# Role of Angiotensin II Type 2 Receptors in oxidative stress and inflammation.

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**A Dissertation Presented to**

**The Department of Pharmacological and Pharmaceutical Sciences,**

**University of Houston**

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**In Partial Fulfillment of**

**The Requirement for the Degree**

**Doctor of Philosophy**

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**By**

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**June 2011**

**Role of Angiotensin II Type 2 Receptors in oxidative stress and inflammation.**

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**ABSTRACT:**

Sodium imbalance, inflammation and oxidative stress are believed to contribute to hypertension in obesity/diabetes. The renin angiotensin system (RAS) is the major hormonal system which affects sodium balance, oxidative stress and inflammation mainly via activation of Angiotensin II- AT1 receptors. We have recently shown that renal AT2 receptors are upreguated in obese animal and contribute to natriuresis. However, the role of AT2 receptors in oxidative stress and inflammation is not well studied. On the other hand, oxidative stress can positively influence sodium retention independently. However the existence of possible cross-talk between oxidative stress, in particular superoxide radicals, and RAS system in terms of renal sodium handling is not known. In order to determine the possible interaction between RAS system and oxidative stress in terms of sodium homeostasis and role of AT2 receptors in oxidative stress and inflammation, we designed acute and chronic studies.

The acute study was designed to investigate the role of NAD(P)H oxidase (NOX), a major superoxide radical producing enzyme, in AT1 and AT2 receptor function on natriuresis/diuresis in Sprague-Dawley (SD), lean Zucker and obese Zucker rats. The results suggested that NAD(P)H oxidase has its independent role in natriuresis. Inhibition of NAD(P)H oxidase had no effect in AT1 receptor

antagonist (candesartan) induced natriuresis in SD and lean Zucker rats suggesting there is no interaction between AT1 receptor and oxidative stress in terms of natriuresis in SD or lean rats. On the other hand inhibition of NAD(P)H oxidase tremendously increased AT2 receptor agonist (CGP-42112A)-mediated natriuresis by increasing the NO/cGMP availability; suggesting that NAD(P)H oxidase can obstruct AT2 receptor function in SD rats. In obese Zucker rats inhibition of NAD(P)H oxidase unexpectedly prevented candesartan induced natriuresis by unknown mechanism.

Secondly, we looked into the effect of chronic activation of AT2 receptors in oxidative stress and inflammation. Lean and obese Zucker rats were treated with AT2 receptor agonist CGP-42112A for 2-weeks and at the end of treatment period level of oxidative stress markers and inflammatory markers/mediators were measured. The results suggested that chronic AT2 receptor activation can reduce oxidative stress and inflammation in obese Zucker rats. AT2 receptor activation decreased hemeoxygenase-1 (HO-1) and gp-91$^{phox}$ expression, increased superoxide dismutase (SOD) activity, decreased TNF-α, IL-1β, IL-6, C-reactive protein (CRP) and monocytes chemoattractant protein -1(MCP-1) levels in obese Zucker rats. On the contrary, chronic AT2 receptor activation may lead to increase oxidative stress (increased gp-91$^{phox}$) and inflammation (increased TNF-α and IL-1β) in lean rats.

Overall the studies demonstrate that superoxides can obstruct AT2 receptor function during physiological condition. However chronic activation of AT2 receptor can have anti-inflammatory and anti-oxidative function in obese Zucker rats but pro-inflammatory and pro-oxidative function in lean Zucker rats.

## TABLE OF CONTENTS

## TABLE OF CONTENTS

## TABLE OF CONTENTS

## TABLE OF CONTENTS

## TABLE OF CONTENTS

## TABLE OF CONTENTS

## TABLE OF CONTENTS

## TABLE OF CONTENTS

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ACE | Angiotensin converting enzyme |
| Ang II | Angiotensin II |
| ANOVA | Analysis of variance |
| AT1R | Angiotensin II type 1 receptor |
| AT2R | Angiotensin II type 2 receptor |
| BCA | Bicinchoninic acid |
| $Ca^{2+}$ | Calcium |
| cAMP | cyclic adenosine monophosphate |
| CAT | Catalase |
| cGMP | cyclic guanosine monophosphate |
| CKD | Chronic kidney disease |
| CRP-1 | C-reactive protein-1 |
| DAG | Diacylglycerol |
| EDTA | Ethylene-diamine-tetra-acetate |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ELISA | Enzyme-linked immunosorbent assay |
| ERK1/2 | Extracellular-signal-regulated kinases ½ |
| FBS | F etal bovine serum |
| $FE_{Na}$ | Fractional excretion of sodium |
| GFR | Glomerular filtration rate |
| GPCR | G-protein coupled receptor |
| GPX | Glutathione peroxidase |
| HEPES | 4-(2-hydroxyethly)-1-piperazineethsanesulfonic acid |
| HRP | Horse radish peroxidase |
| i.v. | Intravenous |
| kD | Kilo Dalton |
| kg | Kilogram |
| IL | Interleukin |
| MAP | Mean arterial pressure |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| MAPK | Mitogen activated protein kinases |
| MCP-1 | Monocyte chemoattractant protein-1 |
| MDA | Malondialdehyde |
| mRNA | messenger Ribonucleic acid |
| Na | Sodium |
| NAD(P)H | nicotinamide adenine dinucleotide phosphate (reduced) |
| NFκB | Nuclear factor kappa B |
| NKA | Sodium/potassium adenosine triphosphatase |
| nM | Nanomolar |
| NO | Nitric oxide |
| PCR | Polymerase chain reaction |
| PE | Polyethylene |
| PI-3K | Phosphoinositol-3-kinase |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| PMSF | Phenylmethanesulfonyl fluoride |
| qRT-PCR | Quantitative reverse transcriptase-polymerase chain reaction |
| RAS | Renin angiotensin system |
| ROS | Reactive oxygen species |
| S.E.M | Standard error of the mean |
| SDS | Sodium dodecyl sulfate |
| SOD | Superoxide dismutase |
| TGF-β | Transforming growth factor- beta |
| TNF-α | Tumor necrosis factor-alpha |
| UF | Urinary flow |
| $U_{Na}V$ | Urinary sodium volume |
| UV | Urinary volume |
| Wt | Weight |

## LIST OF FIGURES

**FIGURE** **PAGE**

## LIST OF FIGURES

## LIST OF FIGURES

## LIST OF FIGURES

**FIGURE** **PAGE**

## LIST OF TABLES

## INTRODUCTION AND STATEMENT OF PROBLEM:

Angiotensin II (AngII) is the main effector peptide of the renin angiotensin system (RAS) (Engeli et al. 2003). Studies have demonstrated that AngII plays a significant role in Na-homeostasis, development of oxidative stress and inflammation (Wolf 2005; Sachse and Wolf 2007). AngII and oxidative stress, alone or together, can lead to the development of inflammation (Suzuki et al. 2003; Kaneto et al. 2010).

The “classical” effects of AngII on blood pressure, electrolyte homeostasis, oxidative stress and inflammation are attributed to the AngII type-1 (AT1) receptors (Fyhrquist and Saijonmaa 2008). The other subtype of AngII receptors, type-2 (AT2) receptors, are less expressed during physiological conditions and mediate opposing effects to those of the AT1 receptors (Schulman and Raij 2008). Recently, we have reported that renal AT2 receptor expression is increased, promotes natriuresis and protects against blood pressure increase in obese animals (Hakam and Hussain 2005; Hakam et al. 2006; Siddiqui et al. 2009).

Obesity is a major risk factor for development of hypertension, insulin resistance, atherosclerosis, dyslipidemia, and diabetes (Elmarakby and Imig 2010; Mathieu et al. 2010). Early-stage obesity is associated with increased sodium retention, oxidative stress and inflammation, which may further contribute

to development of hypertension (Ferder et al. 2006). However the precise role of increased sodium retention, oxidative stress and inflammation in development of obesity-related hypertension is not known.

Abnormal kidney function has been suggested to play a significant role in development of obesity-related hypertension. In the kidney AT1 and AT2 receptors are expressed in various nephron segments with maximal density in the proximal tubules, the site of highest Na reabsorption. Studies have shown increased oxidative stress markers and inflammatory markers/mediators in kidney of obese rats (Lind 2003; Fujiwara and Kobayashi 2005; Inoguchi and Nawata 2005; Ramos et al. 2008; Ndisang 2010). Increased oxidative stress is also shown to positively influence sodium balance (Zou et al. 2001; Lopez et al. 2003) and inflammation. The major superoxide producing enzyme in the kidney is the NAD(P)H oxidase and its activity and expression have been shown to increase in obesity-related metabolic syndrome (Rajagopalan et al. 1996; Haas et al. 1999; Gill and Wilcox 2006).

Although both AngII and oxidative stress are known to be independent contributor for sodium retention and inflammation, it is not known whether these two interact at the molecular level to further influence their effect on sodium balance and inflammation.

In order to determine the presence of interaction between AngII (AT1 and AT2) receptors and oxidative stress, we designed acute and chronic studies. In the acute study we determined how inhibition of NAD(P)H oxidase affects the natriuretic/ anti-natriuretic property of AngII receptors. For this study we used SD rats and lean and obese Zucker rats. In the chronic study, we determined the role of chronic activation of AT2 receptors in inflammation and oxidative stress in lean and obese Zucker rats.

## 2: REVIEW OF LITERATURE

### 2.1: Renin-Angiotensin system (RAS):

RAS plays a pivotal role in blood pressure regulation and cardiovascular homeostasis by maintaining vascular tone, sympathetic nervous system, and electrolyte balance. Traditionally, RAS was viewed as a circulating endocrine system, where the renin from the juxtaglomerular cells of kidney cleaves a liver-derived angiotensinogen to an inactive decapeptide called angiotensin I. The angiotensin I is further cleaved to an active octapeptide called Angiotensin II (Ang II) by angiotensin-coverting enzyme (ACE) (Campbell 1987). In addition to the systemic RAS every tissue is capable of producing their own AngII which can mediate autocrine, intracrine and paracrine effects. The concentration of Ang II found in the kidney proximal tubules is much higher when compared to the plasma Ang II concentration (Seikaly et al. 1990; Navar et al. 1994). Ang II mediates its effects via acting on its receptors.

### 2.2: Angiotensin II receptors:

Ang II mediates its actions mainly via two receptor subtypes the AT1 and AT2 receptors. The pharmacological characterization of Ang II receptor subtypes was made possible by development of selective non-peptide Ang II receptor antagonists, namely losartan (AT1-selective) and PD123319 (AT2-selective)

(Mukoyama et al. 1993; Timmermans et al. 1993). The AT1 and AT2 receptor belongs to the seven transmembrane class of G-protein coupled receptors (GPCRs) (de Gasparo et al. 1995; Goodfriend et al. 1996; Griendling et al. 1996). The AT2 receptors share only 34% sequence homology with the AT1 receptors (Mukoyama et al. 1993), however, Ang II binds with similar affinity to both the receptors (de Gasparo et al. 1995). Like most of the GPCRs, AT1 receptor undergoes desensitization and internalization upon Ang II activation. However AT2 receptors do not follow the same trend (Thomas et al. 1996; Thomas et al. 1996).

**2.3: Receptor distribution and localization:**

Unlike AT1 receptors, AT2 receptors expression, which is abundant during fetal development, goes down rapidly after birth (Lazard et al. 1994; Shanmugam and Sandberg 1996). Both AT1 and AT2 receptors are expressed in the kidney (Zhuo et al. 1997), vasculature (Allen et al. 1990), brain (Song et al. 1992) and adrenals (Zhou et al. 1996). However, the AT1 / AT2 ratio vary in each organ tissue (Bottari et al. 1993).

The expression of AT2 receptor is upregulated in pathophysiological conditions like heart failure, renal failure, myocardial infarction, vascular injury,

brain lesions wound healing, obesity and diabetes (Kimura et al. 1992; Viswanathan et al. 1994; Mukoyama et al. 1995; Nio et al. 1995; Chung et al. 1998; Tsutsumi et al. 1998; Hakam and Hussain 2005)

**2.4: Signal transduction:**

**2.4 (1): AT1 receptors:** Activation of AT1 receptors can initiate signal transduction mechanisms in different ways:

- Stimulation of Phospholipase C coupled to $G_{q/11}$ protein, causing release of intracellular $Ca^{2+}$ and diacylglycerol (DAG) causing vasoconstriction and sodium retention (Capponi 1996; Griendling et al. 1996).
- Stimulation of phospholipase A
- Stimulation of phospholipase D causes release of arachidonic acid which is a precursor for prostaglandins (Capponi 1996; Griendling et al. 1996).
- Inhibition of adenylate cyclase via coupling to $G_{i/o}$ protein and attenuate production of cAMP which is a vasodilator (Jard et al. 1981; Pobiner et al. 1985).

- Opens the L-type $Ca^{2+}$ channels leading to influx of extracellular $Ca^{2+}$ causing aldosterone production and secretion and also vasoconstriction (Apfeldorf and Rasmussen 1988; Macrez et al. 1997).
- Increase tyrosine phosphorylation and activation of mitogen activated proteins (MAP) kinases, Janus kinases (JAK) and signal transducers and activators of transcription (STAT) proteins thereby promoting cellular proliferation and growth (Duff et al. 1995; Marrero et al. 1995; Schieffer et al. 1996).

**2.4 (2): AT2 receptors:** Activation of AT2 receptors leads to the following:

- Activation of nitric oxide and cGMP pathway causing vasodilatation and sodium excretion (Bottari et al. 1992; Widdop et al. 1992; Hakam and Hussain 2006).
- Activation of various phosphatases, such as MAP kinase phosphatase-1 (MKP-1), SH2-domain-conatining phosphatase-1 (SHP-1) and serine/threonine phosphatase 2A. They cause inactivation of extracellular signal-regulated kinase (ERK) leading to opening of $K^+$ channels and inhibition of $Ca^{2+}$ channels (Kang et al. 1993; Bedecs et al. 1997).

- Block activation of MAP kinase via dephosphorylation of tyrosine phosphate by MKP-1, thus blocking cellular proliferation and growth (Carter et al. 1998).
- AT2 via MKP-1 also cause dephosphorylation of Bcl-2 protein which is anti-apoptotic, thus causing cellular apoptosis (Horiuchi et al. 1997).
- Cause release of arachidonic acid via phospholipase-$A_2$ (Kohout and Rogers 1995).
- Coupled to inhibitory G-protein ($G_i$) can lead to reduction in cAMP levels. However, the exact function via this mechanism is not clear (Zhang and Pratt 1996; Zhang and MacLeod 1996; Nouet and Nahmias 2000)

**2.5: Obesity and metabolic syndrome:**

There is a dramatic increase in prevalence of overweight and obesity in recent year approaching epidemic proportions. According to the most recent NHANES (National Health And Nutrition Examination Survey) data, over 68.3% of US population is overweight and 33.8% are obese (BMI≥ 30 kg/$m^2$ ) (Flegal et al. 2010). The increasing prevalence of excess body weight is a major cause of morbidity and mortality (Kopple 2010). Obese individuals are at increased risk for

metabolic syndrome which includes glucose intolerance, central obesity, insulin resistance, dyslipidemia, and hypertension. Obesity is also associated with chronic low grade inflammation and oxidative stress. In fact, oxidative stress had been suggested as a unifying mechanism in development of co-morbidities in obesity (Vincent and Taylor 2006).

**2.5 (1): Obesity related hypertension:**

Obesity is associated with hemodynamic, physiological, structural and pathological changes in the kidney. Abnormal kidney function requiring higher levels of BP to maintain normal sodium and fluid balance is suggested to be the major factor involved in all type of hypertension (Guyton and Coleman 1999). In obesity-associated hypertension, abnormal kidney function secondary to increased tubular sodium reabsorption leads to sodium retention resulting in increased blood volume (Hall 2003). The possible mechanisms behind increased sodium reabsorption in obesity related hypertension are activation of renin-angiotensin-system, defective dopamine receptor function, greater sympathetic nervous (SNS) system activation, and natriuretic peptide effects.

**2.5 (2): RAS in obesity associated hypertension:**

Although obesity is associated with sodium retention and increased extracellular fluid volume, obese subjects usually have elevated plasma renin

activity, plasma angiotensinogen, ACE activity and Ang II levels (Carroll et al. 1995; Hall et al. 1998; Hall et al. 1999).

Ang II via AT1 receptor plays important role in obesity-associated sodium reabsorption, impaired pressure natriuresis and hypertension. This was supported by the finding that treatment with AT1 receptor antagonist or ACE inhibitor blunts sodium retention, volume expansion as well as elevated BP in obese dogs (Robles et al. 1993; Hall 1997). Similar results were demonstrated in obese Zucker rats where AT1 receptor antagonist treatment for seven days caused significant decrease in BP when compared to the lean rats (Alonso-Galicia et al. 1996). In acute studies, use of AT1 receptor antagonist cased significant sodium excretion (natriuresis) in obese Zucker rats (Tallam and Jandhyala 2001; Hakam and Hussain 2005).

The role of AT2 receptor in obesity related hypertension is not clear however, it has been reported that their expression in the kidney of obese Zucker rats is high. Our laboratory and others have shown that stimulation of AT2 receptors with an agonist cause natriuresis and reduction in blood pressure (Siragy and Carey 1999; Siragy et al. 2000; Hakam and Hussain 2006; Hakam and Hussain 2006; Hakam et al. 2006).

## 2.6: Obesity-related oxidative stress and inflammation:

### 2.6 (1): Oxidative stress:

Oxidative stress is defined as increased production or decreased scavenging of reactive oxygen species (ROS). ROS are highly reactive oxygen containing molecules with or without unpaired electrons. ROS include superoxides ($O_2^{\circ -}$), hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH^{\circ}$), and peroxynitrite ($ONOO^-$). Superoxides are neutralized by protective enzymes and antioxidants. Superoxide dismutase (SOD) converts superoxides to $H_2O_2$. SOD has three isoforms, a copper-zinc isoform present in cytosol (Cu/Zn or SOD1), a manganese isoform present in mitochondria (Mn or SOD2), and an extracellular isoform (Ex or SOD3). Catalase (CAT) and glutathione peroxidase (GPX) can further break down $H_2O_2$ to $H_2O$and $O_2$. Glutathione peroxidase also detoxifies lipid peroxides. $H_2O_2$ in presence of enzyme myeloperoxidase (MPO) can form hypochlorous acid (HOCl) (Wolf 2000; Sachse and Wolf 2007).

Low concentrations of ROS are necessary for normal cell function, intracellular signaling and to maintain cell redox status (Yu 1994; Griendling and Ushio-Fukai 2000). However, in some pathological conditions there is excess production of ROS which can cause damage to the DNA, proteins, carbohydrate,

and lipid constituents and alter normal cell function (Yu 1994; Lopes et al. 2003; Vincent and Taylor 2006).

The end product of radical mediated oxidative process is used as a biomarker of oxidative stress. Malondialdehyde (MDA,) thiobarbituric reactive acid substance (TBARS), $F_2$-isoprostane (8-epi $PGE_{2\alpha}$), hydroperoxides and conjugated dienes are indicator of lipid peroxidation. Protein carbonyls, advanced glycation end products are indicator of protein damage by oxidants. DNA oxidation is determined by measuring 8-hydroxy 2'-deoxyguanosine (8-OHdG) (Yu 1994; Vincent and Taylor 2006).

Several enzymatic pathways like xanthine oxidase (Zweier et al. 1988; Phan et al. 1989), cyclooxygenase (Holland et al., 1998; (Kinnula et al. 1993) and cytochrome P450 (Fleming and Busse 2001) can generate superoxides, however, the NAD(P)H oxidase is considered the predominant generator of ROS (Rajagopalan et al. 1996; Hanna et al. 2002)

The NAD(P)H oxidase is composed of six subunits. Two transmembrane bound subunits gp-91 phox and p22 phox and four cytosolic subunits which include p40 phox, p47 phox, p67 phox and Rac. Upon exposure to variety of chemical and biological stimuli the cytosolic subunits shift from the cytosol to the membrane. The assembly of subunits at the membrane causes conformational

changes leading to activation of the enzyme (Hanna et al. 2002; Gill and Wilcox 2006).

**2.6 (2): Obesity and oxidative stress:**

Human cross sectional study by Van Gall et al (1998) showed that the MDA in lipoprotein samples of obese persons was higher than the non-obese subjects. Ozata et al (2002) showed that the group of obese men had higher fasting blood cholesterol and lipoproteins, increased TBARS and lower antioxidant (GPX and SOD) activity compared to non obese men. Urakawa et al (2003) investigated the levels of plasma 8-epi $PGE_{2\alpha}$ in obese and non obese subjects. They found higher 8-epi $PGE_{2\alpha}$ in obese subjects and it was directly proportional to their BMI, body fat weight and total fat area. Furukawa et al (2004) showed similar results in animal model. These studies suggest a significant correlation between obesity and oxidative stress. Hyperglycemia, insulin resistance, elevated tissue lipid levels, hyperleptinemia, inadequate antioxidant defense and chronic low grade inflammation are major cause of obesity related oxidative stress (Vincent et al. 2001; Aronson and Rayfield 2002; Saito et al. 2003).

### 2.6 (3): RAS and oxidative stress:

Ang II via stimulation of AT1 receptors can lead to ROS production by activation of NAD(P)H oxidase (Griendling et al. 2000). Clinical condition in which Ang II levels are increased exhibit increased ROS production with a parallel decrease in nitric oxide (NO) availability. Studies have shown that use of ACEIs and ARBs increased antioxidant defenses and lowered oxidative stress in diabetic animals (de Cavanagh et al. 1997; de Cavanagh et al. 2001). Stimulation of AT1 receptors can increase NFκB nuclear translocation by increasing ROS, which further leads to inflammation and increased ROS production (Radak et al. 2004). Candesartan (ARB) treatment reduces oxidative stress in patients with essential hypertension (Dohi et al. 2003). These reports suggest major role of AT1 receptors in oxidative stress however, the role of AT2 receptors in oxidative stress is not defined.

### 2.6 (4): Inflammation:

Inflammation is an adaptive response triggered by harmful stimuli and conditions like infection and tissue injury (Majno and Joris, 2004). In case of normal acute inflammation the recognition of infection is mediated by resident macrophages and mast cells. They release a variety of pro-inflammatory

mediators like cytokines (TNF-α, IL-6, IL-1 and many others), chemokines (MCP1, RANTES), vasoacitve amines (histamine, serotonine), eicosanoids ($PGE_2$, $PGI_2$) and proteolytic enzymes (Elastin, cathepsins, matrix metalloproteinases) (Qatanani and Lazar 2007). The immediate effect of these mediators is to elicit an inflammatory exudate locally, attracting the plasma proteins and leukocytes (neutrophils) to the site of injury. The activated endothelium of the blood vessel allows selective extravasation of neutrophils and plasma proteins (Pober and Sessa 2007). The neutrophils become activated by direct contact with the pathogen or by the cytokines released by the tissue-resident cells. The neutrophils release toxic substance like cathepsin G, elastase, and ROS which kill the pathogen (Nathan 2006). The neutrophils cannot differentiate between host cell and pathogen and hence the collateral damage to host tissue is unavoidable (Nathan 2002).

Once the infectious agent is eliminated, the resolution and repair phase follows. Resolution and repair is done by the tissue-resident macrophages and newly recruited monocytes which mature into macrophage at the site of injury. The switching of the lipid mediators from pro-inflammatory prostaglandin to anti-inflammatory lipoxins (inhibits recruitment of neutrophils and promote recruitment of monocytes) is very crucial for the transition from inflammation to resolution (Serhan et al. 2007).

Once the neutrophils have done their job they undergo apoptosis (programmed cell death). During apoptosis the cells maintain an intact membrane and, therefore, do not release their toxic agents (Heasman et al. 2003). The apoptotic cells express a repertoire of surface molecules which help phagocytic cells (macrophages) to recognize those (Fadok et al. 2001). After engulfing these neutrophils, marked for apoptosis, macrophages acquire a phenotype and release anti-inflammatory signals like interleukin -10 (IL-10) and transforming growth factor-β (TGF-β) helping in resolution (Huynh et al. 2002). The macrophages after engulfing apoptotic cells leave the tissue by lymphatic drainage (Bellingan et al. 2002). If all the pathways are strictly followed acute inflammation will resolve without causing much damage to the host cells. However, if the clearance of infectious agents or the apoptotic cells from the inflamed tissue fails, the inflammation persists and acquires new characteristics. The neutrophil recruitment is replaced by macrophages and T-cells. If the effect of macrophage and T-cell is ineffective, a chronic inflammatory state ensues which involves formation of granulomas and lymphoid tissue (Kumar et al., 2003; (Drayton et al. 2006). Chronic inflammation can also result from autoimmune responses (persistence of self antigens) or undegradable foreign bodies (Kumar et al., 2003).

## 2.6 (5): Obesity and inflammation:

Recent human studies have confirmed that there is positive association between obesity indices and inflammatory markers, especially, TNF-α, IL-6 and CRP (Wakabayashi 1998; Kern et al. 2001; Pradhan et al. 2001; Shemesh et al. 2007) .

Inflammation associated with obesity is different as it is not associated with infection or autoimmunity or massive tissue injury. Rather the inflammation is metabolically triggered and is called “metaflammation” (Hotamisligil 2006). The dimension of inflammatory activation is not large and hence it is called “low-grade inflammation”. Glucose and fat intake have been shown to increase inflammation potentially by increasing oxidative stress (Tripathy et al. 2003; Dhindsa et al. 2004). Obesity causes expansion of adipocytes by accumulation of lipids. Adipocytes behave as immune cells and are able to synthesize and release huge amount of pro-inflammatory adipokines and cytokines like TNF-α, IL-6, plasminogen activator inhibitor-1 (PAI-1), leptin, resistin, IL-1β, MCP-1, IL-18 and IL-33 (Skurk and Hauner 2005; Qatanani and Lazar 2007; Wood et al. 2009; Libby 2010).

The inflammatory condition associated with overweight and obesity plays an important role in the etiology of the metabolic syndrome and related pathological conditions like atherosclerosis and chronic kidney disease.

**2.6 (6): Inflammatory markers:**

**Tumor necrosis factor-α (TNF- α):** TNF- α is considered one of the major adipocyte cytokines (Dandona et al. 2005), which can influence the synthesis, secretion and activity of other cytokines (Alexandraki et al. 2006). It is abundantly expressed in macrophages, adipose tissue, and endothelial cells. They are over expressed in obesity and can alter insulin sensitivity (Hotamisligil et al. 1995; Uysal et al. 1998).

**Interleukin-6 (IL-6):** Belongs to IL-6 family of cytokines and produced by immune and non-immune cells like endothelial cells, adipocytes, hepatocytes and astrocytes (Kamimura et al. 2003). Inappropriate regulation of IL-6 can have deleterious or protective role in disease characterized by low-grade inflammation (Jones et al. 2001; Pickup 2004; Kolb and Mandrup-Poulsen 2005). IL-6 is considered principal pro-coagulant cytokine as it increases the plasma levels of PAI-1 and CRP ((Willerson and Ridker 2004; Kristiansen and Mandrup-Poulsen 2005). In obesity, adipose tissue is infiltrated with macrophages, but IL-6 expression is more in adipose tissue than in macrophages (Pedersen 2003;

Weisberg et al. 2003). In patients undergoing bariatric surgery the IL-6 levels decrease parallel with reduction in weight loss and improvement in insulin signaling (Kopp et al. 2003).

**Interleukin-1 (IL-1):** One of the important early mediators of inflammation. IL-1 has two isoforms IL-1α and IL-1β. They exert almost similar but not redundant function via the IL-1 receptors (Shoelson et al. 2007). IL-1β expression was found to be increased in high fat diet (HFD)-fed mice and ob/ob mice when compared to low fat fed mice and wild type mice respectively (Bastard et al. 2006). Increased concentration of IL-1 along with IL-6 predicts risk of diabetes in humans (Spranger et al. 2003).

**Monocyte chemoattractant protein-1 (MCP-1):** One of the key chemokines important for recruitment of monocytes to the site of inflammation. MCP-1 activates the inflamed tissue (resident)-macrophages to release cytokines and chemokines which attract the circulating monocytes forming a vicious cycle amplifying the inflammatory status (Alexandraki et al. 2006). MCP-1 levels increases in HFD-fed mice and ob/ob mice when compared to their controls (Chen et al. 2005; Kamei et al. 2006). It is suggested that increase in MCP-1 n obesity precedes the development of hyperglycemia and hyperinsulinemia (Xu et al. 2003; