrol and green bars= SHR treated with resveratrol. Data represents Mean ± SEM from 6-8 rats. *$P<0.05$ vs. SHR using One–way ANOVA followed by Newman-Keuls multiple test.

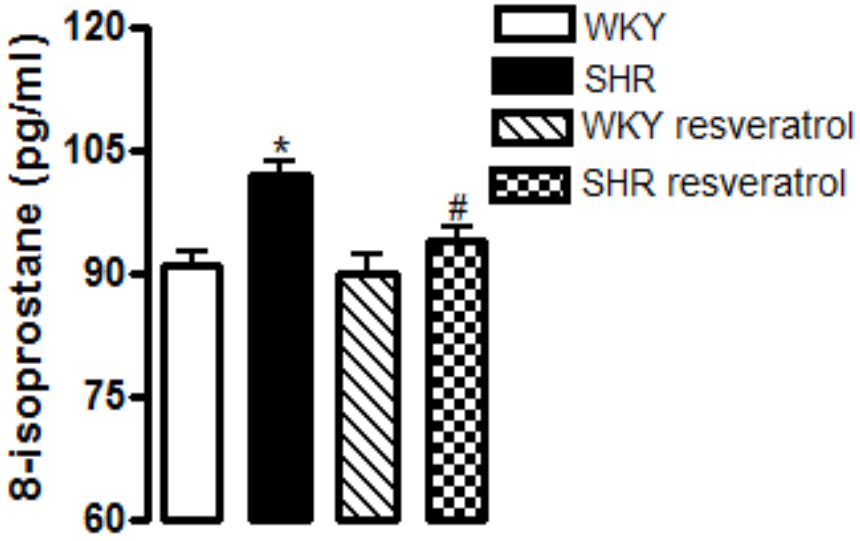


Figure 6: Proximal tubular 8-isoprostane: Proximal tubular 8-isoprostane levels were measured by EIA based kit from Cayman. Data represents Mean ± SEM from 6-8 rats. *$*P<0.05$* vs. WKY, $^{\#}P<0.05$ vs.SHR using One–way ANOVA followed by Newman-Keuls multiple test.

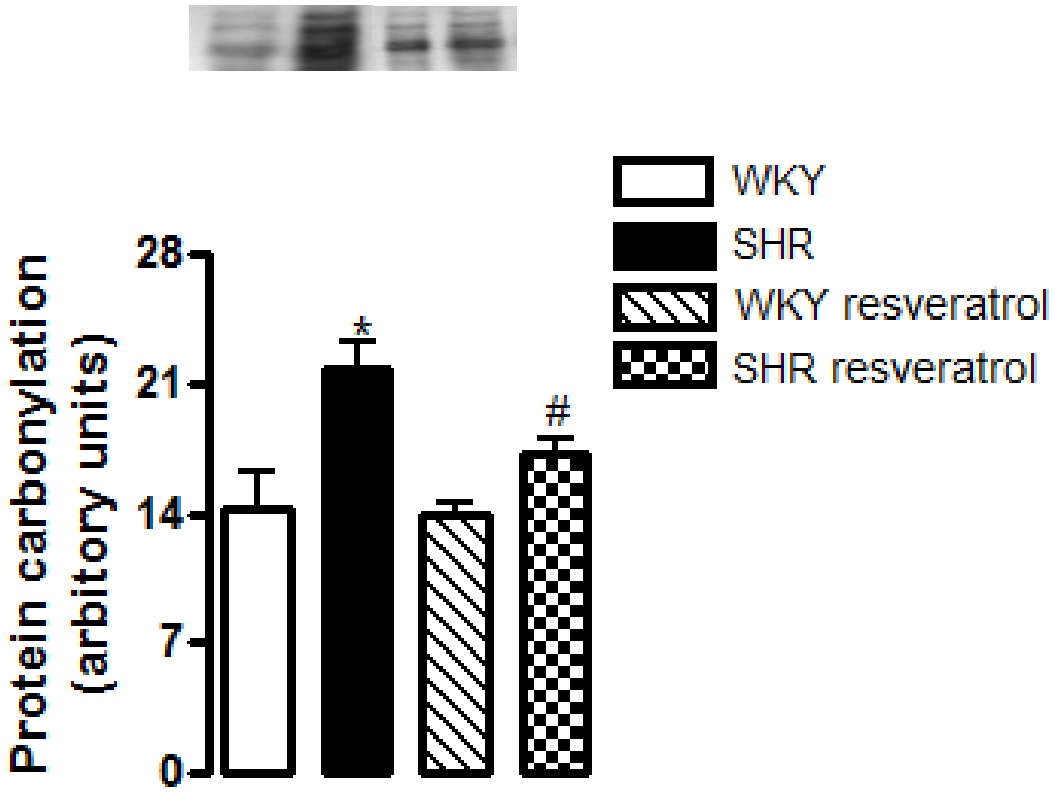


Figure 7: Protein carbonylation levels: Proximal tubular protein carbonylation levels, measured by immunoblotting. Bands represent Western Blot. Data represents Mean ± SEM from 6-8 rats. *$P<0.05$ vs. WKY, $^{\#}P<0.05$ vs.SHR using One–way ANOVA followed by Newman-Keuls multiple test.

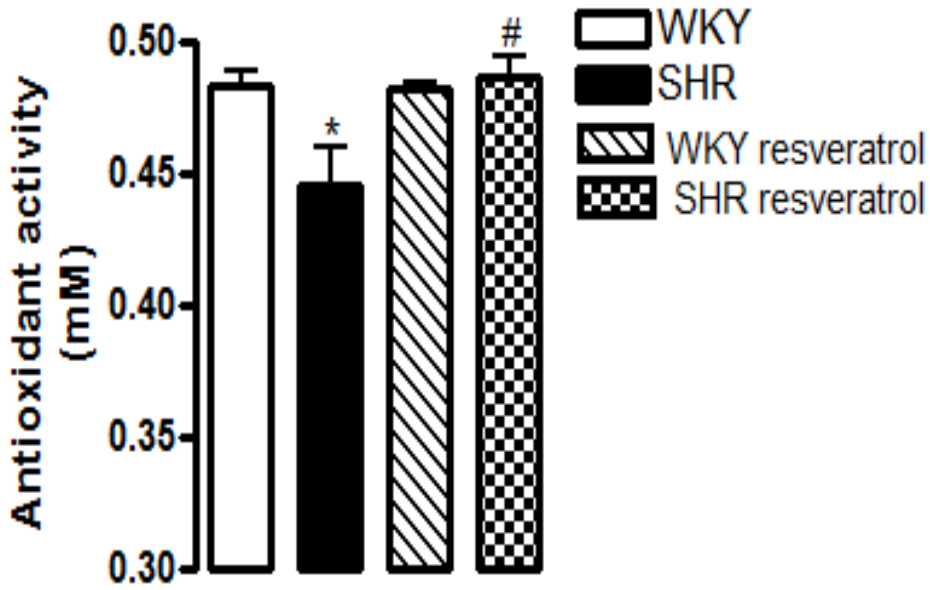


Figure 8: Antioxidant activity: Antioxidant activity in proximal tubular homogenate was measured using commercially available kit from Cayman. Data represents Mean ± SEM from 6-8 rats. *$P<0.05$ vs. WKY, $^{\#}P<0.05$ vs.SHR using One–way ANOVA followed by Newman-Keuls multiple test.

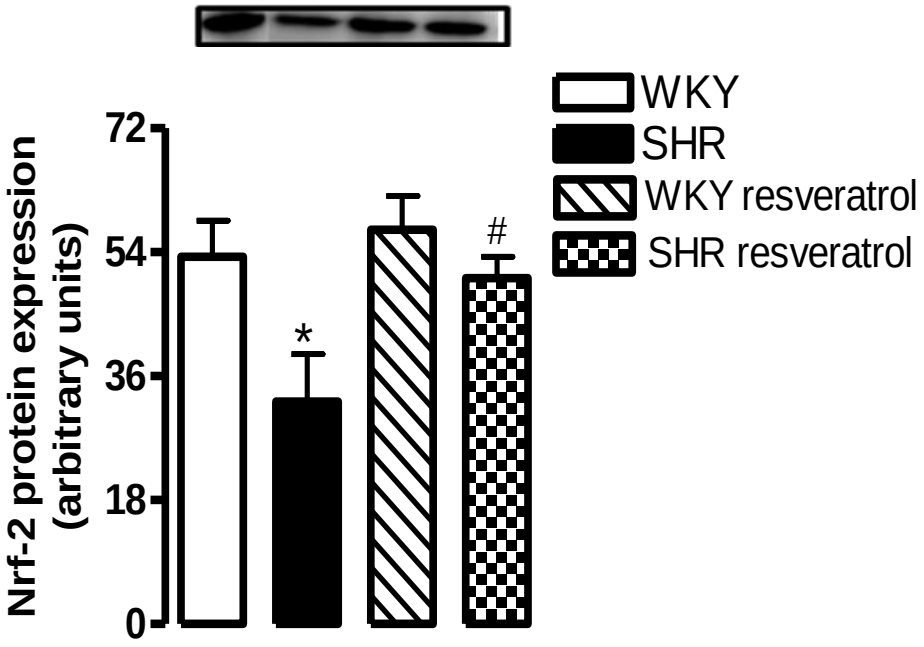


Figure 9: Nrf-2 protein expression: Nuclear proteins were separated by SDS-PAGE and immunoblotted against Nrf-2 specific antibody. Bands are representative Western Blot. Data represents Mean ± SEM from 6-8 rats. $^{*}P<0.05$ vs. WKY, $^{\#}P<0.05$ vs.SHR using One–way ANOVA followed by Newman-Keuls multiple test.

Figure 10: Glutathione-S-transferase (GST) protein expression: Proximal tubular homogenates were used to determine GST protein expression. Bands are representative Western Blot. Data represents Mean ± SEM from 6-8 rats. *$P<0.05$ vs. WKY, $^{\#}P<0.05$ vs.SHR using One–way ANOVA followed by Newman-Keuls multiple test.

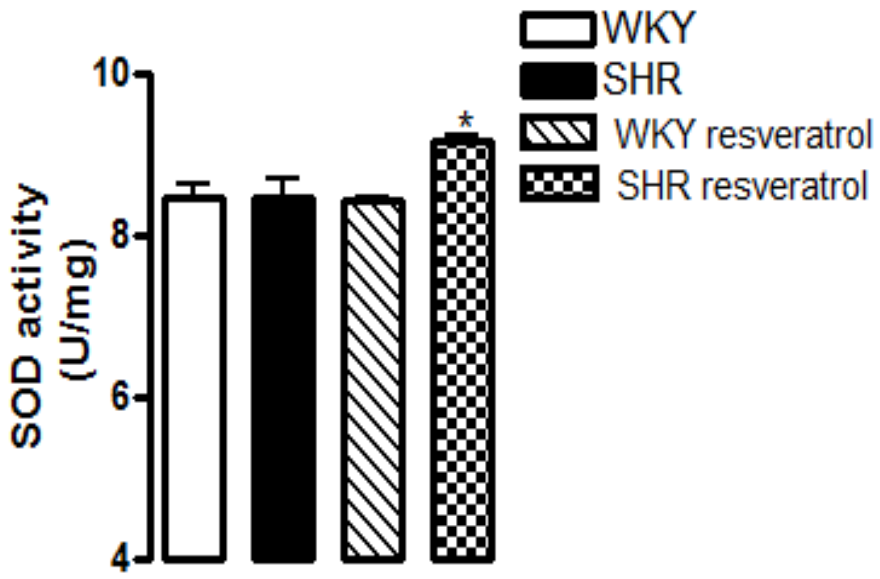


Figure 11: Superoxide dismutase (SOD) activity: Superoxide dismutase activity (SOD) in proximal tubular homogenate was determined using SOD assay kit from Cayman. Data represents Mean ± SEM from 6-8 rats. *$P<0.05$ vs. SHR using One–way ANOVA followed by Newman-Keuls multiple test.

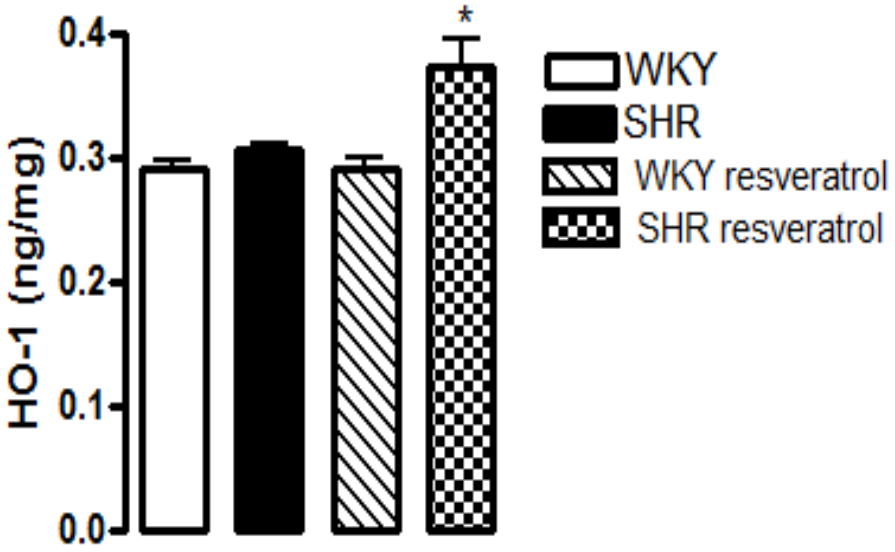
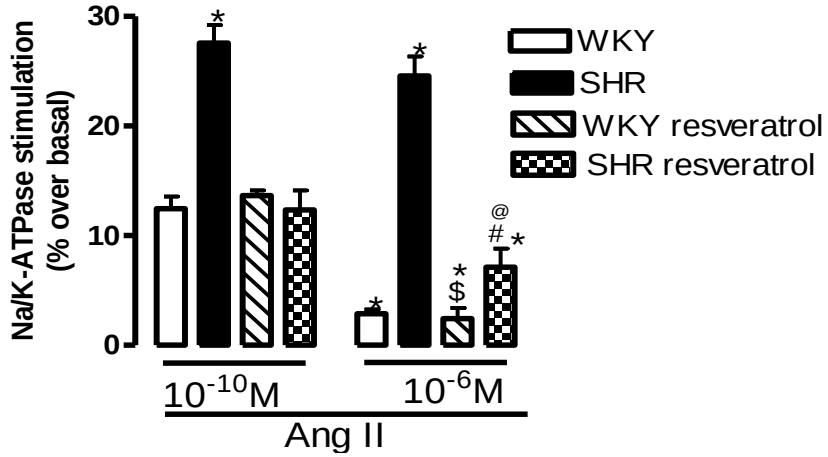


Figure 12: Heme-oxygenase-1 protein levels: Proximal tubular protein levels of heme-oxygenase -1 (HO-1) were determined using ELIZA as described in methods. Data represents Mean ± SEM from 6-8 rats. *$P<0.05$ vs. SHR using One–way ANOVA followed by Newman-Keuls multiple test.

(A)



(B)

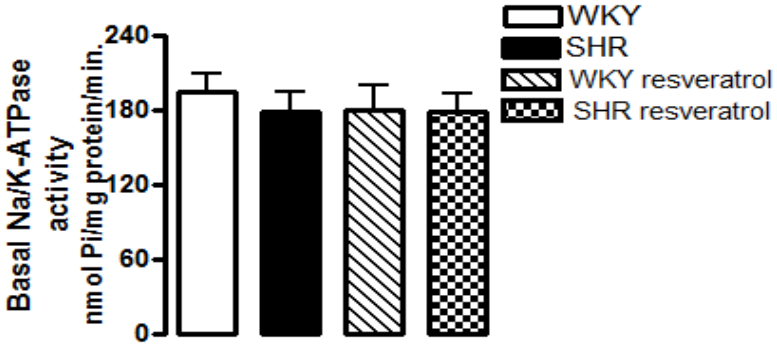


Figure 13: Na/K-ATPase activity: A: Proximal tubules were incubated with indicated doses of Ang II and Na/K-ATPase activity was determined as described in methods section. Data is expressed as % stimulation from the basal. B: Basal Na/K-ATPase activity. Bars represent Mean ± SEM from 6-8 rats. *$*P<0.05$* vs. WKY for Ang II (10^{-10}M), $^{\#}P<0.05$ vs.SHR for Ang II (10^{-6}M), $^{\$}P<0.05$ vs. WKY resveratrol for Ang II (10^{-10}M) and $^{@}P<0.05$ vs. SHR resveratrol for Ang II (10^{-10}M) using One–way ANOVA followed by Newman-Keuls multiple test.

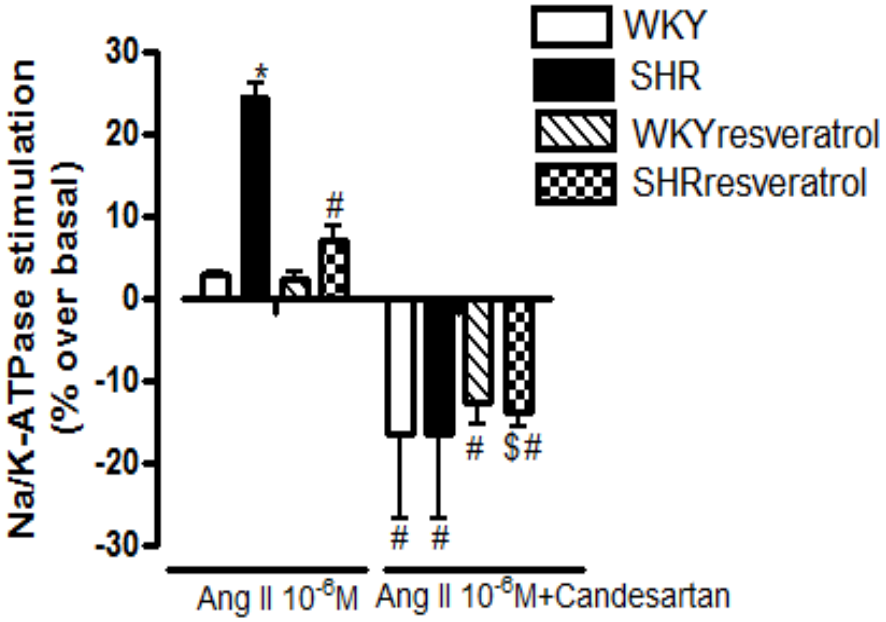


Figure 14: Effect of AT1R antagonist on Ang II-induced Na/K-ATPase activity. Proximal tubules were incubated with candesartan (1µM) for 10 minutes followed by challenge with Ang II (10^{-6}M) and its effect on Na/K-ATPase activity was determined. Data is expressed as % stimulation compared to basal. Bars represent Mean ± SEM from 6-8 rats. *$P<0.05$ vs. WKY for Ang II (10^{-6}M), $^{\#}P<0.05$ vs.SHR for Ang II (10^{-6}M) and $^{\$}P<0.05$ vs.SHR resveratrol for Ang II (10^{-6}M) using One–way ANOVA followed by Newman-Keuls multiple test.

