ROLE OF AGE-ASSOCIATED OXIDATIVE STRESS IN ALTERED RENAL D1 AND AT1 RECEPTOR FUNCTIONS AND HYPERTENSION

A Dissertation Presented to

Department of Pharmacological and Pharmaceutical Sciences

University of Houston

In Partial Fulfillment of the

Requirement for the Degree

Doctor of Philosophy

In

Pharmacology

By

Gaurav Chugh

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ABSTRACT

Blood pressure (BP) and oxidative stress increase with aging. Renal dopamine D1 (D1R) and angiotensin AT1 (AT1R) receptors by maintaining sodium homeostasis regulate blood pressure. Impaired D1R and exaggerated AT1R functions in the kidneys contribute to hypertension in animal models, which also exhibit oxidative stress. However, the role of oxidative stress in age-related hypertension has not been studied. In this study, we hypothesized that age-associated increase in oxidative stress by altering renal D1R and AT1R functions cause high BP in aging.

To test this hypothesis, we measured oxidative stress, BP, and D1 and AT1 receptor functions in adult (3-month) and old (21-month) Fischer 344 X Brown Norway F1 (FBN) rats supplemented without/with antioxidant tempol. We found age-related increases in oxidative stress and blood pressure; which were reduced with tempol treatment in old FBN rats. D1R and AT1R functions were determined by measuring diuretic and natriuretic responses to *SKF-38393* (D1R agonist) and *candesartan* (AT1 receptor antagonist) respectively. Natriuresis in response to D1R activation was impaired in old rats, suggesting an age-associated decline in D1R function in old FBN rats. Increase in G protein coupled receptor kinase (GRK) expression/activity is associated with reduced D1R-G protein coupling and function in humans and animal models with hypertension.

We found age-associated increase in GRK-4 levels accompanied with D1R-G protein uncoupling in the renal proximal tubules of old FBN rats. Tempol treatment reduced GRK-4 levels and restored D1R-G protein coupling in these old rats. Natriuretic and diuretic responses to *candesartan*; however, were exaggerated in old rats, suggesting an age-associated increase in renal AT1R function in old FBN rats. Age-related increases in angiotensin II-mediated G protein coupling leading to exaggerated Na,K-ATPase activity may have caused increased renal AT1R function observed in old FBN rats. Tempol treatment restored angiotensin II-mediated G protein coupling and Na,K-ATPase response and thus reduced *candesartan*-mediated natriuresis and diuresis in old FBN rats.

Our results demonstrate that both diminished renal D1R and exaggerated AT1R functions are associated with high BP in old FBN rats. Furthermore, oxidative stress may cause altered renal D1R and AT1R functions and high BP in these old rats.

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LIST OF ABBREVIATIONS

AChE:	Acetylcholinesterase
Ang II:	Angiotensin II
AT1R:	Angiotensin AT1 receptor
BCA:	Bicinchoninic acid
BP:	Blood Pressure
BSO:	L-buthionine sulfoximine
b.w.:	Body weight
D1R:	Dopamine D1 receptor
ENaC:	Epithelial Na channel
F344:	Fischer 344
FBN:	Fischer 344 X Brown Norway F1
GFR:	Glomerular filtration rate
GRK:	G protein coupled receptor kinase
HO-1:	Hemeoxygenase-1
HRP:	Horseradish peroxidase
L-DOPA:	L-3,4-dihydroxyphenylalanine
MAP:	Mean arterial pressure
NaPi-2:	Type II Na-phosphate cotransporter

NBC1:	Type 1 Na-bicarbonate cotransporter
NCC:	Na-Cl cotransporter
NHE:	Na-H exchanger
NIA:	National Institute on Aging
NKA:	Na,K-ATPase
NKCC2:	Type 2 Na-K-2Cl cotransporter
PKA:	Protein kinase A
PKC:	Protein Kinase C
PLC:	Phospholipase C
PVDF:	Polyvinylidene difluoride
ROS:	Reactive Oxygen Species
SD:	Sprague-Dawley
SHR:	Spontaneously hypertensive rats
SOD:	Superoxide dismutase
WKY:	Wistar-Kyoto

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36. There are no age-associated changes in blood pressure between adult and old F344 rats.
37. Antioxidant tempol restores the age-associated elevated urinary protein excretion levels in old FBN rats.

1. INTRODUCTION AND STATEMENT OF PROBLEM

Hypertension is a major risk factor for cardiovascular morbidity and mortality (1-5). Both genetic and non-genetic factors have been implicated in the development of hypertension (6). Proper sodium handling by kidneys is the key to the maintenance of body's sodium homeostasis and long-term regulation of blood pressure. A close relationship exists between improper renal sodium handling and pathogenesis of hypertension (7-10).

Kidney dopamine and angiotensin II (Ang II) are two key counter-regulatory endogenous compounds that play pivotal roles in the maintenance of sodium homeostasis and blood pressure (11). Dopamine exerts its natriuretic action primarily via D1-like (comprised of D1 and D5 subtypes) receptors and contributes to majority of the sodium excretion during increased sodium intake (12-14). Impairments in natriuretic and diuretic responses to D1-like receptor agonists are linked to hypertension in humans and rodents (15-21). In contrast, Ang II mediates its antinatriuretic effects via angiotensin type 1 (AT1) receptors (22-24), altered functioning of which has also been linked to various forms of hypertension (25, 26). Besides their counter regulatory actions on sodium transporters, recent studies suggest a direct antagonism between the dopamine and renin angiotensin system in regulating sodium balance (11).

Blood pressure increases with advancing age (27-29) and may account for myocardial infarction, stroke, and heart and kidney dysfunctions in the aging population (28). A host of structural and functional changes in the kidney accompany the aging process, which are often associated with an inability of the kidney to regulate sodium balance (30, 31). Aging is also associated with increase in oxidative stress (32) and alterations in renal D1R (33, 34) and AT1R (35) functions. In hypertensive animal models like Spontaneously Hypertensive and Obese Zucker rats, which also exhibit increased oxidative stress, altered renal D1R and AT1R functions have been reported (16, 36-39) . Therefore, we hypothesized that hypertension in aging is associated with alterations in both renal D1 and AT1 receptor functions and age-associated oxidative stress plays causative role in this phenomenon.

We have previously established the role of age-associated oxidative stress in renal dopamine D1R dysfunction in old Fischer 344 (F344) rats (rat aging model) (40, 41). However, these rats do not develop age-associated hypertension. Since our objective was to determine mechanisms for hypertension in aging, F344 rats were not an ideal animal model for our studies. Therefore, in this study, we used Fischer 344 X Brown Norway F1 (FBN) rats; another animal model widely used and recommended for aging research (42). We supplemented adult (3 month) and old (21 month) FBN rats without/with antioxidant tempol and measured age-associated effects on oxidative stress, blood pressure, and

dopamine D1R and angiotensin AT1 receptor functions. While, renal D1R and AT1R functions were determined *in vivo* in response to D1 receptor agonist *SKF-38393* and AT1 receptor antagonist *candesartan* respectively; the underlying mechanisms for D1R and AT1R alterations were studied in the isolated renal proximal tubules in adult and old FBN rats.

We also studied renal AT1 receptor function in normotensive F344 rats, as effects of aging on renal AT1R function in these normotensive rats have not been reported. Thus, we measured renal AT1R function in response to *candesartan* in adult (6 month) and old (24 month) F344 rats.

2. REVIEW OF LITERATURE

2.1 Role of kidney in sodium homeostasis and regulation of blood pressure

Blood pressure is regulated by an integrated action of various physiological systems affecting two major determinants of systemic pressure: cardiac output and peripheral vascular resistance. Control of these two parameters involves distinct contributions from heart, kidney, adrenal gland, brain and peripheral vessels. However, as first suggested by Guyton and now widely recognized, control of sodium excretion by the kidneys plays a critical role and perhaps represents the final common pathway for long-term regulation of blood pressure (43).

2.1.1 Regulation of sodium homeostasis

Kidney maintains sodium and water balance by hormonal regulation of various sodium transporters present along the nephron (functional unit of kidney). For example, NHE (Na-H exchanger), NaPi-2 (Type 2 Na-phosphate cotransporter), NKCC2 (Type 2 Na-K-2Cl cotransporter), NCC (Na-Cl cotransporter) and ENaC (epithelial Na channel) are the major sodium transporters and channels on apical side of the tubular cells in the nephron (44).

Whereas, NBC1 (Type 1 Na-bicarbonate cotransporter) and NKA (Na,K-ATPase) are the two major sodium transporters on the basolateral side of the tubular cells (44). Urinary sodium excretion is regulated in an interactive manner by various natriuretic (e.g. dopamine and atrial natriuretic peptide) and anti-natriuretic hormones (e.g. norepinephrine and angiotensin II) (45). Approximately 70% of the filtered load of sodium is reabsorbed in the proximal tubules, while 20% and 9% is reabsorbed in the loop of Henle/distal tubule and collecting duct respectively, while the rest is excreted (46).

2.1.2 Regulation of blood pressure

The relationship between blood pressure and sodium excretion has been described using the pressure-natriuresis curve. According to this relationship, in normal persons, an increased sodium intake causes appropriate adjustments in various humoral, neural and paracrine mechanisms, which alter systemic and renal hemodynamics to increase sodium excretion without increasing blood pressure. However, a decrease in sodium excretory capability in the face of normal or increased sodium intake can lead to increase in extracellular fluid volume and, therefore, can cause increase in blood pressure. Through the mechanism of pressure natriuresis, increased arterial pressure increases renal

sodium excretion, which restores sodium balance but at the expense of a persistent elevated arterial pressure (47).

2.2 Role of renal dopamine and angiotensin II in sodium balance

Renal sodium excretion is bidirectionally regulated by natriuretic dopamine and antinatriuretic angiotensin II (11).

2.2.1 Renal dopaminergic system

Dopamine produced locally in the kidney is important in the paracrine/autocrine regulation of sodium transport through the renal tubules (20, 48-50). Dopamine exerts its biological effects via the D1-like (D1 and D5; rat homologs: D1A and D1B) and D2-like (D2, D3 and D4) G protein coupled receptors. While D1-like receptors are linked to stimulatory G proteins, $G\alpha_s$, and stimulate adenylyl cyclase, D2-like receptors are linked to inhibitory G proteins, $G\alpha_i/G\alpha_o$, and inhibit adenylyl cyclase (20, 48-50). The D1 receptor also couples to $G\alpha_q$ and stimulates phospholipase C (PLC) resulting in the production of inositol phosphates and diacylglycerol, which activates protein kinase C (PKC) (51-54). The D1-like receptors are present on the renal tubules, juxtaglomerular apparatus and smooth muscles of blood vessels of major body organs. However,

D2-like receptors are present on the glomeruli, renal tubules, postganglionic sympathetic nerve terminals and zona glomerulosa cells of the renal cortex (55-59).

In the kidney, dopamine is produced mainly in the renal proximal tubules, by decarboxylation of L-3,4-dihydroxyphenylalanine (L-DOPA) transported from the circulation (20, 48-50). Under conditions of moderate sodium excess, dopamine via D1-like receptors increase sodium excretion mediated mainly via tubular mechanisms (20, 48-50). This increase in sodium excretion by dopamine is achieved by inhibiting sodium transporters NKA and NHE in proximal tubules and other parts of the nephron (60-62). On the brush border side, dopamine inhibits NHE via cAMP-dependent and cAMP-independent mechanisms (60, 63-65). Dopamine-mediated increase in cAMP activates protein kinase A (PKA), which in turn phosphorylates NHE and subsequently inhibits its activity (60, 66, 67). This D1R-mediated cAMP/PKA pathway has also been implicated in inhibiting NKA activity on the basolateral side in the renal proximal tubules and other segments in the nephron (68-70). In addition, numerous studies have also provided evidence for the role of D1-receptor-mediated PLC/PKC pathway in inhibiting NKA activity in the renal proximal tubules (16, 51, 71-74). It is now recognized that PKC by phosphorylating the NKA pump inhibits NKA activity (75, 76). On the other hand, activation of dopamine D2-like receptors produces antidiuresis and antinatriuresis (77, 78). Activation of mitogen-activated protein

kinase by D2-like receptors stimulates sodium transporter NKA in the renal proximal tubules and thereby, cause sodium retention (79, 80).

2.2.2 Renal tubular effects of Angiotensin II

Similar to dopamine, renin angiotensin system is another important regulator of sodium transport and blood pressure (81). Angiotensin II mediates its sodium regulatory actions primarily via G protein coupled AT1 and AT2 receptors. While AT1 receptors are linked to G proteins Gq/11 and $G\alpha_i/G\alpha_o$, AT2 receptors are coupled to G protein $G\alpha_i2/3$ (82). Angiotensin II via AT1 receptors stimulates sodium transporters, namely NHE3 on the brush border membrane and the NBC1 and NKA on the basolateral membrane (83-85) thereby leading to increase in sodium reabsorption. This stimulation of sodium transporters by Ang II is evident only at lower concentrations (picomolar); higher concentrations (beyond nanomolar) of Ang II, however, have been reported to inhibit these transporters (84, 86, 87). At the cellular level, AT1 receptors by activating Gi proteins cause inhibition of adenylyl cyclase activity and stimulation of sodium transporters (84, 88-90). In the proximal tubules, Ang II-elicited stimulation of the NHE is mediated via the PLC/PKC pathway (86, 91, 92). This phenomenon of AT1 receptor-mediated increase in sodium reabsorption is up regulated during times of low sodium intake in order to conserve body sodium. Although

angiotensin II through AT2 (via NO-cGMP pathway) and AT4 receptors can cause increase in sodium excretion, the major effect of angiotensin II is to retain sodium and is mediated via AT1 receptors (81, 82, 93-95).

In the kidneys, thus, dopamine (via D1-like) and angiotensin II (via AT1 receptors) by modulating sodium transporters serve important functions in maintaining sodium balance, and therefore, in regulating blood pressure.

2.3 Altered renal dopamine and angiotensin AT1 receptor system in hypertension

2.3.1 Defective renal dopaminergic system in hypertension

There is enough evidence for the role of dopamine and the genes that regulate its function in the pathogenesis of hypertension (96). Defective renal dopamine secretion/synthesis has been reported in various forms of human hypertension (97). This deficiency in renal dopaminergic system in human primary hypertension has been attributed to a defect in L-DOPA decarboxylase and/or a decrease in renal tubular uptake of L-DOPA and its conversion to dopamine (96). Also, defective D1-like receptors have been reported in primary cells cultured from hypertensive human proximal tubules (98). A recent study suggested that D1 receptor gene polymorphism is associated with essential

hypertension (99). In another study, single nucleotide polymorphism of GRK4γ resulted in increased GRK activity, causing serine phosphorylation and subsequent D1 receptor-G proteins uncoupling in renal proximal tubules obtained from patients with essential hypertension (100).

In studies in Dahl salt-sensitive rats, researchers have reported a defect in the ability of the D1-like receptor to regulate NKA activity in the renal proximal tubules (13, 14, 101-103). There are also reports of defective ligand-mediated D1-like receptor coupling to G proteins on the basolateral membranes in spontaneously hypertensive rats (SHR) (13, 53). Strong evidence relating defective renal D1A receptor system/signaling with hypertension have come from the cross-breeding studies. In the F2 generation from cross breeds between female Wistar-Kyoto (WKY) and male SHR rats, the inability of dopamine D1-like receptor to inhibit NHE3 in the renal proximal tubules cosegregated with reduced ability to excrete sodium in response to D1-like receptor agonists and hypertension. In another study, where mutant mice lacking functional D1A receptors were generated, there was increased systolic, diastolic and mean arterial pressure (15).

2.3.2 Increased angiotensin AT1 receptor function in hypertension

Angiotensin II is a potent vasoconstrictor and a sodium retaining hormone. Increased responsiveness to angiotensin II in the renal proximal tubules of SHR has been reported to contribute to sodium retention observed during the development of hypertension in these rats (39). Young SHR also express increased AT1 receptors in the renal proximal tubules compared to age-matched WKY rats (104). In Obese Zucker rats, a model for obesity-related hypertension, treatment with an AT1 receptor antagonist cause greater diuresis and natriuresis and reduce blood pressure compared to lean rats (105, 106). Ang II (at lower concentration in pM range) also stimulates NHE and NKA to a greater extent accompanied with greater reduction in cAMP and increased AT1 receptor numbers in the renal proximal tubules of Obese Zucker rats. This up regulated angiotensin AT1 receptor system in the renal proximal tubules cause increase in sodium and water retention and contribute to hypertension in Obese Zucker rats (107).

2.4 Hypertension in elderly

In humans, prevalence of hypertension increases with advancing age. According to the National Health and Nutrition Examination Survey (1999-2004),

about 60% of all adults aged 60-69 years and up to 77% of those aged > 80 years have hypertension (108). In people who are normotensive at the age of 55-65 years, life time risk for developing hypertension exceeds 90% and is mainly accounted for by an age-related increase in systolic blood pressure (29). While the diastolic pressure plateaus in the 50-60 years of life and then slowly declines thereafter, systolic pressure continues to increase and thus, contributes to increases in pulse pressure (109). Age-related vascular, neuronal and humoral changes are important factors leading to the development of hypertension in elderly. As elevated blood pressure is a well recognized risk factor for cerebrovascular accidents, congestive heart failure, coronary artery disease, end stage renal failure and sudden death, lowering blood pressure is important to reduce morbidity and mortality in elderly (110).

2.5 Age-related changes in kidney and sodium regulation

The process of aging is accompanied by profound anatomic and functional changes in the kidney (111). Renal blood flow, GFR and cortical blood flow decrease with advancing age (112, 113). With respect to renal sodium handling, aging kidneys have both reduced capacity to retain sodium in response to sodium restriction and an impaired ability to excrete sodium when challenged with increased sodium load (112). It is likely that sluggish sodium conservation

observed in aging is due to reduction in nephron numbers with age resulting in increased solute load per nephron and reduced levels/responsiveness of the sodium retaining aldosterone (30, 114, 115).

In the normal kidneys, dopamine is produced in response to an increased sodium load and acts on D1-like receptors to excrete excess sodium. However, numerous studies from our laboratory have reported a defective dopaminergic D1-like receptor system in the aging kidneys of old F344 rats. Incubation of renal proximal tubules with dopamine failed to inhibit NKA activity in these old rats. This defect was due to reduced D1 receptor numbers as well as D1 receptor G-protein uncoupling on the membranes and decreased D1 receptor protein expression in the renal proximal tubules (116). However, in old Sprague-Dawley rats, age-associated altered responsiveness to Ang II was suggested to contribute towards blunted renal excretory response observed in these rats (35).

The molecular mechanisms underlying these age-associated changes are currently under active investigation. Amongst many theories that explain cellular senescence, oxidative stress theory of aging suggests that a decline in the organism function with the aging process results from a progressive accrual of oxidative damage to its cellular constituents (117, 118).

2.6 Oxidative stress and regulation of blood pressure

2.6.1 Oxidative stress in physiological and pathophysiological states

Oxidative stress is a complex phenomenon defined as an excess of reactive oxygen species (ROS) resulting from either increased production or decreased degradation of ROS (119-121). ROS are metabolites of oxygen that can strip electrons and oxidize molecules, donate electrons and reduce molecules or react with and cause oxidative modification of the molecules. Superoxide radicals, hydroxyl radicals, lipid peroxy-radicals, alkoxy-radicals, peroxynitrite, hypochlorous acid and hydrogen peroxide are considered as ROS (122). The primary sources of ROS include specific oxidases like the NAD(P)H oxidase, xanthine oxidase, lipoxygenase, cyclooxygenase, cytochrome P450 enzymes, uncoupled nitric oxide synthases and the mitochondrial electron transport chain. Under normal physiological situations, ROS produced are safely neutralized by the antioxidant defense system comprising of numerous antioxidant enzymes {e.g. superoxide dismutase (SOD) and hemeoxygenase}, and endogenous and dietary antioxidant molecules. However, in many pathological situations like hypertension, the levels of ROS elevate either due to increased ROS production or a defective antioxidant defense system causing oxidative stress. This elevated oxidative stress can cause injury and dysfunction

by attacking biomolecules, and by modulating redox-sensitive signal transduction pathways and transcription factors (123).

2.6.2 Oxidative stress in hypertension

Oxidative stress is consistently associated with high blood pressure in the kidney, blood vessels and brain in nearly all forms of acquired and hereditary hypertension (124). The much direct evidence for the role of oxidative stress in hypertension comes from the following experiments: a) induction of oxidative stress cause hypertension in genetically normal animals; b) mice deficient in manganese SOD exhibit salt-sensitive hypertension; and c) binding of Ang II to AT1 receptors cause ROS production via activation of NADPH oxidase in the kidney and vasculature suggesting that ROS is a major mediator for the pressor response of angiotensin II. Also, reduction of oxidative stress using pharmacological doses of antioxidants has been shown to reduce blood pressure in hypertensive, but not in normotensive animals (124).

2.7 Oxidative stress and altered renal D1 and AT1 receptor functions

Impaired natriuretic and diuretic responses to D1-like receptor agonists are usually accompanied with increased oxidative stress in humans with

essential hypertension and rodents with genetic hypertension (12, 13, 15, 17, 19, 102, 103, 125, 126). Treatment of proximal tubules with H_2O_2 (oxidant) increase oxidative stress and results in loss of dopamine D1 receptor-G protein coupling. This loss of coupling is restored by the use of antioxidants suggesting that D1 receptor-G protein coupling modulates in response to changes in redox states (36). Moreover, Obese Zucker rats; a model of type 2 diabetes exhibiting a moderate degree of hypertension and increased oxidative stress also have defective D1 receptor function (38). Similarly in old F344 rats, oxidative stress has been reported to cause dopamine D1 receptor dysfunction (41). Studies from our lab have established that oxidative stress via NFκB-PKC-GRK2 pathway cause dopamine D1 receptor uncoupling from G proteins. In these studies, we have reported that treatment of renal proximal tubules with H_2O_2, induces nuclear translocation of transcription factor, NFκB, increases PKC activity and triggers the translocation of GRK-2 to the membrane (127). This process via phosphorylating D1 receptors cause uncoupling of D1 receptor from G proteins (128).

Studies from our lab and others have also suggested a role of oxidative stress in altering renal AT1 receptor functions. We have previously shown that treatment of Sprague-Dawley rats with a prooxidant, L-buthionine sulfoximine (BSO) increase oxidative stress and cause hypertension. This increase in blood pressure is accompanied by increased renal AT1 receptor protein, numbers, and

a greater stimulation of NKA and NHE3 in response to angiotensin II. Treatment with antioxidant tempol restores the above changes and reduces blood pressure suggesting that oxidative stress via AT1 receptor upregulation cause overstimulation of sodium transporters and subsequently contributes to hypertension (25). Also, in Obese Zucker rats, increase in angiotensin AT1 receptor function coexists with increase in oxidative stress (37, 38). Unpublished studies from our lab have also shown that oxidative stress via changes in G protein expression and/or G protein coupling contributes to increased AT1 receptor function and hypertension in Spontaneously Hypertensive Rats.

