SUPEROXIDE VIA Sp3 TRANSCRIPTION FACTOR UP-REGULATES RENAL AT1 RECEPTOR FUNCTION AND CONTRIBUTES TO HYPERTENSION

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In

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By

Mohammad Saleem

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ABSTRACT

Hypertension is the most prevalent disease condition in the US affecting every 1 in 3 American adults. Renin angiotensin system (RAS) predominately regulates blood pressure and fluid homeostasis in the body. Angiotensin II type 1 (AT1) receptor is the key component of RAS that is implicated in hypertension. Oxidative stress is considered one of the main causes of hypertension. Studies including in our own lab showed that oxidative stress increases renal AT1 receptor function and contributes to hypertension. However, the exact mechanism of oxidative stress mediated increase in blood pressure is not completely known. Recent studies suggested that redox-sensitive transcription factors Sp3 and/or NF-κB regulate basal gene expression of AT1 receptor. Therefore, we designed studies to investigate the role of Sp3 and NF-κB in oxidative stress mediated hypertension or high blood pressure. We also designed studies to investigate the specificity of reactive oxygen species (superoxide vs. hydrogen peroxide) in regulating AT1 receptor function and high blood pressure.

Results from cell culture studies (HK2 cells) demonstrated that SOD-inhibitor DETC but not hydrogen peroxide significantly increased fluorescence

levels of superoxide probe dihydroethidium (DHE). Similarly, H_2O_2, but not DETC, treatment increased the fluorescence of the H_2O_2-sensitive probe dichloro-dihydro-fluorescein (DCFH). In addition, DETC, but not H_2O_2, increased the nuclear accumulation of Sp3 and NF-κB proteins. This effect was attenuated with tempol treatment. Furthermore, DETC increased AT1 receptor mRNA and protein expressions, which were attenuated with tempol treatment. However, H_2O_2 did not have any significant effect on AT1 mRNA expression. Results from transfection studies suggest that Sp3 plasmid increased while

Sp3 siRNA decreased AT1 receptor protein expression. However, NF-κB plasmid failed to increase AT1 receptor protein expression. Immunofluorescence and biotinylation studies demonstrated that DETC treatment increased cell surface AT1 receptor. Moreover, angiotensin II increased PKC activity in vehicle-treated cells that further increased in DETC-treated cells, which was attenuated by AT1R blocker candesartan and SOD-mimetic tempol.

Results from animal studies (SD rats) suggest that DETC treatment increased systolic and diastolic blood pressure, which were attenuated with tempol treatment. Furthermore, DETC treatment increased superoxide levels in kidney superficial cortex, which was attenuated with SOD-mimetic tempol. Candesartan infusion in DETC treated rats produced increased diuresis and

natriuresis compared to controls. Tempol in DETC treated rats attenuated candesartan-induced increased diuresis and natriuresis. Importantly, lysine acetylation of Sp3 protein, an index of Sp3 activation, was significantly higher in DETC treated rats, which was attenuated with tempol treatment.

Our data in both in vivo rat experiments and in vitro cell culture studies suggest that superoxide via Sp3 transcription factor up-regulates renal AT1 receptor function suggesting its role in hypertension. Specific targeting of superoxide and/or Sp3 may provide a selective and better therapeutic target to combat oxidative stress and/or its mediated effects responsible for hypertension and associated cardiovascular diseases.

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LIST OF ABBREVIATION

ACE	Angiotensin converting enzyme
Ang II	Angiotensin II
ANOVA	Analysis of variance
AT1R	Angiotensin II type 1 receptor
BTD	Button head box
DAG	Diacylglycerol
D1R	Dopamine 1 receptor
EDTA	Ethylenediaminetetraacetic acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein coupled receptor
GRK	G protein coupled receptor kinase
GFR	Glomerular filtration rate
H_2O_2	Hydrogen peroxide
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HEK	Human Embryonic Kidney Cells

HK2	Human Kidney 2 cells
HRP	Horseradish peroxidase
IP_3	1,4,5-inositol triphosphate
KHB	Krebs-Henseleit Buffer
NF-κB	Nuclear factor kappa B
NHE	Na, H-Exchanger (sodium hydrogen exchanger)
PBS	Phosphate buffered saline
RAS	Renin angiotensin system
RPTs	Renal proximal tubules
PKC	Protein kinase C
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SD	Sprague Dawley

SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
shRNA	Short hairpin ribonucleic acid
siRNA	Short interfering ribonucleic acid
S.E.M.	Standard error of the mean
SOD	Superoxide dismutase
Sp3	Specificity protein 3

LIST OF FIGURES

1. INTRODUCTION AND STATEMENT OF PROBLEM

Renin angiotensin system (RAS) is a hormonal cascade that regulates blood pressure and fluid homeostasis in the body (Crowley et al., 2006). In recent past, it is one of the most studied systems in the fields of hypertension, atherosclerosis, and cardiovascular and associated diseases. Also, this system is linked to cognitive disorders including Alzheimer disease and dementia (Allum, Hallissey, Ward, & Hockey, 1989). Ang II is an octapeptide that produces most of its physiological and pathophysiological functions by activating AT1 and AT2 receptors, the two key G protein coupled receptors of RAS family (de Gasparo, Catt, Inagami, Wright, & Unger, 2000). However, most of the cellular and physiological functions of Ang II are mediated via AT1 receptor. Recent elegant studies by Crowley and Coffman, Navar, and others confirmed an independent RAS system in the kidney that helps regulate fluid homeostasis and blood pressure in the body (Coffman & Crowley, 2008; Navar, 2005). Hyper-activation of AT1 receptor in the kidney is linked to hypertension, cardiovascular and associated diseases (Crowley et al., 2006; Kobori, Nangaku, Navar, & Nishiyama, 2007). Despite extensive research, the exact mechanism of hyper-activation of renal AT1 receptor contributing to hypertension is not known.

During physiological conditions, normal levels of reactive oxygen species (ROS), as signaling molecules, play an important role in cellular and organ tissue functions including the kidney functions. ROS affect other signaling molecules by modulating intracellular redox state and oxidative modification of cellular proteins (Paravicini & Touyz, 2006). During pathophysiology, imbalances in redox state have been implicated in various diseases including hypertension, diabetes and other cardiovascular disorders. Recent studies in our lab showed that high level of oxidative stress up-regulate AT1 receptor function and high blood pressure in aging rat model (Chugh, Lokhandwala, & Asghar, 2011, 2012). However, molecular mechanism of oxidative stress associated renal AT1 receptor up-regulation and hypertension is not well known.

There are various types of ROS in biological system such as superoxide, hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and peroxynitrite ($ONOO^{-}$) that are linked to oxidative stress associated hypertension and related cardiovascular disorders (Lassegue & Griendling, 2004; Paravicini & Touyz, 2006). Studies in other labs including ours have shown that scavenging ROS with antioxidants lowers blood pressure in hypertensive animal models (Chugh et al., 2011, 2012; Wilcox, 2010). These studies establish a direct link between oxidative stress and hypertension in animal models. Moreover, a number of

studies have demonstrated that superoxide and H_2O_2 are the main ROS, which are responsible for hypertension and related diseases (Landmesser et al., 2002; Vaziri, Dicus, Ho, Boroujerdi-Rad, & Sindhu, 2003; Vaziri, Lin, Farmand, & Sindhu, 2003). In contrast, some other studies suggested that H_2O_2 is a vasodilator and helps reduce high blood pressure (Y. Chen, Pearlman, Luo, & Wilcox, 2007; Park et al., 2015; Schroder et al., 2012). Therefore, determining the specific role of superoxide and H_2O_2 in blood pressure regulation is warranted.

Specificity protein (Sp) including Sp3 belongs to redox-sensitive transcription factors family that regulates the expression of a myriad of genes involved in cell growth and differentiation, cell signaling, and inflammation (Lee et al., 2006; Nair et al., 2014; Vaziri, Lin, et al., 2003). Sp3 protein binds GC rich content of the promoter of these genes. Computer analysis and electrophoretic mobility shift assay (EMSA) suggest that promoter of AT1 receptor gene possesses two distinct GC rich content sites that are the putative binding sites for Sp3 transcription factor (Kambe, Kinjyo, Hiruki, & Kubo, 2004). Similarly, NF-κB is a redox-sensitive transcription factor that has been shown to regulate a myriad of genes involved in cell survival, differentiation and proliferation (Hayden & Ghosh, 2008). In addition, recent studies in our lab showed that oxidative stress

induced activation of NF-κB transcription factor plays an important role in regulating blood pressure (Banday, Fazili, & Lokhandwala, 2007; Bhatt, Lokhandwala, & Banday, 2014; L. George, Lokhandwala, & Asghar, 2009; L. E. George, Lokhandwala, & Asghar, 2012). Also, a number of studies have shown that activation of NF-κB modulates AT1 receptor expression (Cowling et al., 2005). Preliminary computer analysis revealed two putative binding sites at-265 and-2540 at AT1 receptor promoter for NF-κB transcription factor (Z. J. Chen, Parent, & Maniatis, 1996). However, a link between oxidative stress, Sp3/NF-κB and renal AT1 receptor is missing and warrants further investigations.

Therefore, we designed in vivo and in vitro studies to investigate **i) the role of redox-sensitive transcription factor Sp3 and NF-κB in the regulation of renal AT1 receptor expression and function, and ii) Specificity of ROS, i.e. superoxide vs. H_2O_2 and their role in the up-regulation of renal AT1 receptor function and blood pressure.**

These studies were designed in human renal proximal tubule HK2 cell line and Sprague Dawley (SD) rats.

2. REVIEW OF LITERATURE

2.1. Prevalence of hypertension

Hypertension is one of the most common and perhaps the most complex chronic diseases, affecting more than 1 billion people across the globe (Lawes, Vander Hoorn, Rodgers, & International Society of, 2008). It is the most prevalent disease in the US affecting every 1 in 3 American adults. The present cost for the treatment of hypertension is about $46.4 billion in America and there is projection that shows it could rise to 274 billion by 2030 (Go et al., 2014). Moreover, hypertension is a clinical factor and is a major risk factor for cardiovascular diseases, heart attack, cognitive decline and dementia (Birns & Kalra, 2009), this will further increase the economic burden on the US health care system. Therefore, understanding the mechanism of hypertension is needed to better treat and prevent hypertension and associated diseases.

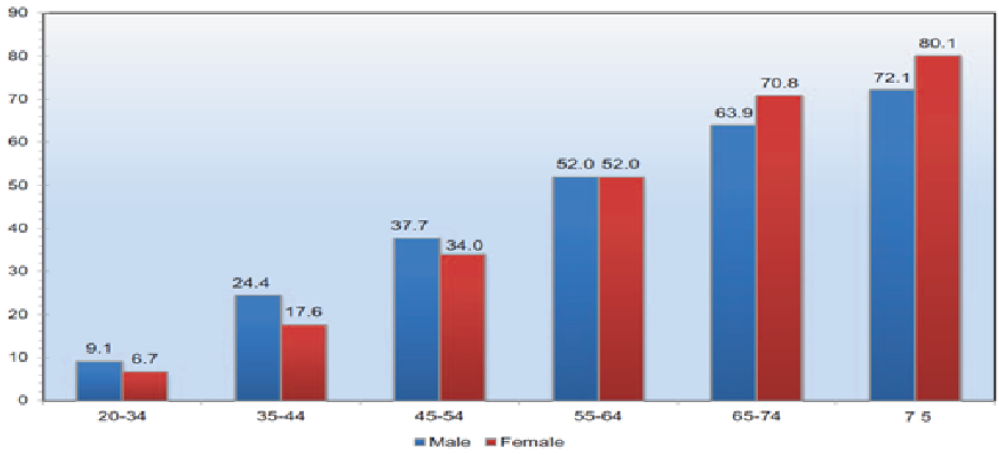


Figure 1. Prevalence of high blood pressure in adults ≥20 years of age by age and sex.

(National Health and Nutrition Examination. Survey: 2007–2010). Source: National Center for Health Statistics and National Heart, Lung, and Blood Institute.

2.2. Role of kidney in blood pressure regulation and fluid homeostasis

Regulation of blood pressure and fluid homeostasis are vital physiological processes for human body to function properly. Regulation of blood pressure is a complex process that requires the integration of several organ system including

central nervous system (CNS), cardiovascular system, kidney, immune system, and adrenal glands. These systems together determine blood pressure levels by modulating cardiac out, fluid volumes, peripheral vascular resistance, and other various extrinsic disturbances. Notably, seminal studies from the groups like Guyton, Coleman and Coffman, and Navar showed that kidney is the predominant organ in the regulation of blood pressure and fluid homeostasis (Coffman & Crowley, 2008; Guyton, 1991; Navar, 2005). The arterial pressure rarely deviates 10 to 15 % from normal in an adult human each day. Such constancy in blood pressure is achieved at three different levels and is corrected by three different systems present in our body: (i) Neuronal receptors react within minutes to correct any abnormal blood pressure, (ii) hormonal system follows the neuronal system within minutes, and (iii) within hours or days, kidney pressure control system comes in and correct the abnormal blood pressure (Coffman & Crowley, 2008; Guyton, 1991). In a series of elegant studies, Lifton et al showed direct evidence of powerful capacity of renal excretory system in blood pressure regulation by conducting genetic studies in humans. These investigators showed that all Mendelian disorders having major impact on blood pressure regulation are caused by genetic variants affecting water and salt reabsorption by renal distal tubule (Lifton, Gharavi, & Geller, 2001).

Kidney transplantation studies in animal models strongly support the concept that kidney plays inextricable role in hypertension. For example, a normotensive recipient of renal graft from genetically hypertensive donor develops posttranslational hypertension (Crowley et al., 2005). These types of transplantation experiments with similar results have been performed at least in four different animal models. Moreover, when genetically hypertensive rats having bilateral nephrectomy receive kidney from normotensive rats show reduced arterial pressure. Similar results have been reported in clinical studies showing that a normotensive patient receives renal graft from a donor with a genetic predisposition to hypertension, develops high blood pressure. These transplantation studies support the concept of kidney playing a primary role in initiating essential hypertension (Navar, 2005).

2.2.1. **Renin angiotensin system (RAS)**

About a century ago in 1989, Robert Tigerstedt and Per Bergman discovered that extract from the kidney cortex produces consistent pressure effect when injected intravenously in rabbits. They termed the substance ‘renin’ (Phillips & Schmidt-Ott, 1999). This study laid the foundation of Renin Angiotensin System (RAS) which is now considered one of the most complex pathways, and the master regulator of blood pressure and fluid homeostasis (Crowley & Coffman, 2012).

This system involves two important enzymes, renin and angiotensin-converting enzyme (ACE). Renin cleaves angiotensinogen, main substrate, into angiotensin I (Ang I) which is converted into angiotensin II (Ang II) by ACE. Notably, Ang (1-12), a product of angiotensinogen, is also cleaved to Ang II largely by chymase in the human heart. Although, appropriate activation of RAS system is indispensible in regulating normal circulatory homeostasis, abnormal activation of RAS has been implicated in hypertension and target organ damage. The importance of RAS system in clinical medicine can be seen by the impressive and specific efficacy of pharmacological agents such ACE inhibitors and AT1 receptor blockers that inhibit the synthesis and function of Ang II respectively resulting in reduced blood pressure (Lewis, Hunsicker, Bain, & Rohde, 1993; Pfeffer et al., 1992). Also, Inhibition of RAS system helps protect target organ damage.

At the cellular levels, Ang II produces its effect by activating angiotensin II type 1, (AT1) and type 2 (AT2) receptors. These two subtypes of receptors have been classified based on their differential affinities towards various non-peptide antagonists. These studies suggest that most of the classically recognized functions of the RAS system are mediated by AT1 receptor (Timmermans et al., 1993). Moreover, elegant gene-deletion studies by Crowley et al confirms this (Crowley, Tharaux, Audoly, & Coffman, 2004).

Distinct role of renal RAS has been established in regulating blood pressure. Hall et al. in 1980s, in a series of elegant articles reported that chronic infusion of low dose of angiotensin II into kidney causes hypertension due to impaired pressure-natriuresis relationship (Hall, 1986). Moreover, Navar and associates postulated that kidney possesses its independent RAS system to regulate Na excretion and blood pressure regulation (Kobori et al., 2007). This group showed that Ang II by activating renal AT1 receptor activates local RAS in the kidney resulting in increased synthesis of Ang II in the lumen of renal tubules and thus activates paracrine and autocrine epithelial transporters (Navar, Harrison-Bernard, Wang, Cervenka, & Mitchell, 1999; Navar, Lewis, Hymel, Braam, & Mitchell, 1994). Most importantly, elegant and uniquely designed studies of kidney cross-transplantation studies in rat by Crowley and Coffman group clearly demonstrate the imperative role of renal RAS in sodium balance and blood pressure regulation (Coffman & Crowley, 2008; Crowley et al., 2005).

2.2.2. **Angiotensin II receptors**

AT1 receptor is a G protein coupled receptor expressed ubiquitously in all tissues. It has seven transmembrane domains, which are extra-cellularly connected to three N-terminus and intra-cellularly with three C-terminus loops. Extracellular loop and transmembrane domain provide the binding site for Ang II.

C-terminus of AT1 receptor is linked to internalization, desensitization and phosphorylation of AT1 receptor. Human AT1 receptor shares 97% homology with rodent and bovine AT1 receptors.

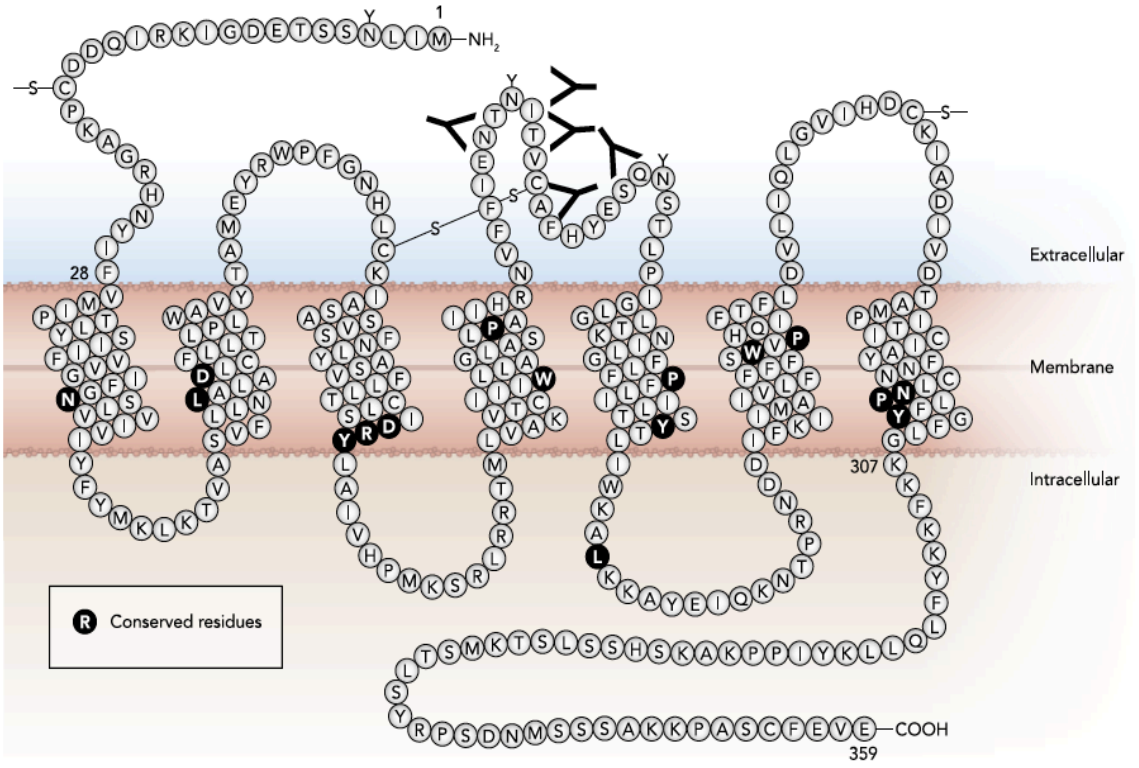


Figure 2. Detailed amino acid sequence of AT1 receptor with antibody binding site.

(de Gasparo et al., 2000).

Although AT1 receptor is expressed ubiquitously, its predominant

expression is found in the key organs such as heart, kidney, blood vessels, adrenal glands and cardiovascular control system in the brain (Shanmugam & Sandberg, 1996). Activation of AT1 receptor in the vascular system causes potent vasoconstriction. In adrenal cortex, their activation causes the release of aldosterone that increases reabsorption of sodium in renal distal tubule. Renal AT1 receptor activation results into reduced natriuresis and increased renal vasoconstriction. In the brain, injection of Ang II into ventricular area causes sudden increases in pressure response mediated by AT1 receptor.

Relative contributions of AT1 receptors in different tissues to control blood pressure and pathophysiology of hypertension are difficult to dissect. A kidney cross-transplantation study using wild-type and AT1 receptor deficient mice showed non-redundant and equal contribution of renal and systemic tissues AT1 receptors in the regulation of blood pressure, suggesting that AT1 receptors of systemic tissues play equally important role in maintaining body fluid volume and thus protect body from circulatory collapse (Crowley et al., 2005). In contrast, another study using cross-transplantation approach showed that AT1 receptors in the kidney plays dominant role in causing hypertension by promoting sodium retention and high blood pressure (Crowley et al., 2006). Coffman and group recently performed conditional deletion of AT1 receptor in the epithelium of renal

proximal tubules (RPTs) and suggested that AT1 receptors in proximal tubule are critical in maintaining blood pressure and Ang II dependent pathogenesis of hypertension. Sodium transporters including NHE3 sodium-proton transporter in RPTs are implicated for this activity (Gurley et al., 2011). Some studies have suggested that Ang II via AT1 receptor modulate solute and fluid reabsorption in the distal nephron (Peti-Peterdi, Warnock, & Bell, 2002; Stegbauer et al., 2011). Moreover, some studies indicated that urine-concentrating mechanism is determined by AT1 receptor present in the collecting duct. To confirm this mechanism, AT1 receptors were deleted from collecting duct in mice using Cre-LoxP technology. These mice exhibit consistent lower urine osmolality following water deprivation or vasopressin infusion. Also, levels of aquaporin-2 (AQP2) protein in inner and outer medulla were significantly reduced in water-deprived mice, but localization to apical membrane was unchanged. This study suggests that AT1 receptors in collecting duct directly affect water reabsorption by modulating the levels of AQP2 and thus play an important role in achieving maximal urinary concentration (Stegbauer et al., 2011). Activation of AT1 receptor causes activation of G protein dependent and independent signaling pathways. Activation of AT1 receptor stimulates phospholipase C (PLC), phospholipase A2 (PLA2) and phospholipase D (PLD) that generates IP3/Ca^{2+}

and diacylglycerol (DAG), arachidonic acid/leukotrienes and phosphatidic acid, respectively. Activation of these diverse molecules induces myriad of downstream signaling pathways. These pathways have been implicated in cell survival, migration, hypertrophy, endothelial dysfunction, contraction, fibrosis and thrombosis. These pathways have been shown to recruit key signaling proteins such as Ras, mitogen-activated protein kinases (ERK ½, P38 MAPK, and stress activated protein kinase), tyrosine kinases, receptor tyrosine kinases (EGFR, PDGFR, IGF) and nuclear factor kappa (NF-κ) B. Although, AT1 receptor in most of the tissues is mainly coupled with Gαq/11 protein resulting in inducing PLC/PKC signaling, this receptor also couples to Gαi/o, and Gα 12/13 in some organ tissues (de Gasparo et al., 2000). Notably, activation of AT1 receptor induces the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This enzymes is considered one of the major sources of ROS that activates pro-inflammatory transcription factors and small G proteins such as Ras, Rac and RhoA (Ushio-Fukai et al., 1999). Also, AT1 receptor stimulation activates β-arrestin and Src-JAK-STAT signaling pathways independent of G proteins (DeWire, Ahn, Lefkowitz, & Shenoy, 2007).

2.2.3. **Regulation of AT1 receptor gene expression**

Studies in others and ours labs have established a direct link between

oxidative stress and hypertension (Chugh, Lokhandwala et al. 2011, Chugh, Lokhandwala et al. 2012). For example, recent studies in our lab demonstrated that oxidative stress plays a causal role in hypertension in FBN rats because treatment with antioxidant tempol resulted in reduced both oxidative stress and high blood pressure. Moreover, this study also showed that renal AT1 receptor is involved in contributing to hypertension because treatment with AT1 receptor blocker candesartan results in increased natriuresis and diuresis in FBN rats (Chugh et al., 2011, 2012). Pioneering work by Guyton and colleagues established kidney as a primary organ in blood pressure regulation and electrolyte homeostasis. Moreover, recent elegant studies from the groups like Crowley and Coffman, Navar, and Hall corroborated and confirmed that renal AT1 receptor plays a unique and non-redundant role in regulating blood pressure, and fluid and electrolyte homeostasis (Crowley et al., 2005; Hall, 1991; Navar, Prieto, Satou, & Kobori, 2011). Furthermore, Gurley et al recently demonstrated that AT1 receptor in the epithelium of renal proximal tubules are critical in maintaining blood pressure and Ang II dependent pathogenesis of hypertension (Gurley et al., 2011). This study suggests that AT1 receptor in renal proximal tubule is cardinal in regulating blood pressure and its hyper-activation contributes to hypertension, chronic kidney disease, and tissue damage.

However, molecular mechanism of renal AT1 receptor regulation is largely unknown.

Over the years, numerous studies have suggested that myriad physiological and pathophysiological factors regulate the expression of AT1 receptor in different cells and tissues (Elton & Martin, 2007). Although molecular mechanism of AT1 receptor gene expression is not well known, a number of stimuli have been shown to modulate its expression. Ang II, interferon-γ, growth factors, tumor necrosis factor (TNF)-α, thyroid hormone, NO, and peroxisome proliferator-activated receptor (PPAR)- γ are some important stimuli that have been shown to suppress AT1 receptor gene expression in cultured cells from rat. On the other hands, interleukin (IL)-6, glucocorticoids, insulin-like growth factor and prostaglandin have been shown to increase the expression of AT1 receptor. Sp3 transcription factors has been shown to regulate basal expression levels of AT1 receptor gene in rodents and humans (Kubo, Kinjyo, Ikezawa, Kambe, & Fukumori, 2003). However, role of Sp3 transcription factor in the up-regulation of AT1 receptor expression and function has not been elucidated yet.

2.3. Specificity protein (Sp) transcription factors family

Specificity protein (Sp) transcription factors are members of Sp/kruppel-like factor

family. Nine different Sp (Sp1-Sp9) proteins have been identified which share highly conserved three zinc finger DNA-binding domains at the C-terminus, the adjacent Button-head box (btd-box) and the N-terminal Sp-box. These proteins play an important role in embryonic and early postnatal development and are extensively studied in the field of cancer research (Safe, Imanirad, Sreevalsan, Nair, & Jutooru, 2014). Sp1 and Sp3 are two important Sp family members, which are expressed ubiquitously and are structurally and evolutionarily closely related and found to be bound to GC and GT rich DNA motifs with similar affinity and specificity (Hagen, Muller, Beato, & Suske, 1992). However, gene deletion studies suggest that functionally these two proteins are different from each other (Kruger et al., 2007). For example, while, Sp1 homozygous knockout mice die in early embryonic development (E10.5 days) (Marin, Karis, Visser, Grosveld, & Philipsen, 1997), Sp3 homozygous knockout mice develop till pregnancy but die immediately after birth due to respiratory failure. In addition, Sp3 knockout mice exhibit distinct defect in late teeth formation and skeleton ossification (Bouwman et al., 2000). Also, Sp3 null mice show defect in erythroid and myeloid cell lineage differentiation.

Sp1 and Sp3 are ubiquitously expressed in mammalian cells, but Sp2 and Sp4 expression is restricted only to certain tissues. For example, Sp4 is highly

expressed in central nervous system and retinal neurons (Lerner, Peng, Gribanova, Chen, & Farber, 2005), Sp2 is expressed in certain cell lines and cancer cells (Phan et al., 2004). A number of studies including in our own lab have reported that Sp3 is a redox-sensitive transcription factor and high levels of ROS modulate its activity (Banday & Lokhandwala, 2015; Jutooru et al., 2014; Lee et al., 2006; Nair et al., 2014; Schafer et al., 2003).

2.3.1. **Modular structure of Sp proteins family**

Sp proteins possess distinct sub-domains each performing specific functions (L. Li, He, Sun, & Davie, 2004). Sp1 and Sp3 both contain two sub-domains A and B, which are called transactivation domain, each of which can stimulate transcription of gene. Button-head box is present in both Sp1 and Sp3 within C domain, which may contribute to their transcription potential. There are three Cis2-His2 zinc “fingers” at carboxyl-terminal of C sub-domain. These zinc fingers help the transcription factors bind to the specific DNA promoter motifs rich in GC content. Sp3 contains an inhibitory domain within C sub-domain in front of the zinc finger, while inhibitory domain for Sp1 is located at N-terminus. The difference in the location of inhibitory domains is believed to be responsible for the factors’ distinct functions (Suske, 1999). Post-synthetic modification of Sp3 makes this protein more complex (Sapetschnig, Koch, Rischitor, Mennenga, &

Suske, 2004).

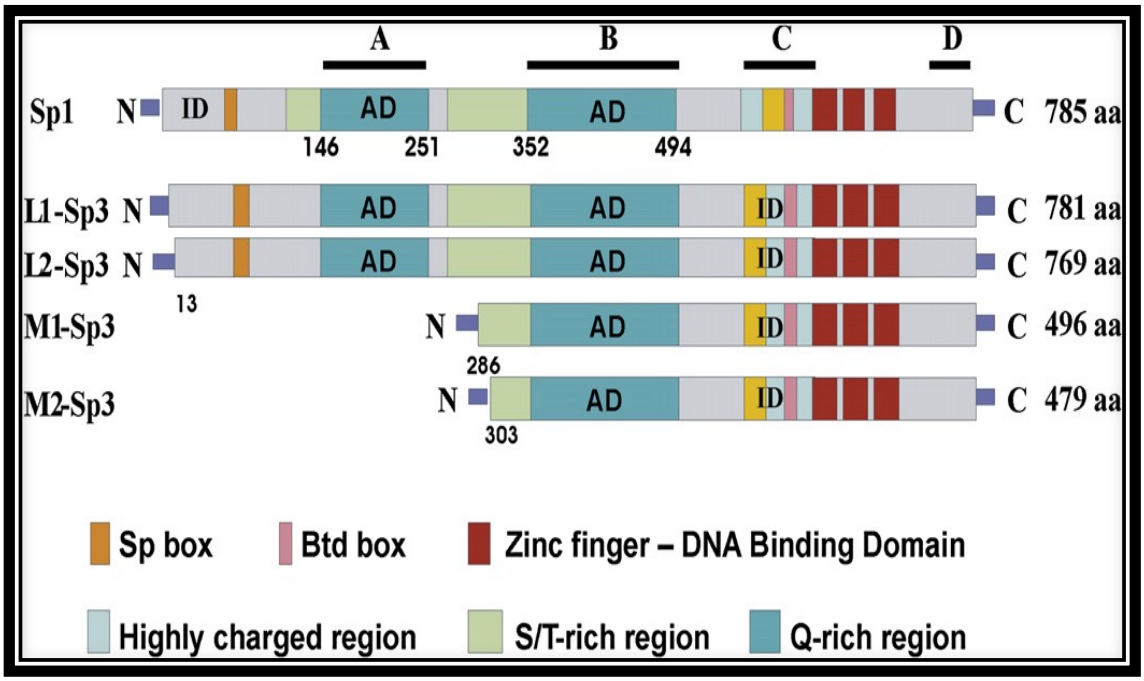


Figure 3. Structural domains of Sp1 and Sp3.

Amino acid length and molecular mass of the Sp1 and Sp3 isoforms are indicated on the right. Colors for Sp box, buttonhead (BTD) box, zinc-finger domain, highly charged region, serine/threonine (S/T)-rich, and glutamine (Q)-rich domains are shown at the bottom. The black bars marked with A, B, C, and D denote the sub-domains. Sp3 long forms (L1- and L2-Sp3) and short forms (M1-, M2-Sp3) are indicated. AD, activation domain; ID, inhibitory domain.

2.3.2. **Sp3 isoforms**

Four isoforms of Sp3 protein have been identified so far, two long (L1-Sp3, L2-Sp3) and two shorts (M1-Sp3, M2-Sp3) that are the products of differential translational initiation sites of their mRNA (Sapetschnig et al., 2004). Long isoform of Sp3 protein is structurally very similar to that of Sp1, except the position of repression domain (Suske, 1999). The long and short isoforms of Sp3 are expressed in all mammalian cells. The short isoforms of Sp3 lack the activation domain A but possess the activation domain B and the inhibitory domains.

2.3.3. **Synergism in Sp protein family**

Sp1 is the perfect example of synergism in Sp family. This protein in promoters containing multiple Sp-binding sites exerts its transcription synergism through direct protein-protein interaction (Mastrangelo, Courey, Wall, Jackson, & Hough, 1991; W. Su, Jackson, Tjian, & Echols, 1991). However, Sp3 does not possess the synergism characteristic as demonstrated in *in vitro* studies (Yu, Datta, & Bagchi, 2003). Sp3 binds as a monomer while Sp1 binds as a multiplier on a single Sp1 binding site. Several studies have also reported that Sp3 competes with Sp1 for binding on gene promoters containing multiple Sp1

binding sites and as a result efficiently represses Sp1-dependent transcription. However, some reports suggest that Sp3 may synergistically potentiate some Sp1-dependent promoters. These and others reports suggest that the repressive function of Sp3 is gene promoter context dependent (L. Li et al., 2004).

2.3.4. **Protein interactions with Sp3**

Sp3 directly or indirectly binds with different transcription factors, transcriptional regulators, and chromatin remodeling factors [e.g. estrogen receptor (ER) α, HDAC1, p300/CBP, SWI/SNF, an ATP-dependent chromatin remodeling complex] to activate or repress the expression of genes (Li, He et al. 2004). Co-immunoprecipitation and indirect immunofluorescence of Sp3 demonstrated that Sp3 associate with HDAC (histone deacetylase)-1 and HDAC2 as well as with ERα in MCF-7 cells (He, Sun, Li, & Davie, 2005). Sp3 acts as a transcriptional repressor protein by recruiting the Sin3A HDAC1/HDAC2 complex and binding to RbAp48 and/or Sin3A (Clem & Clark, 2006), while it acts as a transcriptional activator by recruiting CBP and p300 co-activators. CBP and p300 possess potent histone acetyltransferase (HAT) activity and function as co-activators (Ammanamanchi, Freeman, & Brattain, 2003). Studies reported that estrogen can form complexes with ERα, Sp1 or Sp3

and can induce the expression of estrogen responsive genes (He et al., 2005). On the other hand, repression can be achieved by perturbing the interaction between Sp1/Sp3 and the basal transcription initiation complex (Zhang & Dufau, 2003). These studies suggest that Sp3 can recruit and form complexes with different proteins, which can cause region specific changes in histone acetylation and recruitment of RNA polymerase II within promoters and consequently activate or repress gene expression.

2.3.5. **Sp-protein family and cancer**

Sp-family of transcription factors plays a key role in cell cycle regulation and tissue development. Sp1/Sp3 and other members of this family are over-expressed in various human cancers (Safe & Abdelrahim, 2005). Sp1/Sp3 can bind to the same sites on the oncogene c-myc promoter and other pro-oncogenic genes. One of the mechanisms linking Sp-proteins with cancer suggests that microRNAs regulate the expression of transcriptional repressors such as ZBTB10, ZBTB34 and ZBTB4. In cancer cell lines, expression of Sp1 and Sp3 proteins has been linked to microRNA-dependent regulation of these repressors. These repressors competitively bind to Sp-protein binding sites (GC-rich DNA motifs) at the promoters and repress transcriptional activity of the genes. Recent studies suggest that microRNA 27a (miR-27a) represses the expression of

ZBTB10 while miR-20a and miR-17-5-p repress the expression of ZBTB4 (K. Kim et al., 2012). High levels of ROS (termed oxidative stress) are shown to decrease the expression of miR-27a/miR- 20a:miR-17-5p and thus induces the miR-regulated transcriptional repressors ZBTB10, ZBTB4 and ZBTB34 resulting in down-regulation of Sp proteins (Vizcaino, Mansilla, & Portugal, 2015). This pathway has been exploited to develop a number of anticancer drugs.

2.3.6. **Sp3 protein and AT1 receptor expression**

Although, Sp3 transcription factor and renal AT1 receptor individually have extensively been studied over the years for gene regulation and blood pressure regulation respectively, only a few studies reported that Sp3 transcription factor regulates AT1 receptor. Zhao et al showed that Sp1/Sp3 are required for basal expression levels of AT1 receptor in H295-R cells (Zhao, Martin, & Elton, 2000, 2001). Moreover, Kambe et al showed that Sp1 protein levels were high in hypothalamic cells and inhibition of Sp1 with decoy phosphorothioate oligodeoxynucleotides decreases AT1 receptor expression and blood pressure in SHR (Kubo et al., 2003) suggesting that this family of proteins might be regulating blood pressure by modulating AT1 receptor expression. Also, one more report from the same lab demonstrated that Sp1/Sp3 regulate AT1 receptor expression in a vascular smooth

muscle cell line, A10 cells (Kambe et al., 2004).

2.4. NF-κB p-65 transcription factor and AT1 receptor expression

Nuclear factor-kappa B (NF-κB) protein is a transcription factor, which was first described by H. Singh, R. Sen and D. Baltimore in 1980s (R. Sen & Baltimore, 1986b; Singh, Sen, Baltimore, & Sharp, 1986). It plays an important role in the regulation of innate immunity, cell growth and apoptosis, and inflammatory responses (Atchison & Perry, 1987; X. C. Li & Zhuo, 2008; R. Sen & Baltimore, 1986a). An inhibitory IκB protein binds to NF-κB in the cytoplasm and thus inhibits its activity. Upon stimulation of the cell, IκB protein gets serine-phosphorylated and degraded, resulting in releasing active NF-κB protein that translocates to the nucleus and activates a myriad of genes including genes involved in cancer, cell growth and apoptosis, innate immunity and inflammatory diseases (Hayot & Jayaprakash, 2006; Stancovski & Baltimore, 1997). Studies in others and ours labs have shown that NF-κB is a redox-sensitive transcription factor and contribute to hypertension that impairs renal D1 receptor and AT1 receptor functions and contributes to hypertension (Bhatt et al., 2014; Elton & Martin, 2007; Mitra, Gao, & Zucker, 2010). Also, NF-κB is implicated in diseases like asthma, muscular dystrophy, and hypertension. However, some labs including ours suggested that this transcription factor plays an important role in

up-regulating antioxidants enzymes and protects cells against oxidant damage (L. George et al., 2009; L. E. George et al., 2012). These important and diverse functions make NF-κB one of the most studied transcription factor in biology and related areas.

Numerous studies have shown that oxidative stress activates NF-κB and causes its translocation to nucleus where it binds to AT1 receptor promoter and contributes to hypertension (Banday et al., 2007; Barchowsky, Munro, Morana, Vincenti, & Treadwell, 1995; Bhatt et al., 2014; Toledano & Leonard, 1991). Several studies including in our own lab have demonstrated that AT1 receptor gene possesses the consensus sequence for NF-κB binding (Bhatt et al., 2014; Heinemeyer et al., 1998). However, link between oxidative stress, NF-κB transcription factor, and AT1 receptor is missing. Therefore, we try to investigate the role of oxidative stress mediated NF-κB transcription factor and its role in renal AT1 receptor up-regulation HK2 cells.

2.5. Reactive oxygen species (ROS)

2.5.1. **Classification of ROS**

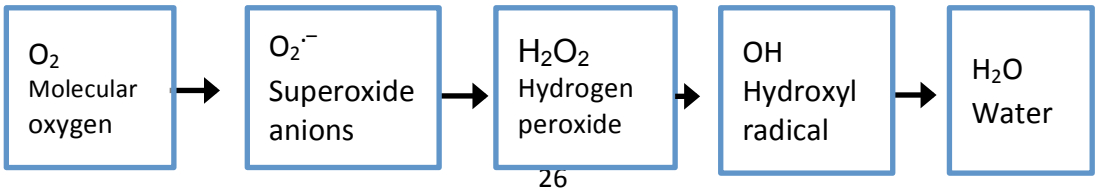
Reactive oxygen species (ROS) is a collective term for free radicals such as superoxide, hydroxyl radical, and alkoxy radicals and non-radicals such as

hydrogen peroxide (H2O2), peroxynitrite, and hypochlorous acid (Harrison & Gongora, 2009). All ROS are products of oxygen metabolism, which can sequester electrons from other molecules (oxidize), donate electrons to molecules (reduce) or react with and become part of other molecules (oxidative modification). Superoxide is particularly important in cardiovascular biology due to the fact that it can serve both as an oxidant and as a reductant and can generate other ROS. Reactive nitrogen species (RNS), another relevant group, includes nitric oxide (NO), nitrogen dioxide radical (NO_2), and nitrosonium cation. Peroxynitrite is considered both as ROS and RNS due to its formation from superoxide and nitric oxide (Paravicini & Touyz, 2006).

2.5.2. **Production**

Oxygen radicals or ROS can be generated by electron-transfer and or energy transfer reactions as shown in boxes below.

Below is the schematic illustration of ROS formation by sequential electron transfer mechanism.



The addition of a single electron to molecular oxygen forms superoxide anion radical. Addition of two electrons to molecular oxygen or one electron to superoxide results into the formation of H_2O_2. It is noteworthy that superoxide is an intermediate molecule in the formation of H_2O_2 from univalent reduction of molecular oxygen. The reaction between superoxide and H_2O_2 generates hydroxyl radical, which is the most reactive ROS in the biological system. Chemically, addition of one electron to H_2O_2 results into the formation of hydroxyl radical and water.

There are a number of sources of ROS in biological systems including enzymes, various types of cells, chemicals, and environmental factors. Xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, NADPH-cytochrome P450 reductase, and, under certain conditions nitric oxidase are the key enzymes that are implicated in the generation of ROS. These enzymes get activated in various disease conditions including atherosclerosis, hypertension, diabetes and renal diseases. Importantly, these enzyme systems can interact with one another, such that ROS produced by one enzyme can activate others. For example, peroxynitrite can oxidize tetrahydrobiopterin, a critical co-factor for nitric oxide synthase, which causes the uncoupling of nitric

oxide synthase (NOS), which as a result produces superoxide instead of nitric oxide (Munzel, Daiber, Ullrich, & Mulsch, 2005). In addition to enzymes, leukocytes and macrophages, mitochondrial electron transfer system, and microsomal monooxygenases are cellular sources of ROS. Ultraviolet light, X-rays, toxic chemicals, pesticides such as paraquat, and chemotherapeutic agents such as quinones are some of the environmental sources of ROS.

Although earlier ROS used to be considered toxic by-product of cell metabolism, recently these have been established as a key signaling molecules in normal cellular functions including growth, remodeling, apoptosis, and migration (Harrison & Gongora, 2009). For example, H_2O_2 has been established as an endothelial-derived hyperpolarizing factor involved in maintaining vascular tone (Miura et al., 2003). The following quote attributed to Hans Selye might well apply to the concept of oxidative stress: “If only stress could be seen, isolated and measured, I am sure we could enormously lengthen the average human life span” (Sies, 2015).