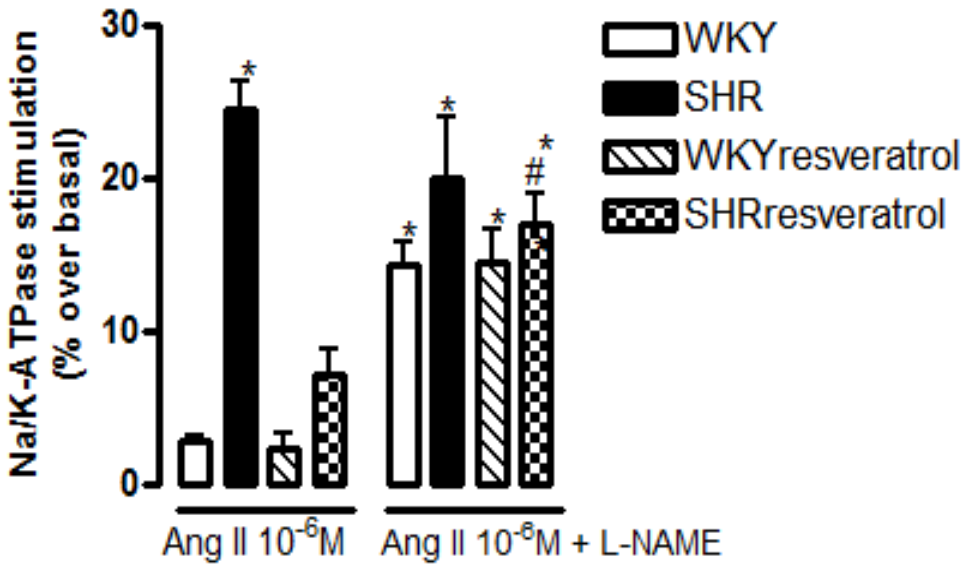


Figure 15: Effect of L-NAME on Ang II-induced Na/K-ATPase activity. Proximal tubules were incubated with or without 1mM L-NAME for 10 minutes followed by challenge with Ang II (10^{-6}M) and Na/K-ATPase activity was determined as described in method's section. Data is expressed as % stimulation compared to basal. Bars represent Mean ± SEM from 6-8 rats. *$P<0.05$ vs. WKY for Ang II (10^{-6}M) and $^{\#}P<0.05$ vs.SHR resveratrol for Ang II (10^{-6}M)) using One–way ANOVA followed by Newman-Keuls multiple test.

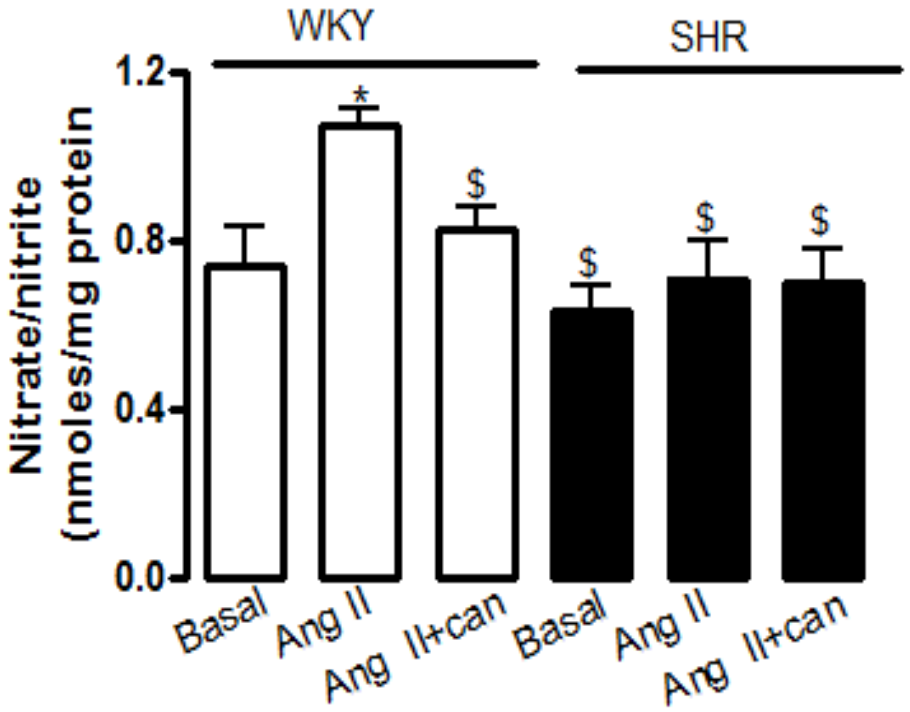
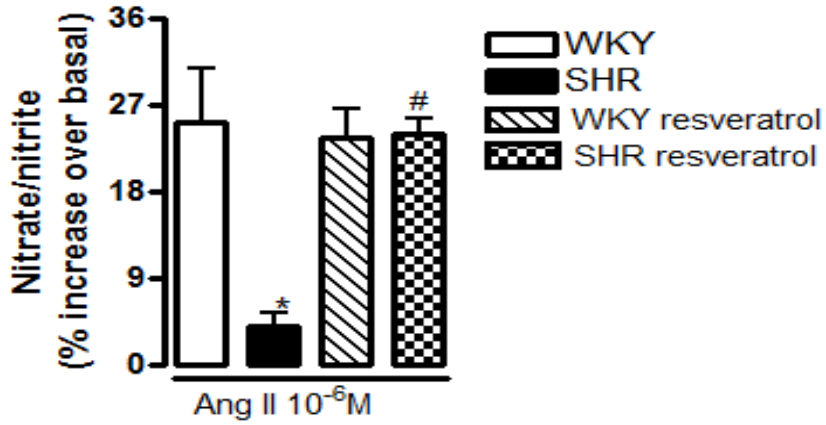


Figure 16: Effect of Ang II (10^{-6}M) on nitric oxide (NO) production: NO production was measured by commercially available kit from Cayman, Proximal tubules of SHR and WKY rats were incubated with or without Ang II (10^{-6}M) to determine NO production in presence or absence of candesartan (1µM). Bars represent Mean ± SEM from 6-8 rats. *$P<0.05$ vs. WKY basal and $^{\$}P<0.05$ vs.WKY for Ang II (10^{-6}M) using One–way ANOVA followed by Newman-Keuls multiple test.

A



B

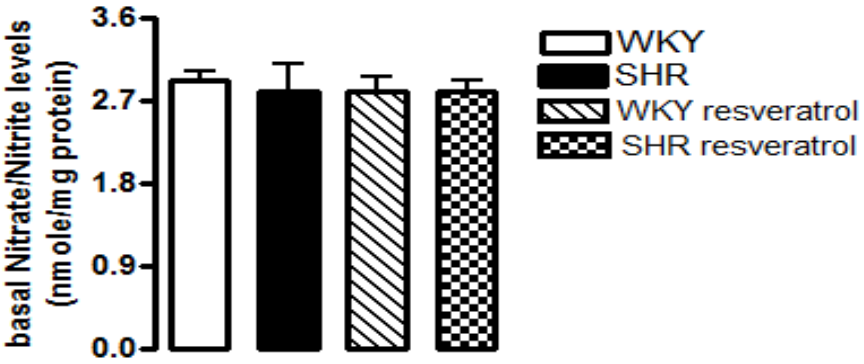


Figure 17: A: Effect of Ang II on NO production: Proximal tubules were incubated with Ang II and its effect on NO production is determined. Data represents % over basal. B: Basal NO production in proximal tubules of resveratrol treated and untreated SHR and WKY rats. Bars represent Mean ± SEM from 6-8 rats. *$*P<0.05$* vs. WKY for Ang II (10^{-6}M) and $^{\#}P<0.05$ vs.SHR for Ang II (10^{-6}M) using One–way ANOVA followed by Newman-Keuls multiple test.

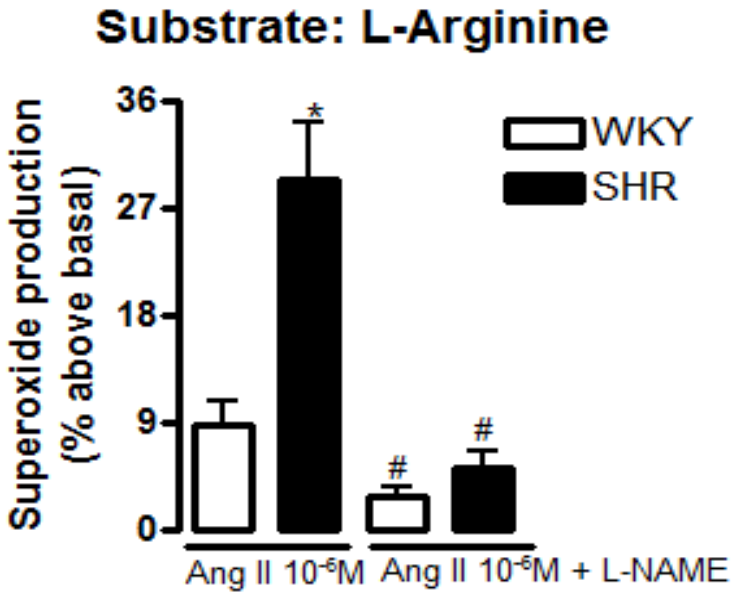


Figure 18: Effect of Ang II (10^{-6}M) on NOS dependent superoxide production: Renal proximal tubules were pre-treated with or without L-NAME (1mM) for 10 minutes and then incubated in presence or absence of Ang II (10^{-6}M) for 15 min followed by challenge with L-arginine (1mM). Data is expressed as % stimulation over basal. Bars represent Mean ± SEM from 6-8 rats. *$P<0.05$ vs. WKY for Ang II (10^{-6}M) and $^{\#}P<0.05$ vs.SHR for Ang II (10^{-6}M) using One–way ANOVA followed by Newman-Keuls multiple test.