2.8 Animal models for studying age-related changes

In the past, researchers have utilized various animal models like Wistar rats, Sprague-Dawley rats and Brown Norway rats to investigate the aging process. However, many of these animal models develop complications that could compromise the observations of age-associated dysfunctions (42). Since 1970's, the National Institute on Aging (NIA) has developed two rodent models, F344 rats and their hybrid cross FBN rats to study age-associated changes. Although, both F344 and FBN rats have been extensively used for aging research; FBN rats, however, is recommended by the National Center for Toxicological Research and NIA as the preferred model for aging rat research

(129-131). FBN rats demonstrate longer maximal life span, normal growth curve and distribution of age-related pathologies occurring relatively late in life, and a greater mean age for 50% mortality compared to F344 rats, thus meeting the NIA goals for an animal model that exhibits age-associated changes as a result of “normal aging process” rather than the simple reflection of an ongoing disease (129-131).

The objective of this study was to determine the role of oxidative stress in alterations in renal D1R and AT1R functions, and hypertension in aging. In the past, we have investigated F344 rats for age-associated changes in oxidative stress, D1R function and blood pressure (41). Although these rats exhibited increased oxidative stress and decline in renal D1R function with age, they did not develop age-associated hypertension. As mentioned before, FBN rats have a distinct advantage over F344 rats as an animal model for aging research. Moreover, the effect of aging on oxidative stress, D1R and AT1R functions and blood pressure have not been reported in these rats. Thus, FBN rats were the preferred choice, and were therefore used to study the effect of aging on these parameters in this study.

3. MATERIALS AND METHODS

3.1 Animals

Three different strains and different ages of rats were included in the study. Adult (3-month), middle age (10-month) and old (21-month) male FBN rats raised by Harlan Sprague-Dawley (Indianapolis, IN) were purchased from the National Institute on Aging (Bethesda, MD). Adult (6-month) and old (24-month) male F344 rats raised by Taconic Farms, Inc. (Hudson, NY) were also purchased from the National Institute on Aging (Bethesda, MD). Adult male Sprague Dawley (SD) rats weighing 250-300 gm were purchased from Harlan Laboratories (Indianapolis, IN). The rats were housed in plastic cages in the University of Houston animal care facility and were used as per the National Institutes of Health guidelines and approved protocols by the Institutional Animal Care and Use Committee. Animals had free access to the standard rodent chow and drinking water.

3.2 Tempol supplementation in FBN rats

FBN rats were randomly divided into four groups, viz. control and tempol-treated (2 each) adult and old rats. In the treated adult and old rat groups, tempol

(Sigma-Aldrich, MO; 1 mM) was supplemented in drinking water for a period of 3-4 weeks while control adult and old rat groups received only drinking water. Water supplemented with tempol was changed twice a day to minimize its oxidation.

3.3 *In-vivo* studies to determine renal dopamine D1 and angiotensin AT1 receptor functions

3.3.1 Animal surgery for measurement of blood pressure and renal function studies

The animals were anesthetized with inactin (150μg/kg b.w. i.p.) and tracheotomy was performed to facilitate breathing. To measure blood pressure and collection of blood samples, left carotid artery was catheterized with PE-50 tubing connected to a pressure transducer. Blood pressure was continuously recorded on a Grass Polygraph (Model 7D; Grass Instrument Company, Quincy, MA) throughout the experiment. The left jugular vein was also catheterized with PE-50 tubing for infusion of drug/saline. A midline abdominal incision was made, bladder urine was collected (for biochemical analyses) with syringe and left ureter was catheterized with PE-10 tubing for urine collection during saline/drug infusion. The rats were continuously infused with normal saline (1% b.w.; ml/hr)

for the duration of the surgery in order to prevent dehydration and maintain a stable urinary output.

3.3.2 Renal Function Studies

3.3.2.1 Renal dopamine D1 receptor function studies using D1 receptor agonist SKF-38393 in adult and old FBN rats

The protocol consisted of stabilizing the animals for 45 min after the surgery followed by three consecutive 30-min urine collection periods viz. C1, C2 and D. Collections C1 and C2 (basal) correspond to periods where only saline was infused whereas D corresponds to the period where *SKF-38393* (1μg/kg/min i.v.) in saline was infused. Urine samples were collected throughout the 30-min period whereas blood samples (250μl) were collected at the end of each C2 and D collection periods. Equal volume of saline was infused to replace the collected blood samples.

3.3.2.2 Renal angiotensin AT1 receptor function studies using specific AT1 receptor antagonist, candesartan.

a) *Candesartan* dose standardization study in SD rats

Candesartan at 100µg/kg dose although produces natriuresis and diuresis but also have renal hemodynamic effects (106, 132). Therefore, preliminary studies in SD rats were carried out to determine the effects of 1/10th lower dose of *candesartan* (10µg/kg) on urine and sodium excretion, and hemodynamic parameters (blood pressure and glomerular filtration rate). In addition, ability of *candesartan* (10µg/kg) in suppressing the pressor response to Ang II was also studied as a part of this dose standardization study.

As above (3.3.2.1), animals were allowed to stabilize for 45 min after the surgery and urine was collected at eight consecutive 20-min periods viz. C1, C2, and D1-D6. During C1 and C2, only saline was infused to determine the basal parameters. *Candesartan* was administered as a bolus dose (10µg/kg b.w. i.v.) and D1-D6 represent collections following drug treatment. Urine samples were collected throughout the 20-min period and blood samples at the end of each D1-D6 periods (250µl; replaced with equal volume of saline) were collected as above.

To determine the effectiveness of *candesartan* (10µg/kg) in attenuating pressor response to Ang II, carotid artery and jugular vein were catheterized under anesthesia as described in section 3.3.1. After allowing the animal to stabilize for 45 min, Ang II (60ng) was administered through jugular vein and increase in blood pressure was recorded. The blood pressure was allowed to return to basal and subsequently, *candesartan* (10µg/kg i.v.) was administered as a bolus dose. Ang II was readministered at 20, 90, 150 and 210 min after *candesartan* administration and changes in blood pressure were recorded.

To ensure that Ang II-mediated pressor response was not diminished on repeated administrations, blood pressure in response to Ang II (60ng i.v.) at 0, 5 and 60 min was recorded in a separate set of SD rats. Blood pressure was recorded in these animals as described above in section 3.3.1.

b) Renal angiotensin AT1 receptor function in adult and old F344 and FBN rats

As above (3.3.2.1), animals were stabilized for 45 min followed by six consecutive 30-min urine collection periods viz. C1, C2, and D1-D4. During C1 and C2, only saline was infused to determine the basal parameters. *Candesartan* was administered as a bolus dose (10µg/kg b.w. i.v.) and D1-D4 represent collections following drug treatment. Urine samples were collected throughout the

30-min period and blood samples at the end of each C2, D2 and D4 collection periods (250µl; replaced with equal volume of saline) were collected as above.

Urine samples collected from above were stored at -80^{0}C for further investigations.

3.3.3 Isolation of plasma from blood

Blood plasma was isolated by centrifuging blood samples at 1,500g for 15 min at 4^{0}C. These plasma samples were also stored at -80^{0}C for further analyses.

3.3.4 Urine and Plasma Analyses

Sodium concentration in urine and plasma was measured using an AAnalyst 400 atomic absorption spectrometer (Perkin Elmer, Waltham, MA). Creatinine levels in plasma and urine were measured using a commercially available assay kit (Catalog # K625-100; BioVision Inc., Mountain View, CA).

3.3.5 Evaluation of Renal Function

Urine volume was measured gravimetrically and urinary flow was assessed (µl/min). Urinary sodium excretion (UNaV; µmol/min) was calculated based on

urine flow and urinary sodium concentration [urine flow (μl/min)*urinary sodium concentration (μmol/μl)]. The glomerular filtration rate (ml/min) was determined based on the clearance of creatinine [{urine flow (ml/min)*urine creatinine (mg/dl)}/plasma creatinine (mg/dl)]. Percent fractional excretion of sodium was also determined [{urinary sodium concentration (μmol/μl)*plasma creatinine (mg/dl)}/{plasma sodium concentration (μmol/μl)*urinary creatinine (mg/dl)}]*100.

3.4 *In-vitro* biochemical studies

3.4.1 Preparation of renal proximal tubules

Another set of adult and old FBN rats treated without and with tempol was used to make renal proximal tubules. An *in situ* enzyme digestion procedure was used to prepare renal proximal tubules. Briefly, a midline abdominal incision was made, aorta was catheterized with PE-50 tubing, and kidneys were perfused with oxygenated Krebs-Henseleit buffer A (KHB-A) containing (in mM) 1.25 $CaCl_2$, 118 NaCl, 4 KCl, 1 KH_2PO_4, 0.12 $MgCl_2$, 27.2 $NaHCO_3$, 5 D-glucose, and 10 HEPES (pH 7.4) at 37^0C using a flow rate of 8ml/min until the kidneys were devoid of blood. A suprarenal ligature was placed on the aorta immediately after initiation of perfusion. This was followed by *in situ* digestion with KHB-A buffer-enzyme solution {containing enzymes collagenase type IV (230U/ml) and hyaluronidase type III (250U/ml) in 40ml buffer}. The kidneys were then excised,

capsules removed and were placed in ice-cold KHB buffer A. Coronal sections of the kidneys were obtained, and superficial cortical tissue slices (rich in proximal tubules) were dissected out with a razor blade. The cortical slices were then chopped into fine pieces and incubated in 20 ml of KHB-A enzyme solution {containing enzymes collagenase type III (460U/ml) and hyaluronidase type IV (500U/ml) in 20ml buffer} at 37^0C with constant oxygenation (95% O_2 and 5% CO_2) until the cortical tissues were uniformly dispersed. The suspension was then centrifuged at 50g for 2 min, following which the pellet was resuspended in 20ml KHB-B buffer containing (in mM) 118 NaCl, 4 KCl, 1 KH_2PO_4, 0.12 $MgCl_2$, 27.2 $NaHCO_3$, 5 D-glucose, and 10 HEPES (pH 7.4). The sample was washed three times with KHB-B buffer by centrifugation to remove residual enzymes. The proximal tubular pellet was re-suspended in 15 ml fresh KHB-B buffer and was centrifuged with 5 ml of 20% Ficoll at 250g for 20 min at 4^0C. The layer at the Ficoll interface (enriched in proximal tubules) was collected and washed three times by centrifugation in 15ml KHB-C buffer containing (in mM) 1.25 $CaCl_2$, 118 NaCl, 4 KCl, 1.2 $MgCl_2$, 27.2 $NaHCO_3$, 5 D-glucose, and 10 HEPES (pH 7.4). The pellet was re-suspended in 10ml of fresh KHB-C buffer and this suspension was used for further studies.

3.4.2 Viability test for renal proximal tubular cells

Trypan blue exclusion test was performed to determine the viability of proximal tubular cells (prepared as above). In this test, an aliquot (10-15µl) from the proximal tubular suspension was mixed with equal volume of 0.15% trypan blue and counted using hematocytometer under the microscope. The proximal tubules were considered viable if more than 95% of proximal tubules excluded the trypan blue dye. These proximal tubules were then included in the further studies.

3.4.3 Protein estimation in renal proximal tubules

Protein estimation was done in renal proximal tubules (prepared as above) by the bicinchoninic acid (BCA) method using protein assay kit (Pierce, Rockford, IL) reagents and bovine serum albumin as protein standards.

3.4.4 Preparation of renal proximal tubular membranes

Renal proximal tubules in KHB-C buffer were homogenized using Wheaton homogenizer followed by centrifuging the homogenate at 2000 rpm for 10 min at 4^0C. The supernatant was further centrifuged at high speed (18000 rpm) for 35 min at 4^0C to obtain a membrane pellet. The pellet containing proximal tubular membrane was resuspended in sucrose buffer (in mM: 10 Tris, 250 sucrose, 1 sodium orthovanadate, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, 1 PMSF and 1X protease inhibitor cocktail; pH 7.4) and washed once in the same

buffer by centrifugation to get rid of cytosolic proteins contamination. Finally, a small volume of sucrose buffer was added to resuspend the membrane pellet and stored at -80^{o}C until further use.

3.4.5 Measurement of oxidative stress

3.4.5.1 Measurement of markers of oxidative stress

a) Superoxide radical producing NADPH-*gp*^{91phox} oxidase

The levels of NADPH-*gp*^{91phox} in the renal proximal tubular homogenate were measured using standard western blotting technique. Briefly, loading samples were prepared by dissolving homogenate proteins in SDS-Laemmli buffer. Samples (20µg) were then resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). The membrane was blocked in 5% milk followed by incubation with specific anti-*gp*^{91phox} antibody (1:1000) (BD Biosciences, San Jose, CA) each for 1hr. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated goat-anti-mouse secondary antibody (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1hr. The bands were detected using chemiluminiscence substrate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) on X-ray films and the band densities were quantified using Kodak ID Image analysis software (Eastman Kodak Company, Rochester, NY). The same blot was stripped and

reprobed for β-actin as loading control using specific anti-β-actin antibody (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by HRP-conjugated goat-anti-mouse secondary antibody (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The bands were detected on X-ray films and quantified as described above.

b) 8-Isoprostane

8-Isoprostane was measured in plasma using a commercially available EIA-based kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's instructions. Briefly, this assay is based on the competition between 8-Isoprostane and an 8-Isoprostane-acetylcholinesterase (AChE) conjugate (8-Isoprostane tracer) for a limited number of 8-Isoprostane-specific rabbit antiserum binding sites. The amount of 8-Isoprostane tracer bound (which is inversely proportional to the concentration of 8-Isoprostane in the sample) to the rabbit antiserum is determined by adding Ellman's reagent (which contains the substrate for AChE) and the intensity of the developed yellow color is measured spectrophotometrically at 405 nm.

3.4.5.2 Measurement of antioxidants

a) Urinary antioxidant capacity

The total antioxidant capacity in bladder urine (diluted 1:10 with deionized water) was determined using a commercially available kit according to the manufacturer's protocol (Catalog # 709001; Cayman Chemical, MI). Briefly, the assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS® (2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABTS^{®,+}$ by metmyoglobin. The capacity of the antioxidants in the sample to prevent ABTS® oxidation is compared with that of Trolox, a water-soluble tocopherol analogue, and is quantified as millimolar Trolox equivalents.

b) Hemeoxygenase-1

Hemeoxygenase-1 (HO-1) and β-actin (loading control) levels were measured in the renal proximal tubular homogenate (15µg protein) by standard western blotting technique as described in section 3.4.5.1(a). Specific anti-HO-1 (1:1000) (Assay Designs, Ann Arbor, MI) and HRP-conjugated goat-anti-mouse secondary (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies were used for this study.

3.4.6 Methods used for dopamine D1 receptor studies

3.4.6.1 Measurement of dopamine D1 receptor levels

a) Measurement of dopamine D1 receptor proteins

Dopamine D1 receptor and β-actin (loading control) levels were measured in the renal proximal tubular homogenate (15µg protein) by standard western blotting technique as described in section 3.4.5.1(a). Specific anti-dopamine receptor D1A (1:500) (Millipore, Billerica, MA) and HRP-conjugated goat-anti-rabbit secondary (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies were used for this study.

b) Measurement of dopamine D1 receptor numbers

Radio-ligand [^{3}H] SCH-23390, a selective D1 receptor antagonist was used to determine dopamine D1-like receptors on the renal proximal tubular membranes (as prepared in section 3.4.4). The binding reaction was carried out in Multiscreen HTS 96 well glass fiber filter plates (Millipore, Billerica, MA). The membrane samples (50µg) were incubated with 20 nM [^{3}H] SCH-23390 (specific activity 73.1 Ci/mmol) in binding buffer (50mM Tris HCl, 2mM $MgCl_2$, 0.2 mM sodium metabisulfite, 0.2 mM PMSF, pH 7.4) at 25^{0}C for 120 min. The reaction was stopped by rapid filtration using Multiscreen HTS vacuum manifold (Millipore, Billerica, MA) following the addition of 100 µl of ice-cold stop solution (50 mM Tris HCl, 2 mM $MgCl_2$, pH 7.4). The wells were washed four times with

250 µl of ice-cold stop solution by rapid filtration as above. The filters were extracted from the plate and incubated overnight in the scintillation fluid. Radioactivity was measured using a liquid scintillation counter (Beckman Coulter, Brea, CA). Non specific binding was determined using 10 µM unlabeled SCH-23390. Specific binding was calculated as the difference between total and non specific binding and was normalized to protein.

3.4.6.2 Measurement of dopamine D1R coupling with G proteins

Binding of dopamine D1R with G proteins in response to D1 receptor agonist *SKF-38393* was determined on renal proximal tubular membranes (as prepared in section 3.4.4) in Multiscreen HTS 96 well glass filter plates (Millipore, Billerica, MA). Membranes (5 µg) were incubated with vehicle (0.1% $Na_2S_2O_3$) or *SKF-38393* (final conc. 10^{-6} M in 0.1% $Na_2S_2O_3$) in the presence of [^{35}S]-GTPγS (final conc. 0.6 nM) and GDP (final conc. 1 µM) at 30^0C for 60 min in 100 µl reaction buffer (25 mM HEPES, 15 mM $MgCl_2$, 1 mM EDTA, 1 mM Dithiothreitol, 100 mM NaCl, pH 8). Non-specific binding was determined in the presence of 100µM cold GTP. The reaction was terminated by rapid filtration following the addition of 100 µl of ice-cold stop/filtering solution (20 mM Tris HCl, 100 mM NaCl, 25 mM $MgCl_2$, pH 8). The filters were washed four times with 250 µl ice-cold stop/filtering solution using Multiscreen HTS vacuum manifold (Millipore, Billerica, MA). The glass filters were removed from the plate and incubated overnight in Beckman

ready safe cocktail and the radioactivity was counted on a liquid scintillation counter (Beckman Coulter, Brea, CA).

3.4.6.3 Measurement of PKC activity

PKC activity was measured in the renal proximal tubules using a commercially available kit (Enzo Life Sciences, Inc., Butler Pike Plymouth Meeting, PA) according to the manufacturer's instructions. Briefly, this assay is based on a solid phase enzyme-linked immunosorbent assay that utilizes a specific synthetic peptide as a substrate for PKC and a polyclonal antibody that recognizes the phosphorylated form of the substrate. In this assay, the substrate, which is readily phosphorylated by PKC, is precoated on 96-wells microtiter plate. The proximal tubular homogenate samples (20 µg) were incubated with ATP at 30^0C for 90 min. The kinase reaction was terminated and the wells were incubated with a phosphospecific antibody that recognizes PKC-mediated phosphorylation on the substrate, at room temperature for 60 min. The wells were then washed four times with wash buffer followed by incubation with an anti-rabbit IgG:HRP conjugate antibody at room temperature for 30 min. Again, the wells were washed four times with wash buffer, HRP substrate tetramethylbenzidine added and incubated at room temperature for 30-60 min. The reaction was terminated using an acid solution and the yellow color developed was read at 450 nm in a microplate reader.

3.4.6.4 Measurement of GRKs

GRK-2, GRK-4 and β-actin (loading control) levels were measured in the renal proximal tubular homogenate (20µg) by standard western blotting technique as described in section 3.4.5.1(a). Specific anti-GRK-2 (1:500) & anti-GRK-4 (1:250) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and HRP-conjugated goat-anti-rabbit (1:1000) & goat-anti-mouse (1:1000) secondary antibodies respectively (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used for this study.

3.4.7 Methods used for angiotensin AT1R studies

3.4.7.1 Measurement of angiotensin AT1R protein

Angiotensin AT1 receptor protein levels were measured in renal proximal tubular homogenate (15µg protein) and membranes (10µg protein) by standard western blotting technique as described in section 3.4.5.1(a). Specific anti-angiotensin AT1 receptor (1:200) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and HRP-conjugated goat-anti-rabbit secondary (1:1500) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies were used for this study. β-actin was measured as a loading control in renal proximal tubular homogenate as described in section 3.4.5.1(a).

3.4.7.2 Measurement of Gα proteins

$G\alpha_{q/11}$ and $G\alpha_i$-1 protein levels were measured on the renal proximal tubular membranes (7.5µg protein for $G\alpha_{q/11}$ and 10µg protein for $G\alpha_i$-1) by standard western blotting technique as described in section 3.4.5.1(a). Specific anti-$G\alpha_{q/11}$ (1:1000), anti-$G\alpha_i$-1 (1:1000) (EMD Chemicals Group, Darmstadt, Germany) and HRP-conjugated goat-anti-rabbit secondary (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies were used for this study.

3.4.7.3 Measurement of angiotensin AT1R coupling with G proteins

Binding of angiotensin AT1R with G proteins was determined on the proximal tubular membranes in Multiscreen HTS 96 well glass filter plates (Millipore, Billerica, MA). Membranes (5 µg) were incubated with vehicle (water) or Ang II (final conc. 10^{-6} M in water) in the presence of [^{35}S]-GTPγS (final conc. 0.6 nM) and GDP (final conc. 1 µM) at 30^0C for 60 min in 100 µl reaction buffer (50 mM Tris HCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM Dithiothreitol, 100 mM NaCl, pH 7.4). Non-specific binding was determined in the presence of 100µM cold GTP. The reaction was terminated by rapid filtration following the addition of 100 µl of ice-cold stop/filtering solution (20 mM Tris HCl, 100 mM NaCl, 25 mM $MgCl_2$, pH 8). The filters were washed four times with 250 µl ice-cold stop/filtering solution using Multiscreen HTS vacuum manifold (Millipore, Billerica, MA). The glass filters were removed from the plate and incubated overnight in Beckman ready

safe cocktail and the radioactivity was counted on a liquid scintillation counter (Beckman Coulter, Brea, CA).

3.4.7.4 Measurement of angiotensin II-mediated Na,K-ATPase activity

Freshly isolated renal proximal tubules were used to determine Na,K-ATPase activity. Briefly, renal proximal tubules (1mg/ml) were incubated with vehicle (distilled water) or angiotensin II (final conc. 10^{-12}, 10^{-10}, 10^{-9} and 10^{-6} M in distilled water) for 15 min at 37^0C. The reaction was terminated by rapidly freezing the renal proximal tubules in liquid nitrogen. The proximal tubular suspension was thawed and Na,K-ATPase activity was determined by measuring end-point phosphate hydrolysis of ATP in the absence and presence of Na,K-ATPase inhibitor, ouabain. The reaction mixture (final volume 1.025 ml) contained 37.5 mM imidazole, 75 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 6 mM NaN_3, 1 mM sodium EGTA, 75 mM Tris-HCl and 100 µl proximal tubular suspension. Ouabain-insensitive ATPase activity was determined in parallel using reaction mix containing 150 mM Tris HCl and 1 mM ouabain. The reaction was initiated by incubating with 4 mM ATP and continued for 15 min at 37^0C. The reaction was terminated by adding 50 µl of ice-cold trichloroacetic acid. The liberated inorganic phosphate was measured by a colorimetric assay (133) and the Na,K-ATPase activity was calculated as the difference between total and ouabain-insensitive ATPase activity.

3.4.8 Measurement of protein in urine

Bladder urine samples were diluted (1:100) with deionized water and protein levels were measured as in section 3.4.3.

3.5 Data Analysis

Data are presented as mean ± SEM. Repeated measures ANOVA followed by Dunnett post hoc test was used to compare variations (from control) within the group. One-way ANOVA followed by Newman-Keuls post hoc test was used to compare variations amongst the groups. Also, paired Student's t test was used wherever appropriate. Statistical analysis was carried out using a software program (GraphPad Prism ver. 5; GraphPad Software, San Diego, CA). The minimum level of significance was considered at $P < 0.05$.

4. RESULTS

4.1 Effect of aging on blood pressure and some of the general body parameters in FBN rats

There was an age-associated increase in systolic, diastolic and mean arterial pressures in FBN rats (old>middle age>adult) (Figure 1). Treatment with tempol reduced blood pressure in old but not in adult rats (Figure 2). The body weight was significantly higher in old compared to adult rats and remained unchanged with tempol treatment. The food and water intake were not different between adult and old control rats. Although, there was no change in food intake, water intake in tempol-supplemented rats was significantly higher in old compared to adult rats (Table 1). Heart rate was not different between adult (306.2 $\pm$ 15.57bpm) and old (330.3 $\pm$ 6.9bpm) rats.

4.2 Effect of aging on oxidative stress in FBN rats

4.2.1 Effect of aging on markers of oxidative stress

a) Renal proximal tubular levels of NADPH-gp^{91phox} oxidase

As shown in Figure 3, the levels of superoxide radical producing enzyme, NADPH-gp^{91phox} oxidase was higher in the renal proximal tubular homogenate of control old compared to control adult rats. Tempol treatment reduced the levels of NADPH-gp^{91phox} oxidase in old but not in adult rats.

b) Plasma 8-Isoprostane levels

Plasma 8-Isoprostane levels were higher in control old compared to control adult rats (Figure 4). The levels of plasma 8-Isoprostane decreased in tempol-treated compared to control old rats. However, tempol treatment did not change the plasma 8-Isoprostane levels in adult rats.

4.2.2 Effect of aging on antioxidant system

a) Urinary antioxidant capacity

As shown in figure 5, the urinary antioxidant capacity was lower in control old than in control adult rats. Tempol treatment increased the urinary antioxidant capacity in old rats. However, tempol treatment did not change the antioxidant capacity in adult rats.

b) Renal proximal tubular levels of HO-1

Figure 6 shows the levels of HO-1 in the renal proximal tubules of adult and old FBN rats. The HO-1 levels were higher in control old than in control adult rats. Tempol treatment did not affect the levels of HO-1 in both adult and old rats.

4.3 Effect of aging on renal dopamine D1 receptor function and signaling cascade in FBN rats

4.3.1 Renal dopamine D1 receptor function in whole animal

As shown in figure 7, D1-like receptor agonist *SKF-38393* increased urine flow in both adult and old rats. However, urinary sodium excretion (UNaV) in response to *SKF-38393* significantly increased only in adult rats (Figure 8). These diuretic

and natriuretic effects of *SKF-38393* were not associated with changes in glomerular filtration rate (Figure 9) and mean arterial pressure (Adult: saline vs. *SKF-38393*, 78.56 ± 2.95 vs. 71.64 ± 2.42 mmHg; Old: saline vs. *SKF-38393*, 107.5 ± 2.86 vs. 99.38 ± 3.79 mmHg).

4.3.2 Dopamine D1 receptor abundance in the renal proximal tubules

There were no age-related differences in the levels of dopamine D1R proteins in the renal proximal tubular homogenates between control adult and control old rats. The levels of D1R protein did not change with tempol treatment in both adult and old rats (Figure 10). However, there was an age-related decline in dopamine D1R numbers on the renal proximal tubular membranes in control old compared to control adult rats. Tempol-treatment restored the age-related decline in the dopamine D1R numbers in old rats (Figure 11).

4.3.3 Dopamine D1 receptor coupling with G proteins in the proximal tubular membranes

D1R-G-protein coupling in response to D1R agonist *SKF-38393* is an index of D1 receptor function in renal proximal tubules (53). Treatment of proximal tubular membranes with *SKF-38393* increased [^{35}S]-GTPγS binding in control and

tempol-treated adult rats. However, the ability of *SKF-38393* to stimulate [^{35}S]-GTPγS binding was attenuated in control old rats. Tempol treatment in old rats increased the *SKF-38393*-mediated [^{35}S]-GTPγS binding (Figure 12). The basal [^{35}S]-GTPγS binding was not different between control and tempol-treated adult and old rats (Figure 13).

4.3.4 PKC activity in the renal proximal tubules

The PKC activity, measured in the renal proximal tubular homogenates, was not different between adult and old rats and remained unchanged with tempol treatment (Figure 14).

4.3.5 Effect of aging on GRK-2 and GRK-4 protein abundance in the renal proximal tubules

The levels of GRK-2 proteins in the renal proximal tubular homogenates were not different between adult and old rats and remained unchanged with tempol treatment (Figure 15). However, the levels of GRK-4 proteins in the renal proximal tubular homogenates increased in an age-dependent manner. Treatment with tempol in old rats reduced the age-related increase in GRK4 to the levels seen in adult rats (Figure 16).

4.4 *Candesartan* dose characterization in adult SD rats

4.4.1 Effect of candesartan (10µg/kg i.v. bolus) on urine flow, urinary sodium excretion and fractional excretion of sodium

Administration of *candesartan* produced a significant increase in urine flow (Figure 17), urinary sodium excretion (Figure 18) and fractional excretion of sodium (Figure 19) compared to basal in SD rats.

4.4.2 Effect of candesartan (10µg/kg i.v. bolus) on glomerular filtration rate and blood pressure

There was no significant change in glomerular filtration rate (Figure 20) and blood pressure (Figure 21) with the administered dose of *candesartan* in SD rats.

4.4.3 Effectiveness of candesartan (10µg/kg i.v. bolus) as an AT1 receptor antagonist

The pressor response to angiotensin II was attenuated in the presence of *candesartan*. The effect of *candesartan*, in terms of its ability to attenuate pressor response of Ang II, lasted for up to 210 min (Figure 22).

4.4.4 Measurement of pressor response to angiotensin II at different time points

Administration of Ang II in the same animal at 0, 5 and 60 min increased blood pressure at each time point suggesting that repeated administrations of Ang II does not lead to tachyphylaxis to pressor response to Ang II (Figure 23).

4.5 Effect of aging on renal angiotensin AT1 receptor function and signaling cascade in FBN rats

4.5.1 Renal angiotensin AT1 receptor function in whole animal

Renal AT1 receptor function was determined by measuring urine flow and urinary sodium excretion (UNaV) in response to AT1 receptor antagonist *candesartan.* Both urine flow (Figure 24) and UNaV (Figure 25) in response to *candesartan* increased in an age-dependent manner in control FBN rats. While tempol treatment did not have any effect on the diuretic and natriuretic response to *candesartan* in adult rats, the exaggerated diuretic (Figure 24) and natriuretic (Figure 25) responses to *candesartan* were reduced with tempol treatment in old rats. These diuretic and natriuretic effects of *candesartan* were not associated with changes in glomerular filtration rate (Figure 26) and mean arterial pressure

(Adult: saline vs. *Candesartan*, 86.08 ± 3.93 vs. 83.69 ± 4.21 mmHg; Old: saline vs. *Candesartan*, 93.79 ± 4.7 vs. 88.57 ± 5.2 mmHg).

4.5.2 Angiotensin AT1R protein abundance in the renal proximal tubules

Angiotensin AT1 receptor protein levels were measured by standard western blotting technique in the renal proximal tubular homogenates and membranes. There were no age-associated changes in the levels of angiotensin AT1 receptor proteins in both homogenates (Figure 27) and membranes (Figure 28) of renal proximal tubules. Treatment with tempol did not affect the levels of AT1 receptor proteins in both homogenates (Figure 27) and membranes (Figure 28) from renal proximal tubules in adult and old rats.

4.5.3 Abundance of Gα subunits in the renal proximal tubules

$G\alpha_i$-1 and $G\alpha_{q/11}$ protein levels were measured on the renal proximal tubular membranes. With respect to $G\alpha_i$-1 protein, there was a moderate but significant age-associated decrease in control old compared to control adult rats. Treatment with tempol did not change the levels in adult and old rats (Figure 29). However, there were no age-associated changes in $G\alpha_{q/11}$ protein levels between control

adult and control old rats, and the protein levels remained unchanged with tempol treatment in these rats (Figure 30).

4.5.4 Angiotensin AT1R coupling with G proteins in the renal proximal tubules

AT1 receptor coupling with G proteins in the proximal tubular membranes was determined by measuring [^{35}S]-GTPγS binding in response to Ang II. The [^{35}S]-GTPγS binding in response to Ang II increased in both control adult and old rats, however, with a much higher degree in control old rats. Tempol treatment in old rats reduced the Ang II-mediated [^{35}S]-GTPγS binding to the levels seen in adult rats (Figure 31). The basal [^{35}S]-GTPγS binding was not different between control and tempol-treated adult and old rats (Figure 32).

4.5.5 Na,K-ATPase activity in the renal proximal tubules of FBN rats

Na,K-ATPase activity was measured in the renal proximal tubules in response to Ang II. While Ang II (10^{-12} and 10^{-9} M) stimulated Na,K-ATPase activity to a greater extent in control old compared to control adult rats, tempol treatment in old rats normalized the Ang II-mediated stimulation of Na,K-ATPase to levels seen in adult rats (Figure 33).

4.6 Effect of aging on renal angiotensin AT1 receptor function and blood pressure in F344 rats

4.6.1 Renal angiotensin AT1 receptor function in whole animal

Administration of *candesartan* significantly increased urine flow (Figure 34) and sodium excretion (Figure 35) compared to basal in both adult and old F344 rats. However, there were no significant differences in diuretic and natriuretic responses to *candesartan* between adult and old rats (Figures 34 & 35).

4.6.2 Blood pressure

Systolic, diastolic and mean arterial pressures were not different between adult and old Fischer 344 rats (Figure 36)

4.7 Effect of aging on proteinuria in FBN rats

Urinary protein levels increase with age and is considered a general marker of kidney dysfunction. There was age-related increase in the levels of urinary proteins, which decreased with tempol treatment in old rats. However, tempol treatment did not affect the levels of urinary proteins in adult rats (Figure 37).

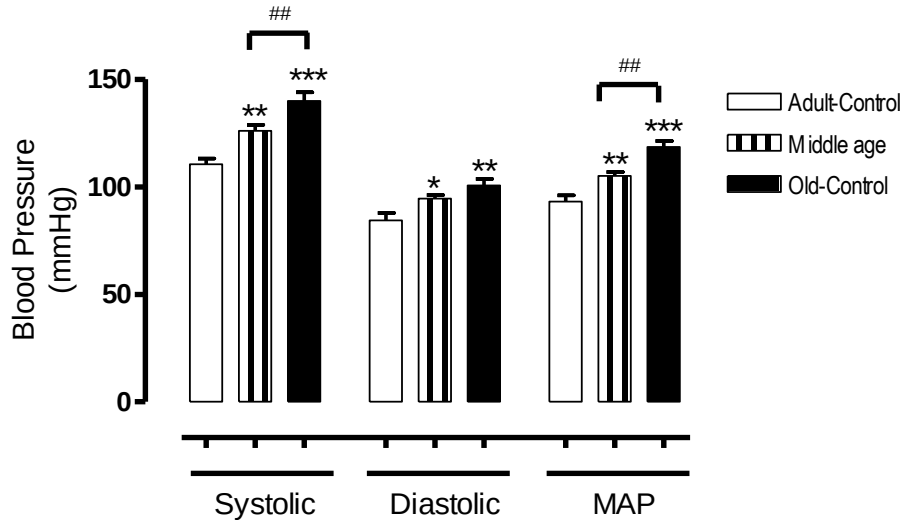

Figure 1: There is an age-associated increase in blood pressure in FBN rats (old>middle age>adult). Blood pressure (systolic, diastolic and mean arterial) was measured in anesthetized rats (details in the methods section). Results are mean ± SEM. (n= 6-10 rats). Significantly different from adult control rats using one-way ANOVA followed by Newman-Keuls post hoc test ($^{***}P<0.001$, $^{**}P<0.01$ and $^{*}P<0.05$). Significantly different from middle age rats using one-way ANOVA followed by Newman-Keuls post hoc test ($^{\#\#}P<0.01$).

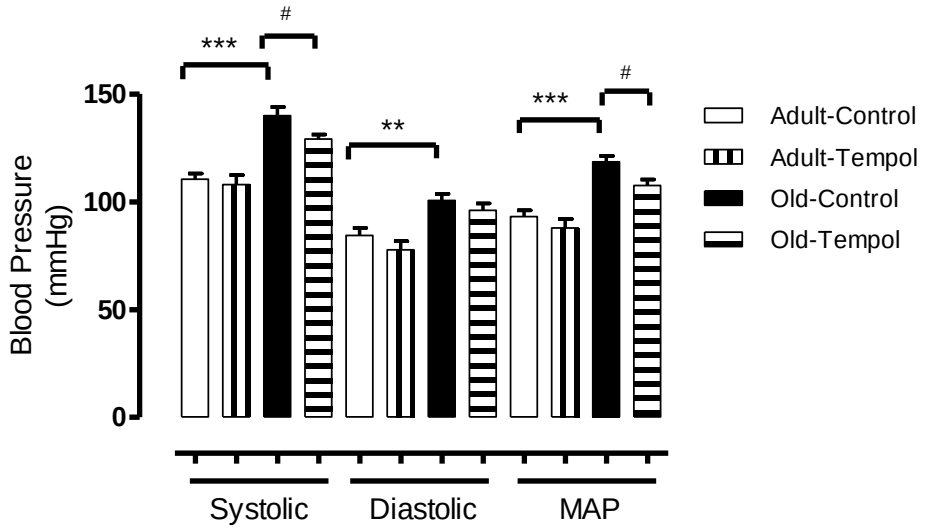

Figure 2: Antioxidant tempol reduces age-related increase in blood pressure in old FBN rats. Blood pressure (systolic, diastolic and mean arterial) was measured in anesthetized rats (details in the methods section). Results are mean ± SEM. (n= 6-10 rats). Significantly different from adult control rats using one-way ANOVA followed by Newman-Keuls post hoc test ($^{***}P<0.001$ and $^{**}P<0.01$). Significantly different from old control rats using one-way ANOVA followed by Newman-Keuls post hoc test ($^{\#}P<0.05$).

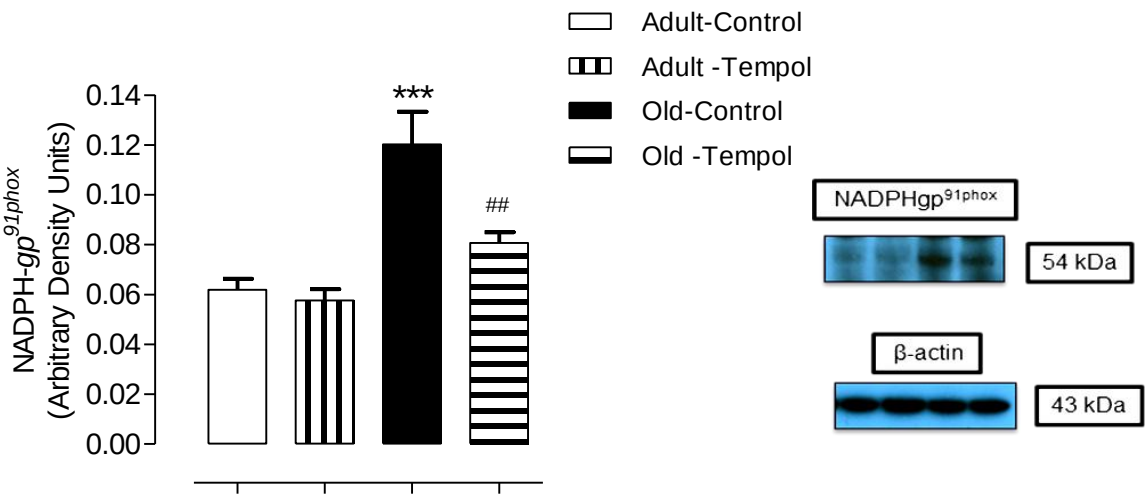

Figure 3: Superoxide radical producing enzyme NADPH-*gp*^{91phox} oxidase increases with aging in FBN rats. Antioxidant tempol decreases age-related increase in NADPH-*gp*^{91phox} in FBN rats. The levels of NADPH-*gp*^{91phox} in renal proximal tubules were measured by western blotting (details in the methods section). The same blot was stripped off and was probed for β-actin as a protein loading control. Right panel: representative blots of NADPH-*gp*^{91phox} and β-actin. In lanes from left to right are adult control, adult tempol, old control and old tempol. Left panel: bars are ratios of the densities between NADPH-*gp*^{91phox} and β-actin protein bands. Results are mean ± SEM (n=4-5 rats in each group). ***Significantly different from adult rats at P<0.001. $^{\#\#}$Significantly different from old control rats at P<0.01 (One-way ANOVA followed by Newman-Keuls post hoc test).

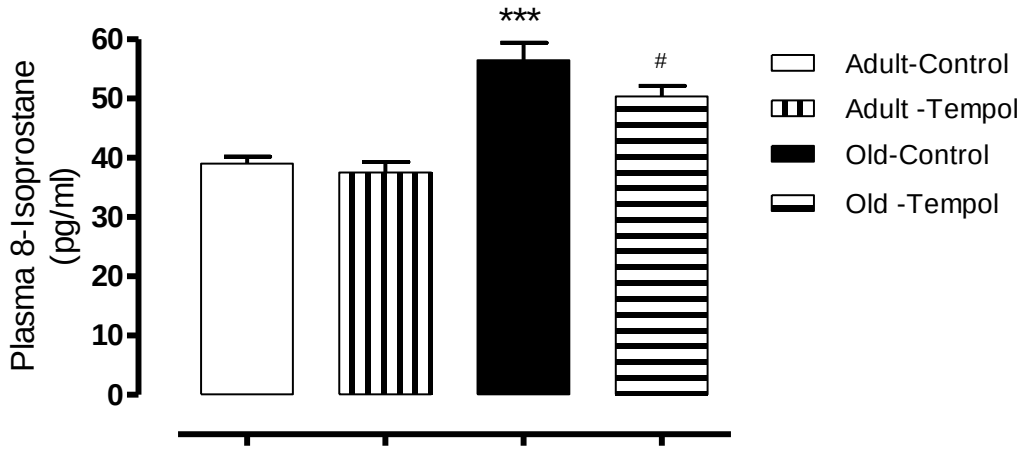

Figure 4: Antioxidant tempol decreases age-related increase in plasma 8-Isoprostane levels in old FBN rats. 8-Isoprostane in plasma was measured using an EIA-based kit assay (details in the methods section). Results are mean ± SEM (n=6-8 rats in each group). ***Significantly different from adult control rats at P<0.001. #Significantly different from old control rats at P<0.05 (One-way ANOVA followed by Newman-Keuls post hoc test).

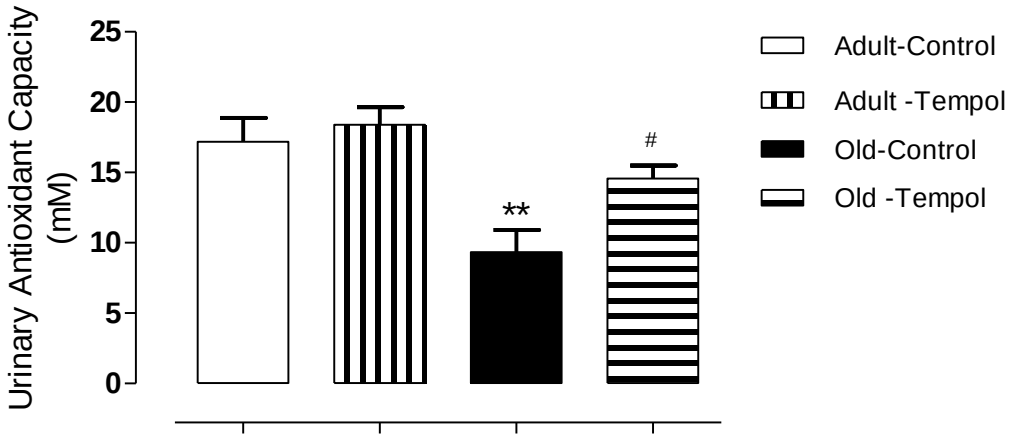

Figure 5: Urinary antioxidant capacity decreases with aging in FBN rats. Antioxidant tempol increases age-related decline in antioxidant capacity in old rats. Total antioxidant capacity in urine was measured using a kit based assay (details in the methods section). Results are mean $\pm$ SEM (n=6 rats in each group). **Significantly different from adult control rats at $P<0.01$. $^{\#}$Significantly different from old control rats at $P<0.05$ (One-way ANOVA followed by Newman-Keuls post hoc test).

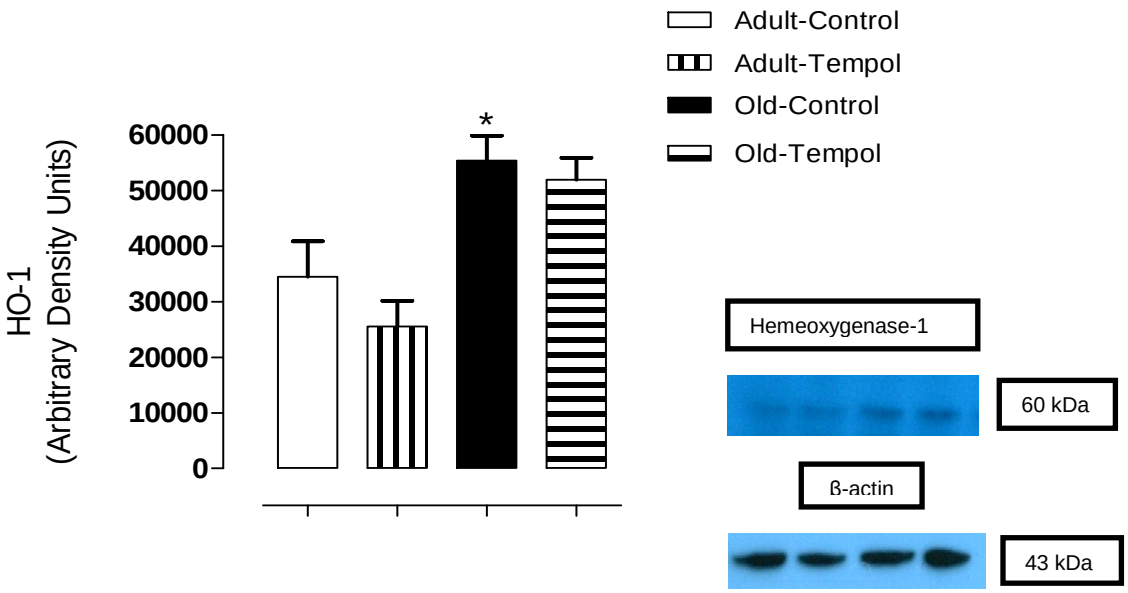

Figure 6: Old control rats exhibit age-associated increase in HO-1 levels compared to adult control rats. Treatment with antioxidant tempol produces no changes in HO-1 levels in adult and old rats. The HO-1 levels in the renal proximal tubules were measured using standard western blotting techniques (details in methods section). The same blot was stripped off and probed for β-actin as a protein loading control. Right panel: representative blots of HO-1 and β-actin. In lanes from left to right are adult control, adult tempol, old control and old tempol. Left panel: bars are ratios of the densities between HO-1 and β-actin protein bands. Results are mean $\pm$ SEM (n=4-5 rats in each group). *Significantly different from adult control rats at $P<0.05$ (One-way ANOVA followed by Newman-Keuls post hoc test).

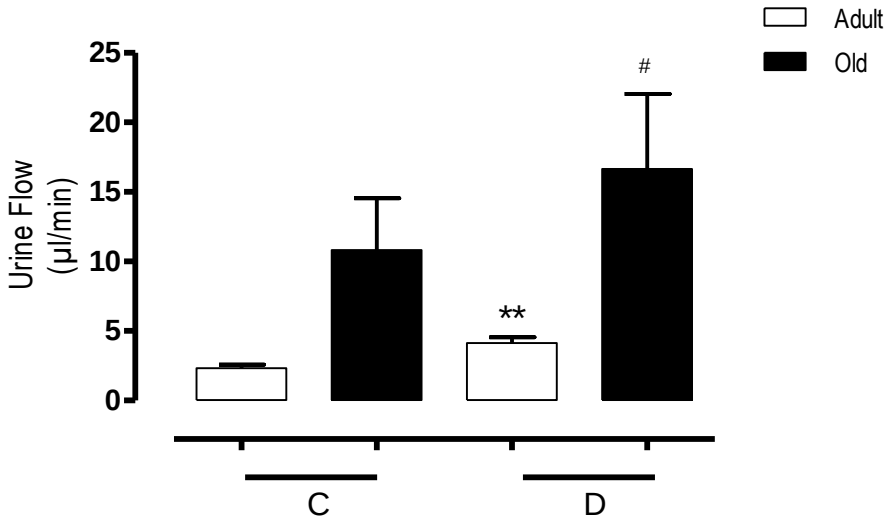

Figure 7: Renal dopamine D1 receptor activation produces diuresis (urine flow) in both adult and old FBN rats. Urine flow in response to D1 receptor agonist *SKF-38393* (1µg/kg/min i.v.) was measured in anesthetized rats (details in the methods section). C represents average of two 30 min control periods where saline only was infused. D represents 30 min drug infusion period where *SKF-38393* in saline was infused. Results are mean ± SEM (n=6 adult and 8 old rats). **Significantly different from C in adult rats at P<0.01. #Significantly different from C in old rats at P<0.05. Statistical significance was achieved using paired t test.

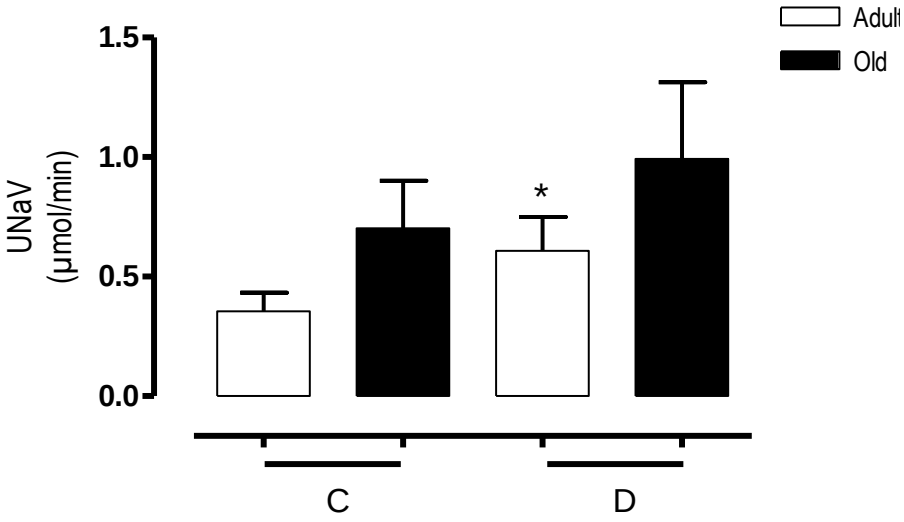

Figure 8: Renal dopamine D1 receptor activation produces natriuresis (UNaV) in adult but not in old FBN rats. UNaV in response to D1 receptor agonist *SKF-38393* (1µg/kg/min i.v.) was determined in anesthetized rats (details in the methods section). C represents average of two 30 min control periods where saline only was infused. D represents 30 min drug infusion period where *SKF-38393* in saline was infused. Results are mean $\pm$ SEM (n=6 adult and 10 old rats). *Significantly different from C in adult rats at $P<0.05$ (paired t test).

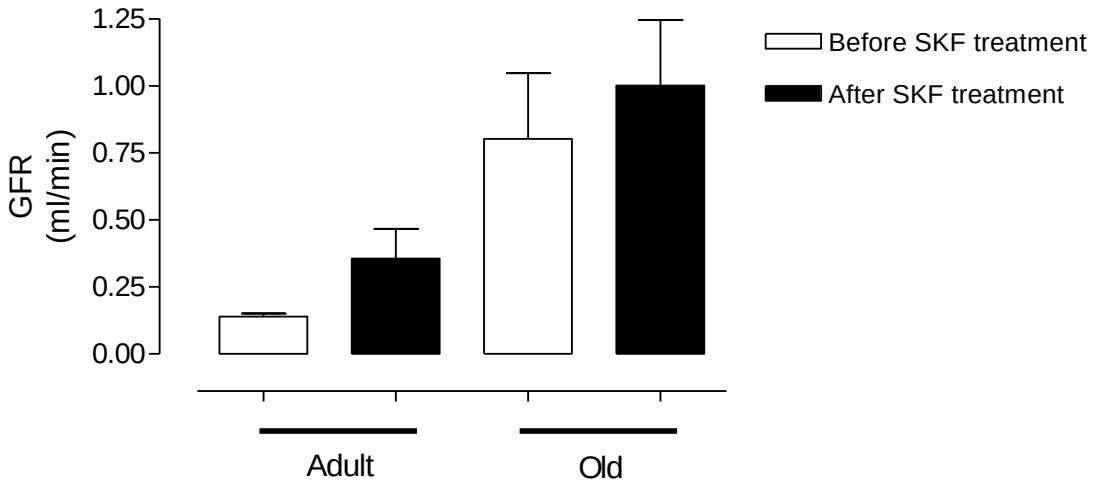

Figure 9: Treatment with D1 receptor agonist *SKF-38393* does not affect glomerular filtration rate (GFR) in adult and old FBN rats. GFR was determined by measuring creatinine clearance (details in the methods section) before and after *SKF-38393* administration in adult and old rats. Results are mean ± SEM (n=5 adult and 8 old rats). P=0.1081 for adult and P=0.1658 for old using paired t test.

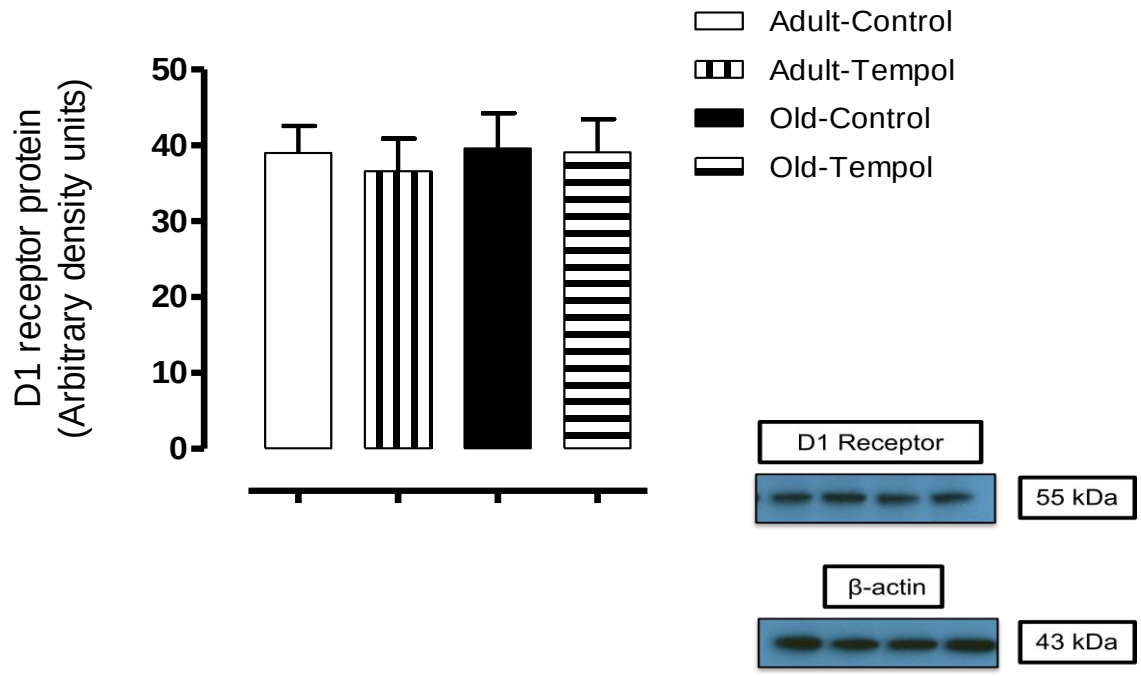

Figure 10: Dopamine D1 receptor protein levels remain unchanged with aging and tempol treatment. Dopamine D1 receptor protein was measured in the renal proximal tubular homogenates using standard western blotting techniques as mentioned in the methods section. The same blot was stripped off and probed for β-actin as a protein loading control. Right panel: representative blots of dopamine D1 receptor and β-actin. In lanes from left to right are adult control, adult tempol, old control and old tempol. Left panel: bars are ratios of the densities between D1 receptor and β-actin protein bands. Results are mean ± SEM (n=5 rats in each group). P=0.9584 (One-way ANOVA).

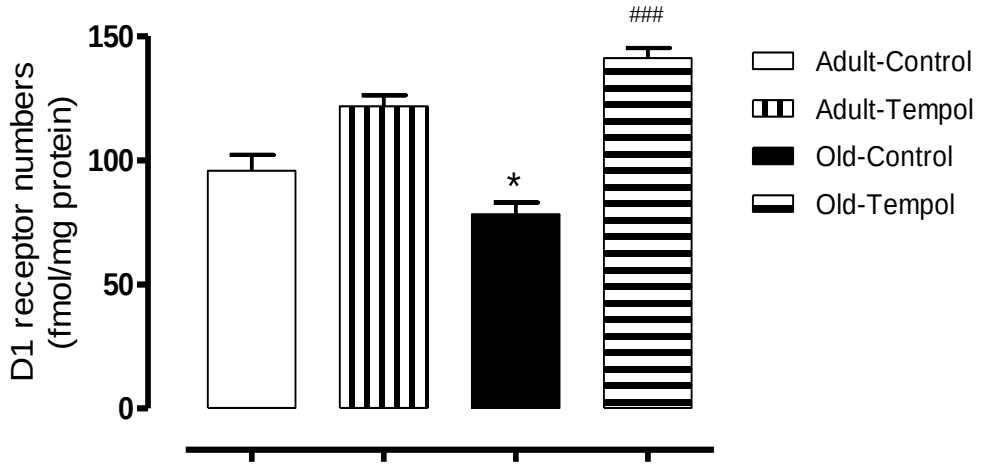

Figure 11: Antioxidant tempol restores the age-associated decline in dopamine D1 receptor numbers in old FBN rats. Dopamine D1 receptor numbers were measured on the renal proximal tubular membranes using radioligand binding assay (details in methods section). Results are mean $\pm$ SEM (n=3-5 rats in each group). *Significantly different from control adult rats at $P<0.05$. $^{###}$Significantly different from control old rats at $P<0.001$ (One-way ANOVA followed by Newman-Keuls post hoc test).

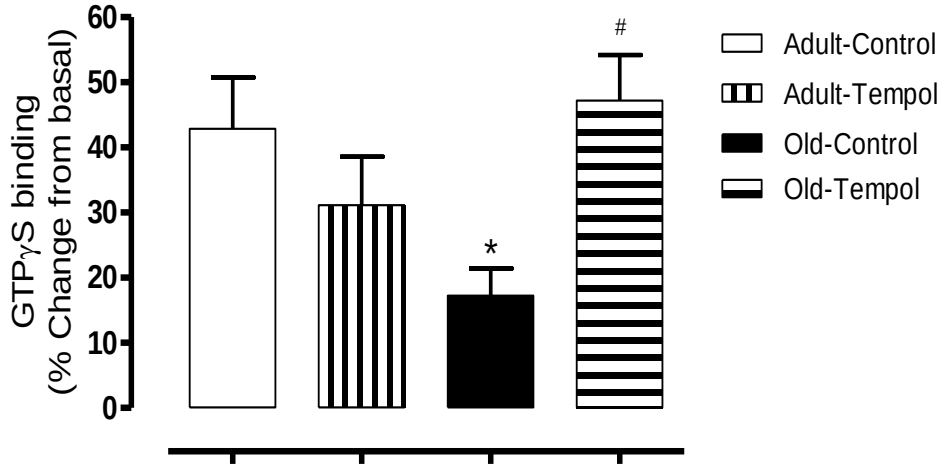

Figure 12: Antioxidant tempol restores the age-related decline in *SKF-38393*-mediated [^{35}S]-GTPγS binding [an index of D1 receptor G-protein coupling] in old rats. [^{35}S]-GTPγS binding in the absence and presence of D1 receptor agonist *SKF-38393* (10^{-6}M) was measured on the renal proximal tubular membranes as described in the methods section. Results are mean ± SEM (n=8-9 rats in each group). *Significantly different from adult control rats at P<0.05. $^{\#}$Significantly different from old control rats at P<0.05 (One-way ANOVA followed by Newman-Keuls post hoc test).

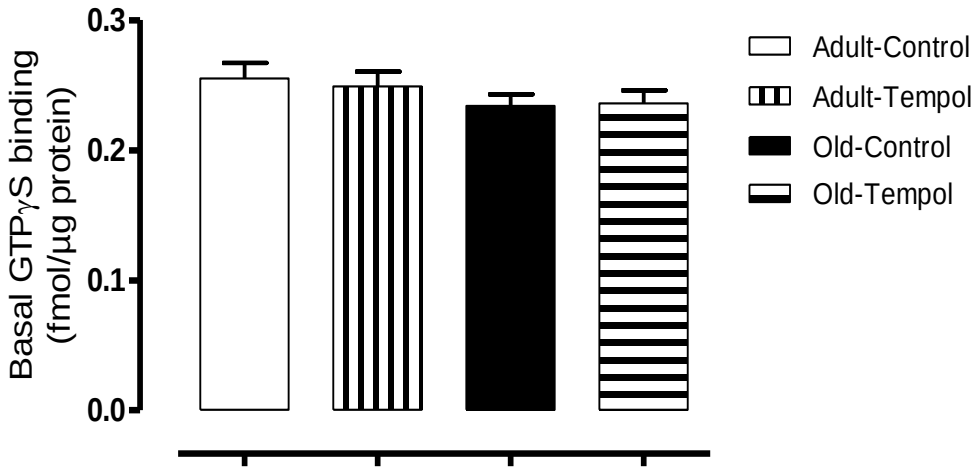

Figure 13: Aging does not affect the basal [^{35}S]-GTPγS binding in FBN rats. The basal GTPγS binding was measured on the renal proximal tubular membranes using radioactive [^{35}S]-GTPγS in the presence of vehicle as described in the methods section. Results are mean ± SEM (n=10 rats in each group). P=0.4462 (One-way ANOVA).

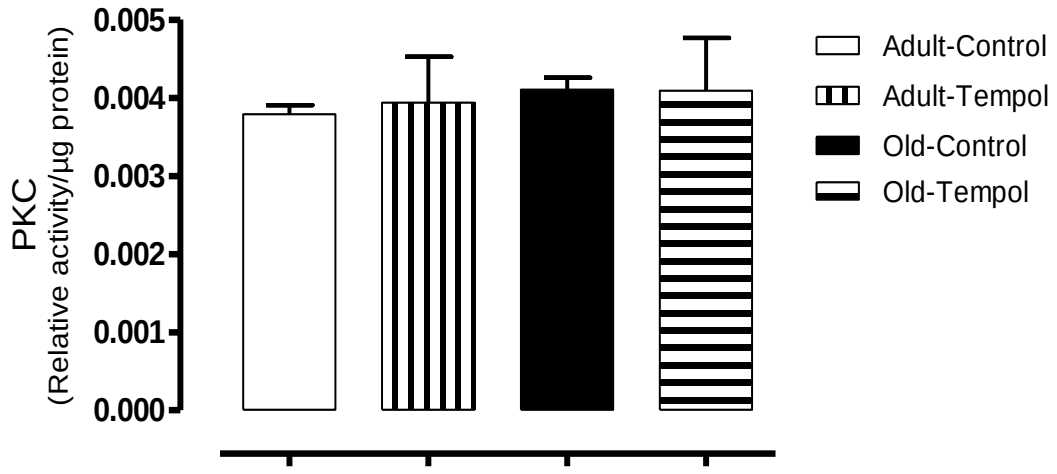

Figure 14: Aging does not affect PKC activity in FBN rats. Tempol treatment in these rats does not change PKC activity. PKC activity was measured in the renal proximal tubules using a commercially available kit (details in methods section). Results are mean ± SEM (n=5-6 rats in each group). P=0.9686 (One-way ANOVA).

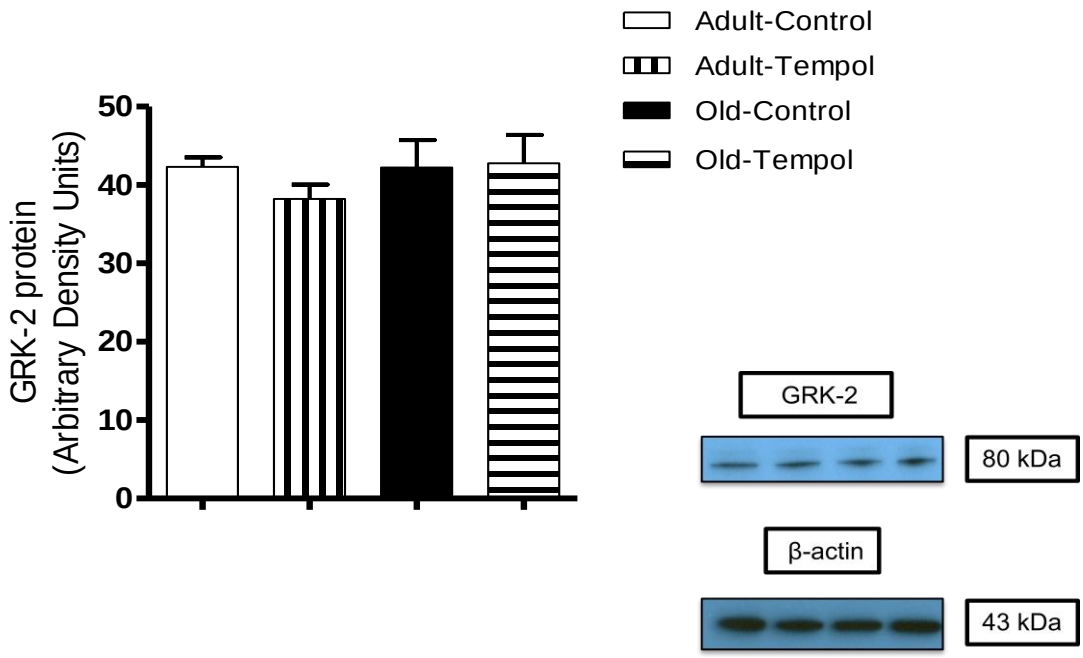

Figure 15: Aging does not affect the levels of GRK-2 protein in FBN rats. Tempol treatment does not change the levels of GRK-2 protein in these rats. GRK-2 levels were measured in the renal proximal tubules using standard western blotting techniques (details in methods section). The same blot was stripped off and probed for β-actin as a protein loading control. Right panel: representative blots of GRK-2 and β-actin. In lanes from left to right are adult control, adult tempol, old control and old tempol. Left panel: bars are ratios of the densities between GRK-2 and β-actin protein bands. Results are mean ± SEM (n=5 rats in each group). P=0.6284 (One-way ANOVA).

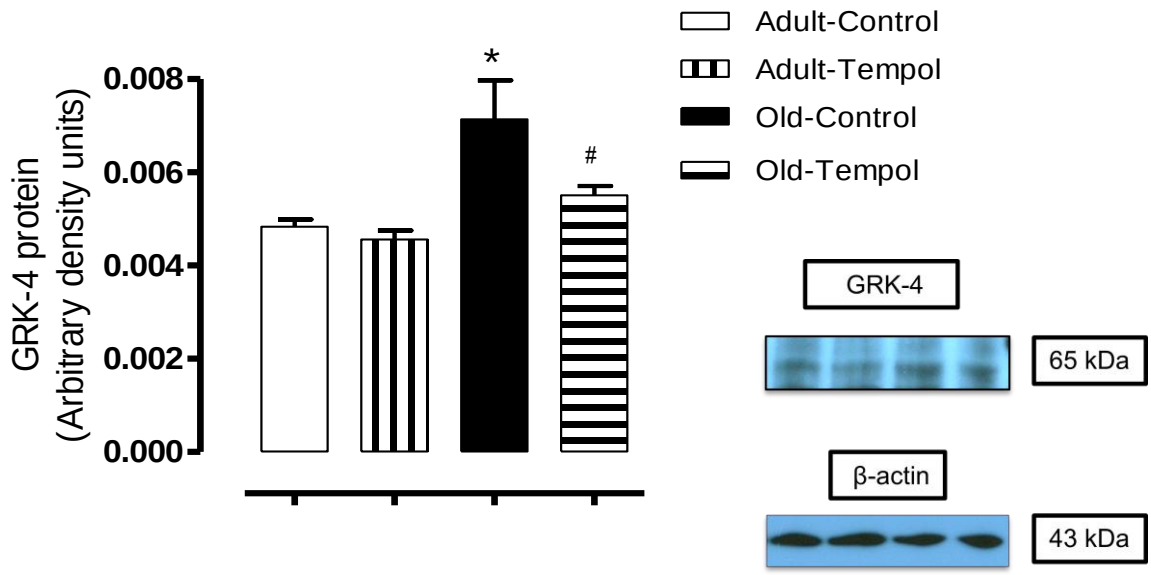

Figure 16: Aging increases the levels of GRK-4 protein in FBN rats. Tempol treatment decreases age-related increase in the GRK-4 protein levels in old FBN rats. GRK-4 protein levels were measured in the renal proximal tubules using standard western blotting techniques (details in methods section). The same blot was stripped off and probed for β-actin as a protein loading control. Right panel: representative blots of GRK-4 and β-actin. In lanes from left to right are adult control, adult tempol, old control and old tempol. Left panel: bars are ratios of the densities between GRK-4 and β-actin protein bands. Results are mean ± SEM (n=4-5 rats in each group). *Significantly different from adult control rats at $P<0.05$. $^{\#}$Significantly different from old control rats at $P<0.05$ (One-way ANOVA followed by Newman-Keuls post hoc test).

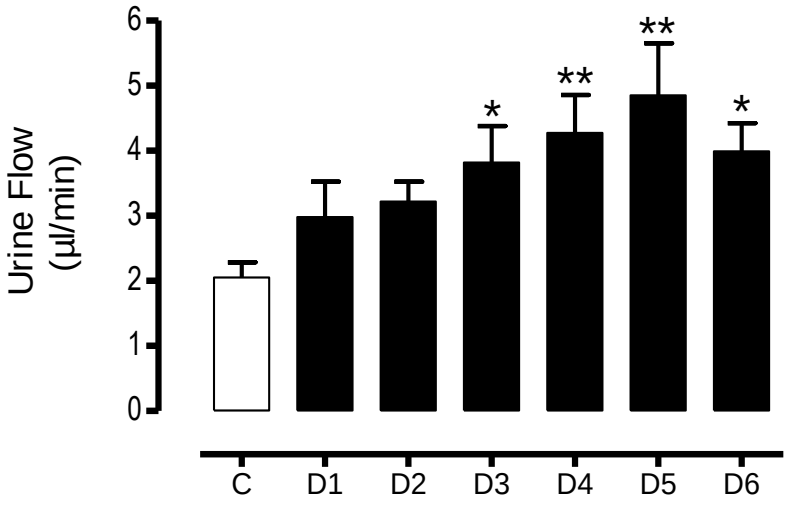

Figure 17: Administration of AT1 receptor antagonist *candesartan* (10µg/kg i.v. bolus) produces significant diuresis (urine flow) in Sprague Dawley rats (details in the methods section). C represents average of two 20 min control periods where saline only was infused. D1-D6 represent drug infusion periods (each 20 min) after *candesartan* was administered as a bolus dose. Results are mean ± SEM (n= 5-10 rats). Significantly different from C (basal) using repeated measures ANOVA followed by Dunnett post-hoc test (**P<0.01 and *P<0.05).

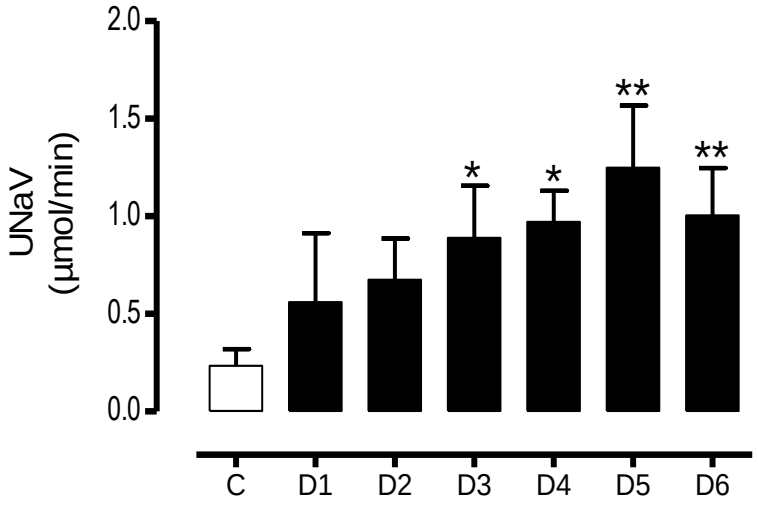

Figure 18: Administration of AT1 receptor antagonist *candesartan* (10µg/kg i.v. bolus) produces significant natriuresis (UNaV) in Sprague Dawley rats (details in the methods section). C represents average of two 20 min control periods where saline only was infused. D1-D6 represent drug infusion periods (each 20 min) after *candesartan* was administered as a bolus dose. Results are mean ± SEM (n= 5-10 rats). Significantly different from C (basal) using repeated measures ANOVA followed by Dunnett post-hoc test (**P<0.01 and *P<0.05).

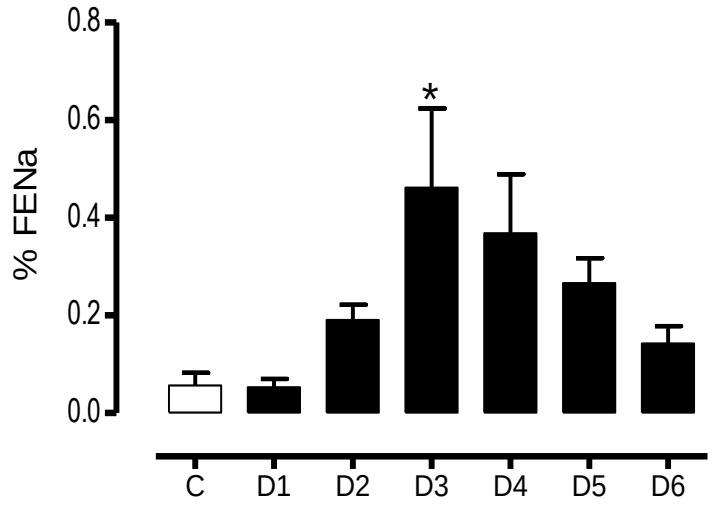

Figure 19: Administration of AT1 receptor antagonist *candesartan* (10µg/kg i.v. bolus) produces significant fractional excretion of sodium (% FENa) in Sprague Dawley rats (details in the methods section). C represents average of two 20 min control periods where saline only was infused. D1-D6 represent drug infusion periods (each 20 min) after *candesartan* was administered as a bolus dose. Results are mean ± SEM (n= 3-5 rats). Significantly different from C (basal) using repeated measures ANOVA followed by Dunnett post-hoc test (*P<0.05).

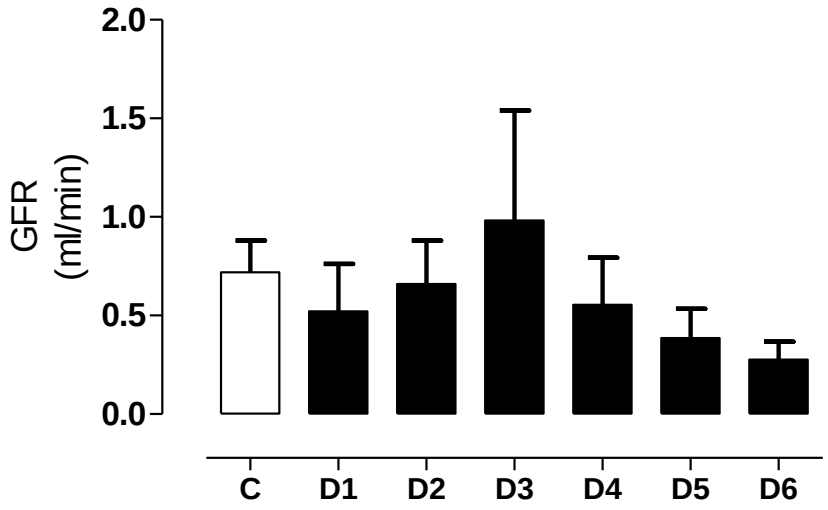

Figure 20: Administration of AT1 receptor antagonist *candesartan* (10µg/kg i.v. bolus) produces no significant change in GFR in Sprague Dawley rats (details in the methods section). C represents average of two 20 min control periods where saline only was infused. D1-D6 represent drug infusion periods (each 20 min) after *candesartan* was administered as a bolus dose. Results are mean ± SEM (n= 4-10 rats). P= 0.1107 using repeated measures ANOVA.

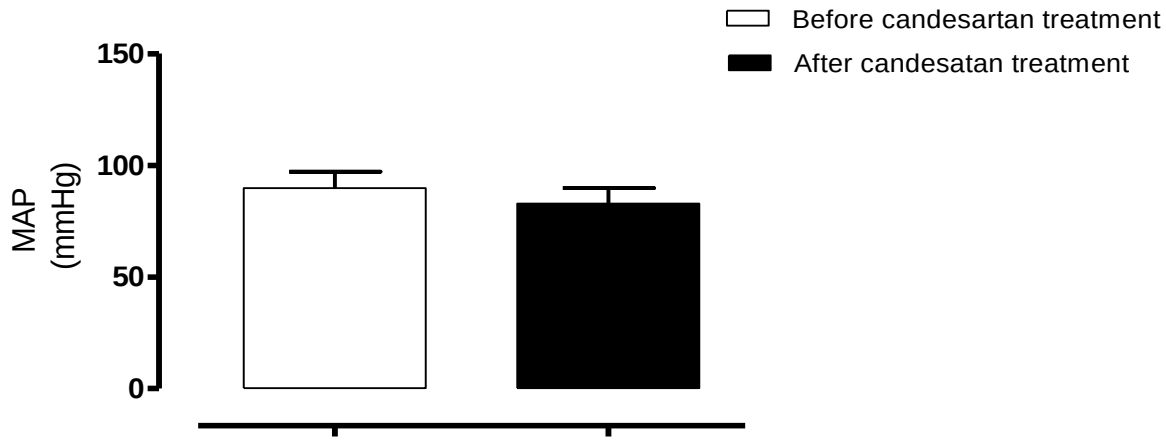

Figure 21: Administration of AT1 receptor antagonist *candesartan* (10µg/kg i.v. bolus) produces no significant change in mean arterial pressure (MAP) in anesthetized Sprague Dawley rats (details in the methods section). Results are mean ± SEM (n= 4 rats in each group). P= 0.1026 using paired t test.

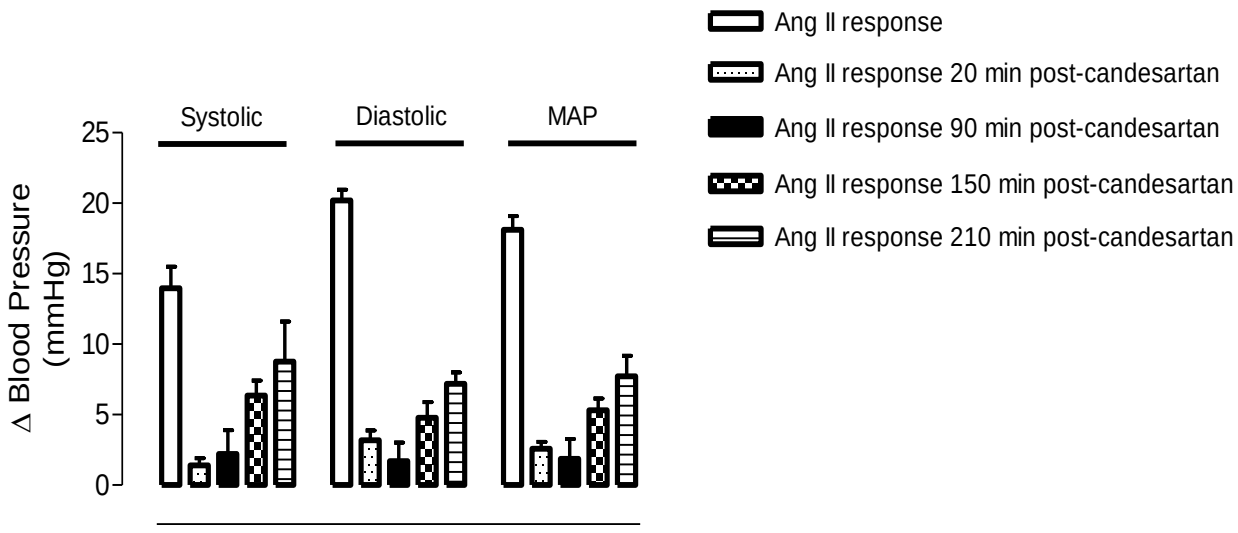

Figure 22: Administration of *candesartan* (10µg/kg i.v. bolus) significantly blunts angiotensin II-mediated increase in systolic, diastolic and mean arterial pressures up to 210 min after giving *candesartan*. Increase in blood pressure (Δ blood pressure) in response to angiotensin II (60ng i.v.) was measured in the absence and 20, 90, 150 and 210 min post-*candesartan* administration. Blood pressure was measured in anesthetized Sprague Dawley rats (details in methods section). Results are mean ± SEM (n= 3-6 rats in each group).

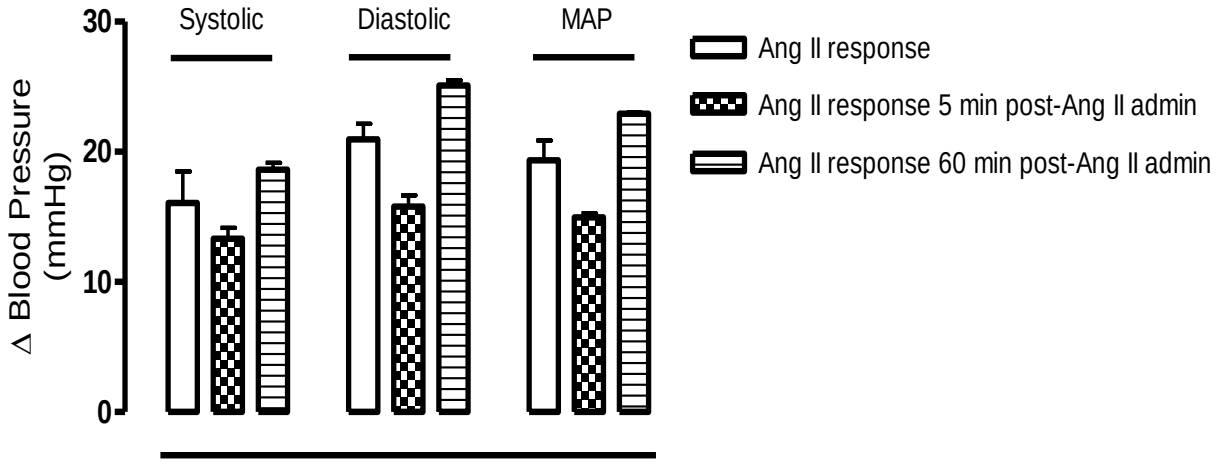

Figure 23: Increase in systolic, diastolic and mean arterial pressures in response to angiotensin II (60ng i.v.) does not change on repeated administrations. Response to angiotensin II (Δ blood pressure) was recorded after 5 and 60 min of an initial increase in blood pressure in response to angiotensin II administration. Blood pressure was measured in anesthetized Sprague Dawley rats as described in the methods section. Results are mean ± SEM (n= 3-6 rats in each group).

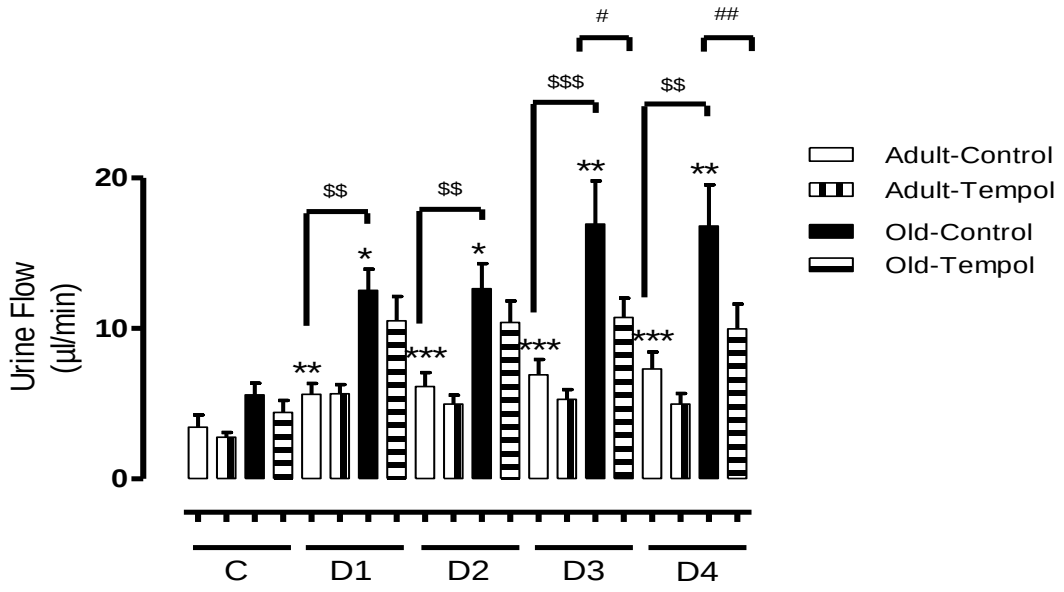

Figure 24: Antioxidant tempol reduces exaggerated *candesartan*-mediated diuresis (urine flow) in old rats. Urine flow in response to AT1 receptor antagonist *candesartan* (10µg/kg i.v. bolus) was determined in anesthetized rats (details in the methods section). C represents average of two 30 min control periods where saline only was infused. D1-D4 represent drug infusion periods (each 30 min) after *candesartan* was administered as a bolus dose. Results are mean ± SEM (n= 5-7 rats). Significantly different from C (basal) using repeated measures ANOVA followed by Dunnett post-hoc test (***P<0.001, **P<0.01 and *P<0.05). Significantly different from adult control rats using one-way ANOVA followed by Newman-Keuls post hoc test ($^{\$\$\$}$P<0.001 and $^{\$\$}$P<0.01). Significantly different from old control rats using one-way ANOVA followed by Newman-Keuls post hoc test ($^{\#\#}$P<0.01 and $^{\#}$P<0.05).

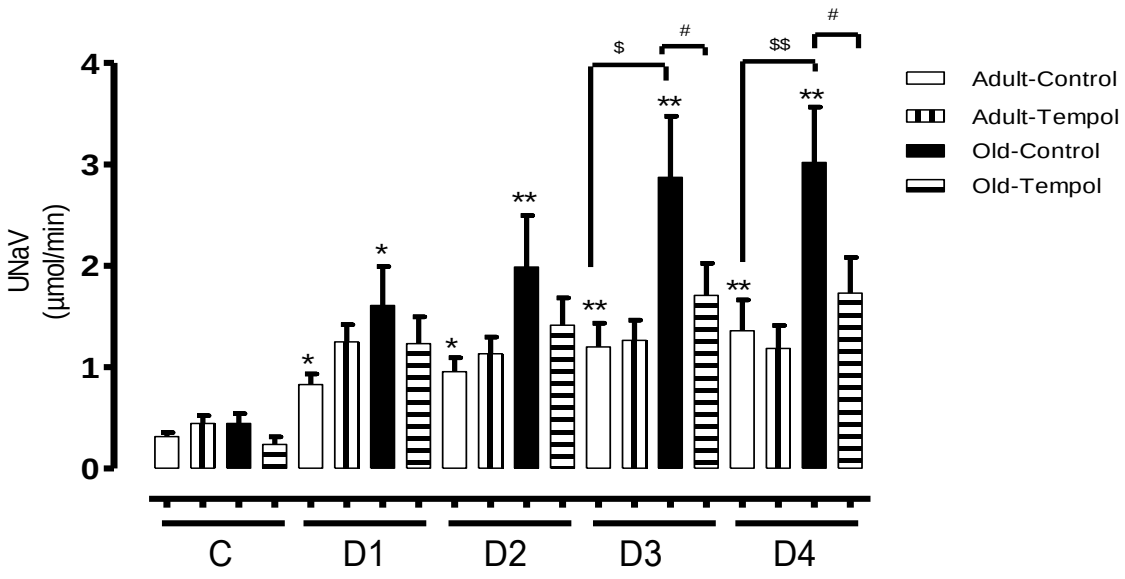

Figure 25: Antioxidant tempol reduces exaggerated *candesartan*-mediated natriuretic response (UNaV) in old rats. UNaV in response to AT1 receptor antagonist *candesartan* (10µg/kg i.v. bolus) was determined in anesthetized rats. C represents average of two 30 min control periods where saline only was infused. D1-D4 represent drug infusion periods (each 30 min) after *candesartan* was administered as a bolus dose. Results are mean ± SEM (n= 5-7 rats). Significantly different from C (basal) using repeated measures ANOVA followed by Dunnett post-hoc test (**P<0.01 and *P<0.05). Significantly different from adult controls using one-way ANOVA followed by Newman-Keuls post hoc test ($^{\$\$}$P<0.01 and $^{\$}$P<0.05). Significantly different from old control rats using one-way ANOVA followed by Newman-Keuls post hoc test ($^{\#}$P<0.05).

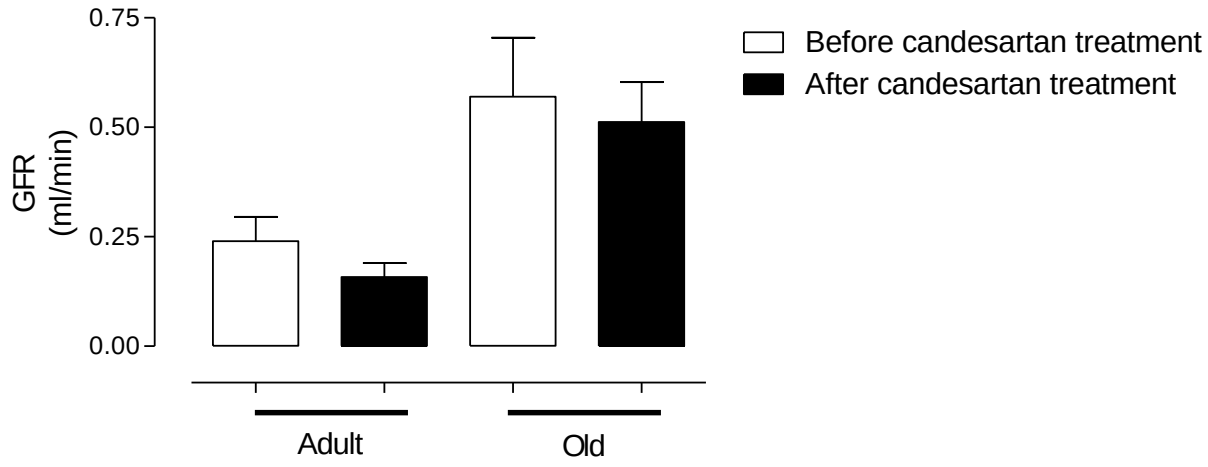

Figure 26: Administration of *candesartan* produces no significant change in GFR in adult and old FBN rats. GFR was determined based on the clearance of creatinine (details in the methods section) before and after *candesartan* administration. Results are mean ± SEM (n=7 adult and 6 old rats). P=0.1245 for adult and P=0.7097 for old using paired t test.

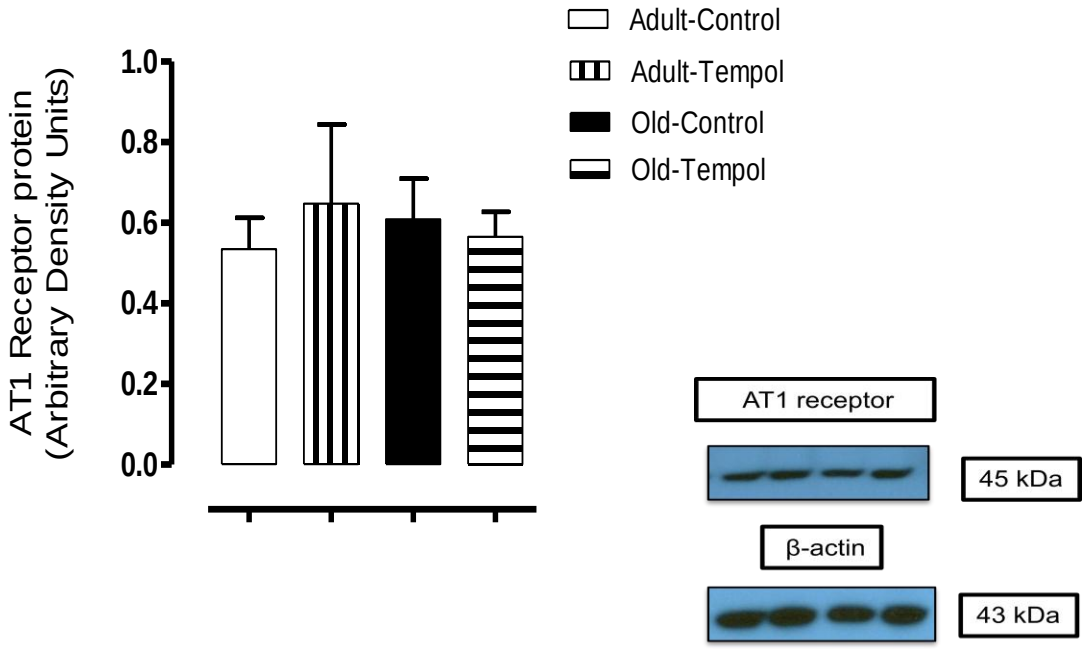

Figure 27: AT1 receptor protein levels are not significantly different between control and tempol-treated adult and old FBN rats. AT1 receptor levels were measured in the renal proximal tubular homogenate using standard western blotting techniques (details in methods section). The same blot was stripped off and probed for β-actin as a protein loading control. Right panel: representative blots of AT1 receptor and β-actin. In lanes from left to right are adult control, adult tempol, old control and old tempol. Left panel: bars are ratios of the densities between AT1 receptor and β-actin protein bands. Results are mean $\pm$ SEM (n=5 rats in each group). P=0.9167 (One-way ANOVA).

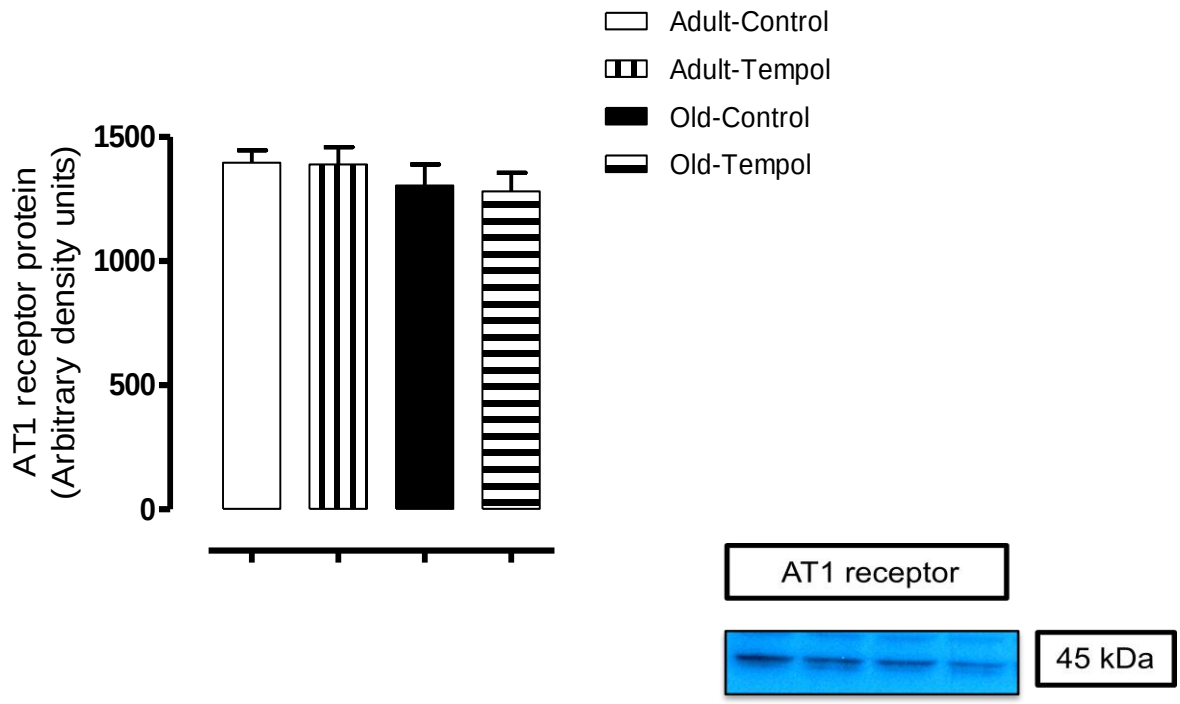

Figure 28: AT1 receptor protein levels on the renal proximal tubular membranes are not significantly different between the four groups. AT1 receptor protein was measured using standard western blotting techniques (details in methods section). Right panel: representative blot of AT1 receptor protein. In lanes from left to right are adult control, adult tempol, old control and old tempol. Left panel: bars are representative of the density of AT1 receptor protein bands. Results are mean ± SEM (n=3-4 rats in each group). P=0.5933 (One-way ANOVA).

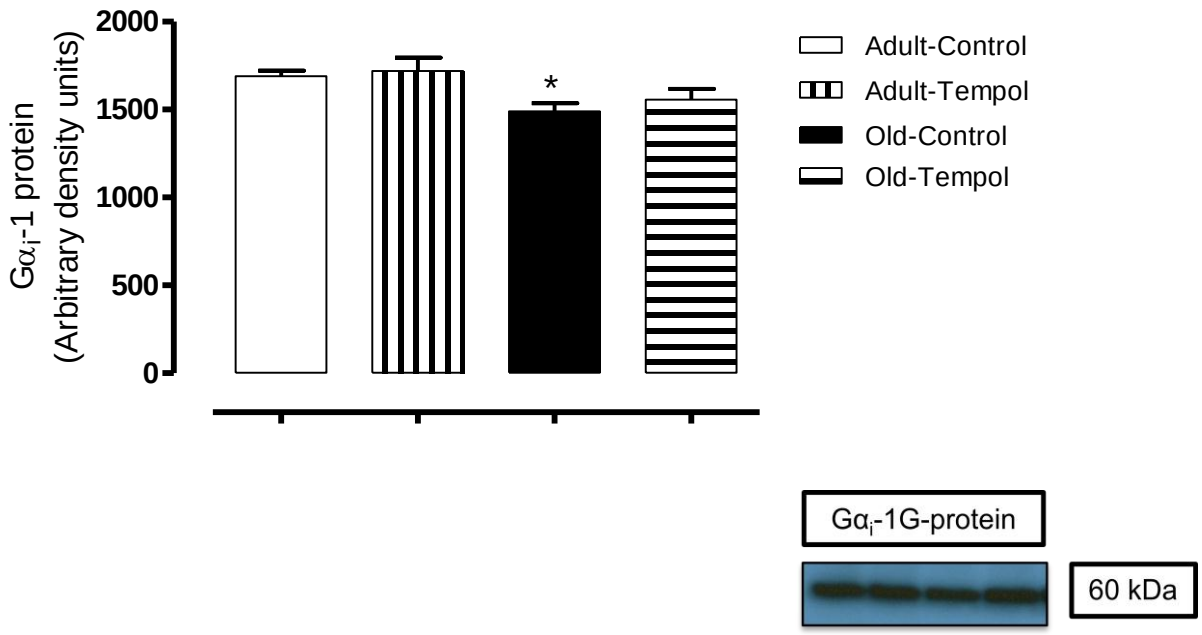

Figure 29: Old control rats have reduced $G\alpha_i$-1 protein levels on the renal proximal tubular membranes compared to adult control rats. Treatment with tempol produces no change in $G\alpha_i$-1 protein levels in adult and old rats. $G\alpha_i$-1 protein levels were measured using standard western blotting techniques as described in the methods section. Right panel: representative blot of $G\alpha_i$-1 protein. In lanes from left to right are adult control, adult tempol, old control and old tempol. Left panel: bars are representative of the density of $G\alpha_i$-1 protein bands. Results are mean $\pm$ SEM (n=3-4 rats in each group). *Significantly different from adult control rats at $P<0.05$ using one-way ANOVA followed by Newman-Keuls post hoc test.

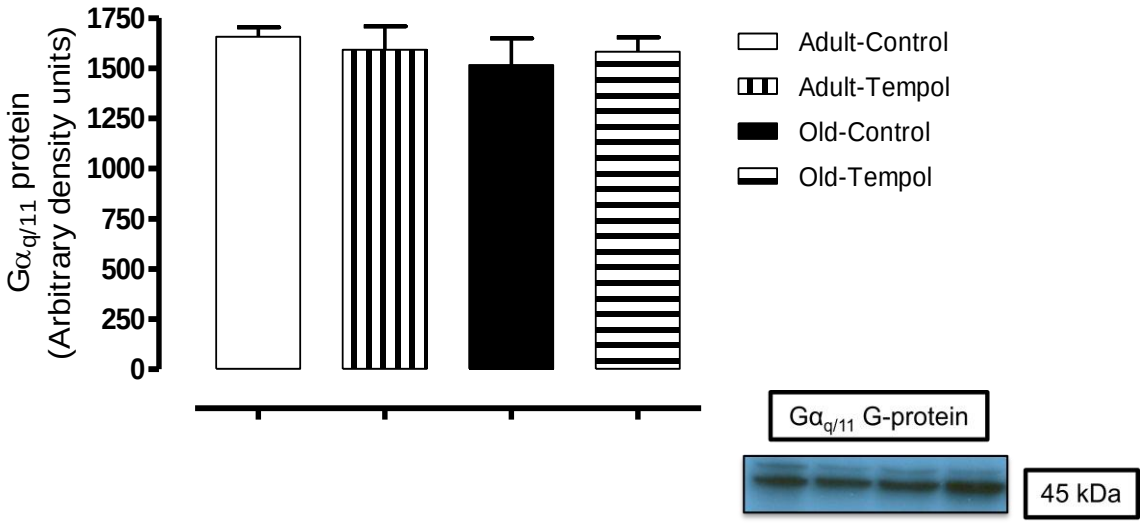

Figure 30: $G\alpha_{q/11}$ protein levels are not significantly different between control and tempol-treated adult and old FBN rats. $G\alpha_{q/11}$ protein levels were measured on the renal proximal tubular membranes using standard western blotting techniques (details in methods section). Right panel: representative blot of $G\alpha_{q/11}$ protein. In lanes from left to right are adult control, adult tempol, old control and old tempol. Left panel: bars are representative of the density of $G\alpha_{q/11}$ protein bands. Results are mean $\pm$ SEM (n=3-4 rats in each group). P=0.7678 (One-way ANOVA).

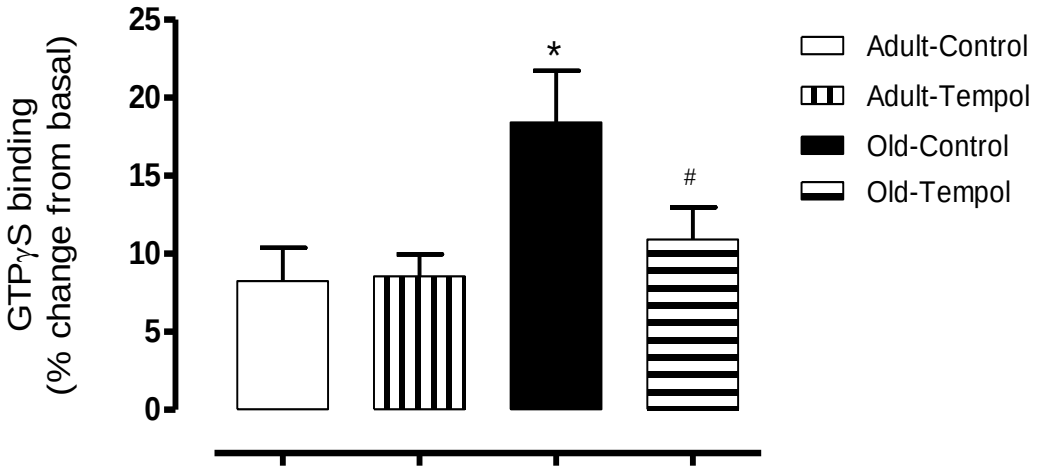

Figure 31: Antioxidant tempol restores age-associated elevated angiotensin II-mediated [^{35}S]-GTPγS binding [an index of AT1 receptor G-protein coupling] in old FBN rats. [^{35}S]-GTPγS binding in the absence and presence of angiotensin II (10^{-6}M) was measured on the renal proximal tubular membranes as described in the methods section. Results are mean ± SEM (n=6-7 rats in each group). *Significantly different from adult control rats at P<0.05. $^{\#}$Significantly different from old control rats at P<0.05 (One-way ANOVA followed by Newman-Keuls post hoc test).

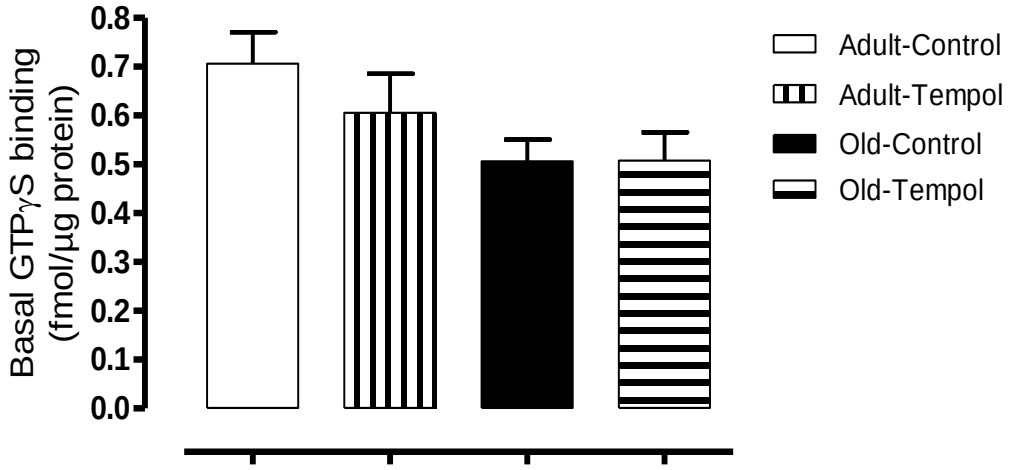

Figure 32: Aging does not affect the basal [^{35}S]-GTPγS binding in FBN rats. The basal GTPγS binding was measured on the renal proximal tubular membranes using radioactive [^{35}S]-GTPγS in the presence of vehicle as described in the methods section. Results are mean ± SEM (n=6-8 rats in each group). P=0.1559 (One-way ANOVA).

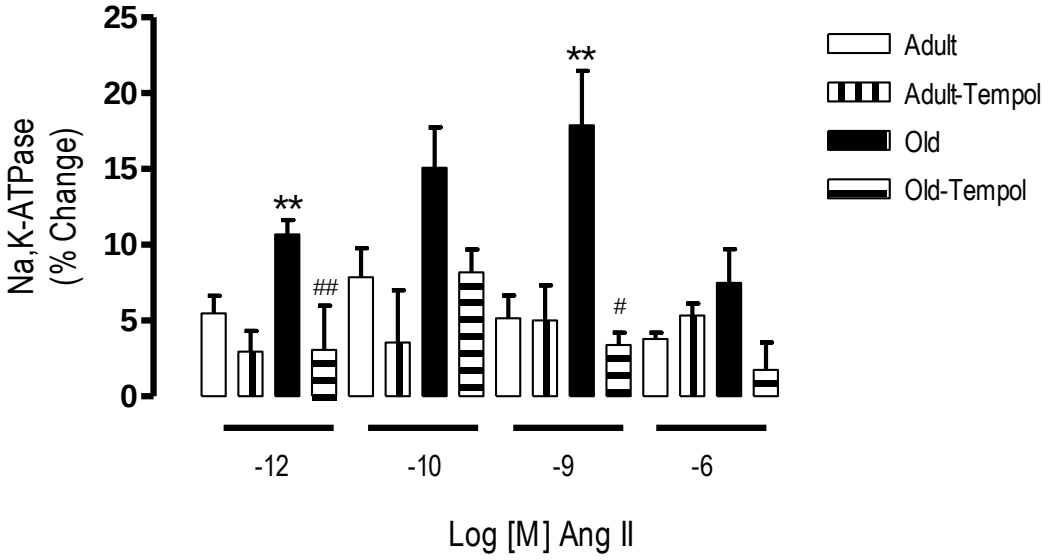

Figure 33: Old control rats have an age-associated increase in angiotensin II-mediated Na,K-ATPase activity (10^{-12} and 10^{-9} M) compared to adult control rats. Treatment with tempol restores the exaggerated angiotensin II-mediated response in old rats. The basal Na,K-ATPase activity (nmolPi/mg/min) was not different between adult (323.9 $\pm$ 20.33) and old (364.7 $\pm$ 31.96) rats. Na,K-ATPase activity was measured in the freshly isolated renal proximal tubules by a colorimetric assay as described in the methods section. Results are mean $\pm$ SEM (n=4-10 rats in each group). Significantly different from adult control rats using one-way ANOVA followed by Newman-Keuls post hoc test (**P<0.01). Significantly different from old control rats using one-way ANOVA followed by Newman-Keuls post hoc test ($^{\#}$P<0.05, $^{\#\#}$P<0.01).

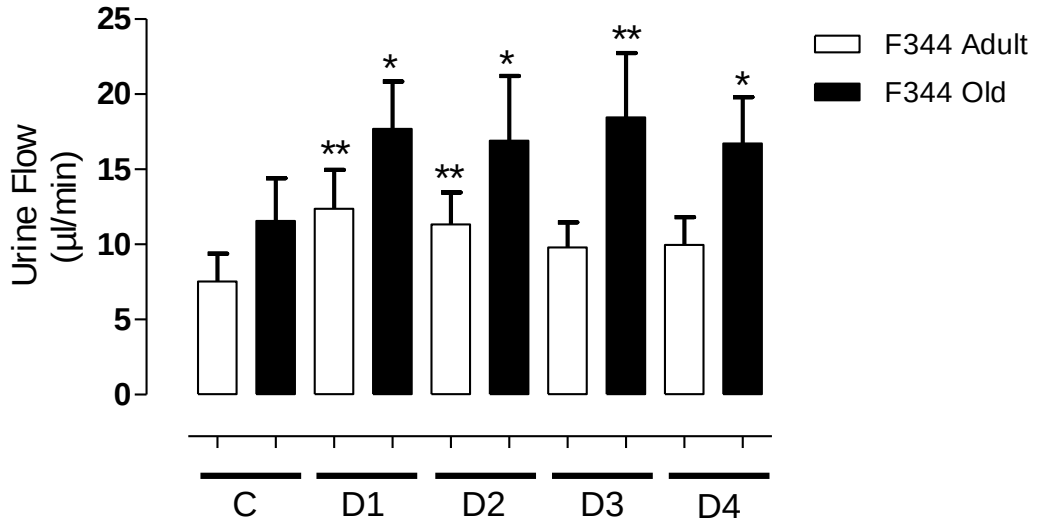

Figure 34: There are no age-associated changes in diuretic response (urine flow) to *candesartan* between adult and old F344 rats. Urine flow in response to AT1 receptor antagonist *candesartan* (10µg/kg i.v. bolus) was determined in adult (6 month) and old (24 month) rats (details in the methods section). C represents average of two 30 min control periods where saline only was infused. D1-D4 represent drug infusion periods (each 30 min) after *candesartan* was administered as a bolus dose. Results are mean $\pm$ SEM (n= 7 adult and 5 old rats). Significantly different from C (basal) using repeated measures ANOVA followed by Dunnett post-hoc test (**P<0.01 and *P<0.05). Using unpaired t test, no significant difference was observed between adult and old rats.

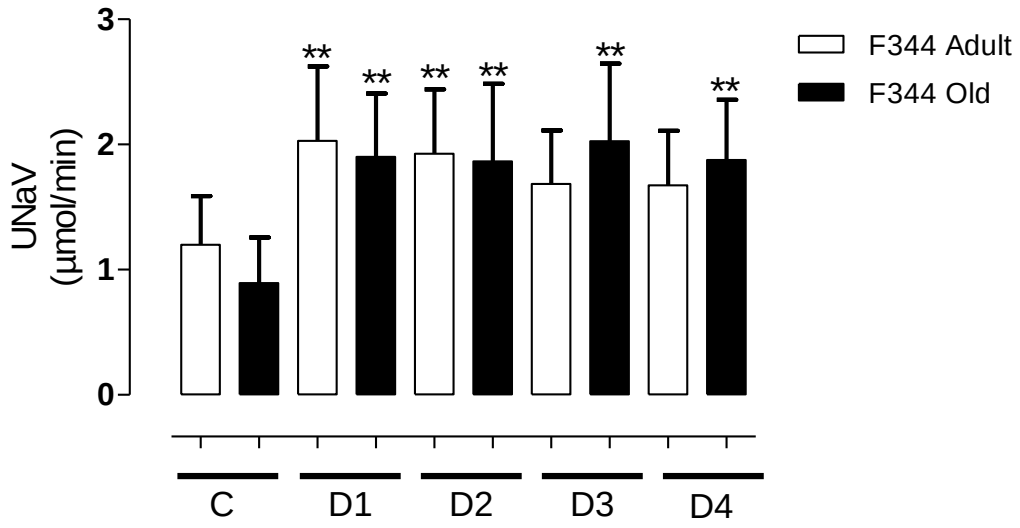

Figure 35: There are no age-associated changes in natriuretic response to *candesartan* between adult and old F344 rats. Urinary sodium excretion (UNaV) in response to AT1 receptor antagonist *candesartan* (10µg/kg i.v. bolus) was determined in adult (6 month) and old (24 month) rats (details in the methods section). C represents average of two 30 min control periods where saline only was infused. D1-D4 represent drug infusion periods (each 30 min) after *candesartan* was administered as a bolus dose. Results are mean ± SEM (n= 7 adult and 5 old rats). Significantly different from C (basal) using repeated measures ANOVA followed by Dunnett post-hoc test (**P<0.01). Using unpaired t test, no significant difference was observed between adult and old rats.

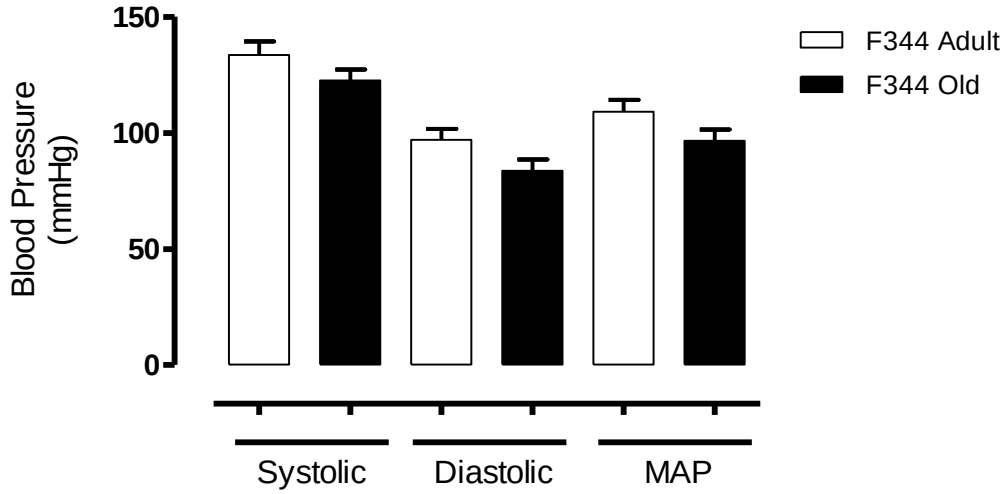

Figure 36: There are no age-associated changes in blood pressure between adult and old F344 rats. Blood pressure (systolic, diastolic and mean arterial) was measured in anesthetized F344 rats (details in the methods section). Results are mean ± SEM. (n= 7 adult and 6 old rats). Using unpaired t test, there was no difference in systolic (P= 0.1822), diastolic (P= 0.0795) and mean arterial (P= 0.1061) pressures between adult and old rats.

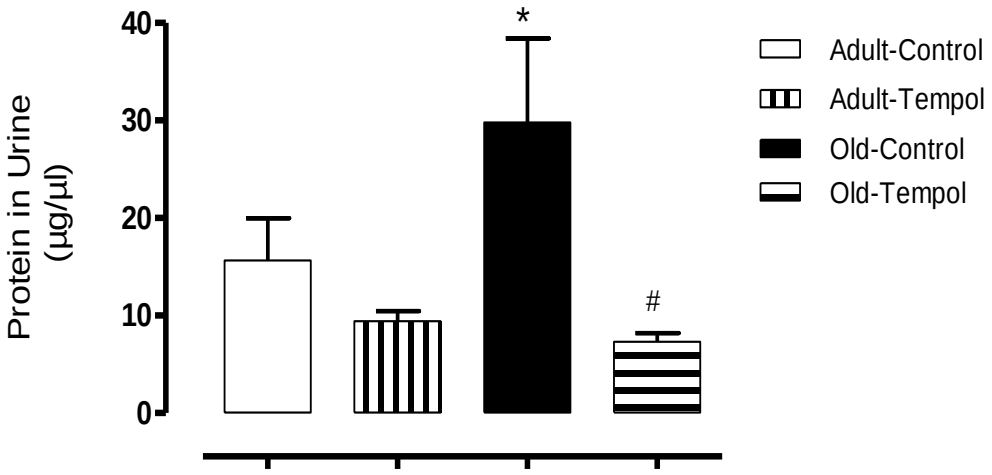

Figure 37: Antioxidant tempol restores the age-associated elevated urinary protein excretion levels in old FBN rats. Urinary protein was measured by BCA method as described in the methods section. Results are mean ± SEM. (n= 5-6 rats). Significantly different from adult control rats using one-way ANOVA followed by Newman-Keuls post hoc test (*P<0.05). Significantly different from old control rats using one-way ANOVA followed by Newman-Keuls post hoc test ($^{\#}$P<0.05).

General Parameters	Adult-Control	Old-Control	Adult-Tempol	Old-Tempol
Body Weight (gm)	302.5 ± 8.4 (n=8)	561.3 ± 16.84*** (n=8)	305.6 ± 7.53 (n=8)	552.5 ± 14.49*** (n=8)
Food Intake (gm/rat/day)	21.9 ± 0.9 (n=7-8)	21.81 ± 0.95 (n=7-8)	18.38 ± 1.64 (n=8)	21 ± 1.22 (n=8)
Water Intake (ml/rat/day)	29.44 ± 1.54 (n=7-8)	25.12 ± 1.74 (n=7-8)	26.3 ± 1.12 (n=12)	30.99 ± 1.33* (n=12)

Significantly different from adult rats (*P<0.05; ***P<0.001)

Table 1: Effect of aging on general body parameters in FBN rats

5. DISCUSSION

Aging is frequently associated with high blood pressure (hypertension) and its accompanied cardiovascular complications like myocardial ischemia, stroke and heart failure (28). The exact mechanism of patho-biology of age-associated hypertension is not known. However, since sodium retention is a hallmark for essential hypertension, growing number of studies have focused on abnormal renal sodium handling in the pathogenesis of hypertension (7-10). In the kidneys, there are two key receptor systems namely dopamine D1 and angiotensin AT1 receptors, which by counter regulating proximal tubular sodium transporters maintain sodium homeostasis and regulate blood pressure (11). Dopamine D1 (96) and angiotensin AT1 (25, 26) receptor dysfunctions have been reported in humans and animal models with hypertension. In FBN rats; an animal model widely used in aging research, we found age-related increase in blood pressure (old>middle aged>adult) and reduced renal D1 and exaggerated renal AT1 receptor functions. It is likely that abnormal sodium handling as a result of altered D1 and AT1 receptor functions may have caused age-associated hypertension in old FBN rats.

We further explored the cause and mechanisms of altered renal D1 and AT1 receptors functions contributing to hypertension in old FBN rats. There is emerging evidence that oxidative stress contributes to increase in blood pressure

in various animal models of hypertension (25, 124, 134). Also, aging is associated with an increased state of oxidative stress (32). However, in the kidneys, role of oxidative stress in the development of high blood pressure as it relates to aging has not been clearly established. We have previously studied age-associated oxidative stress in renal dopamine D1 receptor dysfunction in old F344 rats (41). Although age-related or oxidative stress mediated alterations in renal AT1 receptor function were not known, we did not investigate these animals in this study as they do not develop age-associated hypertension. Instead, we used FBN rats (rat aging model that exhibits hypertension) to determine the contribution of oxidative stress in age-related impairments in renal D1 and AT1 receptor functions and hypertension. We found a causal role of oxidative stress in the development of age-related high blood pressure in old FBN rats; most likely mediated via alterations in renal D1 and AT1 receptor functions.

To examine the role of oxidative stress in age-associated hypertension in FBN rats, we treated adult and old rats with antioxidant tempol and determined BP, oxidative stress, and renal D1 and AT1 receptor functions. Tempol is a known antioxidant with SOD mimetic properties and has been used previously to reduce oxidative stress and high blood pressure in spontaneously hypertensive rats (135). We began our studies by determining the effects of aging and 3-4 week tempol treatment on blood pressure and some of the general parameters such as body weight and food and water intake in FBN rats. We found age-

associated increase in blood pressure in old compared to adult FBN rats; which was reduced with tempol treatment in old rats. The old FBN rats were overweight compared to adult FBN rats despite a similar amount of food and water intake in these rats. This observation was similar to an earlier report by Turturro et al (1999) (131). Although tempol intake was higher in old than in adult rats it did not alter body weight or food-intake in adult and old FBN rats.

Furthermore, the effects of aging and tempol on oxidative stress were studied by determining some of the markers of oxidative stress and antioxidant defenses. The antioxidant markers were studied so as to determine their contribution to age-associated oxidative stress. NADPH-gp^{91phox} enzyme and 8-isoprostane were studied as oxidative stress markers while HO-1 enzyme and total antioxidant capacity were included in the study as antioxidant markers. NADPH-gp^{91phox} enzyme produces superoxide radicals (136) whereas 8-isoprostane levels are an index of lipid oxidation (137). We found higher levels of NADPH-gp^{91phox} in the renal proximal tubules and 8-isoprostane in the plasma in old FBN rats. Tempol treatment in old FBN rats decreased the levels of NADPH-gp^{91phox} and 8-isoprostane. With regard to antioxidant defenses, we found that there was an age-associated reduction in total urinary antioxidant capacity in old FBN rats, which increased with tempol treatment. However, antioxidant enzyme HO-1 levels in the renal proximal tubules were higher in old FBN rats. HO-1 is an inducible enzyme that is expressed in response to oxidative stress (138), thus

greater levels of HO-1 enzyme in old FBN rats further confirms increased age-associated oxidative stress in these animals. Reduction in oxidative stress with tempol did not reduce HO-1 levels in old rats. The reasons for increased HO-1 levels despite reduced oxidative stress in tempol-treated old rats are unclear. However, increased HO-1 expression with tempol treatment has been previously reported (139). Taken together, these findings suggest that there is an age-related increase in oxidative stress in FBN rats and tempol is an effective antioxidant in this animal model.

We also found an age-associated defect in renal D1 receptor function in FBN rats as activation of dopamine D1 receptor failed to increase sodium excretion in old rats. In the present study, we did not measure natriuretic response to D1 receptor activation in tempol-supplemented adult and old rats. This was decided based upon our previous finding in F344 rats, where tempol treatment restored a similar decline in D1 receptor function in old rats (41).

To study the mechanism(s) for impaired renal D1 receptor function with aging in FBN rats, we measured the levels of dopamine D1 receptors and its associated signaling molecules in the renal proximal tubules of control and tempol-treated adult and old rats. We found reduced D1 receptor numbers on the proximal tubular membranes of old rats. The levels of D1 receptor proteins in the proximal tubular homogenate; however, were not changed in old rats. These are

interesting findings as similar observations have been reported in hypertensive Obese Zucker rats, which are also associated with oxidative stress (38). Our results in old FBN rats suggest that perhaps a similar mechanism of D1 receptor dysfunction exists in these rats as in Obese Zucker rats. G protein coupling in response to D1 receptor activation was also reduced in old rats. The uncoupling of D1 receptor from its G protein effector complex involves activation of GRKs (98, 100, 140-142). GRKs 2-6 have been reported to cause D1 receptor desensitization in heterologous expression systems (100, 141-143). Previous work from our laboratory has established a role of GRK-2 mediated serine phosphorylation in D1 receptor uncoupling from G proteins in old F344 rats. This study suggested a role of elevated PKC levels in this phenomenon (41). In renal proximal tubules from FBN rats, we found no age-associated changes in PKC and GRK-2 levels; however, the levels of GRK-4 were elevated in old rats. While PKC and GRK-2 levels remained unchanged with tempol treatment, GRK-4 levels were reduced in renal proximal tubules of old FBN rats. GRK-4 is considered important than some other GRKs (including GRK-2) in the desensitization of D1 receptor in the renal proximal tubules (144). GRK-4 gene variants are also associated with salt sensitive hypertension (145, 146); however, we are not aware of any such report in FBN rats. Phosphorylation of D1 receptor by GRK-4 causes D1 receptor G-protein uncoupling resulting in reduced D1 receptor responsiveness (145). Although we did not measure D1 receptor

phosphorylation in this study; higher levels of GRK-4 probably may have caused a decline in D1 receptor coupling to G proteins resulting in D1 receptor dysfunction as observed in old FBN rats. Furthermore, reduction of oxidative stress with tempol in old FBN rats increased D1 receptor numbers and restored D1 receptor function, in terms of D1 receptor G-protein coupling, suggesting a role of age-associated oxidative stress in impaired renal D1 receptor function in old FBN rats. These results in FBN rats are in agreement with our previous finding in F344 rats, where tempol while reducing oxidative stress also restored age-associated decline in dopamine D1 receptor numbers and G-protein coupling (41).

We also measured the effect of aging and tempol treatment on angiotensin AT1 receptor function in response to AT1 receptor antagonist, *candesartan*, in adult and old FBN rats. However, before measuring renal AT1 receptor function in FBN rats, we did some preliminary studies using *candesartan* in adult SD rats. The objective of these preliminary studies was to characterize an appropriate dose for *candesartan*, as natriuretic and diuretic responses to a previously reported dose (100µg/kg) of *candesartan* were associated with hemodynamic effects (106, 132). Using 1/10th (10µg/kg) of the reported dose, we found that *candesartan* significantly increased sodium excretion without affecting hemodynamic parameters such as blood pressure and glomerular filtration rate. Pressor response to Ang II was also attenuated in the presence of

candesartan (10µg/kg), suggesting that the *candesartan* dose used was effective for AT1 receptor antagonism. The attenuated pressor response to Ang II was not due to desensitized AT1 receptor function but indeed, due to AT1 blocking effect of *candesartan* at this dose.

After establishing the dose of *candesartan*, the diuretic and natriuretic responses to *candesartan* were studied in control and tempol-treated adult and old FBN rats. Both diuresis and natriuresis in response to *candesartan* administration were greater in old compared to adult FBN rats, suggesting that there is exaggerated renal AT1 receptor function in old FBN rats. These exaggerated diuretic and natriuretic responses were normalized in old FBN rats treated with tempol. With regard to understanding the mechanism(s) of exaggerated renal AT1 receptor function in old FBN rats, we measured levels of angiotensin AT1 receptor proteins and its signaling components in the renal proximal tubules of these rats. We found that there were no age-associated changes in AT1 receptor protein levels in the renal proximal tubules in these animals. Previously, increases in $G\alpha_i$ and $G\alpha_{q/11}$ protein expression have been attributed to increased renal AT1 receptor responses in Obese Zucker rats (26, 37). However, we found no age-associated increase in expression levels of these G proteins in the renal proximal tubules of adult and old FBN rats. In fact, there was a modest decrease in $G\alpha_i$-1 protein levels in old rats. However, Ang II-mediated G protein coupling was higher (twice as compared to adults) in old rats.

Similarly, there was a greater response to Ang II in activating Na,K-ATPase in the renal proximal tubules of old compared to adult rats. Increase in Ang II-mediated G protein coupling in part may have contributed to greater Na,K-ATPase stimulation and exaggerated renal AT1 receptor function in old rats. Treatment with tempol restored Ang II-mediated both G protein coupling and Na,K-ATPase activity; which in turn may have restored renal AT1 receptor function in old FBN rats. As tempol treatment by reducing AT1 receptor mediated signaling in the renal proximal tubules restored renal AT1 receptor function, we suggest a causative role of oxidative stress in the development of exaggerated renal AT1 receptor function in old FBN rats.

In normotensive F344 rats that have defective renal D1 receptor function, the effects of aging on renal AT1 receptor function have never been reported. Therefore, we measured age-associated changes in renal AT1 receptor function using *candesartan* in adult and old F344 rats. We found that the diuretic and natriuretic responses to *candesartan* were not different between adult and old F344 rats; suggesting that aging does not alter renal AT1 receptor function in F344 rats. This finding was contrary to our results in FBN rats and perhaps explains the normotensive phenotype, despite age-associated defects in renal D1 receptor function in old F344 rats.

Taken together, our studies suggest that both diminished D1 and exaggerated AT1 receptor function in the aging kidneys probably contributes to increase in blood pressure observed in old FBN rats. Age-associated oxidative stress seems to play a causative role in this phenomenon as reducing oxidative stress with antioxidant tempol restores both D1 and AT1 receptors functions and reduces blood pressure in old FBN rats.

In addition to determining renal D1 and AT1 receptor functions and blood pressure, we also measured urinary protein levels in adult and old FBN rats. Proteinuria is an index of general kidney dysfunction. The urinary protein levels increased in an age-dependent manner but decreased with antioxidant tempol treatment in old FBN rats. These studies suggest that reducing age-associated oxidative stress also improves general kidney function in old FBN rats.

In summary, to our knowledge for the first time, we show causative role of age-associated oxidative stress in the development of high blood pressure in FBN rats. The mechanism by which oxidative stress mediates age-related hypertension most likely involves both impaired D1 and exaggerated AT1 receptor functions in the aging kidneys. However, defective D1 receptor function, per se, as found in F344 rats, is not sufficient to cause hypertension during aging. These altered renal D1 and AT1 receptor functions by increasing sodium reabsorption may contribute to age-associated hypertension in FBN rats. The

research presented here provides a mechanism for the development of age-related hypertension and suggests a beneficial role of antioxidant therapy in reducing blood pressure and subsequently, improving the overall health and quality of life in the aging population.

6. SUMMARY AND CONCLUSIONS

1. There is increased oxidative stress and high blood pressure with aging in old FBN rats.
2. Natriuretic response to D1R activation is impaired with aging suggesting age-associated decline in D1R function in old FBN rats.
3. Reduced D1 receptor numbers and GRK-4 mediated D1R-G protein uncoupling may contribute to age-associated impairments in D1R function in old FBN rats.
4. Natriuretic and diuretic response to *candesartan* (AT1 receptor antagonist) is exaggerated with aging suggesting age-associated increase in renal AT1R function in old FBN rats.
5. Increase in angiotensin II-mediated G protein coupling and Na,K-ATPase activity may cause exaggerated renal AT1R function with aging in old FBN rats.
6. Antioxidant tempol treatment alleviates age-associated increase in oxidative stress and reduces blood pressure in old FBN rats.
7. Tempol treatment increase D1R numbers, reduces GRK-4 levels and restores D1R-G protein coupling in old FBN rats.
8. Tempol treatment reduces age-associated increase in G protein coupling and Na,K-ATPase activity in response to AT1 receptor activation in old FBN rats.

9. Age-associated increases in natriuretic and diuretic responses to candesartan were also restored with tempol in old FBN rats.
10. Contrary to FBN rats, renal AT1R function remains unchanged with aging in normotensive F344 rats, underscoring alterations in both D1R and AT1R functions for the development of age-associated hypertension.
11. Impaired D1R and exaggerated AT1R functions in the aging kidneys contribute to age-associated hypertension in old FBN rats. As antioxidant tempol treatment restores renal D1 and AT1 receptor functions and reduces blood pressure, it is likely that oxidative stress is involved in this phenomenon.

7. REFERENCES

1. Castelli WP. Epidemiology of coronary heart disease: the Framingham study. *Am J Med* 1984; **76:** 4-12.
2. MacMahon S, Peto R, Cutler J, Collins R*, et al.* Blood pressure, stroke, and coronary heart disease. Part 1, Prolonged differences in blood pressure: prospective observational studies corrected for the regression dilution bias. *Lancet* 1990; **335:** 765-774.
3. Thom TJ. International mortality from heart disease: rates and trends. *Int J Epidemiol* 1989; **18:** S20-28.
4. Uemura K, Pisa Z. Trends in cardiovascular disease mortality in industrialized countries since 1950. *World Health Stat Q* 1988; **41:** 155-178.
5. Whelton PK. Epidemiology of hypertension. *Lancet* 1994; **344:** 101-106.
6. Kurokawa K, Okuda T. Genetic and non-genetic basis of essential hypertension: maladaptation of human civilization to high salt intake. *Hypertens Res* 1998; **21:** 67-71.
7. Elliott P, Stamler J, Nichols R, Dyer AR*, et al.* Intersalt revisited: further analyses of 24 hour sodium excretion and blood pressure within and across populations. Intersalt Cooperative Research Group. *Bmj* 1996; **312:** 1249-1253.

8. Hall JE. Mechanisms of abnormal renal sodium handling in obesity hypertension. *Am J Hypertens* 1997; **10:** 49S-55S.

9. Hollenberg NK, Moore T, Shoback D, Redgrave J*, et al.* Abnormal renal sodium handling in essential hypertension. Relation to failure of renal and adrenal modulation of responses to angiotensin II. *Am J Med* 1986; **81:** 412-418.

10. Kuller LH. Salt and blood pressure: population and individual perspectives. *Am J Hypertens* 1997; **10:** 29S-36S.

11. Gildea JJ. Dopamine and angiotensin as renal counterregulatory systems controlling sodium balance. *Curr Opin Nephrol Hypertens* 2009; **18:** 28-32.

12. Carey RM. Theodore Cooper Lecture: Renal dopamine system: paracrine regulator of sodium homeostasis and blood pressure. *Hypertension* 2001; **38:** 297-302.

13. Hussain T, Lokhandwala MF. Renal dopamine receptors and hypertension. *Exp Biol Med (Maywood)* 2003; **228:** 134-142.

14. Jose PA, Eisner GM, Felder RA. Renal dopamine and sodium homeostasis. *Curr Hypertens Rep* 2000; **2:** 174-183.

15. Albrecht FE, Drago J, Felder RA, Printz MP*, et al.* Role of the D1A dopamine receptor in the pathogenesis of genetic hypertension. *J Clin Invest* 1996; **97:** 2283-2288.

16. Chen C, Beach RE, Lokhandwala MF. Dopamine fails to inhibit renal tubular sodium pump in hypertensive rats. *Hypertension* 1993; **21:** 364-372.

17. Chen CJ, Lokhandwala MF. An impairment of renal tubular DA-1 receptor function as the causative factor for diminished natriuresis to volume expansion in spontaneously hypertensive rats. *Clin Exp Hypertens A* 1992; **14:** 615-628.

18. Damasceno A, Santos A, Serrao P, Caupers P*, et al.* Deficiency of renal dopaminergic-dependent natriuretic response to acute sodium load in black salt-sensitive subjects in contrast to salt-resistant subjects. *J Hypertens* 1999; **17:** 1995-2001.

19. Felder RA, Seikaly MG, Cody P, Eisner GM*, et al.* Attenuated renal response to dopaminergic drugs in spontaneously hypertensive rats. *Hypertension* 1990; **15:** 560-569.

20. Jose PA, Eisner GM, Felder RA. Renal dopamine receptors in health and hypertension. *Pharmacol Ther* 1998; **80:** 149-182.

21. Zeng C, Eisner GM, Felder RA, Jose PA. Dopamine receptor and hypertension. *Curr Med Chem Cardiovasc Hematol Agents* 2005; **3:** 69-77.

22. Kaschina E, Unger T. Angiotensin AT1/AT2 receptors: regulation, signalling and function. *Blood Press* 2003; **12:** 70-88.

23. Kobori H, Nangaku M, Navar LG, Nishiyama A. The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. *Pharmacol Rev* 2007; **59:** 251-287.

24. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol* 2007; **292:** C82-97.

25. Banday AA, Lokhandwala MF. Oxidative stress-induced renal angiotensin AT1 receptor upregulation causes increased stimulation of sodium transporters and hypertension. *Am J Physiol Renal Physiol* 2008; **295:** F698-706.

26. Shah S, Hussain T. Enhanced angiotensin II-induced activation of Na+, K+-ATPase in the proximal tubules of obese Zucker rats. *Clin Exp Hypertens* 2006; **28:** 29-40.

27. Najjar SS, Scuteri A, Lakatta EG. Arterial aging: is it an immutable cardiovascular risk factor? *Hypertension* 2005; **46:** 454-462.

28. Pestana M. Hypertension in the elderly. *Int Urol Nephrol* 2001; **33:** 563-569.

29. Vasan RS, Beiser A, Seshadri S, Larson MG*, et al.* Residual lifetime risk for developing hypertension in middle-aged women and men: The Framingham Heart Study. *JAMA* 2002; **287:** 1003-1010.

30. Epstein M. Aging and the kidney. *J Am Soc Nephrol* 1996; **7:** 1106-1122.

31. Percy CJ, Power D, Gobe GC. Renal ageing: changes in the cellular mechanism of energy metabolism and oxidant handling. *Nephrology (Carlton)* 2008; **13:** 147-152.

32. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci U S A* 1993; **90:** 7915-7922.

33. Beheray S, Kansra V, Hussain T, Lokhandwala MF. Diminished natriuretic response to dopamine in old rats is due to an impaired D1-like receptor-signaling pathway. *Kidney Int* 2000; **58:** 712-720.

34. Zemel MB, Sowers JR. Salt sensitivity and systemic hypertension in the elderly. *Am J Cardiol* 1988; **61:** 7H-12H.

35. Baylis C. Renal responses to acute angiotensin II inhibition and administered angiotensin II in the aging, conscious, chronically catheterized rat. *Am J Kidney Dis* 1993; **22:** 842-850.

36. White BH, Sidhu A. Increased oxidative stress in renal proximal tubules of the spontaneously hypertensive rat: a mechanism for defective dopamine D1A receptor/G-protein coupling. *J Hypertens* 1998; **16:** 1659-1665.

37. Becker M, Umrani D, Lokhandwala MF, Hussain T. Increased renal angiotensin II AT1 receptor function in obese Zucker rat. *Clin Exp Hypertens* 2003; **25:** 35-47.

38. Banday AA, Marwaha A, Tallam LS, Lokhandwala MF. Tempol reduces oxidative stress, improves insulin sensitivity, decreases renal dopamine D1 receptor hyperphosphorylation, and restores D1 receptor-G-protein coupling and function in obese Zucker rats. *Diabetes* 2005; **54:** 2219-2226.

39. Javkhedkar AA, Lokhandwala MF, Banday AA. Higher renal AT1 receptor affinity exaggerates Ang II induced Na/K-ATPase stimulation in spontaneously hypertensive rats. . *FASEB J* 2010 **24:** lb707

40. Asghar M, Hussain T, Lokhandwala MF. Higher basal serine phosphorylation of D1A receptors in proximal tubules of old Fischer 344 rats. *Am J Physiol Renal Physiol* 2002; **283:** F350-355.

41. Fardoun RZ, Asghar M, Lokhandwala M. Role of oxidative stress in defective renal dopamine D1 receptor-G protein coupling and function in old Fischer 344 rats. *Am J Physiol Renal Physiol* 2006; **291:** F945-951.

42. Rice KM, Wu M, Blough ER. Aortic aging in the Fischer 344 / NNiaHSd x Brown Norway / BiNia Rat. *J Pharmacol Sci* 2008; **108:** 393-398.

43. Crowley SD, Coffman TM. In hypertension, the kidney rules. *Curr Hypertens Rep* 2007; **9:** 148-153.

44. Knepper MA, Brooks HL. Regulation of the sodium transporters NHE3, NKCC2 and NCC in the kidney. *Curr Opin Nephrol Hypertens* 2001; **10:** 655-659.

45. Holtback U, Kruse MS, Brismar H, Aperia A. Intrarenal dopamine coordinates the effect of antinatriuretic and natriuretic factors. *Acta Physiol Scand* 2000; **168:** 215-218.

46. Lote CJ. *Principles of renal physiology*, 4 edn. Kluwer Academic Publishers, 2000.

47. Navar LG. The kidney in blood pressure regulation and development of hypertension. *Med Clin North Am* 1997; **81:** 1165-1198.

48. Jose PA, Eisner GM, Felder RA. Role of dopamine receptors in the kidney in the regulation of blood pressure. *Curr Opin Nephrol Hypertens* 2002; **11:** 87-92.

49. Hussain T, Lokhandwala MF. Renal dopamine receptor function in hypertension. *Hypertension* 1998; **32:** 187-197.

50. Aperia AC. Intrarenal dopamine: a key signal in the interactive regulation of sodium metabolism. *Annu Rev Physiol* 2000; **62:** 621-647.

51. Felder CC, Jose PA, Axelrod J. The dopamine-1 agonist, SKF 82526, stimulates phospholipase-C activity independent of adenylate cyclase. *J Pharmacol Exp Ther* 1989; **248:** 171-175.

52. Bertorello A, Aperia A. Na+-K+-ATPase is an effector protein for protein kinase C in renal proximal tubule cells. *Am J Physiol* 1989; **256:** F370-373.

53. Hussain T, Lokhandwala MF. Renal dopamine DA1 receptor coupling with G(S) and G(q/11) proteins in spontaneously hypertensive rats. *Am J Physiol* 1997; **272:** F339-346.

54. Lezcano N, Mrzljak L, Eubanks S, Levenson R*, et al.* Dual signaling regulated by calcyon, a D1 dopamine receptor interacting protein. *Science* 2000; **287:** 1660-1664.

55. Felder CC, McKelvey AM, Gitler MS, Eisner GM*, et al.* Dopamine receptor subtypes in renal brush border and basolateral membranes. *Kidney Int* 1989; **36:** 183-193.

56. Sokoloff P, Giros B, Martres MP, Bouthenet ML*, et al.* Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. *Nature* 1990; **347:** 146-151.

57. Felder RA, Blecher M, Eisner GM, Jose PA. Cortical tubular and glomerular dopamine receptors in the rat kidney. *Am J Physiol* 1984; **246:** F557-568.

58. Missale C, Pizzi M, Memo M, Picotti GB*, et al.* Postsynaptic D1 and D2 dopamine receptors are present in rabbit renal and mesenteric arteries. *Neurosci Lett* 1985; **61:** 207-211.

59. Lokhandwala MF, Amenta F. Anatomical distribution and function of dopamine receptors in the kidney. *FASEB J* 1991; **5:** 3023-3030.

60. Felder CC, Campbell T, Albrecht F, Jose PA. Dopamine inhibits Na(+)-H+ exchanger activity in renal BBMV by stimulation of adenylate cyclase. *Am J Physiol* 1990; **259:** F297-303.

61. Chen C, Lokhandwala MF. Inhibition of Na+,K(+)-ATPase in rat renal proximal tubules by dopamine involved DA-1 receptor activation. *Naunyn Schmiedebergs Arch Pharmacol* 1993; **347:** 289-295.

62. Satoh T, Cohen HT, Katz AI. Different mechanisms of renal Na-K-ATPase regulation by protein kinases in proximal and distal nephron. *Am J Physiol* 1993; **265:** F399-405.

63. Albrecht FE, Xu J, Moe OW, Hopfer U*, et al.* Regulation of NHE3 activity by G protein subunits in renal brush-border membranes. *Am J Physiol Regul Integr Comp Physiol* 2000; **278:** R1064-1073.

64. Felder CC, Albrecht F, Eisner GM, Jose PA. The signal transducer for the dopamine-1 regulated sodium transport in renal cortical brush border membrane vesicles. *Am J Hypertens* 1990; **3:** 47S-50S.

65. Felder CC, Albrecht FE, Campbell T, Eisner GM*, et al.* cAMP-independent, G protein-linked inhibition of Na+/H+ exchange in renal brush border by D1 dopamine agonists. *Am J Physiol* 1993; **264:** F1032-1037.

66. Beheray SA, Hussain T, Lokhandwala MF. Dopamine inhibits na,h-exchanger via D1-like receptor-mediated stimulation of protein kinase a in renal proximal tubules. *Clin Exp Hypertens* 2000; **22:** 635-644.

67. Hu MC, Fan L, Crowder LA, Karim-Jimenez Z*, et al.* Dopamine acutely stimulates Na+/H+ exchanger (NHE3) endocytosis via clathrin-coated vesicles: dependence on protein kinase A-mediated NHE3 phosphorylation. *J Biol Chem* 2001; **276:** 26906-26915.

68. Bertorello AM, Katz AI. Short-term regulation of renal Na-K-ATPase activity: physiological relevance and cellular mechanisms. *Am J Physiol* 1993; **265:** F743-755.

69. Horiuchi A, Takeyasu K, Mouradian MM, Jose PA*, et al.* D1A dopamine receptor stimulation inhibits Na+/K(+)-ATPase activity through protein kinase A. *Mol Pharmacol* 1993; **43:** 281-285.

70. Bertorello A, Aperia A. Inhibition of proximal tubule Na(+)-K(+)-ATPase activity requires simultaneous activation of DA1 and DA2 receptors. *Am J Physiol* 1990; **259:** F924-928.

71. Felder CC, Blecher M, Jose PA. Dopamine-1-mediated stimulation of phospholipase C activity in rat renal cortical membranes. *J Biol Chem* 1989; **264:** 8739-8745.

72. Vyas SJ, Eichberg J, Lokhandwala MF. Characterization of receptors involved in dopamine-induced activation of phospholipase-C in rat renal cortex. *J Pharmacol Exp Ther* 1992; **260:** 134-139.

73. Efendiev R, Bertorello AM, Pedemonte CH. PKC-beta and PKC-zeta mediate opposing effects on proximal tubule Na+,K+-ATPase activity. *FEBS Lett* 1999; **456:** 45-48.

74. Asghar M, Hussain T, Lokhandwala MF. Activation of dopamine D(1)-like receptor causes phosphorylation of alpha(1)-subunit of Na(+),K(+)-ATPase in rat renal proximal tubules. *Eur J Pharmacol* 2001; **411:** 61-66.

75. Asghar M, Kansra V, Hussain T, Lokhandwala MF. Hyperphosphorylation of Na-pump contributes to defective renal dopamine response in old rats. *J Am Soc Nephrol* 2001; **12:** 226-232.

76. Chibalin AV, Ogimoto G, Pedemonte CH, Pressley TA*, et al.* Dopamine-induced endocytosis of Na+,K+-ATPase is initiated by phosphorylation of Ser-18 in the rat alpha subunit and Is responsible for the decreased activity in epithelial cells. *J Biol Chem* 1999; **274:** 1920-1927.

77. Siragy HM, Felder RA, Howell NL, Chevalier RL*, et al.* Evidence that dopamine-2 mechanisms control renal function. *Am J Physiol* 1990; **259:** F793-800.

78. Siragy HM, Felder RA, Peach MJ, Carey RM. Intrarenal DA2 dopamine receptor stimulation in the conscious dog. *Am J Physiol* 1992; **262:** F932-938.

79. Narkar V, Hussain T, Lokhandwala M. Role of tyrosine kinase and p44/42 MAPK in D(2)-like receptor-mediated stimulation of Na(+), K(+)-ATPase in kidney. *Am J Physiol Renal Physiol* 2002; **282:** F697-702.

80. Yamaguchi I, Walk SF, Jose PA, Felder RA. Dopamine D2L receptors stimulate Na+/K(+)-ATPase activity in murine LTK- cells. *Mol Pharmacol* 1996; **49:** 373-378.

81. Navar LG, Harrison-Bernard LM, Nishiyama A, Kobori H. Regulation of intrarenal angiotensin II in hypertension. *Hypertension* 2002; **39:** 316-322.

82. Inagami T, Kambayashi Y, Ichiki T, Tsuzuki S*, et al.* Angiotensin receptors: molecular biology and signalling. *Clin Exp Pharmacol Physiol* 1999; **26:** 544-549.

83. Geibel J, Giebisch G, Boron WF. Angiotensin II stimulates both Na(+)-H+ exchange and Na+/HCO3- cotransport in the rabbit proximal tubule. *Proc Natl Acad Sci U S A* 1990; **87:** 7917-7920.

84. Bharatula M, Hussain T, Lokhandwala MF. Angiotensin II AT1 receptor/signaling mechanisms in the biphasic effect of the peptide on proximal tubular Na+,K+-ATPase. *Clin Exp Hypertens* 1998; **20:** 465-480.

85. Garvin JL. Angiotensin stimulates bicarbonate transport and Na+/K+ ATPase in rat proximal straight tubules. *J Am Soc Nephrol* 1991; **1:** 1146-1152.

86. Harris PJ, Hiranyachattada S, Antoine AM, Walker L*, et al.* Regulation of renal tubular sodium transport by angiotensin II and atrial natriuretic factor. *Clin Exp Pharmacol Physiol* 1996; **23 Suppl 3:** S112-118.

87. Houillier P, Chambrey R, Achard JM, Froissart M*, et al.* Signaling pathways in the biphasic effect of angiotensin II on apical Na/H antiport activity in proximal tubule. *Kidney Int* 1996; **50:** 1496-1505.

88. Douglas JG, Hopfer U. Novel aspect of angiotensin receptors and signal transduction in the kidney. *Annu Rev Physiol* 1994; **56:** 649-669.

89. Woodcock EA, Johnston CI. Inhibition of adenylate cyclase by angiotensin II in rat renal cortex. *Endocrinology* 1982; **111:** 1687-1691.

90. Liu FY, Cogan MG. Angiotensin II stimulates early proximal bicarbonate absorption in the rat by decreasing cyclic adenosine monophosphate. *J Clin Invest* 1989; **84:** 83-91.

91. Liu FY, Cogan MG. Role of protein kinase C in proximal bicarbonate absorption and angiotensin signaling. *Am J Physiol* 1990; **258:** F927-933.

92. Schelling JR, Linas SL. Angiotensin II-dependent proximal tubule sodium transport requires receptor-mediated endocytosis. *Am J Physiol* 1994; **266:** C669-675.

93. Handa RK. Characterization and signaling of the AT(4) receptor in human proximal tubule epithelial (HK-2) cells. *J Am Soc Nephrol* 2001; **12:** 440-449.

94. Haithcock D, Jiao H, Cui XL, Hopfer U*, et al.* Renal proximal tubular AT2 receptor: signaling and transport. *J Am Soc Nephrol* 1999; **10 Suppl 11:** S69-74.

95. Carey RM, Howell NL, Jin XH, Siragy HM. Angiotensin type 2 receptor-mediated hypotension in angiotensin type-1 receptor-blocked rats. *Hypertension* 2001; **38:** 1272-1277.

96. Banday AA, Lokhandwala MF. Dopamine receptors and hypertension. *Curr Hypertens Rep* 2008; **10:** 268-275.

97. O'Connell DP, Ragsdale NV, Boyd DG, Felder RA*, et al.* Differential human renal tubular responses to dopamine type 1 receptor stimulation are determined by blood pressure status. *Hypertension* 1997; **29:** 115-122.

98. Sanada H, Jose PA, Hazen-Martin D, Yu PY*, et al.* Dopamine-1 receptor coupling defect in renal proximal tubule cells in hypertension. *Hypertension* 1999; **33:** 1036-1042.

99. Sato M, Soma M, Nakayama T, Kanmatsuse K. Dopamine D1 receptor gene polymorphism is associated with essential hypertension. *Hypertension* 2000; **36:** 183-186.

100. Felder RA, Sanada H, Xu J, Yu PY*, et al.* G protein-coupled receptor kinase 4 gene variants in human essential hypertension. *Proc Natl Acad Sci U S A* 2002; **99:** 3872-3877.

101. Jose PA, Raymond JR, Bates MD, Aperia A*, et al.* The renal dopamine receptors. *J Am Soc Nephrol* 1992; **2:** 1265-1278.

102. Zeng C, Zhang M, Asico LD, Eisner GM*, et al.* The dopaminergic system in hypertension. *Clin Sci (Lond)* 2007; **112:** 583-597.

103. Nishi A, Eklof AC, Bertorello AM, Aperia A. Dopamine regulation of renal Na+,K(+)-ATPase activity is lacking in Dahl salt-sensitive rats. *Hypertension* 1993; **21:** 767-771.

104. Cheng HF, Wang JL, Vinson GP, Harris RC. Young SHR express increased type 1 angiotensin II receptors in renal proximal tubule. *Am J Physiol* 1998; **274:** F10-17.

105. Alonso-Galicia M, Brands MW, Zappe DH, Hall JE. Hypertension in obese Zucker rats. Role of angiotensin II and adrenergic activity. *Hypertension* 1996; **28:** 1047-1054.

106. Tallam LS, Jandhyala BS. Significance of exaggerated natriuresis after angiotensin AT1 receptor blockade or angiotensin- converting enzyme inhibition in obese Zucker rats. *Clin Exp Pharmacol Physiol* 2001; **28:** 433-440.

107. Hussain T. Renal angiotensin II receptors, hyperinsulinemia, and obesity. *Clin Exp Hypertens* 2003; **25:** 395-403.

108. Ostchega Y, Dillon CF, Hughes JP, Carroll M*, et al.* Trends in hypertension prevalence, awareness, treatment, and control in older U.S. adults: data from the National Health and Nutrition Examination Survey 1988 to 2004. *J Am Geriatr Soc* 2007; **55:** 1056-1065.

109. National High Blood Pressure Education Program Working Group Report on Hypertension in the Elderly. National High Blood Pressure Education Program Working Group. *Hypertension* 1994; **23:** 275-285.

110. Kannel WB, Gordan T. Evaluation of cardiovascular risk in the elderly: the Framingham study. *Bull N Y Acad Med* 1978; **54:** 573-591.

111. Epstein M. Effects of aging on the kidney. *Fed Proc* 1979; **38:** 168-171.

112. Epstein M, Hollenberg NK. Age as a determinant of renal sodium conservation in normal man. *J Lab Clin Med* 1976; **87:** 411-417.

113. Hollenberg NK, Adams DF, Solomon HS, Rashid A*, et al.* Senescence and the renal vasculature in normal man. *Circ Res* 1974; **34:** 309-316.

114. Weidmann P, De Myttenaere-Bursztein S, Maxwell MH, de Lima J. Effect on aging on plasma renin and aldosterone in normal man. *Kidney Int* 1975; **8:** 325-333.

115. Crane MG, Harris JJ. Effect of aging on renin activity and aldosterone excretion. *J Lab Clin Med* 1976; **87:** 947-959.

116. Kansra V, Hussain T, Lokhandwala MF. Alterations in dopamine DA1 receptor and G proteins in renal proximal tubules of old rats. *Am J Physiol* 1997; **273:** F53-59.

117. Harman D. Aging: a theory based on free radical and radiation chemistry. *J Gerontol* 1956; **11:** 298-300.

118. Harman D. Aging and oxidative stress. *J Int Fed Clin Chem* 1998; **10:** 24-27.

119. Paravicini TM, Touyz RM. Redox signaling in hypertension. *Cardiovasc Res* 2006; **71:** 247-258.

120. Wilcox CS. Oxidative stress and nitric oxide deficiency in the kidney: a critical link to hypertension? *Am J Physiol Regul Integr Comp Physiol* 2005; **289:** R913-935.

121. Dikalov S, Griendling KK, Harrison DG. Measurement of reactive oxygen species in cardiovascular studies. *Hypertension* 2007; **49:** 717-727.

122. Harrison DG, Gongora MC. Oxidative stress and hypertension. *Med Clin North Am* 2009; **93:** 621-635.

123. Vaziri ND, Rodriguez-Iturbe B. Mechanisms of disease: oxidative stress and inflammation in the pathogenesis of hypertension. *Nat Clin Pract Nephrol* 2006; **2:** 582-593.

124. Vaziri ND. Causal link between oxidative stress, inflammation, and hypertension. *Iran J Kidney Dis* 2008; **2:** 1-10.

125. Zeng C, Villar VA, Yu P, Zhou L*, et al.* Reactive oxygen species and dopamine receptor function in essential hypertension. *Clin Exp Hypertens* 2009; **31:** 156-178.

126. Zeng C, Sanada H, Watanabe H, Eisner GM*, et al.* Functional genomics of the dopaminergic system in hypertension. *Physiol Genomics* 2004; **19:** 233-246.

127. Asghar M, Banday AA, Fardoun RZ, Lokhandwala MF. Hydrogen peroxide causes uncoupling of dopamine D1-like receptors from G proteins via a mechanism involving protein kinase C and G-protein-coupled receptor kinase 2. *Free Radic Biol Med* 2006; **40:** 13-20.

128. Fardoun RZ, Asghar M, Lokhandwala M. Role of nuclear factor kappa B (NF-kappaB) in oxidative stress-induced defective dopamine D1 receptor signaling in the renal proximal tubules of Sprague-Dawley rats. *Free Radic Biol Med* 2007; **42:** 756-764.

129. Lipman RD, Chrisp CE, Hazzard DG, Bronson RT. Pathologic characterization of brown Norway, brown Norway x Fischer 344, and Fischer 344 x brown Norway rats with relation to age. *J Gerontol A Biol Sci Med Sci* 1996; **51:** B54-59.

130. Sprott RL. Development of animal models of aging at the National Institute of Aging. *Neurobiol Aging* 1991; **12:** 635-638.

131. Turturro A, Witt WW, Lewis S, Hass BS*, et al.* Growth curves and survival characteristics of the animals used in the Biomarkers of Aging Program. *J Gerontol A Biol Sci Med Sci* 1999; **54:** B492-501.

132. Hiranyachattada S, Saetew S, Harris PJ. Acute effects of candesartan on rat renal haemodynamics and proximal tubular reabsorption. *Clin Exp Pharmacol Physiol* 2005; **32:** 714-720.

133. Taussky HH. A microcolorimetric determination of creatine in urine by the Jaffe reaction. *J Biol Chem* 1954; **208:** 853-861.

134. de Champlain J, Wu R, Girouard H, Karas M*, et al.* Oxidative stress in hypertension. *Clin Exp Hypertens* 2004; **26:** 593-601.

135. Wilcox CS, Pearlman A. Chemistry and antihypertensive effects of tempol and other nitroxides. *Pharmacol Rev* 2008; **60:** 418-469.

136. Chamulitrat W. Activation of the superoxide-generating NADPH oxidase of intestinal lymphocytes produces highly reactive free radicals from sulfite. *Free Radic Biol Med* 1999; **27:** 411-421.

137. Ogawa F, Shimizu K, Muroi E, Hara T*, et al.* Serum levels of 8-isoprostane, a marker of oxidative stress, are elevated in patients with systemic sclerosis. *Rheumatology (Oxford)* 2006; **45:** 815-818.

138. Nath KA, Grande JP, Haggard JJ, Croatt AJ*, et al.* Oxidative stress and induction of heme oxygenase-1 in the kidney in sickle cell disease. *Am J Pathol* 2001; **158:** 893-903.

139. Rosenberger C, Khamaisi M, Abassi Z, Shilo V*, et al.* Adaptation to hypoxia in the diabetic rat kidney. *Kidney Int* 2008; **73:** 34-42.

140. Kim OJ, Gardner BR, Williams DB, Marinec PS*, et al.* The role of phosphorylation in D1 dopamine receptor desensitization: evidence for a novel mechanism of arrestin association. *J Biol Chem* 2004; **279:** 7999-8010.

141. Rankin ML, Marinec PS, Cabrera DM, Wang Z*, et al.* The D1 dopamine receptor is constitutively phosphorylated by G protein-coupled receptor kinase 4. *Mol Pharmacol* 2006; **69:** 759-769.

142. Tiberi M, Nash SR, Bertrand L, Lefkowitz RJ*, et al.* Differential regulation of dopamine D1A receptor responsiveness by various G protein-coupled receptor kinases. *J Biol Chem* 1996; **271:** 3771-3778.

143. Premont RT, Macrae AD, Stoffel RH, Chung N*, et al.* Characterization of the G protein-coupled receptor kinase GRK4. Identification of four splice variants. *J Biol Chem* 1996; **271:** 6403-6410.

144. Watanabe H, Xu J, Bengra C, Jose PA*, et al.* Desensitization of human renal D1 dopamine receptors by G protein-coupled receptor kinase 4. *Kidney Int* 2002; **62:** 790-798.

145. Jose PA, Soares-da-Silva P, Eisner GM, Felder RA. Dopamine and G protein-coupled receptor kinase 4 in the kidney: role in blood pressure regulation. *Biochim Biophys Acta* **1802:** 1259-1267.

146. Sanada H, Yatabe J, Midorikawa S, Hashimoto S*, et al.* Single-nucleotide polymorphisms for diagnosis of salt-sensitive hypertension. *Clin Chem* 2006; **52:** 352-360.