2.5.3. **ROS, kidney and hypertension**

Oxidative stress is a state when there is an imbalance between ROS producing systems and anti-oxidative defense mechanism of the body. Higher

activity of either ROS producing and/or lower activity of antioxidant defense systems can cause a state of oxidative stress. A large body of evidence suggests that elevated levels of ROS contribute to hypertension in animal models. Scavenging ROS using different approaches attenuated hypertension in animals model, suggesting ROS are a contributing factor in causing hypertension (Nakazono et al., 1991). Moreover, a number of studies have shown high levels of oxidative stress in the kidneys of hypertensive animals suggesting their role in blood pressure. Several studies have shown virtually every cells in the kidney including vessels, glomeruli, podocytes, interstitial fibroblast, macula densa, medullary thick ascending limb (mTAL), the distal tubules, and the collecting ducts express NADPH oxidase (Chabrashvili et al., 2002). The main targets of oxidant stress include the proximal tubule, the afferent arteriole, the glomerulus and the cortical collecting duct. Importantly, superoxide in the afferent arteriole reduces the levels of nitric oxide (NO) resulting in increased vasoconstriction and reduced GFR. Studies both in whole animals and isolated afferent arteriole have shown that superoxide generated by NADPH oxidase causes endothelial dysfunction in afferent arteriole (Fellner & Arendshorst, 2005; Wang et al., 2003). Moreover, scavenging superoxide induces vaso-relaxation and improves kidney perfusion, thus alleviates hypertension (Kopkan, Castillo, Navar, & Majid, 2006).

Furthermore, Dahl salt-sensitive rats, a hypertensive rat model, exhibit overexpression of NADPH oxidase subunits nox4 and $p22^{phox}$ in glomerulus with podocyte injury. Superoxide scavenger tempol reduces proteinuria and glomerular sclerosis in these rats, further supporting the role of ROS, especially superoxide, in the glomerular injury (Meng, Cason, Gannon, Racusen, & Manning, 2003).

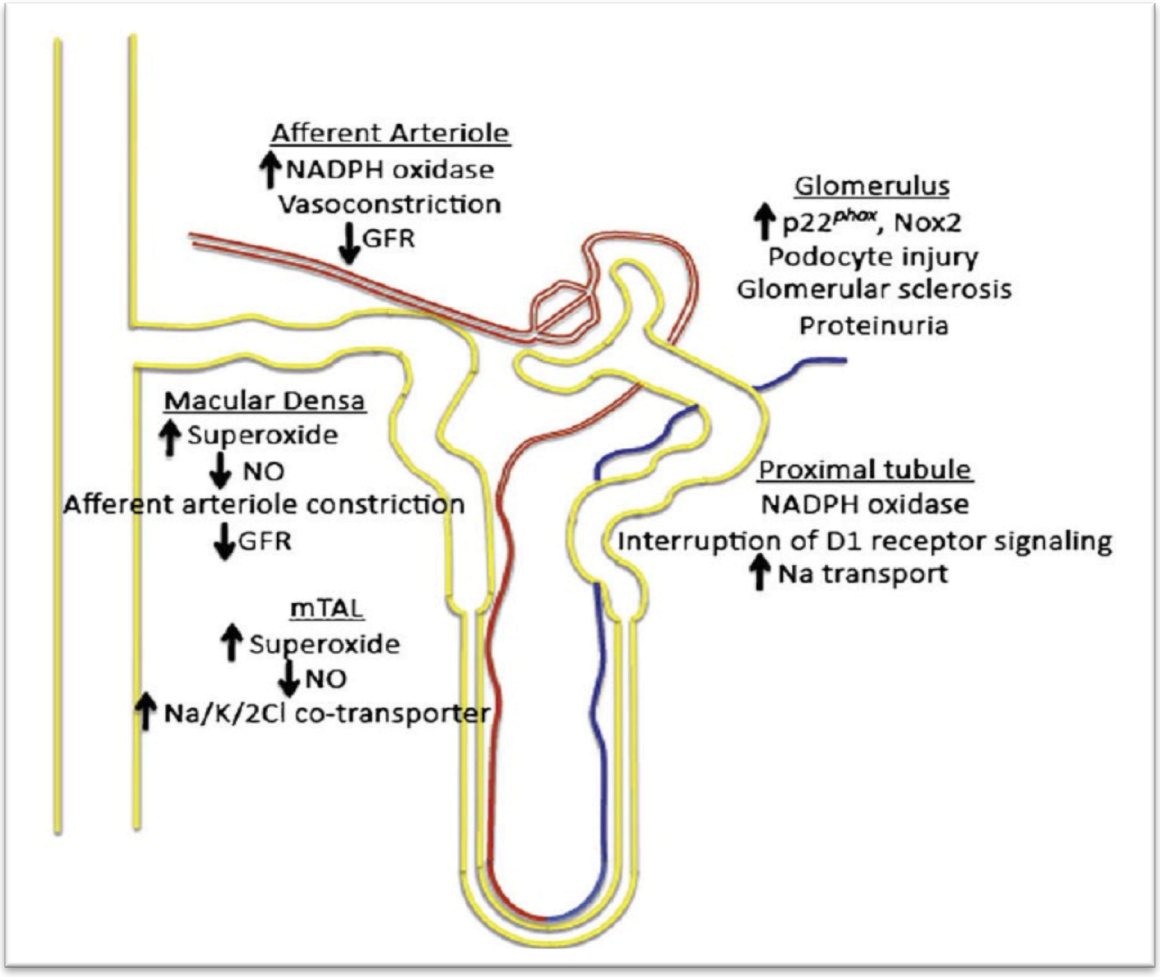


Figure 4. Schematic representation of a juxtamedullary glomerulus showing sites of ROS production and potential roles in sodium transport, reabsorption, and blood pressure regulation.

D1, Dopamine type 1 receptor; GFR, glomerular filtration rate; mTAL, medullary thick ascending limb; Na, sodium; NO, nitric oxide (Harrison & Gongora, 2009).

Renal proximal tubular cells possess inactive NADPH oxidase especially in lipid rafts, which can be activated by either disrupting lipid rafts or application of Ang II. Recent studies have shown that ROS and NADPH oxidase modulate levels of sodium transport by altering the activity of Na/K ATPase and Na/H exchange present on basal and apical membranes, respectively, in proximal tubular cells (Banday et al., 2007; Banday, Lau, & Lokhandwala, 2008; Banday & Lokhandwala, 2008a). These studies demonstrated that Ang II and dopamine respectively stimulate and inhibit sodium transport. Also, these studies suggest that oxidative stress potentiates Ang II response while inhibits dopamine response, that results in increased sodium transport in proximal tubular cells. Challenging question is how oxidative stress affects proximal tubular cells and contributes to hypertension. One of the mechanisms is that ROS by modulating tubuloglomerular feedback may modulate sodium transport in the cortex and as a result controls blood pressure. For detailed description, please read (Liu, Ren, Garvin, & Carretero, 2004).

ROS also modulate sodium transport in renal medulla that is implicated in blood pressure regulation. Medullary epithelial cells of mTAL and pericytes in the vasa recta equally contribute to the production of superoxide and nitric oxide. Comparative studies suggested that NO production in medulla is higher than that

of cortex, therefore, it is likely that regulation of cortex and medulla is independent of each other ((Wu, Park, Cowley, & Mattson, 1999). Studies from Cowley et al. demonstrated that nitric oxide released by mTAL diffuses to nearby pericytes of adjacent vasa recta that in turn dilates these vessels. As a result, medullary and interstitial Starling flow are alleviated that in turn promotes sodium movement to the tubules and thus increasing natriuresis and diuresis (Cowley, Mori, Mattson, & Zou, 2003; Dickhout, Mori, & Cowley, 2002). Renal medullary cells also contain all components of NADPH oxidase that can be activated by locally or systemically produced Ang II that results into increased production of superoxide. Elevated levels of superoxide cause vasoconstriction of vasa recta and reduce Starling forces causing sodium movement into vasa recta, reduced natriuresis, and increased blood pressure (Mori & Cowley, 2003).

In summary, it seems that normal levels of ROS play indispensible role in controlling renal physiology, while high levels of ROS have been implicated in the pathophysiology of a number of diseases including hypertension, atherosclerosis and other cardiovascular disorders. Importantly, sodium and water retaining quality of kidney during sodium-restricted diet is an extremely important function in terrestrial mammals, without which survival would be impossible. It is likely that superoxide and other ROS are generated in the terrestrial mammals to maintain

sodium and water balance.

2.5.4. **Oxidative stress in animal models of hypertension**

A number of hypertensive animal models including genetic forms (SHR, stroke-prone SHR), endocrine induced (Ang II, and deoxycorticosterone acetate), experimental pulmonary hypertension (Dorfmuller et al., 2011), and aging Fisher 344 X Brown Norway (FBN) (Chugh et al., 2011) rat models exhibit high level of oxidative stress. It is reported that oxidative stress does fetal programming in SHR to cause hypertension because in this rat model oxidative stress precedes the development of hypertension. Experimental hypertensive rats exhibit reduced levels of antioxidant enzymes and NO, increased activation of xanthine oxidase, NADPH oxidases and other ROS generating enzymes, elevated organ tissue levels of superoxide and H_2O_2, and increased levels of oxidative stress markers such as 8-isoprostane, thiobarbituric acid reactive substances. Evidence for oxidative stress as a contributing factor in hypertension, several studies have demonstrated that antioxidants SOD mimetic [tempol, (4- hydroxy-2,2,6,6-tetramethyl piperidinoxyl)], vitamins (A and E), and tetrahydrobiopterin attenuate or prevent hypertension in hypertensive animal models (Raijmakers, Dechend, & Poston, 2004; Rey, Cifuentes, Kiarash, Quinn, & Pagano, 2001; Wilcox, 2010).

2.5.5. **Oxidative stress and hypertension in humans**

Although, there is ample experimental evidence supporting the causative role of oxidative stress in human hypertension, no single study confirms that oxidative stress is the primary cause of hypertension in humans. In spite of that, patient with essential hypertension, malignant hypertension, cyclosporine-induced hypertension, reno-vascular hypertension, pre-eclampsia, and salt-sensitive hypertension exhibit higher levels of ROS (K. Chen et al., 2011; Raijmakers et al., 2004; Rodrigo et al., 2007; Ward et al., 2004). Most of the clinical studies showed increased levels of oxidative stress biomarkers such as lipid peroxidation thiobarbituric acid reactive substances and 8-epi-isoprostane (K. Chen et al., 2011; Murphey et al., 2003). Also, levels of superoxide generated by polymorphonuclear leucocyte and platelet are increased in hypertensive patients (Yasunari, Maeda, Nakamura, & Yoshikawa, 2002).

Patients with hypertension exhibit increased production of plasma H_2O_2 than normotensive persons. Moreover, normotensive persons with family history of hypertension exhibit higher production of H_2O_2 than the blood pressure-matched normotensive persons without a family history of hypertension. This study suggested that genetic component might be involved in H_2O_2 production (Lacy, Kailasam, O'Connor, Schmid-Schonbein, & Parmer, 2000; Lacy,

O'Connor, & Schmid-Schonbein, 1998). Vascular smooth muscle cells of resistant artery from hypertensive patients show increased activity of NADPH oxidase and ROS production.

Low levels of antioxidants have been shown to contribute to oxidative stress in patients with hypertension. These patients show reduced levels and activity of antioxidant enzymes such as SOD, catalase, and glutathione peroxidase. Lower levels of vitamins A, C, and E have also been reported in untreated hypertensive patients. Additionally, there exists an inverse relationship between SOD activity and blood pressure in hypertensive patients. Further, support for a role of oxidative stress in hypertension comes from a study that reported increased levels of serum protein carbonyls (an indicator of protein oxidation), and reduced levels of enzymatic antioxidants in patients with white coat hypertension compared with normotensive persons (Caner et al., 2006).

2.5.6. **Molecular and cellular mechanisms of oxidative stress in hypertension**

At cellular level, mechanism of ROS induced hypertension involves activation of redox-sensitive signaling pathways, particularly in the vascular system. In the vasculature, increased ROS, particularly superoxide and H_2O_2, reduced the

levels of vasodilator NO and causes vasoconstriction and contributing to hypertension. In addition, ROS are implicated in activating various redox-sensitive transcription factors including NF-κB, Sp3, HIF-1 and AP-1, inactivating protein tyrosine phosphatases (PTP), and increasing intracellular free calcium concentration ($[Ca^{2+}]_i$). Furthermore, ROS are implicated in the overexpression of proto-oncogenes and inflammatory genes (Al Ghouleh et al., 2011; Banday & Lokhandwala, 2015; Droge, 2002; Zinkevich & Gutterman, 2011). These changes in intracellular signaling may cause endothelial dysfunction, increased constriction, reduced vasodilation, and structural remodeling of vasculature that results in increased peripheral resistance and hypertension (Touyz & Briones, 2011). In the kidney, activation of redox-sensitive pathway is implicated in sodium and water retention, glomerular damage, proteinuria, and nephron loss, all of which are associated with the development of hypertension (Wilcox, 2005).

2.5.7. **Antioxidants against oxidative stress**

Antioxidants are agents that at low concentrations prevent or inhibit the oxidation of biomolecules such as DNA, lipid, and protein by scavenging elevated levels of ROS. A number of defense systems have evolved in biological system to scavenge elevated levels of ROS. These are enzymatic, non-enzymatic, and external antioxidants, which show beneficial and protective effect against

oxidative damage caused by ROS. Superoxide dismutase (SOD), catalase, glutathione peroxidase, thioredoxin, and peroxiredoxin are main enzymatic antioxidants (Gongora et al., 2006). While ascorbic acid, tocopherol, glutathione, bilirubin, and uric acid constitute non-enzymatic antioxidants (X. Chen, Touyz, Park, & Schiffrin, 2001). Low bioavailability of antioxidants results in cellular oxidative damage that has been implicated in cardiovascular and renal tissue damage and hypertension. It is reported that patients with hypertension exhibit lower activity of SOD, catalase, and glutathione peroxidase and higher levels of oxidized and reduced glutathionein plasma and circulating cells (Redon et al., 2003).

Superoxide is a substrate for SOD, which converts it into H_2O_2 and O_2. There are two types of SOD: manganese (Mn) SOD is present in mitochondrial matrix while copper/zinc Cu/Zn) SOD is present in cytosol, mitochondrial inter-membrane and extracellular spaces (Miriyala et al., 2012). Extracellular SOD or Cu/Zn SOD is important as it determines oxidative status in the vascular interstitium. In addition, SOD prevents the formation of peroxynitrite by inhibiting the inactivation of NO, thereby helps maintains the endothelial function (Fukai & Ushio-Fukai, 2011). Catalase mainly resides in peroxisome and catalyzes H_2O_2 into water and O_2 and thus protects cellular damage arising from H_2O_2.

Glutathione peroxidase also converts H_2O_2 into water and O_2 and helps protect cellular injury from H_2O_2 (Goyal & Basak, 2010).

Vitamin E, vitamin A and glutathione are major non-enzymatic biological antioxidants. Vitamin E (tocopherols and tocotrienoles) is the lipid-soluble while vitamin C (ascorbic acid) is water-soluble antioxidant, which is involved in scavenging radicals generated by oxidation of vitamin E. Vitamins C and E, at high concentrations, function as pro-oxidants that can be damaging to the cell. A number of clinical trials have attributed the negative impact of these vitamins on cardiovascular physiology (Montezano & Touyz, 2012).

Carotenoids, uric acid and bilirubin are small-molecules that function as biological antioxidants. Carotenoids are naturally occurring lipophilic compounds with β-carotene being present abundantly. These compounds possess conjugated double bonds, which are attributed for their antioxidant capacity. Bilirubin by inhibiting lipid oxidation and free radical formation may act as antioxidants and provide protection against cardiovascular disorders. Studies have suggested bilirubin as a potential clinical biomarker for cardiovascular diseases because clinical studies showed a clear inverse relationship between bilirubin levels and cardiovascular risk factors such as hypertension, diabetes

and metabolic disorders (Nagao, 2011).

A number of animal studies and a few small clinical trials have shown protective effect of antioxidant on cardiovascular diseases including hypertension. Therefore, much effort has been put into developing synthetic and naturally occurring antioxidants as therapeutic agents to prevent and/or treat patients with hypertension or related cardiovascular diseases. However, due to various reasons, many large clinical trials have failed to show protective effects of antioxidants in hypertensive humans (Kizhakekuttu & Widlansky, 2010). This indicates that a more focused study could be undertaken regarding role of antioxidants in combating hypertension and associated cardiovascular diseases in patients.

2.5.8. **Why antioxidants may have failed in patients to treat hypertension**

As mentioned earlier, use of antioxidants to treat cardiovascular diseases including hypertension have been disappointing despite the promising results from animal studies. Reasons of failure may be multifold: (i) selection and dose of antioxidants, (ii) design of trials and, (iii) patient cohorts selected for the study. In these clinical trials, the dose and type of antioxidants was never demonstrated to reduce the levels of oxidative stress. In addition, several studies have shown

that vitamins C and E may act as pro-oxidants resulting in increase in oxidative stress-induced damage (Gori & Munzel, 2011). Moreover, either bioavailability of antioxidants with oral administration is not sufficient to scavenge ROS, or ROS generated in specific cellular organelles or compartments might not be inaccessible to antioxidants (Farbstein, Kozak-Blickstein, & Levy, 2010; Talaulikar & Manyonda, 2011). Importantly, antioxidants scavenge those ROS which are already generated and do not inhibit their production. There are no large clinical trials so far in which patients were included based on elevated oxidative stress levels. Also, these clinical trials were not specifically designed to study the effect of antioxidants on hypertension. It is worth mentioning here that there is no validated biomarker exists in the clinical setting that can verify that hypertensive patients have high oxidative stress and treatment with antioxidants reduces it. An analogy would be to test the effect of cholesterol-lowering drug for the treatment of coronary artery disease but without measuring cholesterol levels (Gori & Munzel, 2011).

3. MATERIAL AND METHODS

3.1. Methods used in cell culture studies

3.1.1. **Cell culture**

Human Kidney (HK2) cells were purchased from American Tissue Culture Collection (ATCC® CRL-2190™, ATCC, Manassas, VA), cultured and used in the studies using our published methods (I. D. Pokkunuri, Chugh, & Asghar, 2013). Briefly, cells were cultured in DMEM/F12 culture media supplemented with fetal bovine serum (FBS, 10% vol/vol), epidermal growth factor (EGF, 5ng/ml), bovine pituitary extract (BPE, 50µg/ml), and cocktail of antibiotics and antimycotics in a humidified cell culture incubator maintained at 37^0C under 5% CO_2. HK2 cells were grown up to 90-95% confluency, starved for 2 hr in DMEM/F12 media without FBS, BPE, and EGF before any treatments and used in the study unless otherwise stated. Cell passages between 4-17 were used.

3.1.2. **Detection of superoxide and hydrogen peroxide in HK2 cells**

Levels of intracellular superoxide radicals were determined using cell permeable fluorescent probe, dihydroethidium [(DHE), Life technologies, Eugene, Oregon, USA]. Briefly, cells were grown, detached using trypsin/EDTA (0.5/0.2 g/L), suspended in Krebs-Henseleit (KH, pH 7.4) buffer and loaded in triplicate in a 96-

well transparent plate. Subsequently, 25μM DHE probe was loaded in each well and incubated for 5 min at room temperature. Thereafter, cells were treated with 500μM SOD-inhibitor diethyldithiocarbamate (DETC) in the absence and presence of tempol, and 50μM H_2O_2 at room temperature and fluorescence intensity was recorded immediately using excitation (490 nm) and emission (610 nm) wavelengths in a spectrofluorometer (Varioskan, Thermo Scientific, Rockford, IL, USA). Pre-treatment of tempol was carried out for 10 min before adding DETC and remained there in the reaction. DHE fluorescence was measured for 30 min at 5 min time-intervals and no significant difference among the readings was found. Data presented was at 5 min.

Levels of intracellular H_2O_2 were determined using cell permeable DCFHDA probe (DCFHDA, Life technologies, Eugene, Oregon, USA). Briefly, cells were grown, detached using trypsin/EDTA (0.5/0.2 g/L), suspended in Krebs-Henseleit (KH, pH 7.4) buffer, and loaded in triplicate in a 96-well transparent plate. Subsequently, 10μM DCFHDA probe was loaded in each well and incubated for 30 min at room temperature. Thereafter, cells were treated with 50μM H2O2 and DETC (500μM) for 30 min and with 10mM 3-amino-1,2,4-triazole (3-AT, a catalase inhibitor), for 60 min. Subsequently, DCFHDA fluorescence intensity was recorded using excitation (490 nm) and emission (520

nm) wavelengths.

3.1.3. **Toxicity measurement in DETC, H_2O_2, and tempol treated cells**

Toxicity was determined by a colorimetric assay using a commercially available kit (CellTiter 96® Aqueous One Solution Assay, Promega) and following manufacturer's instructions. Briefly, adherent cells were treated with SOD-inhibitor DETC (500μM) in the absence and presence of tempol (1mM), and H_2O_2 (50μM) separately. Tempol was added 10 min before adding DETC. Subsequently, cells were detached using trypsin/EDTA (0.5/0.2 g/L), suspended in KH buffer, and loaded equally in triplicate in a 96-well transparent plate. Thereafter, CellTiter96® AQ_{eous} One Solution Reagent (20μl) was added to each well, and incubated for 2 h at 37^0C and absorbance was read at 490 nm. Absorbance is directly proportional to the number of viable cells.

Moreover, trypan blue exclusion test was performed to determine the cell viability before conducting each experiment in HK2 cells (PMID: 3039859). Briefly, HK2 cells suspension was mixed with equal volume of trypan blue (0.15% in KHB) followed by monitoring trypan blue uptake by HK2 cells under microscope. Almost 90- 95% cells excluded trypan blue uptake suggesting 90-95% cells were viable used in the study.

3.1.4. **Isolation of nuclear proteins**

Adherent cells were treated with DETC (500μM, 2 hr), H_2O_2 (50μM, 30 min) in the absence and presence of tempol (1 mM). Pre-treatment of tempol was carried out for 10 min before the addition of DETC and H_2O_2. Subsequently, cells were washed twice with cold PBS and detached using trypsin/EDTA (0.5/0.2 g/L). Thereafter, cells were washed twice with media by centrifugation (300 g, 4 min). Finally, cell palettes were used to isolate nuclear proteins using a commercial kit following manufacturer's instructions (NE-PER kit, ThermoFisher scientific, Rockford, IL USA).

3.1.5. **Cells transfections**

3.1.5.1 *Sp3 plasmid and siRNA transfections*

Sp3 plasmid was a generous gift from Dr. Douglous A Weigent (University of Alabama, Birmingham, AL) that was used to overexpress Sp3 protein in HK2 cells. A 2.3 Kb Sp3 construct was cloned into pCMV vector for the synthesis of Sp3 plasmid. FuGENE®6 (Promega Corp. Madison, WI) was used as the transfection reagent following manufacturer's instructions. Briefly, cells were grown up to 65% confluency in a 6-well plate and transfected with 1 μg Sp3 plasmid for 24 hr in a humidified cell culture incubator maintained at 37^0C under

5% CO_2. Control cells were transfected with empty pCMV vector. For siRNA studies, cells were transfected with Sp3 siRNA (100 ng) for 24 hr. Control cells were transfected with Allstar control siRNA (Qiagen, Valencia, CA USA). Subsequently, cells were washed with PBS twice and lysed with lysis buffer (20mM tris-HCl pH 7.5, 150mM NaCl, 1mM EGTA, 2.5mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na_3VO_4, 1mM PMSF and protease inhibitor cocktail) for 10 min on ice. Thereafter, cells were sonicated on ice for 3 sec, cell lysates obtained and used to determine the levels of Sp3 and AT1 receptor proteins by Western blotting.

3.1.5.2 *NF-κB plasmid and shRNA transfections*

An NF-κB p-65 plasmid in a pShooter vector (pEF/myc/nuc-NF-κB) with a nuclear translocation domain was used for the transfection. Briefly, cells were grown up to 65% confluency in a 6-well plate in antibiotic free media supplemented with FBS, transfected with 2 µg NF- κB p-65 plasmid for 24 hr. Control cells were transfected with pEF/myc/nuc (pShooter) vector (L. E. George et al., 2012). For NF-κB shRNA, cells were transfected with NF-κB shRNA (Santa Cruz Biotechnology) following manufacturer's protocol. Briefly, control and NF-κB shRNA plasmids (each 10 µl) were mixed with transfection medium (90 µl) separately (solution A), In a separate tube 1 µl of transfection reagent (sc-

108061) was mixed with 99 µl normal growth media (solution B). Thereafter, solutions A and B were mixed together, incubated for 30 min and used to transfect HK2 cells. Cell lysates were obtained as mentioned above and used to determine the levels of NF-kB and AT1 receptor proteins by Western blotting. All the experiments were conducted 24 hr post transfection.

3.1.6. **Western blotting procedure**

Western blot was performed according to our published protocol (PMID: 23698121). Cell homogenates, nuclear extracts, and membrane proteins (15-20µg) were resolved on 8-16% Tris-glycine gels (ThermoFisher scientific, Rockford, IL USA) by SDS-PAGE and transblotted onto PVDF membranes for 2.5 hr at 4^{o}C. Subsequently, PVDF membranes were blocked with 5% BSA in TBST for 1 hr at room temperature (RT). Thereafter, membranes were washed with TBST thrice (each wash 5 min), and probed with respective primary antibodies in TBST overnight at 4 ^{o}C. After three washes with TBST, 5 min each, membranes were incubated with corresponding secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology). The protein bands were visualized with chemiluminescent reagent and quantified using G:BOX image station (Syngene, Frederick, MD) integrated software. Primary antibodies dilutions used were 1:500 for Sp3 and AT1 (both from SCBT, Dallas, TX), histone

3 and lamin B [(1:1000) nuclear protein loading control, Abcam, Cambridge, MA, USA)]. GAPDH (1:1000) (Millipore, Temecula, CA, USA) as protein loading control was used for cell homogenates.

3.1.7. **AT1 receptor mRNA isolation and PCR**

Different drug treatments were carried out as mentioned in 'isolation of nuclear proteins' section. Following treatment, cellular mRNA was isolated and purified using Qiagen mini-kit following manufacturer's protocol (Qiagen, Valencia, CA). As per the MIQE (Minimum Information for Publication of Quantitative Real- time PCR experiments) accepted guidelines, 1 µg total mRNA was reverse transcribed to cDNA using Advantage-RT-for-PCR kit (Clontech Laboratories, Mountain View, CA). Obtained cDNA was diluted 5 times and 9 µl of it was used for qPCR reaction. TaqMan human-specific primers for AT1 receptor (ID#Hs00258938_m1) and 18S ribosome internal control (ID# Hs99999901-s1) were purchased from Life Technologies (Grand Island, NY) and used in qPCR. The assay mixture consisted of 10 µl TaqMan® Gene Expression Master Mix, 1 µl AT1 receptor or 18S ribosome TaqMan primers, and 9 µl of diluted cDNA in a final volume of 20 µl. Reactions were performed in Applied Biosystems® 7300 Real Time PCR system using manufacturer's software. AT1 receptor mRNA was quantified by relative quantification using Delta-Delta Ct method (I. Pokkunuri,

Chugh, Rizvi, & Asghar, 2015).

3.1.8. **Immunofluorescence imaging**

HK2 cells were grown to 70-80% on to glass-chambered slides (ThermoFisher scientific, Rockford, IL USA) and treated with DETC (500μM, 2 hr) in the absence and presence of tempol (1 mM).Control cells were treated with vehicle (water). Subsequently, cells were washed with cold PBS, fixed with formalin for 20 min and blocked with 5% normal goat serum (NGS in TBS) for 1 hr at RT. After washing with PBS, cells were incubated with rabbit AT1 receptor antibody that recognizes extracellular loop of AT1 receptor (1:500 in TBS) overnight at 4C. After washing with (TBS), cells were incubated with Alexa-488 conjugated goat anti-rabbit secondary antibody for 1 hr at RT in the dark. Cells were washed three times, each 10 min, with TBS and mounted with glass cover slips with DAPI mount media. Thereafter, the glass slides were dried overnight in the dark and scanned under a microscope (Olympus) using green filter and 20X objective.

3.1.9. **Isolation of cell-surface proteins using biotin-streptavidin approach**

Cell surface proteins were cross-linked with EZ-LINK Sulfo-NHS-SS-biotin and isolated with streptavidin magnetic beads (ThermoFisher scientific, Rockford, IL

USA). Briefly, cells were treated with DETC (500μM, 2 hr) in the absence and presence of tempol (1 mM). Subsequently, cells were washed twice with cold PBS and immediately removed and incubated with EZ-LINK Sulfo-NHS-SS-biotin (250μg/ml) for 30 min at 4^{o}C while shaking. Subsequently, 100 mM glycine was added to quench the reaction. Cells were centrifuged at 500g for 4 min at 4^{o}C, cell pellet obtained and lysed with 500 μl lysis buffer [50mM sodium phosphate buffer (pH 8.0), 150mM NaCl, 1% Nonidet P-40, 0.5% Triton X-100, 1mM PMSF and protease inhibitor cocktail] followed by sonication on ice. The cell lysate was incubated with streptavidin-coated magnetic beads (0.5mg/50ul) overnight with end-over-end (360^{o}) shaking at 4^{o}C. Biotinylated proteins-streptavidin magnetic bead complex was isolated using magnetic stand (DynaMag-Spin, Invitrogen, Oslo, Norway). Supernatant was carefully removed and magnetic bead complex was washed with lysis buffer. The biotinylated proteins-streptavidin complex was dissociated with 40 μl Laemmeli buffer (125mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 5% β-mercaptoethanol) by heating at 90-95^{o}C for 10 min. After vortexing, magnetic beads were separated as above, and the supernatant (30μl) was used to determine AT1 receptor protein by Western blotting as mentioned above.

3.1.10. **Measurement of PKC activity**

Cells were pre-treated with tempol (1mM) and AT1 receptor blocker candesartan (1μM), separately, for 10 min followed by treatment with DETC (500μM, 2 hr). DETC treatment in the absence of tempol and candesartan was also carried out separately. Control cells were treated with vehicle (DMSO). Thereafter, cells were treated with angiotensin II (1μM, 10 min). Cells were taken in lysis buffer (mM: 20 MOPS, 50 β-glycerolphosphate, 50 sodium fluoride, 1 sodium orthovanadate, 5 EGTA, 2 EDTA, 1% Triton-100X, 1 dithiothreitol, 1 PMSF and protease inhibitor cocktail) and passed 4-5 times through a 23-gauge needle. PKC activity in cell homogenate (10μg) was measured using ELISA-based assay kit (Enzo Life Sciences, Farmingdale, NY).

3.2. Methods used in Animal studies

3.2.1. **Animal procurement**

Male Sprague Dawley (SD) rats weighing 200-250gm were purchased from Harlan laboratories (Indianapolis, IN). The rats were housed in plastic cages in the University of Houston animal care facility. Rats were acclimatized for one week and used as per the National Institute of Health guidelines and approved protocols by Institutional Animal Care and Use Committee (IACUC). Animals

were provided with rodent chow and water ad libitum.

3.2.2. **Animal treatments**

Animals were divided into four groups: Control group, DETC treated group, DETC + tempol treated group, and tempol treated group. DETC groups received intra-peritoneal (Phillips & Schmidt-Ott) injection of DETC (10mg/kg BW/daily) for two weeks. DETC was dissolved in saline and sterilized by filtering through 0.2 µm filters each day. Control and tempol groups were injected with sterilized saline. DETC plus tempol, and tempol groups of rats received tempol (2mM, Santa Cruz Biotechnology) in drinking water. Control and DETC groups of rats were given drinking water only (without tempol). Rats had had free access of rat chow and water supplemented with and without tempol. Tempol-supplemented water was changed daily to minimize its oxidation. Water and food intake were recorded every day.

3.2.3. **Animal surgery**

Animals were first knocked-down with isoflurane and then anesthetized with Inactin (150µg/kg BW, IP). Under deep anesthesia tracheotomy was performed to facilitate spontaneous breathing. Right carotid artery was cannulated with pressure transducer to measure blood pressure using software PowerLab

(ADInstruments, Colorado Springs, CO). After measuring blood pressure, left jugular vein was catheterized with PE-50 tubing for saline and candesartan infusions. A midline abdominal incision was made and urinary bladder was catheterized with PE-10 tubing to collect urine. Rats were infused with saline (1% BW ml/h) to prevent dehydration and to maintain stable urinary output.

3.2.4. **Blood pressure measurement**

Blood pressure was measured in anaesthetized rats. After cannulating right carotid artery, blood pressure was recorded for 30 minutes using PowerLab software (ADInstruments, Colorado Springs, CO). A stable blood pressure from this recording was chosen and reported.

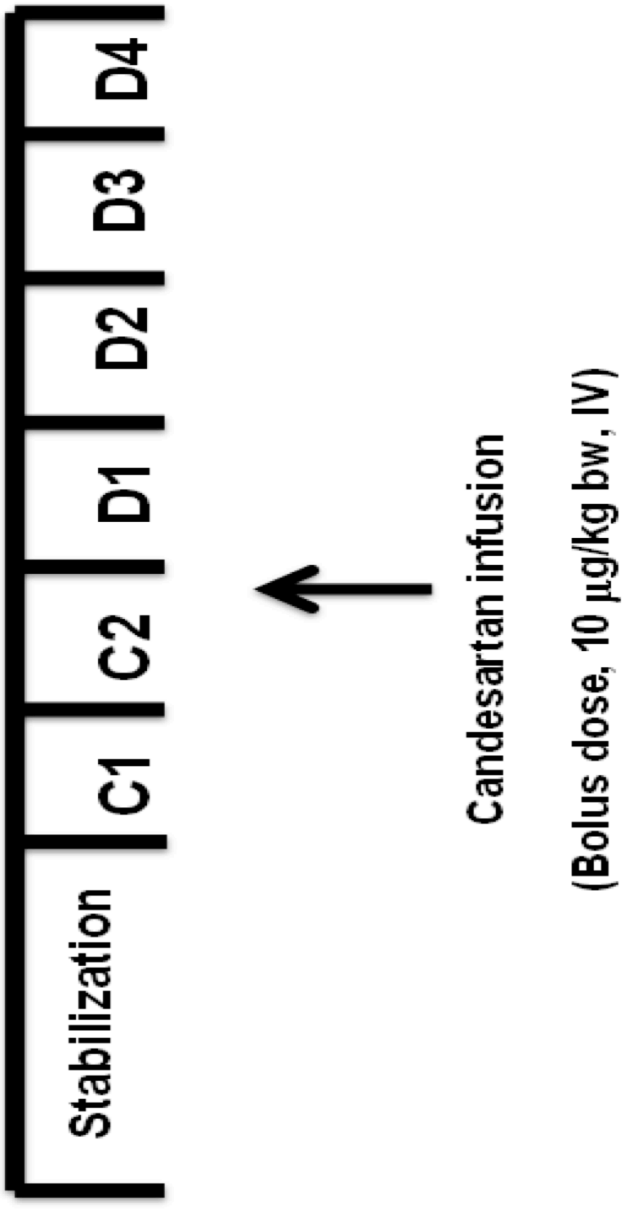


Figure 5. Protocol for renal function studies.

3.2.5. **Urine and blood collection**

Two urine samples (C1 and C2, each 30 min period) from cannulated bladder were collected during saline infusion. Thereafter, a bolus dose of candesartan in saline (10 μg/kg bw, IV) was given and four urine samples (D1, D2, D3 and D4, each for 30 min period) were collected. Blood samples were collected through heart with 1 ml syringe. Blood samples were centrifuged at 2,000g for 10 min at 4 ^{o}C and blood plasma obtained. Urine and blood plasma samples were stored at -80 ^{o}C for further analyses. Urine and plasma samples were used to measure creatinine and sodium.

3.2.6. **Measurement of sodium and creatinine in urine and plasma**

Sodium concentration in urine and plasma was measured using AAnalyst 400 Atomic Absorption Spectrometer [(AAS), Perkin Elmer, Waltham, MA]. Creatinine concentration in urine and plasma was measured using a creatinine assay kit (BioVision, Cat # K625) to determine glomerular filtration rate (GFR). Natriuretic and diuretic responses to AT1 receptor blocker candesartan are represented as urinary sodium excretion ($U_{Na}V$) and urine flow (μL/min), respectively in the result section. Creatinine clearance ($CL_{clearance}$) was determined as an index of GFR.

$U_{Na}V$, and urine flow were calculated using the following formulae:

Urine flow [(UF) (μL/min)] = Urine volume (UV)/30

$U_{Na}V$ (μmol/min) = UF (μL/min) X Urinary sodium concentration (μmol/μL)

3.2.7. **Removal of the kidneys and separation of superficial cortex**

Another set of male Sprague Dawley (SD) rats (weighing 200-250gm) was purchased from Harlan laboratories (Indianapolis, IN). These rats were maintained and drug treatments carried out as mentioned above. Thereafter, rats were knockdown with isoflurane and anesthetized with inactin (150μg/kg BW, i.p.). A midline abdominal incision was made with a sharp scissor; kidneys were removed and placed in KH buffer (pH 7.5). The kidneys were sliced coronally and superficial cortical tissues (rich in proximal tubules) were obtained using razor blade.

3.2.8. **ROS measurement**

Renal tissues (100mg) were homogenized in tissue lysis buffer using Shredder SG3 (Pressure Biosciences Inc., South Easton, MA) and homogenates obtained. Homogenate samples (30μg proteins) were loaded in triplicates in a 96-well transparent plate. Superoxide probe dihydroethidium (DHE, 20μM, Life

technologies, Eugene, Oregon, USA) was mixed well in Krebs-Henseleit (KH, pH 7.4) buffer, added in each well (final volume 200µl), and incubated for 30 min at room temperature. The plate was shaken for 5 min and DHE fluorescence signal measured using excitation (490 nm) and emission (610 nm) wavelengths in a spectrofluorometer (Varioskan, Thermo Scientific, Rockford, IL, USA).

To measure H_2O_2 levels, samples (30µg proteins) were loaded in triplicates in a 96 well transparent plate. Thereafter, H_2O_2 probe dichloro-dihydro-fluorescein diacetate (DCFHDA, 10µM, Life technologies, Eugene, Oregon, USA) was added and mixed well in KH buffer. After that, 200µl KH buffer containing DCFHDA was added in each well and incubated for 30 min at RT. DCFH fluorescence signal was measured using excitation (490nm) and emission (520nm) wavelengths in a spectrofluorometer (Varioskan, Thermo Scientific, Rockford, IL, USA).

3.2.9. **Immunoprecipitation (IP) of nuclear Sp3 protein**

Kidney superficial cortex was homogenized and used to separate the nuclear extract using a commercial kit following manufacturer's instructions (NE-PER kit, ThermoFisher scientific, Rockford, IL USA). Nuclear samples (250 µg protein, total volume 400 µl) were mixed with 10µg Sp3 antibody (Santa Cruz

Biotechnology, Dallas, USA) followed by 2 h end-to-end shaking at 4 ^{o}C. Subsequently, 90µl sepharose A/G (Santa Cruz Biotechnology) was washed in 1 ml IP buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1% Triton X 100, 1X protease inhibitor cocktail] and centrifuged (14,000 rpm, 20 sec) and Sp3 antibody complex was added into it and incubated at end-to-end shaker overnight 4 ^{o}C. Next day, whole complex was washed with IP buffer and tris-HCL separately by centrifuging (14,000 rpm, 1 min) at 4 ^{o}C. Laemmli buffer (60µl) was added in obtained pelleted beads and heated for 1 h at 37 ^{o}C followed by vortexing and centrifuging again as previously. Supernatant was collected; bromophenol blue dye (5 µl) was added and used for Western blotting.

3.2.10. **Statistical analysis**

Data are presented as means ± SE. One-way ANOVA followed by Newman-Keul's post hoc test was used to compare the differences among the groups. Two-way ANOVA was employed for multiple group comparisons. Student's *t*-test was used wherever appropriate. The P value of less than 0.05 was considered statistically significant. GraphPad prism software (GraphPad software ver. 5, San Diego, CA) was used to analyze the data.

3.3. Materials

A 2.3 Kb Sp3 construct cloned into pCMV vector was a generous gift from Dr. Douglas A. Weigent, University of Alabama, Birmingham, AL. The sources of all other materials and chemicals purchased are written in parenthesis. Cell toxicity assay kit Cell Titer 96® Aqueous One Solution Assay, and PKC activity assay kits, (Promega, Madison, WI); Dihydroethidium, and dichloro-dihydro-fluorescein diacetate (Life technologies, Eugene, Oregon); Immobilon P memebrane (Millipore, Temecula, CA); FuGENE®6 transfection reagent, and protease inhibitor cocktail (Roche, Indianapolis, CA); Nuclear and cytosolic extraction kit (Pierce, Rockford, IL); cDNA Advantage-RT-for-PCR kit (Clontech Laboratories, Mountain View, CA); immunofluorescence-glass chambered slides, AT1R siRNA catalog # 4392420, Lipofectamine RNAiMAX transfection reagent, EZ-LINK Sulfo-NHS-SSbiotin**,** streptavidin magnetic beads, protein measuring BCA kit (ThermoFisher scientific, Rockford, IL); DMEM/F12, fetal bovine serum (FBS), epidermal growth factor (EGF), bovine pituitary hormone (BPE), and anti-biotic mixture (Invitrogen, Waltham, MA); Tempol, diethyldithiocarbamate (DETC), chemiluminescent reagent, (Santa Cruz Biotechnology, Dallas, Texas); Allstar control siRNA, and RNA extraction Qiagen mini kit (Qiagen, Valencia, CA); TaqMan human-specific primers for AT1 receptor (ID#Hs00258938_m1), and 18S

ribosome internal control (ID# Hs99999901-s1) (Life Technologies, Grand Island, NY); magnetic stand-DynaMag-Spin (Invitrogen, Oslo, Norway); Candesartan (gift from AstraZeneca, Cheshire, UK); Ang II (Sigma Aldrich, St. Louis, MO); PKC assay kit (Enzo Life Sciences, Farmingdale, NY).

4. RESULTS

Results section is sub-divided in (4.1.) Results from cell culture studies; and (4.2.) Results from animal studies.

4.1. RESULTS FROM CELL CULTURE STUDIES

4.1.1. **Toxicity measurement**

To determine whether the treatments of DETC, H_2O_2, and tempol affect cells viability, cell toxicity assay was performed. Viable cell number did not decrease and hence toxicity did not increase with DETC, tempol or H_2O_2 treatment in HK2 cells (Fig. 6).

4.1.2. **Dihydroethidium (DHE) probe detects superoxide radicals in a cell free system**

4.1.2.1 *Effect of superoxide producing system xanthine/ xanthine oxidase on DHE fluorescence levels*

To determine if DHE dye specifically detects superoxide, a cell free system that generates superoxide was employed. Xanthine and xanthine oxidase (XO) that produce superoxide radicals were added in KH buffer, which contains DHE probe. DHE fluorescence intensity increased 2-fold in xanthine/xanthine

oxidase than in controls (Fig. 7) Moreover, addition of DHE in KH buffer, which contains H_2O_2, did not show any significant increase in DHE fluorescence (Fig. 7). This study clearly shows that DHE probe specifically detects superoxide but not H_2O_2.

4.1.3. **Diethyldithiocarbamate [(DETC) an SOD inhibitor] treatment increases superoxide levels in HK2 cells**

4.1.3.1 *Effects of SOD-inhibitor DETC and H_2O_2 on the levels of DHE fluorescence*

The following experiments were conducted to determine whether DETC, an SOD inhibitor, generates superoxide in HK2 cells. To achieve this, HK2 cells were treated with DETC in the presence and absence of tempol. DETC treatment significantly increased DHE fluorescence levels compared to vehicle treated cells (Fig. 3). However, H_2O_2 treatment did not have any significant effect on DHE fluorescence, suggesting that DHE specifically detects superoxide in a cell system (Fig. 8). In addition, this study also showed that H_2O_2 is not converted to superoxide by HK2 cells (Fig. 8). Pretreatment of cells with tempol (SOD-mimetic) abolished DETC-mediated increase in DHE fluorescence levels, suggesting that DETC produces superoxide and tempol is a potent antioxidant

that specifically scavenges superoxide (Fig. 8).

4.1.3.2 *Effects of H2O2, 3-AT and SOD-inhibitor DETC on the levels of DCFHDA fluorescence*

To determine exogenously added and endogenously produced H_2O_2 levels, DCFHDA fluorescent probe was used. DCFHDA is a non-fluorescent molecule, which is converted into DCF by intracellular esterases. Upon oxidation by ROS, such as H2O2 it fluoresces. Treatment of cells with either exogenous H_2O_2 or catalase inhibitor 3-AT, that produces endogenous H_2O_2, significantly increased DCF fluorescence levels compared to vehicle treated cells (Fig. 9). These results suggest that H_2O_2 when added exogenously as well as produced endogenously increased intracellular levels of H_2O_2. DETC treatment, that produces superoxide, did not have any effect on DCF fluorescence (Fig. 9), suggesting that this probe specifically detect H_2O_2.

4.1.4. DETC treatment increases nuclear accumulation of Sp3 and NF-κB proteins in HK2 cells

4.1.4.1 *Effect of SOD-inhibitor DETC and H_2O_2 on nuclear accumulation of Sp3 protein*

To determine if superoxide and H_2O_2 affect nuclear translocation of Sp3 protein, HK2 cells were treated with DETC, H_2O_2 and vehicle. Western blotting showed the presence of three bands for Sp3 protein (115, 100 & 78 kDa) in the nuclear extract of HK2 cells (Fig. 10). Sp3 gene is expressed into three isoforms of Sp3 protein (Davie et al., 2008). Densitometric analysis of Sp3 protein bands showed that DETC treatment significantly increased nuclear levels of Sp3 compared to vehicle treated cells. Pretreatment of the cells with tempol attenuated this effect. However, H_2O_2 did not show any significant effect on nuclear accumulation of Sp3 protein levels (Fig. 10). This experiment suggests that Sp3 is a redox-sensitive transcription factor, which can be activated by high levels of superoxide radicals.

4.1.4.2 *Effect of SOD-inhibitor DETC and H_2O_2 on nuclear accumulation of NF-κB protein*

To determine the effect of superoxide and H_2O_2 on NF-κB nuclear accumulation, Western blotting was conducted. Densitometric analysis of nuclear NF-κB protein bands showed that DETC treatment significantly increased nuclear levels of NF-κB compared to vehicle treated cells (Fig. 11). Pretreating the cells with tempol attenuated this effect (Fig. 11). However, H_2O_2 did not show any effect on nuclear accumulation of NF-κB protein (Fig. 11). These results suggest

that superoxide activates another redox-sensitive transcription factor NF-κB, which can be activated by high levels of superoxide radicals.

4.1.5. **DETC treatment increases AT1 receptor mRNA, and protein expressions in HK2 cells**

4.1.5.1 *Effects of SOD-inhibitor DETC and H_2O_2 on AT1 receptor mRNA expression*

To demonstrate the effect of superoxide and H_2O_2 on gene expression of AT1 receptor, levels of AT1 receptor mRNA were measured by RT-qPCR. Results showed that DETC treatment significantly increased mRNA levels of AT1 receptor compared to vehicle treated cells (Fig. 12). Pretreatment of cells with tempol attenuated this effect (Fig. 12). H_2O_2 treatment did not show any significant effect on the AT1 receptor mRNA levels (Fig. 12).

4.1.5.2 *Effect of SOD-inhibitor DETC on AT1 receptor protein levels*

To examine the effects of superoxide on the levels of AT1 receptor protein, Western blotting was conducted. Western blotting demonstrated a single 41-kDa protein band for AT1 receptor. Densitometric analyses of bands showed that DETC treatment significantly increased the levels of AT1 receptor protein

compared to controls (Fig. 13). This effect was attenuated by tempol treatment (Fig. 13).

4.1.6. **Sp3 plasmid transfection increases Sp3 and AT1 receptor proteins in HK2 cell**

4.1.6.1 *Effect of Sp3 transfection on the levels of Sp3 and AT1 receptor proteins*

To determine the effect of Sp3 plasmid on AT1 receptor protein levels, cells were transfected with Sp3 plasmid and empty vector. Western blot image showed the presence of three distinct bands of 115, 100, and 78-kDa for Sp3 protein and a single band of 41-kDa for AT1 receptor. Densitometric analysis of the bands demonstrated that Sp3 plasmid transfection significantly increased the expression levels of Sp3 and AT1 receptor proteins compared to empty vector (Fig. 14A, B).

4.1.7. **Sp3 siRNA transfection decreases Sp3 and AT1 receptor proteins in HK2 cells**

4.1.7.1 *Effect of Sp3 siRNA on the expressions of Sp3 and AT1 receptor proteins levels*

To confirm that Sp3 regulates AT1 receptor protein expression, HK2 cells

were transfected with Sp3 siRNA and all-star negative control siRNA. Sp3 siRNA transfection significantly reduced the expression levels of AT1 receptor protein compared to all-star negative control siRNA treated cells. Densitometric analysis of the bands showed that Sp3 siRNA transfection significantly attenuated the expression levels of Sp3 and AT1 receptor proteins compared to all-star negative control siRNA treated cells (Fig. 15A, B).

4.1.8. **Sp3 siRNA decreases AT1 receptor protein expression in the presence of DETC in HK2 cells**

4.1.8.1 *Effect of Sp3 siRNA on AT1 receptor protein expression in the presence of SOD-inhibitor DETC*

To confirm whether superoxide mediated up-regulation of renal AT1 receptor protein and function is mediated via Sp3 transcription factor, cells were transfected with Sp3 and control siRNA in the presence DETC, and AT1 receptor protein was measured. Western blotting results showed a single band of 41-kDa for AT1 receptor. Densitometric analysis of the bands showed that Sp3 siRNA decreased AT1 receptor protein expression in the presence of DETC compared to control siRNA treated cells (Fig. 16). DETC alone increased AT1 receptor protein expression compared to control siRNA treated cells (Fig. 16). This

experiment confirms that basal AT1 receptor protein expression is regulated by Sp3 transcription factor. Additionally, this experiment also demonstrates that superoxide (DETC-induced) via Sp3 transcription factor up-regulates renal AT1 receptor protein expression.

4.1.9. DETC treatment increases membranous AT1 receptor protein and function in HK2 cells

4.1.9.1 *Effect of SOD-inhibitor DETC on cell surface AT1 receptor protein levels*

To determine if superoxide affects the levels of membranous AT1 receptor levels, HK2 cells were treated with DETC followed by the detection of cell surface AT1 receptors by i) immunofluorescence imaging and ii) performing Western blot of the biotinylated cross-linked cell surface proteins.

i) **Immunofluorescence imaging of AT1 receptor protein expression**: DETC treatment increased membranous AT1 receptor protein expression in impermeabilized cells as detected by immunofluorescence technique (Fig. 17b). This effect was attenuated by tempol treatment (Fig. 17c).

ii) **Western blotting the biotinylated cross-linked cell surface proteins**: Membranous protein isolated with biotin-streptavidin

approach used for Western blotting. Densitometric analysis of the bands showed that DETC treatment significantly increases membranous AT1 receptor protein levels compared to control cells (Fig. 18). Western blot image showed the presence of a single 41-kDa band for AT1 receptor protein. This effect was attenuated by tempol treatment (Fig. 18). Biotin-streptavidin approach corroborated immunofluorescence-imaging results.

4.1.9.2 *Effect of SOD-inhibitor DETC on PKC (AT1 receptor function) activity*

Activation of AT1 receptor in response to Ang II results in the activation of downstream signaling molecules including PKC. Therefore, PKC activity has been used as an indirect measurement of AT1 receptor function. AT1 receptor ligand angiotensin II (1μM) treatment increased PKC activity in naïve cells (absence of DETC) compared to control naive cells (Fig. 19). Angiotensin II further increased PKC activity in DETC-treated cells. The effect of angiotensin II on PKC was attenuated by AT1 receptor blocker candesartan and SOD-mimetic tempol in DETC treated cells (Fig. 19). This experiment demonstrates that superoxide mediated up-regulation of AT1 receptor protein expression is translated to higher renal AT1 receptor function.

4.1.10. **NF-κB p-65 plasmid transfection increases NF-κB protein but not AT1 receptor in HK2 cells**

4.1.10.1 *Effect of NF-κB p-65 vector on the levels of NF-κB and AT1 receptor proteins*

To determine the effect of NF-κB p-65 vector on AT1 receptor protein levels, HK2 cells were transfected with NF-κB p-65 and empty vectors. Western blot images showed the presence of a single 65-kDa and 41-kDa band for NF-κB p-65 and AT1 receptor respectively. Densitometric analysis of the bands showed that NF-κB p-65 vector transfection significantly increased the expression levels of NF-κB p-65 protein (20A) but did not have any significant effect on AT1 receptor protein compared to empty vector treated HK2 cells (Fig. 20B).

4.1.11. **NF-κB p-65 shRNA transfection and PDTC decreases NF-κB protein levels but overexpress AT1 receptor mRNA in HK2 cells**

4.1.11.1 *Effect of NF-κB p-65 shRNA on the levels of NF-κB protein and AT1 receptor mRNA*

To confirm the role of NF-κB in the regulation of AT1 receptor expression, if any, HK2 cells were treated with NF-κB and control shRNAs and the levels of NF-κB

protein and AT1 receptor mRNA expression were measured by western blotting and RT-qPCR, respectively. Densitometric analyses of protein bands showed that NF-κB shRNA decreased NF-κB protein levels compared to control shRNA treated cells. RT-qPCR results revealed that NF-κB shRNA significantly increased the expression levels of AT1 receptor mRNA compared to control shRNA treated cells (Fig 21A). This experiment suggests that NF-κB transcription factor has an inhibitory effect on AT1 receptor mRNA expression in HK2 cells.

4.1.11.2 *Effect of PDTC on AT1 receptor mRNA expression*

Similar experiment was designed to confirm an inhibitory effect of NF-κB on AT1 receptor mRNA expression. In this experiment, HK2 cells were treated with PDTC (an NF-κB inhibitor). RT-qPCR data analysis showed that PDTC significantly increased the expression levels of AT1 receptor mRNA compared to vehicle treated cells (21B). This experiment again corroborates that NF-κB transcription factor has an inhibitory effect on AT1 receptor mRNA expression in HK2 cells.

4.2. RESULTS FROM ANIMAL STUDIES

4.2.1. **DETC treatment did not have any significant effect on body weight, food and water intake in Sprague Dawley (SD) rats**

4.2.1.1 *Effect of SOD-inhibitor DETC on body weight, food and water intake*

DETC treatment with and without tempol did not show any significant effect on body weight, food, and water intake compared to control groups of rats (Fig 22A,B,C). Tempol-alone treatment also did not show any significant effect on these parameters (Fig 22A,B,C).

4.2.2. **DETC treatment increases blood pressure in SD rats**

4.2.2.1 *Effect of SOD-inhibitor DETC on blood pressure*

DETC treatment in rats significantly increased systolic and diastolic blood pressures compared to control group of rats (Fig. 23). DETC-induced increase in blood pressure was reduced in tempol treated rats. Tempol treatment alone did not significantly affect blood pressure in rats (Fig. 23).

4.2.3. **DETC treatment increases superoxide levels in kidney superficial cortex of SD rats**

4.2.3.1 *Effect of SOD-inhibitor DETC on DHE and DCFHDA fluorescence*

Kidney superficial cortex, rich in proximal tubules, from the rats treated with DETC showed higher levels of superoxide compared to control groups (Fig. 24), which decreased in rats treated with tempol (Fig. 24). Rats treated with tempol-only did not show any significant difference in superoxide levels compared to control rats. The levels of H_2O_2 measured as DCFHDA fluorescence did not change in kidney cortex from these rats (Fig. 25).

4.2.4. **DETC treatment causes greater magnitude of diuresis and natriuresis in response to candesartan in SD rats**

4.2.4.1 *Effect of SOD-inhibitor DETC on diuresis and natriuresis*

There were significantly greater increases in diuresis and natriuresis in response to AT1 receptor antagonist candesartan in DETC treated compared to control rats (Fig. 26, 27), which reduced in tempol treated rats (Fig. 26, 27). This experiment demonstrated that superoxide (DETC-induced) increases AT1 receptor function in SD rats because i) blocking AT1 receptor with candesartan

increases excretion of sodium (natriuresis) and water (diuresis), and ii) tempol treatment in response to candesartan exhibits reduced natriuresis and diuresis in DETC treated rats. However, there was no effect in response to candesartan on diuresis and natriuresis in rats treated with tempol alone (Fig. 26, 27).

4.2.5. **DETC treatment causes increased acetylation of lysine of Sp3 protein in SD rats**

4.2.5.1 *Effect of SOD-inhibitor DETC on acetylation of lysine of Sp3 protein*

To investigate whether acetylation is one of the causes of nuclear translocation of Sp3, immunoprecipitation-Western blotting (IP-WB) experiments were performed in the nuclear extracts of KSC of SD rats. Nuclear extracts from DETC rats showed increased pan-acetylation of lysine of Sp3 protein compared to control or tempol-only treated rats (Fig. 28A). Importantly, tempol treatment significantly attenuated this effect compared to DETC treated rats (Fig. 28A). There was no effect in response to candesartan on diuresis and natriuresis in rats treated with tempol alone (Fig. 28A). These results demonstrate that acetylation of lysine could be one of the mechanisms of Sp3 protein nuclear accumulation/translocation in DETC treated rats.

4.2.6. **DETC treatment did not have any effect on Sp3 protein phosphorylation in SD rats**

4.2.6.1 *Effect of SOD-inhibitor DETC on Sp3 protein phosphorylation*

To investigate the role of phosphorylation in nuclear accumulation/translocation of Sp3, immunoprecipitation-Western blotting experiments were performed in the nuclear extracts of KSC of SD rats. Nuclear extracts from DETC rats did not show any significant increment in phosphorylation of Sp3 protein compared to control or tempol-only treated rats (Fig 28B). Importantly, tempol treatment significantly attenuated this effect compared to DETC treated rats (Fig 28B). There was no effect in response to candesartan on diuresis and natriuresis in rats treated with tempol alone (Fig. 28B). These results demonstrate that phosphorylation of Sp3 protein is not involved in nuclear translocation of Sp3 in DETC treated rats.

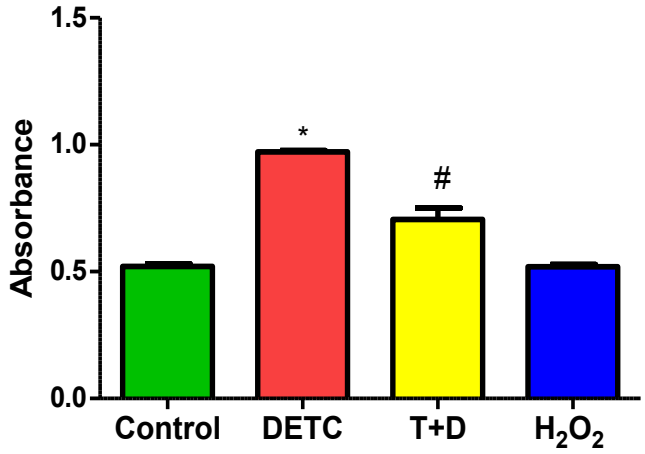


Figure 6. SOD-inhibitor DETC, H_2O_2 and tempol do not cause toxicity in HK2 cells.

Cells were treated with DETC (500μM, 2 h), H_2O_2 (50μM, 30 min), and tempol (1mM, 2 h). Cells were pre-treated with tempol for 10 min before adding DETC. DETC = Diethyldithiocarbamate, T = Tempol, D = DETC, H_2O_2 = Hydrogen peroxide. Commercial kit as described in Methods was used to detect cell toxicity. Absorbance at 490 nm is plotted as bar graph. Bars represent results as mean ± SEM, *P<0.05*. *significantly different from control, #significantly different from DETC. Data were analyzed by one-way ANAOVA followed by post hoc Newman-Keuls analysis. N = 3 separate experiments.

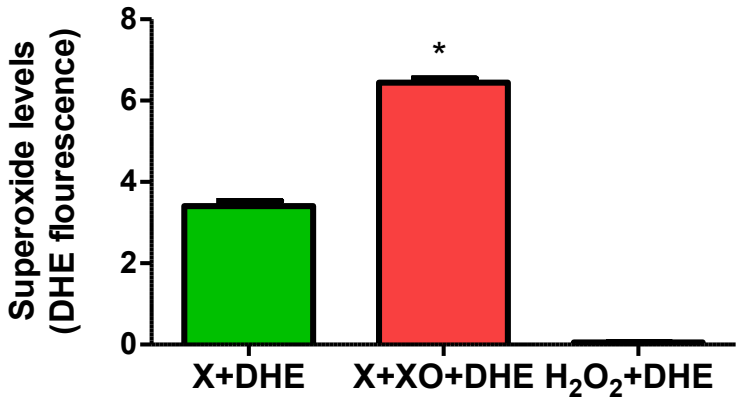


Figure 7. Superoxide producing system xanthine/xanthine oxidase increases DHE fluorescence levels in cell-free system.

Xanthine and XO were dissolved in KH buffer containing DHE probe (25 μM) and fluorescence was measured by spectrofluorometer using excitation 490 and emission 610 nm wavelengths. (Detail in methods). X = Xanthine, XO = Xanthine oxidase, DHE = Dihydroethidium, H_2O_2 = Hydrogen peroxide. Bars represent results as mean ± SEM. *P<0.05,* *significantly different from X+DHE. Data were analyzed by one-way ANAOVA followed by post hoc Newman-Keuls analysis. N = 3 separate experiments.

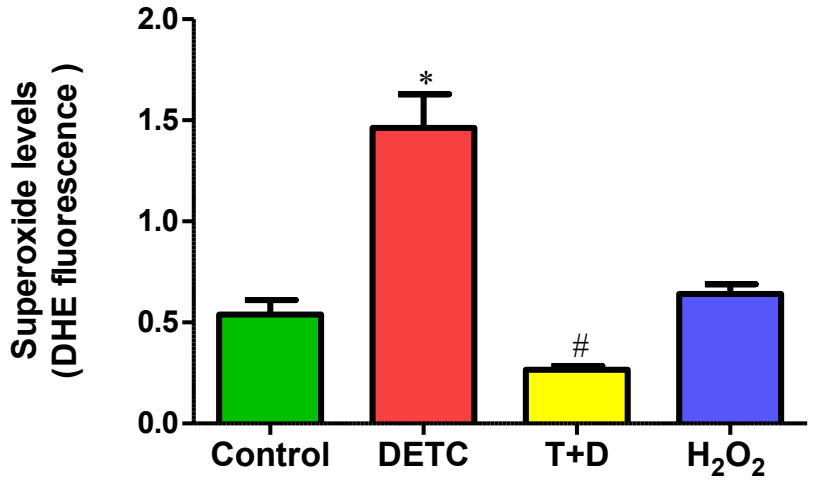


Figure 8. SOD- inhibitor DETC increases DHE fluorescence (superoxide levels) in HK2 cells.

Cells were loaded with DHE probe followed by treatment with SOD- inhibitor DETC in the absence and presence of tempol, and H_2O_2 and DHE fluorescence levels were measured. (Drug treatments and DHE measurement are described in Methods). DETC = Diethyldithiocarbamate, T = Tempol, D = DETC, H_2O_2 = Hydrogen peroxide, DHE = Dihydroethidium. Bars represent results as mean ± SEM, *P<0.05*. *significantly different from control, #significantly different from DETC. Data were analyzed by one-way ANAOVA followed by post hoc Newman-Keuls analysis. N = 5 separate experiments.

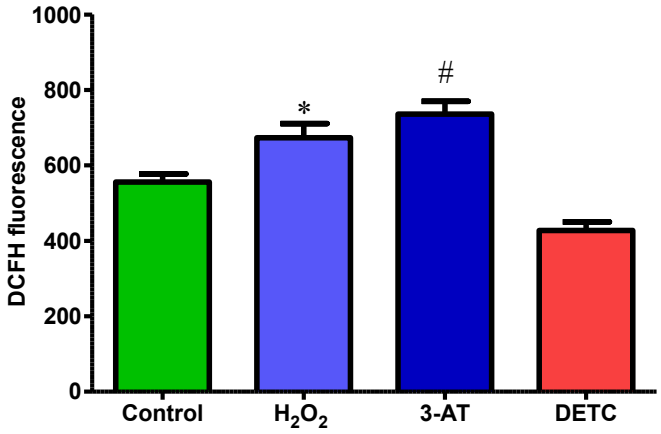


Figure 9. SOD- inhibitor DETC does not increase DCFH fluorescence in HK2 cells.

Cells were loaded with DCFHDA probe followed by treatment with H_2O_2, catalase inhibitor 3-AT, and DETC, and DCFH fluorescence levels were measured (Drug treatments, and DCFHDA measurements are described in Methods). DETC = Diethyldithiocarbamate, T = Tempol, D = DETC, H_2O_2 = Hydrogen peroxide, DCFHDA = Dichloro-dihydro-fluorescein diacetate, 3-AT = 3-Amino-1,2,4-triazole. Bars represent results as mean ± SEM, *P<0.05*. Data were analyzed by one way ANAOVA followed by post hoc Newman-Keuls analysis. *Significantly different from control, #significantly different from control. N = 5 separate experiments.

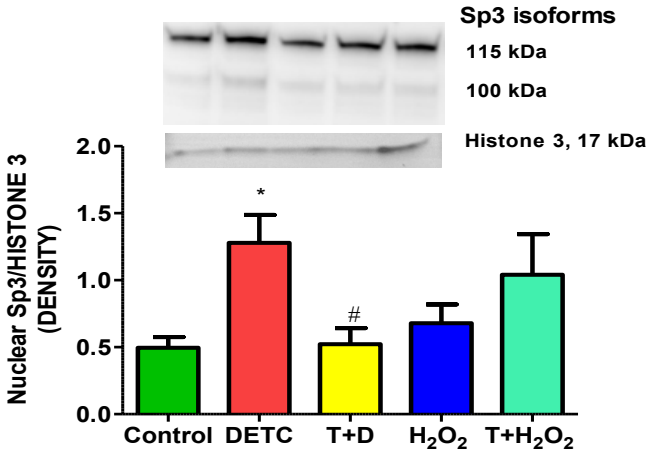


Figure 10. SOD- inhibitor DETC increases nuclear levels of Sp3 protein in HK2 cells.

Cells were treated with DETC, and H_2O_2 in the absence and presence of tempol, nuclear proteins isolated and Sp3 levels determined by Western blotting (*Details in methods*). Upper panel: representative blot of Sp3 (top) and nuclear protein loading control histone 3 (bottom). Lower panel: bars represent ratios of the densities of Sp3 and histone 3 proteins bands. Both Sp3 protein bands were considered for quantification. DETC = Diethyldithiocarbamate, T = Tempol, D = DETC, H_2O_2 = Hydrogen peroxide. Results are mean ± SEM. *P<0.05*, *significantly different from control, #significantly different from DETC. Data were analyzed by one-way ANAOVA followed by post hoc Newman-Keuls analysis. N = 5 separate experiments.

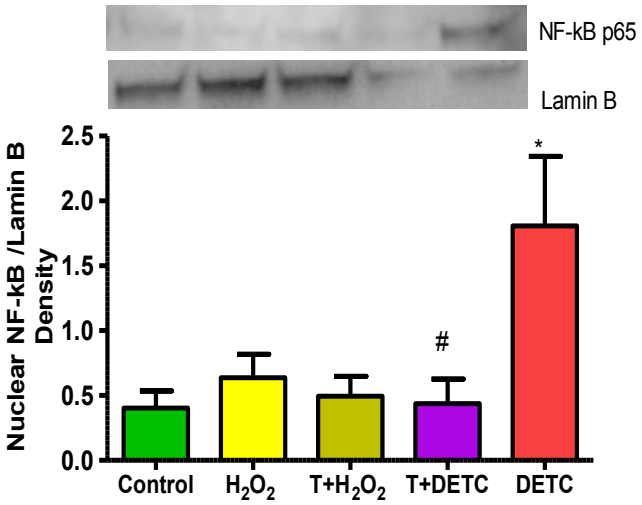


Figure 11. SOD- inhibitor DETC increases nuclear levels of NF-κB accumulation in HK2 cells.

Cells were treated with DETC, and H_2O_2 in the absence and presence of tempol, nuclear proteins isolated and NF-κB levels determined by Western blotting (*Details in methods*). Upper panel: representative blot of NF-κB (top) and nuclear protein loading control lamin B (bottom). Lower panel: bars represent ratios of the densities of NF-κB and histone 3 proteins bands. DETC = Diethyldithiocarbamate, T = Tempol, D = DETC, H_2O_2 = Hydrogen peroxide. Results are mean ± SEM. *P<0.05*, *significantly different from control, #significantly different from DETC. Data were analyzed by one-way ANAOVA followed by post hoc Newman-Keuls analysis. N = 4 separate experiments.

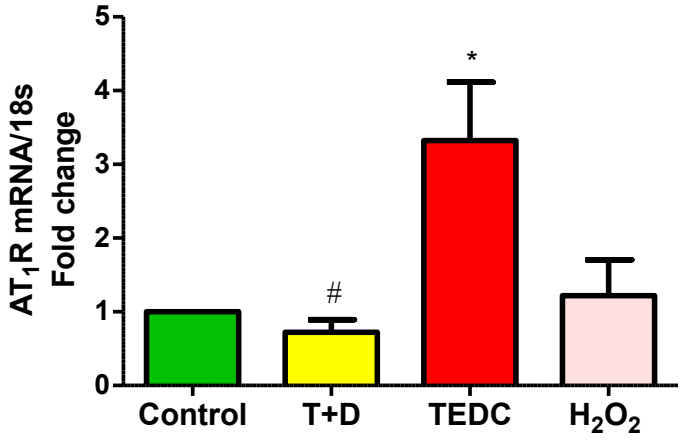


Figure 12. SOD- inhibitor DETC increases AT1 receptor mRNA expression in HK2 cells.

Cells were treated with DETC and H_2O_2 in the absence and presence of tempol and AT1 receptor mRNA was determined by RT-qPCR (*Details in methods*). Reference gene 18S was used to normalize the data. DETC = Diethyldithiocarbamate, T = Tempol, D = DETC, H_2O_2 = Hydrogen peroxide. Bars represent results as mean ± SEM. *P<0.0.05*, *significantly different from control, #significantly different from DETC. Data were analyzed by one-way ANAOVA followed by post hoc Newman-Keuls analysis. N = 4 separate experiments.

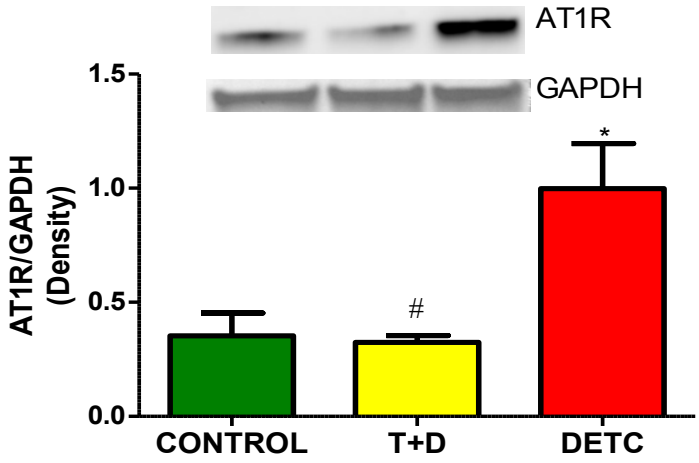


Figure 13. SOD- inhibitor DETC increases AT1 receptor protein levels in HK2 cells.

Cells were treated with DETC in the absence and presence of tempol and cell lysate was used to measure AT1 receptor protein by Western blotting (*Details in methods*). Upper panel: representative blot of AT1 receptor (top) and loading control GAPDH (bottom). Lower panel: bars represent ratios of the densities of AT1 receptor and GAPDH proteins bands. DETC = Diethyldithiocarbamate, T = Tempol, D = DETC. Results are mean ± SEM. *P<0.05*, *significantly different from control, #significantly different from DETC. Data were analyzed by one-way ANAOVA followed by post hoc Newman-Keuls analysis. N = 4 separate experiments.

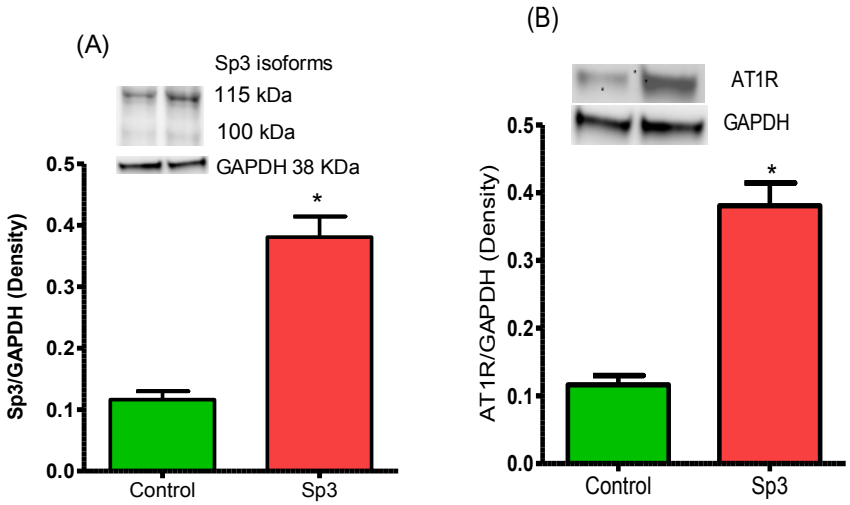


Figure 14. Sp3 overexpression increases AT1 receptor protein expression in HK2 cells.

HK2 cells were transfected with Sp3 plasmid and cell lysate was used to measure Sp3 (A) and AT1 receptor (B) proteins by Western blotting (*Details in methods*). Upper panels: representative blots of Sp3 (A), AT1 receptor (B) and protein loading controls GAPDH (A, B). Lower panels: bars represent ratios of the densities between Sp3 and GAPDH (A) and AT1 receptor and GAPDH (B). Both the Sp3 protein bands were considered for quantification (A). Results are mean ± SEM, *P<0.05*. Data were analyzed by Student's t-test. *Significantly different from control. N = 4 separate experiments.

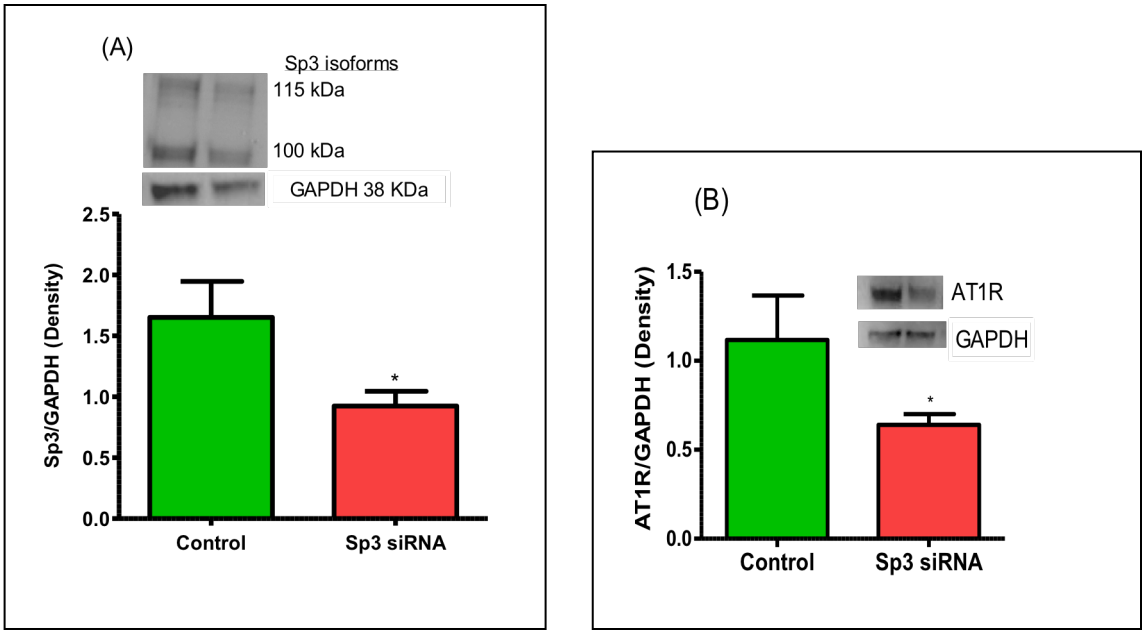


Figure 15. Sp3 siRNA decreases AT1 receptor protein expression in HK2 cells.

HK2 cells were transfected with Sp3 and control siRNAs and cell lysate was used to measure protein levels of Sp3 (A) and AT1 receptor (B) by Western blot (*Details in methods*). Upper panels: representative blots of Sp3 (A), AT1 receptor (B) and protein loading control GAPDH (A, B). Lower panels: bars represent ratios of the densities between Sp3 and GAPDH (A) and AT1 receptor and GAPDH (B). Both the Sp3 protein bands were considered for quantification (A). Results are mean ± SEM, *P<0.05.* Data were analyzed by Student's t-test. *significantly different from control. N = 3-5 separate experiments.

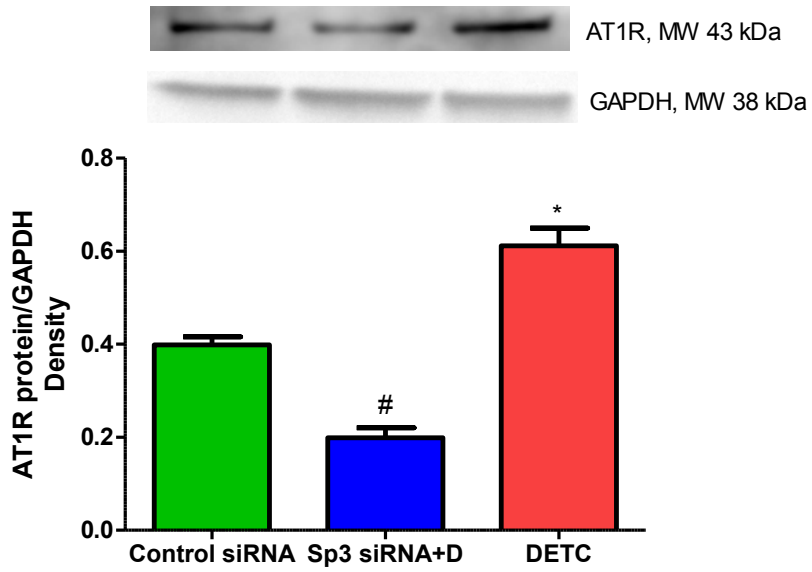


Figure 16. Sp3 siRNA in the presence of SOD- inhibitor DETC decreases AT1 receptor protein expression in HK2 cells.

Cells were transfected with Sp3 and control siRNAs (100ng, 24 h) followed by DETC treatment (500μM, 2 h) and cells lysate was used to measure protein levels of AT1 receptor and Sp3 (data not shown) by Western blotting. Upper panel: representative blot of AT1 receptor and loading control GAPDH. Lower panel: bars represent ratios of the densities of AT1 receptor and GAPDH proteins bands. DETC = Diethyldithiocarbamate. Results are mean ± SEM. *P<0.05*, *significantly different from control, #significantly different from DETC, N = 3 separate experiments.

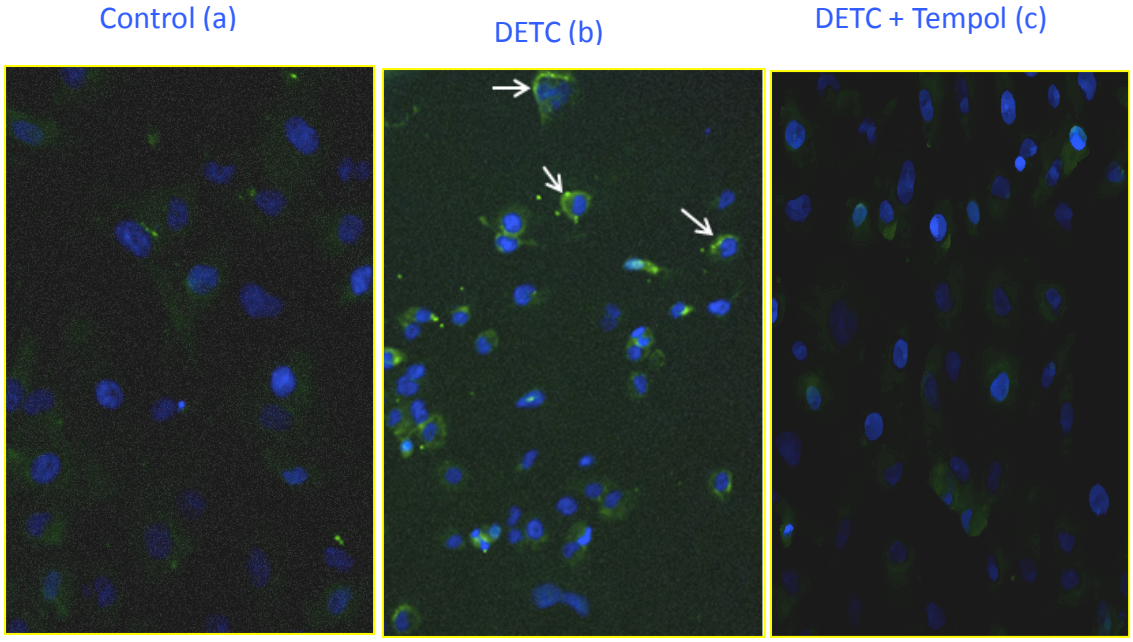


Figure 17. SOD- inhibitor DETC increases cell surface AT1 receptor levels in HK2 cells.

Cells were treated with vehicle (control) and DETC in the absence and presence of tempol (*Details in the methods)*. Cells were fixed with formalin followed by labeling with primary AT1 receptor and secondary Alexa488 conjugated antibodies. Cells were mounted with media containing nuclear dye DAPI. The AT1 receptor antibody used recognizes extracellular loop of the receptor. Arrows in panel b show cell surface AT1 receptor (green). Nuclei are blue stained with DAPI (panels: a, b and c). DETC = Diethyldithiocarbamate, N = 4 separate experiments.

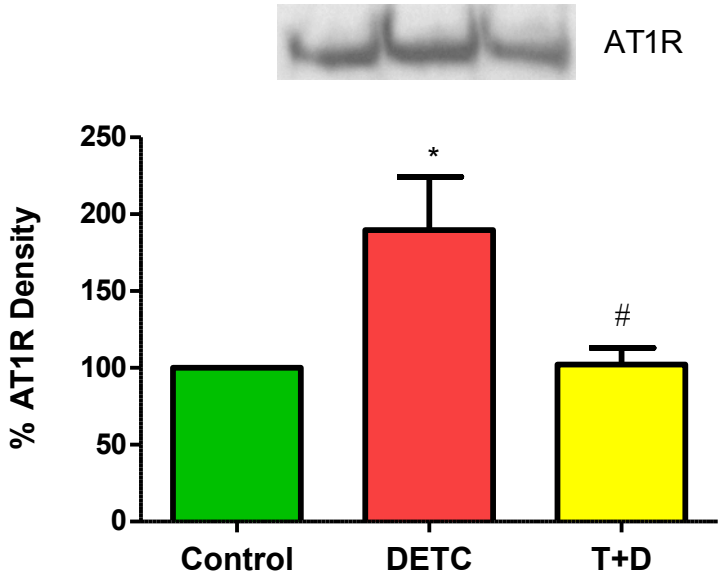


Figure 18. SOD- inhibitor DETC increases cell surface AT1 receptor levels in protein in HK2 cells.

Cell surface proteins were biotinylated and isolated with streptavidin-conjugated magnetic beads followed by measuring AT1 receptor proteins by Western blotting (*details in methods*). Upper panel: representative blot of AT1 receptor. Lower panel: bars represent the densities of AT1 receptor protein bands. DETC = Diethyldithiocarbamate, T = Tempol, D = DETC. Results are mean ± SEM. *P<0.05*, Data were analyzed by one-way ANAOVA followed by post hoc Newman-Keuls analysis. *significantly different from control, #significantly different from DETC. N = 5 separate experiments.

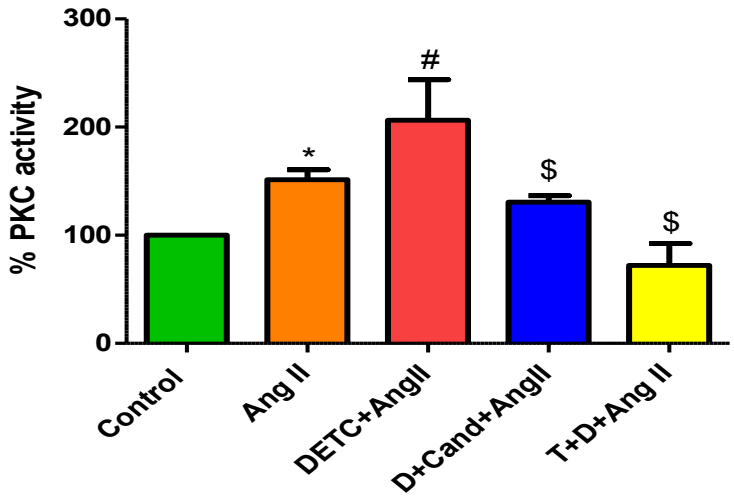


Figure 19. SOD- inhibitor DETC increases angiotensin II mediated PKC activity in HK2 cells.

Cells were treated with vehicle and DETC in the absence and presence of candesartan (Cand), and tempol. Thereafter, cells were treated with or without angiotensin II (Ang II), lysates obtained and used to measure PKC activity (*Details in methods*). Ang II = Angiotensin II, Cand = Candesartan, DETC = Diethyldithiocarbamate, T = Tempol, D = DETC. Bars represent results as mean ± SEM. Data were analyzed by one-way ANAOVA followed by post hoc Newman-Keuls analysis. *P<0.05*, *significantly different from control, #significantly different from Ang II, $significantly different from DETC+Ang II. N = 3-6 separate experiments.

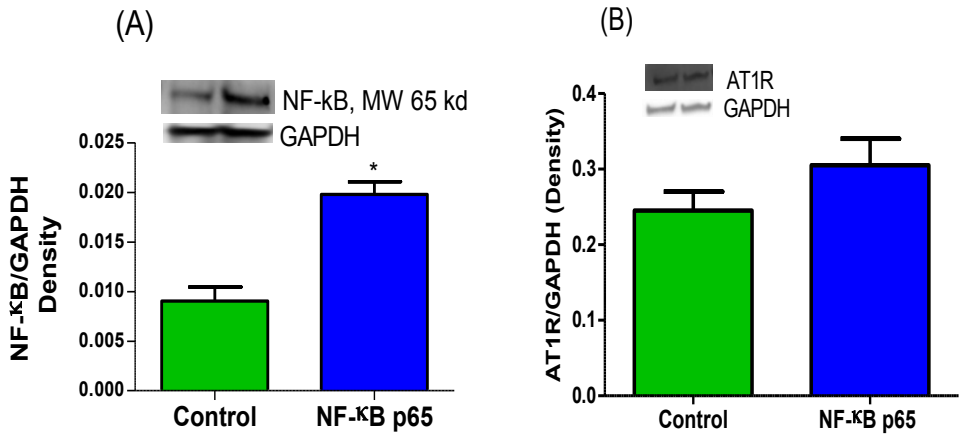
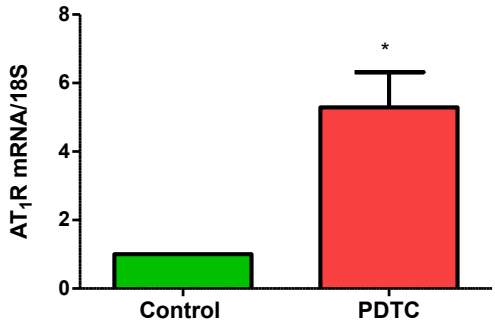


Figure 20. NF-κB p-65 overexpression does not increase AT1 receptor protein expression in HK2 cells.

HK2 cells were transfected with NF-κB p-65 plasmid and cell lysate was used to measure NF-κB (A) and AT1 receptor (B) proteins measured by Western blotting (*Details in methods*). Upper panels: representative blots of NF-κB (A), AT1 receptor (B) and protein loading controls GAPDH (A, B). Lower panels: bars represent ratios of the densities between NF-κB and GAPDH (A) and AT1 receptor and GAPDH (B). Results are mean ± SEM. *P<0.05*, Data were analyzed by Student's t-test. *significantly different from control. N = 3 separate experiments.

(A)



(B)

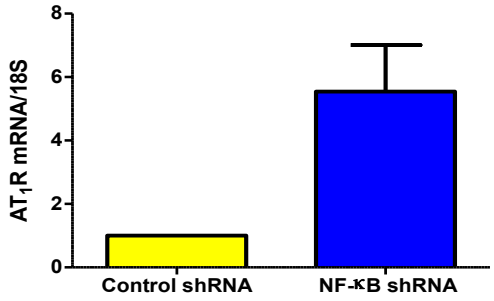
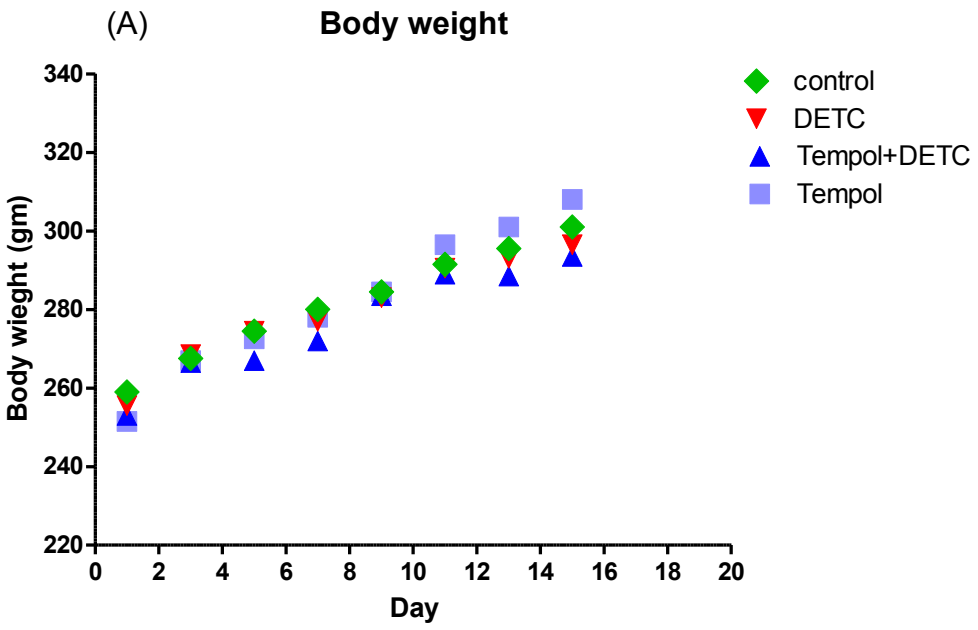
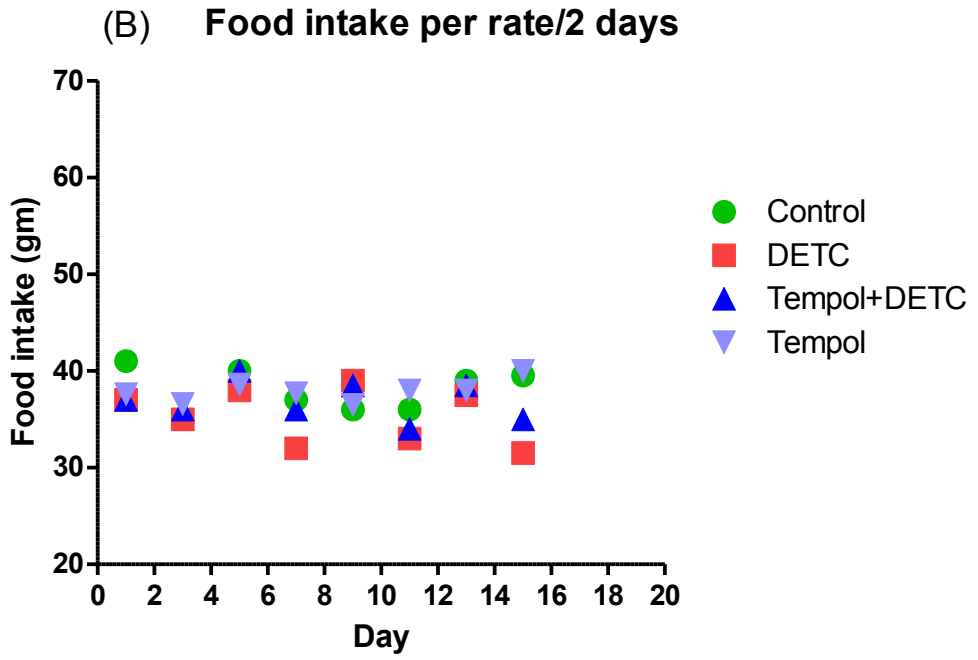


Figure 21. NF-κB shRNA PDTC increases AT1 receptor mRNA expression in HK2 cells.

Cells were treated with PDTC [(a) an NF-κB inhibitor] and NF-κB shRNA (b) and AT1 receptor mRNA was determined by RT-qPCR (*Details in methods*). Reference gene 18S was used to normalize the data. PDTC = Pyrrolidinedithiocarbamate. Bars represent results as mean ± SEM. *P<0.05*, *significantly different from control, Data were analyzed by Student's t-test. N = 3-4 separate experiments.


(A)
Body weight
Body wieght (gm)
340
320
300
280
260
240
220
0
2
4
6
8
10
12
14
16
18
20
Day
control
DETC
Tempol+DETC
Tempol


(B)
Food intake per rate/2 days
Food intake (gm)
70
60
50
40
30
20
0
2
4
6
8
10
12
14
16
18
20
Day
Control
DETC
Tempol+DETC
Tempol

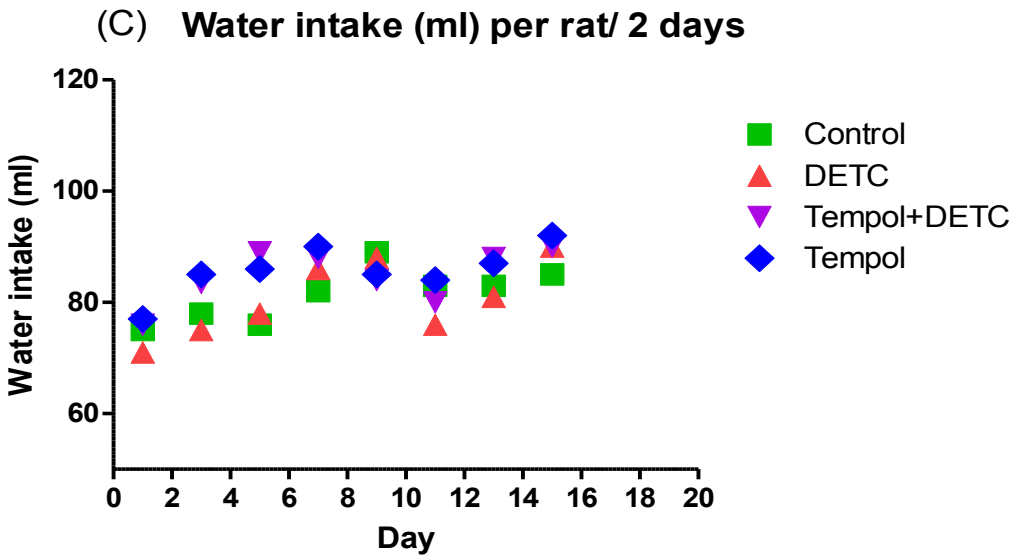


Figure 22. (A, B, C). SOD- inhibitor DETC with or without tempol does not affect body weight, food or water intake in SD rats.

SD rats were treated with vehicle and DETC in the absence and presence of tempol, and body weight, food, and water intake were measured every alternate day. (*Details in methods*). DETC = Diethyldithiocarbamate. Results as mean ± SEM, $P<0.05$. Data were analyzed by one-way ANAOVA followed by Bonferroni post-test analysis. N = 10 rats in each group.

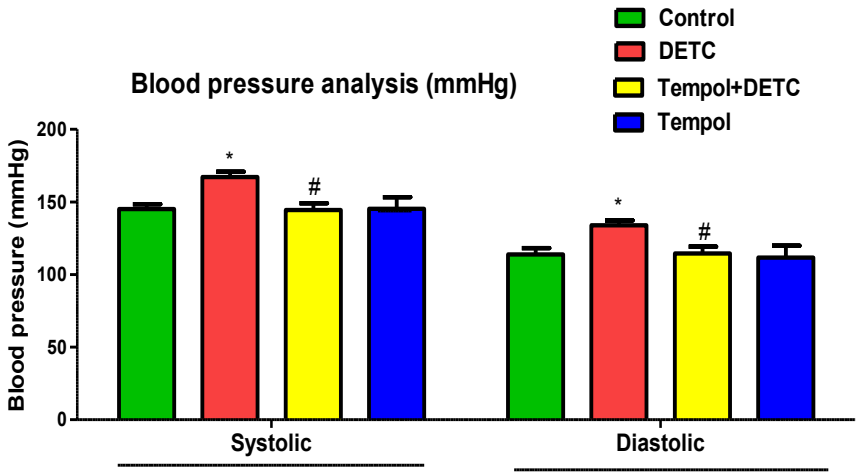


Figure 23. SOD- inhibitor DETC increases blood pressure in SD rats.

SD rats were treated with vehicle and DETC in the absence and presence of tempol and blood pressure (systolic and diastolic) was measured in anesthetized rats. (*Details in methods*). DETC = Diethyldithiocarbamate. Bars represent results as mean ± SEM, *P<0.05*. Data were analyzed by two-way ANOVA followed by Bonferroni post-test analysis. *significantly different from control, #significantly different from DETC. N = 5-8 rats in each group.

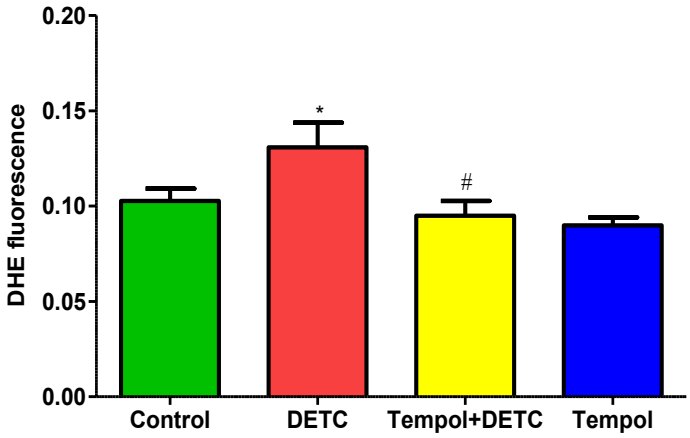


Figure 24. SOD- inhibitor DETC increases DHE fluorescence in renal tissues of SD rats.

SD rats were treated with vehicle and DETC in the absence and presence of tempol, tissue homogenate prepared and used to measure superoxide levels using DHE probe. (*Details in methods*). DETC = Diethyldithiocarbamate, DHE = Dihydroethidium. Bars represent results as mean ± SEM, *P<0.05*. Data were analyzed by one-way ANAOVA followed by post hoc Newman-Keuls analysis. *Significantly different from control, #significantly different from DETC, N = 4-6 rats in each group.

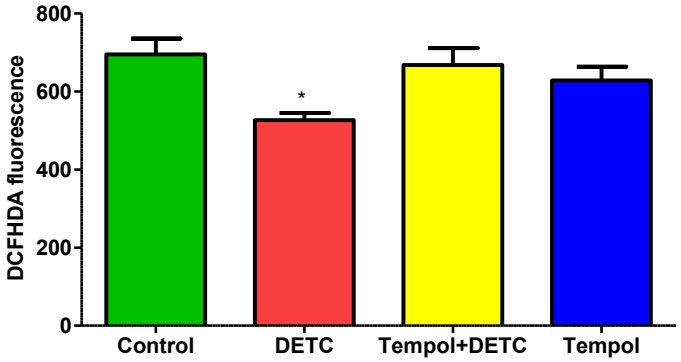


Figure 25. SOD- inhibitor DETC does not increase DCFH fluorescence in renal tissues of SD rats.

SD rats were treated with vehicle and DETC in the absence and presence of tempol, tissue homogenate prepared and used to measure H_2O_2 levels using DCFHDA probe. (*Details in methods*). DETC = Diethyldithiocarbamate, H_2O_2 = Hydrogen peroxide, DCFHDA = Dichloro-dihydro-fluorescein diacetate. Bars represent results as mean ± SEM, *P<0.05*. Data were analyzed by one-way ANAOVA followed by post hoc Newman-Keuls analysis. *Significantly different from control, N = 6 rats in each group.

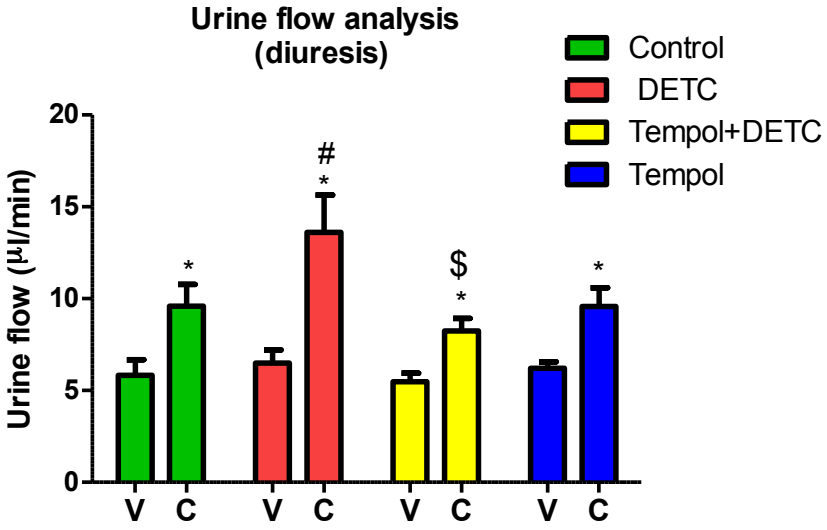


Figure 26. SOD-inhibitor DETC increases urine flow (diuresis) in response to *candesartan* in SD rats.

Urine flow in response to AT1 receptor antagonist candesartan (10 µg/kg iv; bolus) was determined in anesthetized rats. (*Details in methods*). V = average of two 30-min saline infusion periods. C = average of four 30-min drug (candesartan) infusion periods. DETC = Diethyldithiocarbamate. Bars represent results as mean ± SEM, *P<0.05*. Data were analyzed by two-way ANAOVA followed by Bonferroni post-test analysis. *Significantly different from V (vehicle), #significantly different from control C, $ significantly different from DETC C. N = 5-8 rats in each group

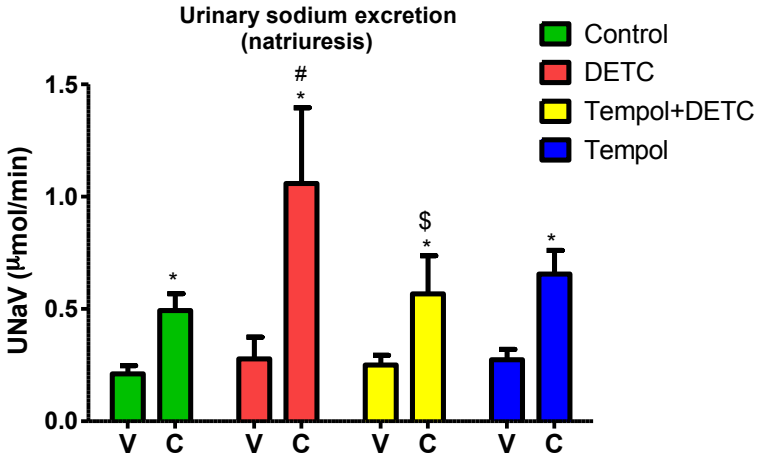


Figure 27. SOD-inhibitor DETC increases natriuresis (UNaV) in response to candesartan in SD rats.

Natriuresis or urinary sodium excretion (UNaV) in response to AT1 receptor antagonist candesartan (10 µg/kg iv bolus) was determined in anesthetized rats. (*Details in methods*). V = average of two 30-min period of saline infusion. C = average of four 30-min drug infusion after a bolus dose of candesartan was infused. DETC = Diethyldithiocarbamate. Bars represent results as mean ± SEM, *P<0.05*. Data were analyzed by two-way ANAOVA followed by Bonferroni post-test analysis. *Significantly different from V (vehicle), #significantly different from control C, $ significantly different from DETC C, N = 5-8 rats in each group.

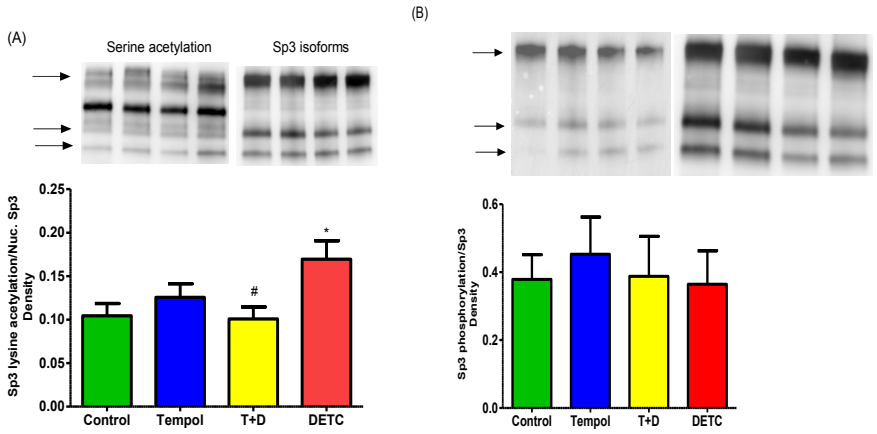


Figure 28. SOD- inhibitor DETC increases lysine acetylation and not phosphorylation of Sp3 in renal tissues of SD rats.

Nuclear extracts were prepared from renal tissues and used to immunoprecipitate Sp3 with Sp3 antibody. Sp3 protein-antibody complexes were separated by SDS-PAGE and analyzed by Western blotting using anti-pan-acetyl lysine (A) and anti-phosphorylated (B) antibodies (*Details in methods*). Subsequently, blots were stripped and re-probed with polyclonal anti-Sp3 antibody. (A) Upper panels: representative blots of pan-acetyl lysine of Sp3 (*left)* and Sp3 protein (*(right)*. Sp3 acetylated (left) and Sp3 protein (right) bands are indicated with arrows. (B) Upper panels: representative blots of phosphorylated Sp3 *(left)* and Sp3 protein *(right)*. Phosphorylated Sp3 (left) and Sp3 protein bands are indicated with arrows. Lower panels (A, B): Quantification of acetylated-Sp3 (A) and phosphorylated-Sp3 (B). Bars are ratios between the

densities of acetylated-Sp3 and Sp3 protein (A), and phosphorylated-Sp3 and Sp3 proetin bands (B). Results are mean ± SEM, *P<0.05*. Data were analyzed by one-way ANOVA followed by post hoc Newman-Keuls analysis. N = 3-4 rats. DETC = Diethyldithiocarbamate, T = Tempol, D = DETC. *Significantly different from control, #significantly different from DETC, N = 3-4 rats in each group.

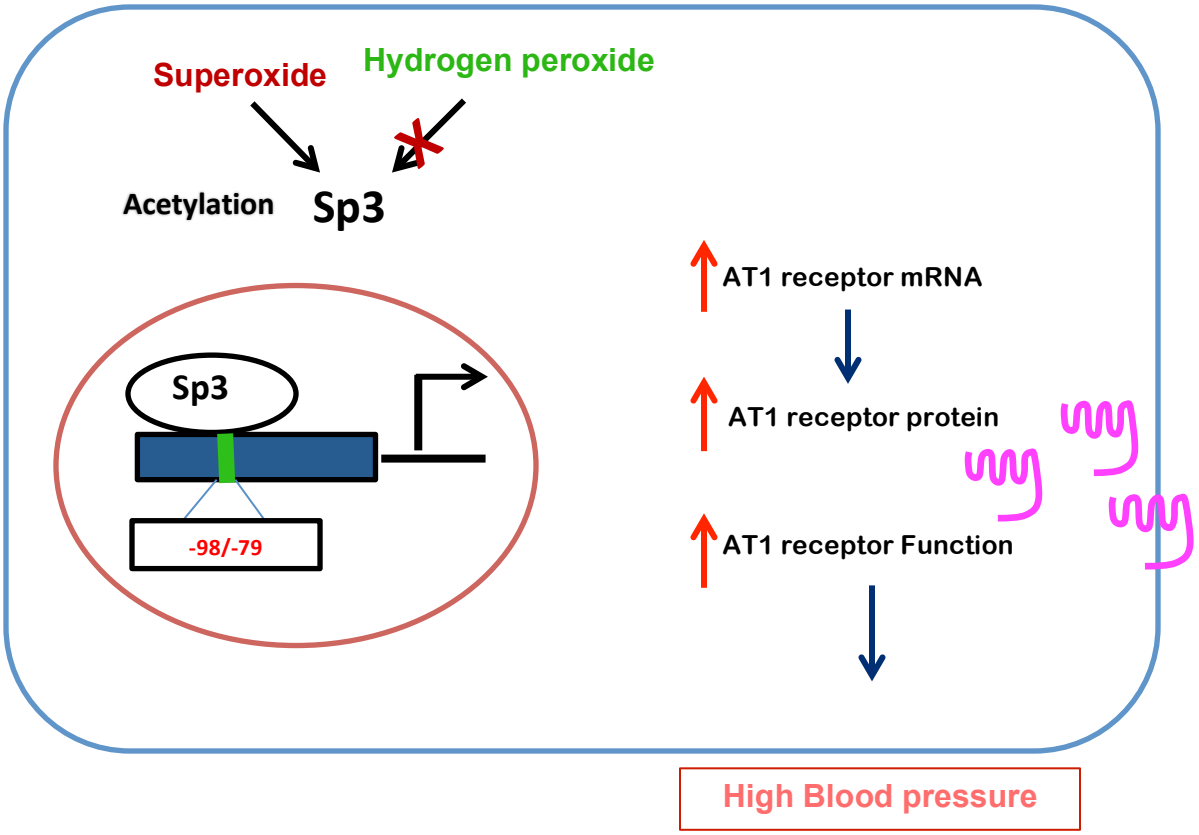


Figure 29. RESULTS SUMMARY

5. DISCUSSION

Reactive oxygen species (ROS) burden (also known as oxidative stress) is known to increase renal AT1 receptor function that contributes to hypertension (Chugh et al., 2011, 2012; Montezano & Touyz, 2014; Nickenig & Harrison, 2002). However, it is not known which of the ROS, namely superoxide or H_2O_2, mediates this effect. Especially there are contrasting reports regarding the role of H_2O_2 in hypertension (Y. Chen et al., 2007; Park et al., 2015; Schroder et al., 2012). Further, AT1 receptor gene promoter possesses putative binding sites for redox-sensitive Sp3 and NF-κB transcription factors (TFs) (Cowling et al., 2005; Kambe et al., 2004), however, which one of them is involved in regulating renal AT1 receptor function is not known. Therefore, present studies were designed to examine roles and mechanisms of ROS (superoxide vs. H_2O_2) as well as TFs (Sp3 vs. NF-κB) in regulating renal AT1 receptor function. First, renal cells (HK2 cells) were used to determine ROS and TFs specificity in regulation of AT1 receptor function. Herein, we investigated the roles of superoxide and H_2O_2 in renal AT1 receptor mRNA and protein expressions and found that superoxide (DETC effect) but not H_2O_2 increases AT1 receptor mRNA and protein levels. Superoxide (DETC effect) also increased membranous abundance of AT1 receptors. In addition, PKC activity was measured as an index of AT1 receptor

function. As indicated earlier that AT1 receptor promoter possesses binding sites for Sp3 and NF-κB, we elucidated their roles in AT1 receptor protein expression. For that, we transfected HK2 cells with Sp3 plasmid, and Sp3 siRNA separately and AT1 protein levels were measured. We found that increasing Sp3 protein levels increases while its depletion decreases AT1 receptor proteins in HK2 cells. Similar approach was employed to identify the role of NF-κB in the protein expression of AT1 receptor and found that NF-κB plasmid although increases its protein levels but fails to increase the levels of AT1 receptors. Taken together, studies in HK2 cells suggest that superoxide by involving Sp3 causes up-regulation of renal AT1 receptor function. We further investigated the effect of superoxide radical on renal AT1 receptor function and blood pressure in Sprague Dawley (SD) rats. We also determined lysine-acetylation and serine-phosphorylation of Sp3 in renal cortex as these have been linked in Sp3 activation (Ehlting, Haussinger, & Bode, 2005; Pages, 2007).

To investigate the effects of ROS and their specificity (superoxide vs. H_2O_2) in the activation of transcription factors (Sp3 and NF-κB) and renal AT1 receptor up-regulation, we used DETC (an SOD inhibitor) to increase superoxide levels in HK2 cells. SOD is a potent antioxidant enzyme, which scavenges superoxide radicals and contributes to maintain cellular redox homeostasis

(Valdivia, Perez-Alvarez, Aroca-Aguilar, Ikuta, & Jordan, 2009). DETC is an SOD inhibitor and previously has been used to increase superoxide levels in cell culture studies (A. D. Kim et al., 2014; Lapo et al., 2014). First, we confirmed the specificity of DHE probe for the detection of superoxide radical using xanthine and xanthine oxidase in a cell free system. Xanthine and xanthine oxidase system has previously been employed to generate superoxide radicals (Galbusera, Orth, Fedida, & Spector, 2006; Lacy, Gough, & Schmid-Schonbein, 1998). Our results demonstrated that xanthine and xanthine oxidase significantly increased DHE fluorescence (Fig. 7). Moreover, we treated HK2 cells with DETC, and H_2O_2 and measured superoxide levels with DHE probe. Our results showed that DETC significantly increased superoxide levels in HK2 cells compared to control (Fig. 8). Importantly, tempol treatment significantly reduced the levels of superoxide in DETC treated cells (Fig. 8), suggesting that tempol efficiently scavenges superoxide. Tempol is a known antioxidant with SOD mimetic properties, which has extensively been used to scavenge superoxide in cells culture as well as in hypertensive animal models (Tawa, Shimosato, Iwasaki, Imamura, & Okamura, 2015; Wilcox, 2010). Enrique Cadenas and others have suggested that H_2O_2 might convert into superoxide, which can potentially increase superoxide levels. H_2O_2 treatment of HK2 cells did not increase DHE

fluorescence levels (Fig. 8) suggesting that H_2O_2 does not get converted into superoxide in HK2 cells and DHE is a specific probe for superoxide detection. Further, to determine cellular levels of H_2O_2, DCFHDA probe was used. DCFHDA is taken up by cells and is converted to DCFH by diesterases, which then gives fluorescence. Since there is controversy regarding DFCHDA as specific probe for H_2O_2 (L. E. George et al., 2012; Gough & Cotter, 2011; Lasagni Vitar et al., 2015; Taguchi, Ogura, Takanashi, Hashizoe, & Honda, 1996; Takanashi, Ogura, Taguchi, Hashizoe, & Honda, 1997), HK2 cells were treated with H_2O_2, 3-AT catalase inhibitor that increases endogenous H_2O_2 levels, and DETC separately. We found that cells treated with H_2O_2, and 3-AT showed increased DCFH fluorescence compared to control (Fig. 9). However, DETC treatment did not increase DCFH fluorescence, suggesting DCFHDA is a specific probe for H_2O_2 (Fig. 9).

Many studies have demonstrated that ROS (termed oxidative stress) regulate a myriad of genes by modulating the activity of various redox-sensitive transcription factors such as nuclear factor kappa B p-65 (NF-κB), Sp3, NRF2, and activator protein (AP)-1 (Banday & Lokhandwala, 2015; Bhatt et al., 2014; Lavrovsky, Chatterjee, Clark, & Roy, 2000). However, specificity of ROS (superoxide vs. H_2O_2) in their activation has never been addressed. Our results

demonstrated that superoxide (DETC effect) but not H_2O_2 increased the nuclear accumulation of Sp3 and NF-κB proteins (index of their activation) in HK2 cells (Fig. 10, 11). Moreover, SOD-mimetic tempol treatment attenuated nuclear accumulation of both of these transcription factors, suggesting role of superoxide in this phenomenon. There are reports attributing either lysine acetylation and/or serine phosphorylation as being the mechanism of nuclear translocation of transcription factors (Ehlting et al., 2005; Pages, 2007). Therefore, we determined lysine-acetylation and phosphorylation of Sp3 in nuclear fractions of renal tissues and found lysine-acetylation of Sp3 as a mechanism of its activation in response to superoxide (DETC effect).

Modulation of redox-sensitive transcription factors has been implicated in pathological conditions such as atherosclerosis, hypertension, diabetes and other cardiovascular disorders (C. K. Sen & Packer, 1996). Recent in vitro studies evidenced that oxidative stress activates Sp3 transcription factor (Lee et al., 2006; Ryu et al., 2003) that in turn increases AT1 receptor transcription. However, the link between Sp3 and AT1 receptor up-regulation is not well known. Computer analysis and EMSA assays have demonstrated that transcription factor Sp3 binds at the AT1 receptor promoter and regulates its gene expression (Kambe et al., 2004). We also found that Sp3 plasmid

increases, while its depletion with Sp3 siRNA decreases AT1 protein levels in HK2 cells (Fig. 14, 15). Studies conducted in Zhao et el lab reported similar results showing that Sp3 transcription factor regulate basal AT1 receptor expression in H295-R cell line (Zhao et al., 2000, 2001).

NF-κB is another redox-sensitive transcription factor involved in the modulation of myriad of genes responsible for stress and immune responses, apoptosis, and cell proliferation and survival (Hayden & Ghosh, 2004). Recent studies including ours have shown that NF-κB is also involved in cellular antioxidant defenses (Gomez-Cabrera, Domenech, & Vina, 2008; Laughlin et al., 1990; Lavrovsky, Song, Chatterjee, & Roy, 2000; Morceau et al., 2004) and helps attenuates blood pressure by improving kidney function (L. George et al., 2009; L. E. George et al., 2012). Numerous studies have implicated NF-κB in the regulation of renal AT1 receptor gene (Bhatt et al., 2014). Therefore, we became interested to determine role of NF-κB in the regulation of AT1 receptor in HK2 cells. Our transfection studies with NF-κB plasmid showed no increment in renal AT1 receptor protein expression, suggesting that NF-κB transcription factor might not be involved in the regulation of renal AT1 receptor at least in HK2 cells (Fig. 20). In order to further investigate the effect of NF-κB on AT1 receptor mRNA expression, we knockdown NF-κB using NF-κB shRNA and NF-κB inhibitor

PDTC. To our surprise we found that depletion of NF-κB gene expression results into increased AT1 receptor mRNA expression (Fig. 21), suggesting an inhibitory effect of NF-κB transcription factor on the regulation of AT1 gene expression in human kidney cells. Further studies are warranted to confirm the role of NF-κB in renal AT1 receptor gene regulation.

As mentioned earlier that a number of studies reported that superoxide and H_2O_2 are the main ROS in causing oxidative stress contributing to high blood pressure or hypertension (Jutooru et al., 2014; Landmesser et al., 2002; Vaziri, Dicus, et al., 2003; Vaziri, Lin, et al., 2003). Moreover, a recent study including ours have shown that oxidative stress up-regulates renal AT1 receptor function and contributes to high blood pressure or hypertension (Chugh et al., 2011, 2012; Q. Su et al., 2016; Sungkaworn, Lenbury, & Chatsudthipong, 2011). In contrast, some other studies reported that H_2O_2 is a vasodilator and attenuates hypertension (Y. Chen et al., 2007; Park et al., 2015; Schroder et al., 2012). To our knowledge, specificity of ROS (superoxide vs. H_2O_2) in the up-regulation of renal AT1 receptor function has not been investigated. To test the specificity of superoxide and H_2O_2 in the up-regulation of renal AT1 receptor expression and function, we conducted studies in HK2 cells. First, we measured AT1 receptor mRNA expression and found that superoxide (DETC effect) increased mRNA

expression (Fig. 12). This effect was attenuated by SOD-mimetic tempol suggesting role of superoxide in over-expression of renal AT1 receptor mRNA (Fig. 12). H_2O_2 failed to increase AT1 receptor mRNA levels suggesting that H_2O_2 perhaps does not play a role in this phenomenon in HK2 cells (Fig. 12) To correlate mRNA data we measured AT1 receptor protein levels and found that superoxide (DETC effect) also increased the levels of AT1 receptor protein (Fig. 13). This effect was attenuated with SOD-mimetic tempol (Fig. 13). Interestingly, superoxide (DETC) mediated Sp3 activation was associated with an increase in renal AT1 receptor mRNA and protein expressions in HK2 cells. H_2O_2 failed to cause Sp3 activation and AT1 receptor expressions. Taken together, these results suggest that superoxide via Sp3 transcription factor regulates renal AT1 receptor expression.

Functional receptors reside on the cell membranes. Therefore, we determined whether DETC (superoxide effect) affects the abundance of membranous AT1 receptor levels and its function. The levels of membranous AT1 receptors were determined by biotinylation and imaging methodological approaches. Cell surface membrane proteins were isolated by cross-linking with biotin followed by determining AT1 receptor by Western blotting. Our results showed that superoxide (DETC effect) increased membranous AT1 receptor

protein levels that were attenuated with SOD-mimetic tempol (Fig. 17). Moreover, imaging studies demonstrated that superoxide (DETC effect) increased the fluorescence levels of AT1 receptor protein in the cell membranes, and SOD-mimetic tempol attenuated this effect of superoxide [(DETC effect) (Fig. 18)]. These results suggest that superoxide (DETC effect) increases cellular as well as membranous levels of AT1 receptor. Finally, to examine whether superoxide-induced increase in the levels of membranous AT1 receptor is functional, PKC activity in response to AT1 receptor ligand ang II was measured. Measuring PKC activity in response to ang II is an index of AT1 receptor function (Mehta and Griendling 2007, (I. D. Pokkunuri et al., 2013). We found that ang II increased PKC activity in the absence of DETC, which was more pronounced in DETC treated cells (Fig. 19). This effect was attenuated by SOD-mimetic tempol and AT1 receptor blocker candesartan (Fig. 19). These results suggest that superoxide induced (DETC effect) increase in the levels of membranous AT1 receptors are functional. Also, these results suggest that increase in PKC activity is superoxide and AT1 receptor specific as SOD-mimetic tempol and candesartan attenuate ang II response on PKC, respectively (Fig. 15). To determine whether superoxide (DETC effect) involves Sp3 transcription factor in regulating expression of renal AT1 receptor protein, HK2 cells were transfected

with and without Sp3 siRNA followed by treatment with DETC. We found that superoxide (DETC effect) in the absence of Sp3 siRNA increased the levels of AT1 receptor proteins, which were attenuated in the presence of Sp3 siRNA (Fig. 16). These results suggest that superoxide via Sp3 transcription factor increases renal AT1 receptor protein levels.

Cell culture systems are powerful tool to corroborate findings in animal models. However, cell culture systems pose limitations when it comes to measuring blood pressure. Therefore, we became interested in examining whether superoxide (DETC effect) regulates blood pressure and renal AT1 receptor function in an animal model. Sprague Dawley rats were treated with DETC that by inhibiting SOD increases superoxide levels. Many labs have used DETC to generate superoxide in rats (Gupta & Sharma, 2014; Matsumura et al., 2001). Therefore, we first determined superoxide levels in renal tissues of DETC treated rats using superoxide probe DHE as used in cell culture studies. We found higher levels of superoxide in DETC-treated compared to vehicle-treated rats (Fig. 24). DETC induced increase in superoxide levels were reduced in renal tissues of rats treated with SOD mimetic tempol (Fig. 24). SOD mimetic tempol has been used as an antioxidant in various hypertensive animal models (Chugh et al., 2011; Wilcox, 2010). Further, using H_2O_2 probe DCFHDA we found that

DETC does not increase H_2O_2 levels in renal tissues of DETC treated rats (Fig. 25). Again, these results suggest that DETC specifically increases superoxide levels in renal tissues as well as in HK2 in cultures (Figs. 24, 8).

In order to determine role of superoxide in regulation of blood pressure and renal AT1 receptor function we measured blood pressure and renal AT1 receptor function (diuresis and natriuresis in response to AT1 receptor blocker candesartan) in vehicle and DETC treated rats. Determining natriuresis and diuresis in response to candesartan is an index of renal AT1 receptor function in rats (Chugh et al., 2011). We found high blood pressure in vehicle-treated control rats, which further increased in DETC treated rats (Fig. 23). DETC (superoxide effect) induced increase in blood pressure was attenuated in rats treated with SOD mimetic tempol (Fig. 23). These results suggest that superoxide increases blood pressure in rats. We do not know the reason for high blood pressure in control rats. It could be due to batch of the rats employed in the study.

Studies from Crowley and Coffman and other groups have demonstrated a predominant role of renal AT1 receptor in blood pressure regulation (Coffman & Crowley, 2008; Crowley et al., 2005). Furthermore, studies in our lab have shown that AT1 receptor blocker candesartan increases diuresis and natriuresis in an

aging rat model, aged FBN rats, associated with increased reactive oxygen species (ROS, oxidative stress) and high blood pressure (Chugh et al., 2011, 2012). These studies suggest that increase in the levels of ROS increases renal AT1 receptor function and blood pressure. Therefore, we became interested in determining role of superoxide (one of the ROS) in the regulation of renal AT1 receptor function and blood pressure in adult SD rats. We found that AT1 receptor blocker candesartan increased diuresis and natriuresis in DETC treated SD rats, suggesting that AT1 receptor function is exaggerated in these rats (Figs. 26, 27). Interestingly, SOD-mimetic tempol reduced diuresis and natriuresis in response to candesartan in DETC treated rats (Figs. 26, 27), suggesting involvement of superoxide in exaggerated AT1 receptor function. Previous studies from our lab reported that rats exhibiting high levels of oxidative stress showed Na/K ATPase reduction upon candesartan treatment thereby increased sodium excretion or natriuresis in renal proximal tubules (RPTs) (Banday & Lokhandwala, 2008b). Based on these studies, we speculate that increased natriuresis upon candesartan treatment is due to the reduction of exaggerated Na/K ATPase activity in RPTs of DETC rats.

Lysine acetylation (Ehlting et al., 2005) and serine-73 phosphorylation (Pages, 2007) are attributed to the activation of Sp3 transcription factors.

Therefore, we determined lysine-acetylation and serine-phosphorylation of Sp3 in the renal nuclear extracts of rats. We measured pan-serine-phosphorylation of Sp3, phosphoserine-73 specific antibody was not available. We found increased lysine-acetylated Sp3 in DETC treated rats compared to control rats. SOD-mimetic tempol reduced the levels of lysine-acetylated Sp3 in DETC -treated rats (Fig 28A). On the other hand, we did not find any change in the phosphorylation levels of Sp3 in DETC-treated compared to vehicle-treated or tempol-treated rats (Fig 28B). These results suggest that superoxide (DETC effect) by acetylating lysine residue of Sp3 activates the transcription factor.

Based upon our findings from cell culture and animal studies, we propose that superoxide by lysine-acetylating Sp3 activates the transcription factor. Activated Sp3 translocates to the nucleus and increases AT1 receptor mRNA and protein levels. Increased levels of cellular AT1 receptors increase membranous levels of AT1 receptor that amplifies AT1 receptor response and contributes to hypertension.

6. SUMMARY AND CONCLUSIONS

In vitro studies (HK2 cells)

1. Our results demonstrate that DHE and DCFHDA probes specifically detect superoxide and hydrogen peroxide respectively.
2. Superoxide but not hydrogen peroxide increases nuclear levels of Sp3, and NF-κB transcription factors.
3. Sp3 plasmid increases while Sp3 siRNA decreases AT1 receptor protein expression.
4. Superoxide increases the levels of AT1 receptor mRNA and protein expressions. However, hydrogen peroxide fails to increase AT1 receptor mRNA expression.
5. Sp3 siRNA reduced AT1 receptor protein expression even in the presence of increased superoxide levels.
6. Superoxide in response to Ang II exaggerates PKC activity (an index of measuring AT1 receptor function), which is attenuated both with AT1 receptor blocker candesartan and tempol.
7. NF-κB transcription factor does not show any significant effect on AT1 receptor protein expression, suggesting that this transcription factor does

not up-regulate AT1 receptor protein in HK2 cells.

Animal studies (SD rats)

8. DETC treatment increases superoxide levels in KSC as measured by DHE probe. However, levels of hydrogen peroxide are not higher in DETC treated rats as measured by DCFHDA probe. These results are in consensus with our cell culture studies.
9. Superoxide caused increased acetylation of Sp3 transcription factor in KSC.
10. Candesartan infusion increases diuresis and natriuresis in DETC treated rats, suggesting high levels of superoxide increases AT1 receptor function.
11. Superoxide (DETC treatment) increases blood pressure in SD rats as measured carotid artery catheterization.

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