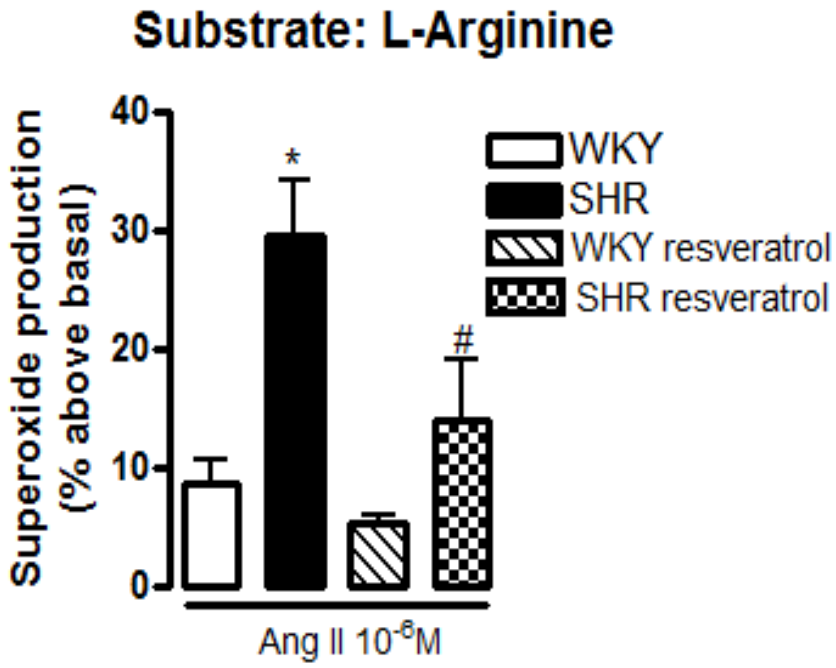


Figure 19: Effect of resveratrol on Ang II (10^{-6}M) induced NOS mediated superoxide production: Renal proximal tubules were incubated in presence or absence of Ang II (10^{-6}M) for 15 min followed by challenge with L-arginine (1mM). Data is expressed as percentage of stimulation over basal. Bars represent Mean ± SEM from 6-8 rats. *$P<0.05$ vs. WKY for Ang II (10^{-6}M) and $^{\#}P<0.05$ vs.SHR for Ang II (10^{-6}M) using One–way ANOVA followed by Newman-Keuls multiple test.

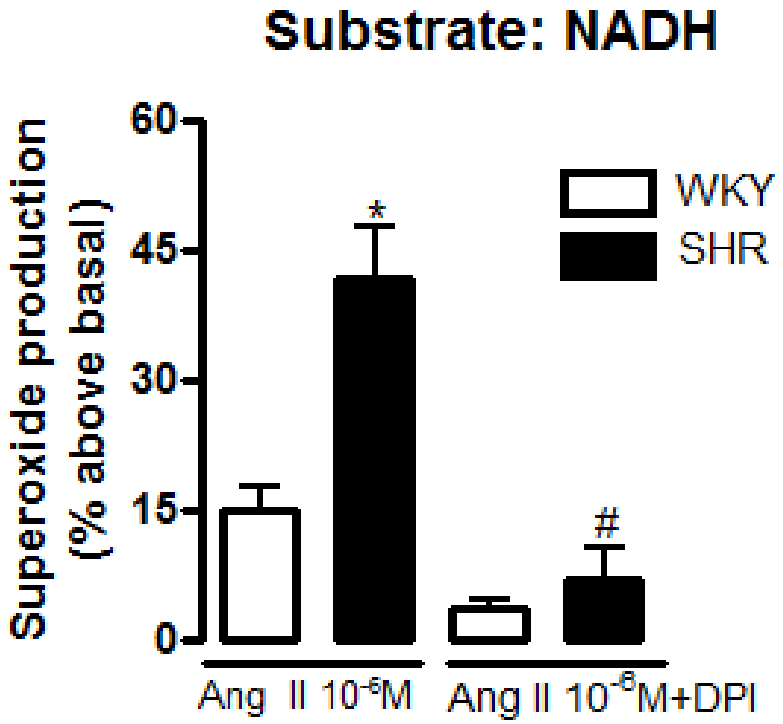


Figure 20: Effect of Ang II (10^{-6}M) on NADPH oxidase dependent superoxide production: Renal proximal tubules were pre-treated with or without diphenyleneiodonium (DPI, 0.1mM) and then incubated in presence or absence of Ang II (10^{-6}M) for 15 min followed by challenge with NADH (0.1mM). Data is expressed as % increase over basal. Bars represent Mean ± SEM from 6-8 rats. *$P<0.05$ vs. WKY for Ang II (10^{-6}M) and $^{\#}P<0.05$ vs.SHR for Ang II (10^{-6}M) using One–way ANOVA followed by Newman-Keuls multiple test.

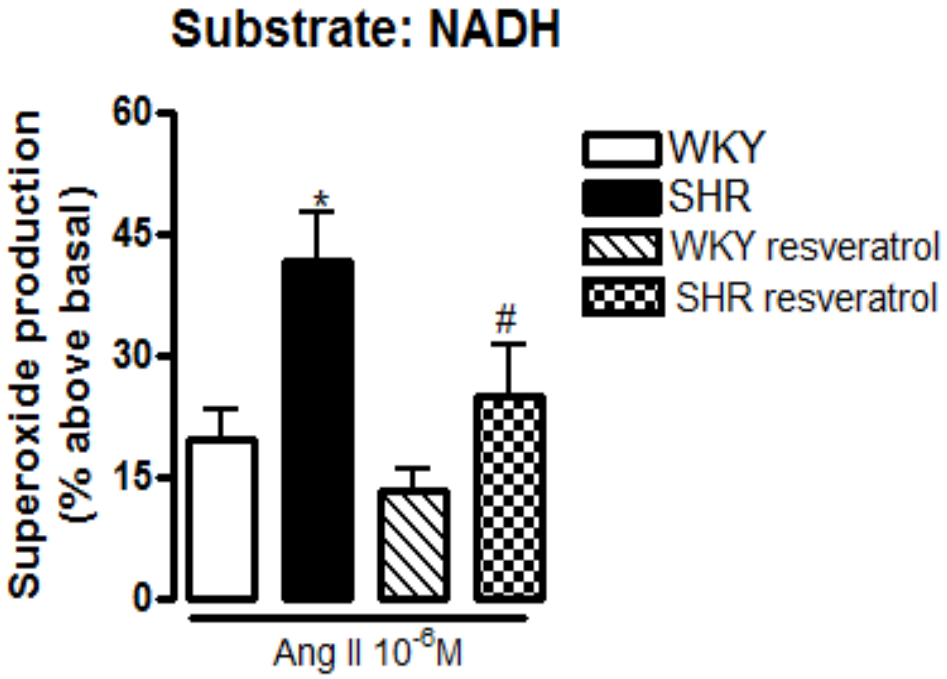


Figure 21: Effect of resveratrol on Ang II (10^{-6}M)-induced NADPH oxidase dependent superoxide production: Renal proximal tubules were incubated in presence or absence of Ang II (10^{-6}M) for 15 min followed by challenge with NADH (0.1mM). Data is expressed as % increase over basal. Bars represent Mean ± SEM from 6-8 rats. *$*P<0.05$* vs. WKY for Ang II (10^{-6}M) and $^{\#}P<0.05$ vs.SHR for Ang II (10^{-6}M) using One–way ANOVA followed by Newman-Keuls multiple test.

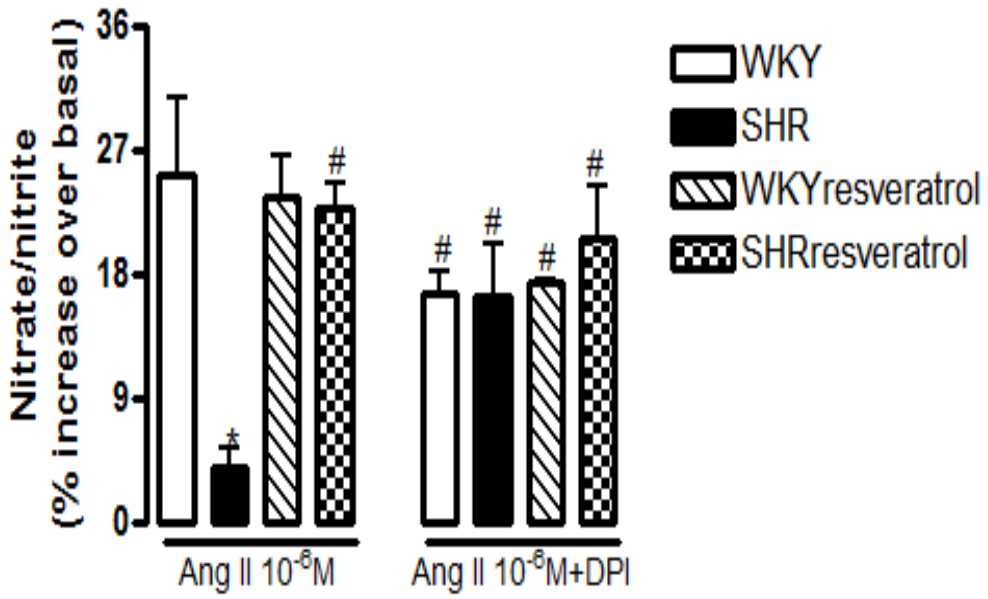


Figure 22: Effect of DPI (0.1mM) pretreatment on Ang II-induced nitric oxide production: Proximal tubules were pre-treated with or without DPI (0.1mM) for 10 minutes and then incubated in presence or absence of Ang II (10^{-6}M). Data is expressed as % increase over basal. Bars represent Mean ± SEM from 6-8 rats. *$P<0.05$ vs. WKY for Ang II (10^{-6}M) and $^{\#}P<0.05$ vs.SHR for Ang II (10^{-6}M) using One–way ANOVA followed by Newman-Keuls multiple test.

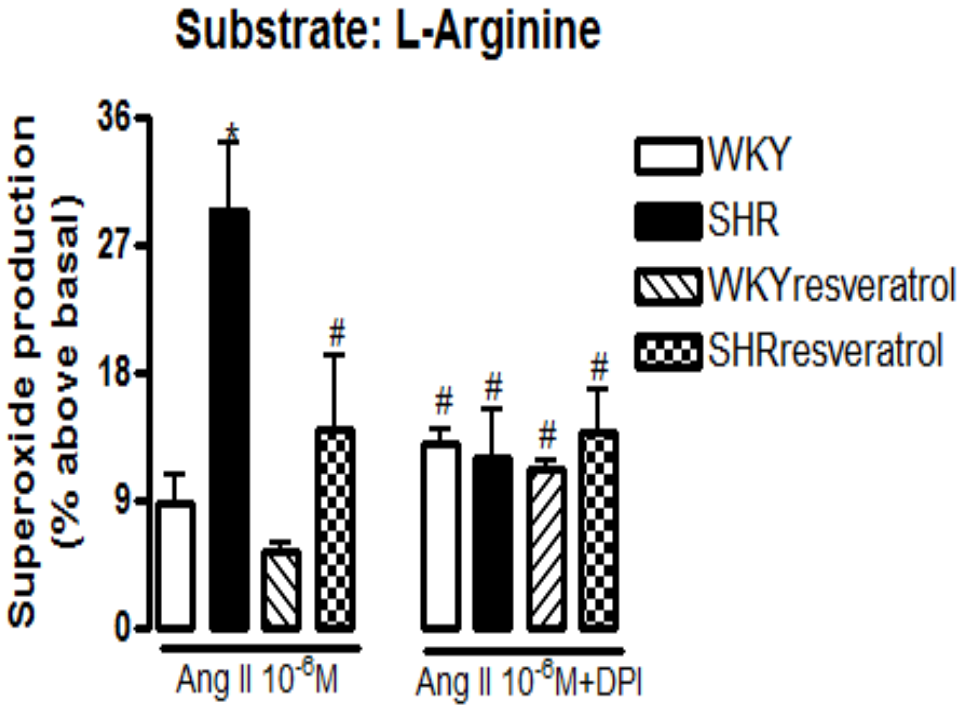


Figure 23: Effect of DPI pretreatment Ang II-induced NOS mediated superoxide production: Proximal tubules were pre-treated with or without DPI (0.1mM) and then incubated in presence or absence of Ang II (10^{-6}M) treatment followed by challenge with L-arginine (1mM). Data is expressed as % increase over basal. Bars represent Mean ± SEM from 6-8 rats. *$P<0.05$ vs. WKY for Ang II (10^{-6}M) and $^{\#}P<0.05$ vs.SHR for Ang II (10^{-6}M) using One–way ANOVA followed by Newman-Keuls multiple test.

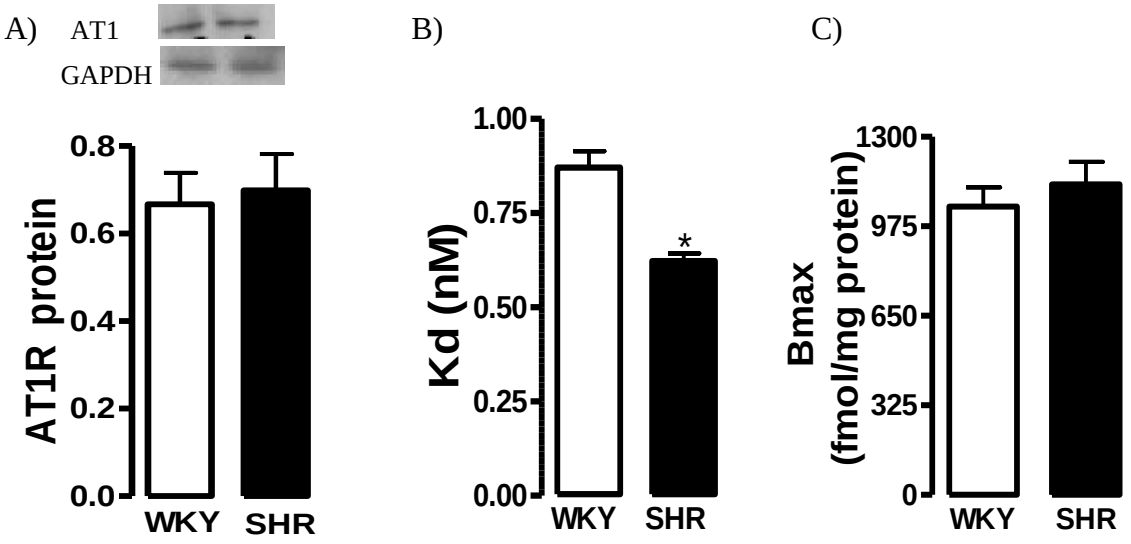
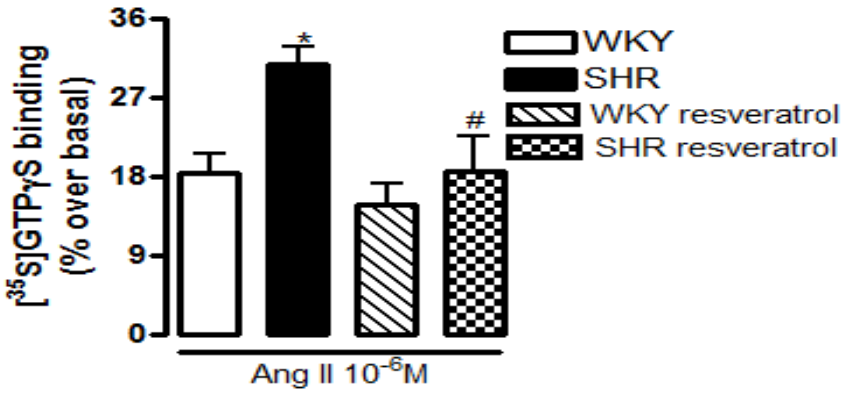


Figure 24: AT1R expression, affinity and number: A: Proximal tubular homogenate proteins were separated using SDS-PAGE and immunoblotted with specific antibodies against AT1R and GAPDH. Bands are representative Western Blots. B and C: AT1R affinity and receptor numbers respectively were determined by saturation binding using ^{125}I-sar-Ang II. Data represents Mean ± SEM from 6-8 rats. *$P<0.05$ vs. WKY using *student's t-test.*

A



B

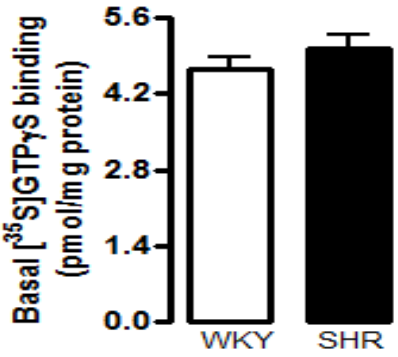


Figure 25: Ang II-induced [^{35}S] GTPγS binding: A) Proximal tubules were incubated with or without Ang II (10^{-6}M) and [^{35}S] GTPγS was determined as described in methods section. Data is expressed as percentage of stimulation over basal B) Represents basal [^{35}S] GTPγS binding in SHR and WKY rats. Bars represent Mean ± SEM from 6-8 rats. *$P<0.05$ vs. WKY for Ang II (10^{-6}M) and $^{\#}P<0.05$ vs.SHR for Ang II (10^{-6}M) using One–way ANOVA followed by Newman-Keuls multiple test.

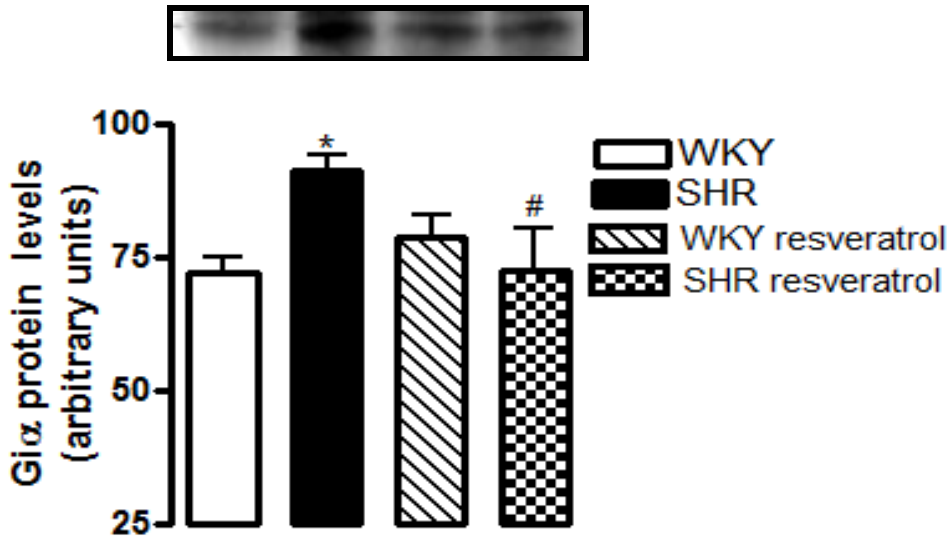


Figure 26: Giα protein expression in proximal tubular membranes: Proximal tubular membrane proteins were separated using SDS-PAGE and immunoblotted against Giα specific antibodies. Bands are representative Western Blots. Bars represent Mean ± SEM from 6-8 rats. *$P<0.05$ vs. WKY and $^{\#}P<0.05$ vs.SHR using One–way ANOVA followed by Newman-Keuls multiple test.

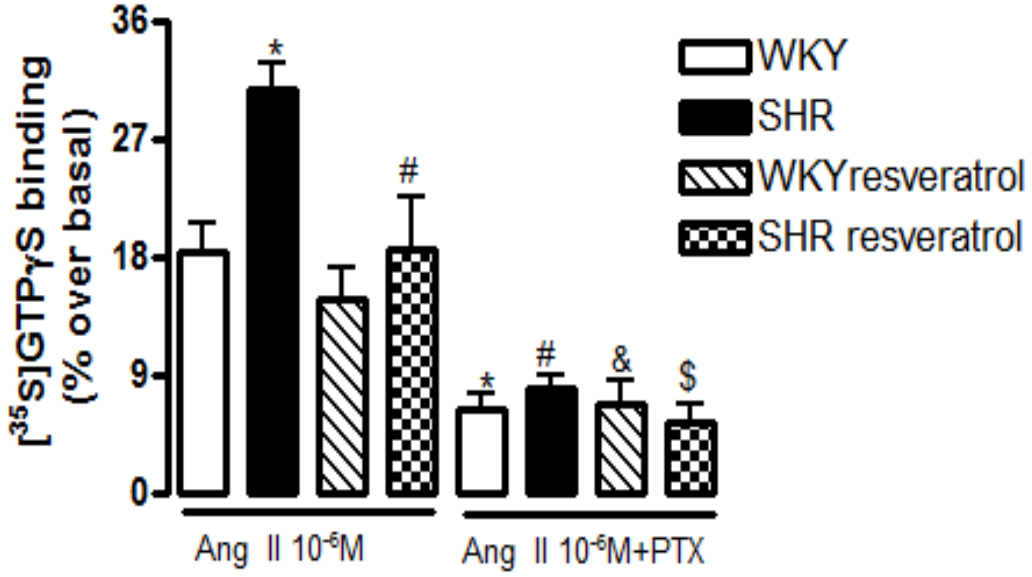


Figure 27: Effect of pertussis toxin treatment on Ang II-induced [^{35}S] GTPγS binding. Proximal tubular membranes were pretreated with or without pertussis toxin (100ng/mg protein) followed by incubation in presence or absence of Ang II (10^{-6}M) and [^{35}S] GTPγS binding was determined as described in methods section. Data represents % over basal. Bars represent Mean ± SEM from 6-8 rats. *$^{*}P<0.05$ vs. WKY for Ang II (10^{-6}M), $^{\#}P<0.05$ vs.SHR for Ang II (10^{-6}M), $^{\&}P<0.05$ vs. WKY treated with resveratrol for Ang II (10^{-6}M) and $^{\$}P<0.05$ vs. SHR treated with resveratrol for Ang II (10^{-6}M) using One–way ANOVA followed by Newman-Keuls multiple test.

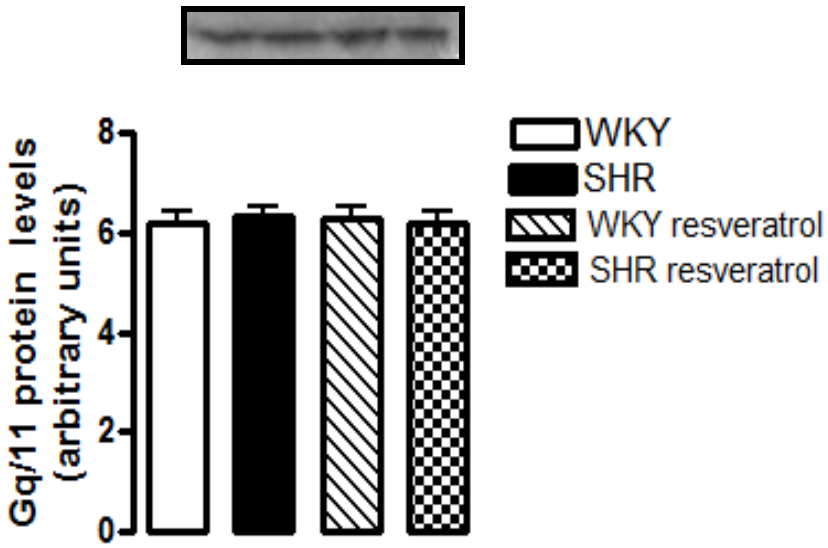


Figure 28: Gq/11 protein expression in proximal tubular membranes: Proximal tubular membrane proteins (40µg) were separated using SDS-PAGE and immunoblotted against Gq/11 specific antibodies. Bands are representative Western Blots. Bars represent Mean ± SEM from 6-8 rats.

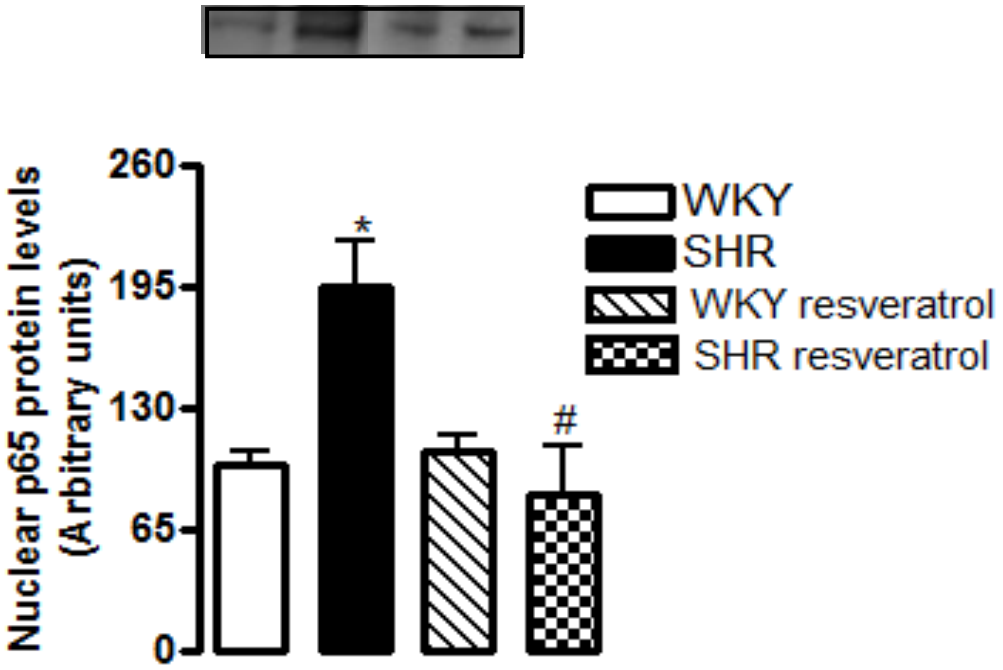


Figure 29: Nuclear protein expression of p65: Proximal tubular nuclear fractions were separated using SDS-PAGE and immunoblotted with antibody specific against p65. The bands are representative Western Blot. Bars represent Mean ± SEM from 6-8 rats. *$P<0.05$ vs. WKY and $^{\#}P<0.05$ vs.SHR using One–way ANOVA followed by Newman-Keuls multiple test.

5 DISCUSSION

The present study was designed to understand a link between renal oxidative stress and hypertension. The project was divided into 2 sections—1) To determine if proximal tubular oxidative stress precedes development of hypertension in SHR and 2) To determine if chronic treatment of antioxidant resveratrol at early age in SHR can prevent development of hypertension.

5.1 Studies in 3-4 weeks old SHR and WKY rats

Studies in various experimental models of hypertension suggest that oxidative stress alters renal AT1R mediated Na/K-ATPase regulation and contribute to the development of hypertension (8, 9, 73, 76). Yet it is not known if oxidative stress and altered renal AT1R function precedes development of hypertension or is the cause of hypertension. Although large number of studies performed in adult SHR show that oxidative stress contributes to hypertension by altering renal AT1R function, very few studies are reported in prehypertensive 3-4 weeks old SHR to establish such a link (77-79). Thus in first part of our studies we determined if oxidative stress and renal AT1R mediated Na/K-ATPase regulation is altered at 3-4 weeks old SHR and WKY rats.

We found that mean arterial pressure (MAP) was similar in 3-4 weeks old SHR and WKY rats. The proximal tubular protein carbonylation levels, protein oxidation marker, were similar in both SHR and WKY rats. We found that NADPH oxidase (subunit gp91,

phox) protein expression was similar in proximal tubules of SHR and WKY rats. Superoxide dismutase (SOD), an important antioxidant enzyme, activity was similar in proximal tubules of both SHR and WKY rats. These results suggest absence of proximal tubular oxidative stress in 3-4 weeks old SHR when compared to WKY rats.

We found that there was equal AT1R protein expression in SHR and WKY rats at 3-4 weeks of age. Studies in our lab show that changes in activities of intermediate molecules activated by AT1R such as, protein kinase C (PKC) and MAP Kinase are involved in modulating Na/K-ATPase activity which can further contribute to hypertension (67, 75). We observed similar PKC activity in proximal tubules of SHR and WKY rats. Furthermore, incubation of proximal tubules with Ang II produced similar stimulation of Na/K-ATPase in SHR and WKY rats.

Thus, to summarize the results of first part of our studies, we found that at 3-4 weeks of age SHR were normotensive, did not exhibit proximal tubular oxidative stress when compared to WKY rats. We also found similar AT1R protein expression, PKC activity and Ang II-induced Na/K-ATPase stimulation in proximal tubules of SHR and WKY rats at 3-4 weeks of age.

5.2 Studies in adult SHR and WKY rats

After establishing that at 3-4 weeks of age SHR are normotensive, do not exhibit oxidative stress and have similar Ang II-induced Na/K-ATPase stimulation compared to

WKY rats, we determined if early antioxidant resveratrol treatment in SHR ameliorates hypertension. We started resveratrol treatment in 3-4 weeks old SHR and WKY rats, and determined blood pressure by radiotelemetry. We continued with the treatment till the blood pressure difference between resveratrol treated and untreated SHR remained constant.

5.3 Effect of antioxidant resveratrol treatment on blood pressure and markers of oxidative stress

Increased oxidative stress contributes to the development of hypertension and antioxidant treatment decreases hypertension in various animal models of hypertension including SHR (8, 80, 81). Our results in SHR are consistent with these studies regarding increased blood pressure and oxidative stress. As expected MAP was significantly higher in SHR compared to WKY rats. There was redox imbalance in proximal tubules of SHR such that markers of oxidative stress (high protein carbonylation levels and high 8-isoprostane levels) were elevated and antioxidant capacity was decreased, compared to WKY rats. We found that seven weeks of resveratrol treatment in SHR significantly decreased MAP when compared to untreated SHR and by 9-10 weeks of treatment there was a fall of about 20 mmHg MAP in resveratrol treated SHR. Resveratrol treatment in SHR decreased oxidative stress and increased antioxidant activity. These results show that oxidative stress plays an important role in increasing blood pressure in SHR and antioxidant treatment by lowering oxidative stress decreases blood pressure in SHR.

5.4 Resveratrol mediated Nrf-2 activation

Studies show that one of the mechanisms of antioxidant effect of resveratrol is via activation of transcription factor Nrf-2. Redox sensitive transcription factor Nrf-2 increases transcription of antioxidant enzymes that protect cells against oxidative damage (82, 83). Under normal cell resting condition Nrf-2 is present in cytoplasm and upon receiving stimulus such as antioxidants, gets translocated to the nucleus (84). Despite severe oxidative stress in SHR, Nrf-2 activation as measured by protein expression in nuclear fractions was significantly lower in SHR compared to WKY rats. These results suggest that diminished Nrf-2 activation in SHR could be a contributor for increased oxidative stress. Nrf-2 activation has been shown to be impaired in various rat models of oxidative stress and inflammation (85). Antioxidant resveratrol treatment caused a significant increase in Nrf-2 protein expression in proximal tubular nuclear fraction of SHR compared to untreated SHR. It is worth noting that resveratrol treatment in WKY rats did not activate Nrf-2, suggesting that Nrf-2 activation requires a combination of oxidative stress and antioxidant stimuli. This requirement for activation of Nrf-2 might prevent excess levels of antioxidants enzymes in the body, because increase in antioxidant enzyme levels under normal conditions may not have significant beneficial effects.

Nrf-2 mediated activation of antioxidant enzymes such as glutathione-S-transferase (GST), Heme-Oxygenase-1 (HO-1) and superoxide dismutase (SOD) plays an important

role in protecting cells against oxidative damage. Our lab has recently shown that Nrf-2 mediated activation of these antioxidant enzymes is beneficial for decreasing pathology associated with enhanced oxidative stress during aging (86). We found that GST protein expression was significantly decreased in proximal tubules of SHR compared to WKY rats. Resveratrol treatment increased GST expression in proximal tubules of SHR compared to untreated SHR. We observed that there was a decrease in GST protein expression in proximal tubules of untreated SHR whereas resveratrol treatment significantly increased GST expression in SHR, a pattern similar to Nrf-2 activation in these groups. Proximal tubular HO-1 protein levels were equal in SHR and WKY rats and resveratrol treatment in SHR caused a significant increase in HO-1 protein levels compared to untreated SHR. We found that SOD activity in proximal tubules of SHR was equal to WKY rats and resveratrol treatment increased SOD activity in proximal tubules of SHR compared to untreated SHR. These results suggest that in resveratrol treated SHR, Nrf-2 has activated antioxidant defense machinery which might have reduced high levels of proximal tubular oxidative stress and decreased blood pressure when compared to untreated SHR.

5.5 Na/K-ATPase regulation

The regulation of Na/K-ATPase in response to Ang II is biphasic or dual, such that picomolar- nanomolar concentrations of Ang II stimulate Na/K-ATPase whereas at micromolar concentration, Ang II either inhibits or causes loss of stimulation of Na/K-

ATPase (7, 23). In agreement with these studies we observed stimulation of Na/K-ATPase at low concentrations of Ang II (10^{-10}M) and loss of stimulation at high concentration of Ang II (10^{-6}M) in WKY rats. However in SHR, there was loss of such biphasic effect of Ang II on Na/K-ATPase such that, Ang II (10^{-10}M) produced enhanced Na/K-ATPase stimulation, compared to WKY rats, and this stimulation was continued even at high Ang II concentrations (10^{-6}M). Resveratrol treatment in SHR normalized biphasic effect of Ang II-induced Na/K-ATPase. Effect of Ang II (10^{-6}M) on Na/K-ATPase regulation was sensitive to AT1R blacker candesartan in untreated SHR and resveratrol treated SHR and WKY rats suggesting that effect of Ang II on Na/K-ATPase regulation is mediated via AT1R.

Navar et al (25, 26) have reported that proximal tubular concentration of Ang II is much higher than plasma concentration because there are two sources of Ang II in proximal tubules, 1) local synthesis and 2) glomerular filtration. There is also further increase in Ang II concentrations during pathological conditions such as hypertension and there are reports of increased Ang II levels in proximal tubules of SHR (27). Thus Ang II (10^{-6}M) mediated regulation of Na/K-ATPase may be important because increase in intrarenal Ang II levels can contribute to sodium imbalance and hypertension if sodium transporter stimulation is maintained at high concentration. Therefore, it is possible that stimulation of Na/K-ATPase at both low and high concentration of Ang II may cause increase in sodium reabsorption and contribute to hypertension in SHR.

5.6 Role of NO in regulating Ang II-induced Na/K-ATPase activity

Stimulatory response at low concentration of Ang II on Na/K-ATPase activity is widely studied and is shown to be mediated by various protein kinases including MAP kinase and PKC (7-9). However, the mechanism that causes inhibition or loss of stimulation of Na/K-ATPase at high concentrations of Ang II is currently unknown. There are reports of involvement of Ang II-induced NO-cGMP signaling in abolishing the stimulation of Na/K-ATPase (9, 87). Therefore, we studied the role of NO signaling in Ang II (high concentrations) mediated Na/K-ATPase regulation by pre-treating the proximal tubules with NOS inhibitor L-NAME. Our results show that L-NAME treatment resulted in Ang II-induced stimulation of Na/K-ATPase in proximal tubules of WKY rats. L-NAME treatment did not alter exaggerated Ang II induced Na/K-ATPase stimulation in untreated SHR. These results suggest that Ang II via NO signaling could be responsible for loss of stimulation of Na/K-ATPase in WKY rats and this pathway might be defective in SHR. Similar to WKY rats, resveratrol treated SHR showed a significant Ang II-induced Na/K-ATPase stimulation after L-NAME treatment suggesting that resveratrol normalized Ang II-Na/K-ATPase regulation.

To further investigate the mechanism for defective NO signaling in SHR, we studied the effect of Ang II on NO production and determined renal NO levels in proximal tubules of SHR and WKY rats. Our results show that Ang II treatment increased NO production in WKY rats and this increase was sensitive to AT1R antagonist candesartan. However,

Ang II failed to increase NO levels in proximal tubules of SHR suggesting that NO production in response to Ang II is defective. Interestingly, resveratrol treatment restored Ang II–induced NO production in SHR suggesting that oxidative stress in SHR could be responsible for decreasing NO production in response to Ang II.

One of the mechanisms that can reduce NO production is NOS uncoupling. It is known that uncoupled NOS produce superoxides instead of NO. To investigate if NOS is uncoupled in proximal tubules of SHR, we determined Ang II-induced NOS dependent superoxide production in SHR and WKY rats. Our results showed that Ang II produced L-arginine dependent superoxides in SHR, which was sensitive to NOS inhibitor L-NAME suggesting NOS mediated superoxide production. In proximal tubules of resveratrol treated SHR, Ang II failed to produce L-arginine dependent superoxides suggesting that resveratrol treatment restored NOS coupling in SHR.

Previous studies from our lab have shown that Ang II via NADPH oxidase activation can lead to superoxide production which in turn can cause NOS uncoupling (9). Herein, we found that treatment of proximal tubules with Ang II caused NADPH oxidase activation in both SHR and WKY rats. However, Ang II-induced NADPH oxidase activation was significantly higher in SHR compared to WKY rats. Resveratrol treatment normalized Ang II-induced NADPH oxidase activation in proximal tubules of SHR compared to untreated SHR. These results suggest a role of oxidative stress in causing overactivation

NADPH oxidase in response to high concentrations of Ang II in SHR and this in turn could have lead to NOS uncoupling.

To determine whether enhanced activation of NADPH oxidase via superoxide production causes NOS uncoupling which further decreases NO levels in SHR, we treated proximal tubules of SHR and WKY rats with NADPH oxidase inhibitor DPI and determined its effect on NOS uncoupling and NO production. Pretreatment of proximal tubules with NADPH oxidase inhibitor DPI prevented Ang II-induced, NOS dependent superoxide production in SHR. Interestingly, DPI treatment in SHR normalized NO production in response to Ang II. DPI treatment had no effect on Ang II-induced NOS mediated superoxide production and NO levels in proximal tubules of resveratrol treated SHR and WKY rats. These results suggest that overactivation of NADPH oxidase by Ang II could cause NOS uncoupling and lead to defective Ang II-induced NO production in SHR.

5.7 AT1R levels

There are reports of oxidative stress-mediated exaggerated AT1R signaling in SHR; however the mechanisms are not known (9, 73, 88). Our results also suggest that there is exaggerated AT1R signaling in SHR compared to WKY rats, because—1) exaggerated stimulation of Na/K-ATPase at low concentration of Ang II and 2) overactivation of NADPH oxidase. Resveratrol treatment in SHR decreased oxidative stress and normalized AT1R signaling as suggested by decrease in Ang II (low concentrations)-induced Na/K-ATPase stimulation and NADPH oxidase activation. These results suggest

that oxidative stress plays a major role in increasing AT1R signaling in SHR. However, the mechanism for oxidative stress mediated exaggerated AT1R signaling is not yet known. Studies in our lab show that oxidative stress can upregulate AT1R protein expression and thus increase AT1R signaling (9). To determine whether there are any changes in AT1R protein, affinity or numbers we performed AT1R immunoblotting and saturation binding studies using radiolabeled ^{125}I [Sar]-Ang II. We found no changes in AT1R protein in SHR compared to WKY rats. There was a modest but significant increase in AT1R affinity but similar receptor number (Bmax) in SHR compared to WKY rats. This suggests that oxidative stress in SHR has not changed AT1R expression or numbers.

5.8 G protein coupling and expression

Studies show that changes in agonist induced G protein-coupling can modify receptor signaling (72, 73). Thus, to determine if changes in Ang II-induced G protein-coupling could result in enhanced AT1R signaling in SHR, we performed [^{35}S] GTPγS binding in response to Ang II. We found that there was significant increase in Ang II-induced [^{35}S] GTPγS binding in proximal tubules of SHR compared to WKY rats. Interestingly, resveratrol treatment in SHR normalized [^{35}S] GTPγS binding compared to untreated SHR. These results suggest that oxidative stress plays an important role in increasing AT1R-G protein-coupling in SHR.

Studies done by Hussain et al show that enhanced G protein expression in proximal tubules of obese Zucker rats contributes to exaggerated AT1R signaling (89). AT1R is coupled to Gq and Gi proteins and thus to investigate if there are any changes in G protein expression, we determined G protein expression by immunoblotting. We found that there were no changes in Gq/11 protein expression in proximal tubular membranes of SHR compared to WKY rats. However, we found a significant increase in Gi protein expression in proximal tubular membranes of SHR compared to WKY rats. Interestingly, resveratrol treatment in SHR decreased Gi protein expression compared to untreated SHR. To confirm if enhanced Gi protein expression could be responsible for increased Ang II-induced G protein-coupling, we pretreated proximal tubules with Gi inhibitor pertussis toxin. We found that treatment of proximal tubules with pertussis toxin decreased Ang II-induced [^{35}S] GTPγS binding in treated and untreated SHR and WKY rats. It is interesting to note that after pertussis toxin treatment Ang II-induced [^{35}S] GTPγS binding was equal in SHR and WKY rats. This suggests that enhanced Gi protein expression is indeed responsible for increased G protein coupling in SHR.

5.9 Role of NFkappa B in increasing Gi expression

Our results, so far suggests that oxidative stress by increasing Gi protein expression leads to enhanced AT1R- G protein coupling and signaling in SHR. Thus next step was to determine the mechanisms by which oxidative stress increases Gi protein expression. Studies done in K562 cells show that oxidative stress via activation of transcription factor

NFkappa B causes an increase in Gi protein expression (90). To determine if NFkappa B is activated in SHR, we performed immunoblotting for NFkappaB subunit p65 in proximal tubular nuclear fractions. It is reported that under normal resting cell conditions NFkappa B is located in the cyotsol (91). Upon receiving a stimulus, NFkappa B gets translocated to the nucleus where it carries out the transcription of target genes. Our results show that there was enhanced expression of p65 subunit in nuclear fraction of proximal tubules in SHR compared to WKY rats. Resveratrol treatment in SHR decreased p65 subunit expression in proximal tubular nuclear fractions. These results suggest that enhanced activation of NFkappa B in SHR could possibly have increased Gi expression, whereas antioxidant resveratrol treatment in SHR decreases NFkappa B activation which decreases Gi expression.

In conclusion, our results show that oxidative stress in SHR via NFkappa B activation increases Gi protein expression. This leads to enhanced Ang II-induced G protein-coupling and signaling leading to increased activation of NADPH oxidase which could cause NOS uncoupling. This results in enhanced stimulation of Na/K-ATPase at both the concentrations of Ang II, which could cause decrease in sodium excretion and lead to hypertension in SHR. Antioxidant resveratrol treatment, via Nrf-2 activation causes increase in antioxidant enzymes and decreases oxidative stress in SHR. The decrease in oxidative stress reduces NFkappa B activation which restores Gi expression and Ang II-induced G protein coupling and signaling. Antioxidant treatment in SHR also prevents NOS uncoupling and restores biphasic effect of Ang II on Na/K-ATPase. These

phenomenon could have decreased sodium reabsorption and cause amelioration of hypertension in SHR.

6 SUMMARY AND CONCLUSIONS

1. At 3-4 weeks of age, SHR are normotensive, do not exhibit proximal tubular oxidative stress and have normal Ang II (10^{-10}M)-induced Na/K-ATPase regulation.

2. Adult SHR have high blood pressure and redox imbalance such that there is enhanced proximal tubular oxidative stress and decrease in antioxidant activity, compared to WKY rats.

3. In WKY rats, Ang II exerts a dual or biphasic effect on Na/K-ATPase such that at low concentrations (10^{-10}M) Ang II stimulates Na/K-ATPase whereas at high concentration (10^{-6}M) there is loss of stimulation. However in SHR, Ang II (10^{-10}M) causes enhanced stimulation of Na/K-ATPase when compared to stimulation observed in WKY rats at same Ang II concentration. At high concentration (10^{-6}M), Ang II continues to stimulate Na/K-ATPase, suggesting loss of biphasic effect of Ang II on Na/K-ATPase in SHR.

4. In WKY rats, Ang II (10^{-6}M) exerts its effect on Na/K-ATPase via NOS and this pathway is defective in SHR.

5. In SHR, Ang II causes enhanced NADPH oxidase activation and NOS uncoupling and leads to defective NO signaling. This impairs Ang II induced Na/K-ATPase regulation in SHR.

6. There is exaggerated AT1R signaling in SHR due to increased Gi protein expression caused by enhanced activation of NFkappaB.

7. Antioxidant resveratrol treatment in SHR decreases blood pressure, increases Nrf-2 mediated antioxidant activity and decreases oxidative stress levels.

8. Antioxidant resveratrol treatment decreases Ang II-induced NADPH oxidase activation, restores NOS coupling and biphasic effect on Na/K-ATPase in SHR.

9. Antioxidant resveratrol treatment further decreased NFkappa B activation, normalized Gi protein expression and AT1R signaling in SHR.

7 PUBLICATIONS AND ABSTRACTS

In preparation: **Javkhedkar A,** Lokhandwala MF, Banday A A, Defective Nitric Oxide Production Impairs Angiotensin II-Induced Na/K-ATPase Regulation In Spontaneously Hypertensive Rats.

In preparation: **Javkhedkar A**, Lokhandwala MF, Banday A A, Oxidative Stress Impairs Ang II-Induced Na/K-ATPase Regulation And Contributes To Hypertension In Spontaneously Hypertensive Rats.

Abstracts

- **Javkhedkar A**., Lokhandwala M.F., Banday AA. : Oxidative stress impairs renal nitric oxide signaling and Ang II-mediated biphasic Na/K-ATPase regulation in spontaneously hypertensive rats. *Hypertension*, 52: e164, 2010

- **Javkhedkar A**., Lokhandwala MF and Banday AA. : Higher renal AT1 receptor affinity exaggerates Ang II induced Na/K-ATPase stimulation in spontaneously hypertensive rats. *FASEB J24*, LB707, 2010.

- **Javkhedkar A.,** Lokhandwala MF, Banday AA. : Oxidative stress upregulates renal Angiotensin II type I receptors via activation of transcription factor nuclear factor-kappaB. *FASEB J 23*, LB149, 2009

- Lokhandwala, M.F., Banday, A.A., Bhatt, S. and **Javkhedkar, A**. : Oxidative stress and transcriptional regulation of angiotensin AT1 receptors in hypertension. *Physiol. Res.* 57: 54P, 2008.

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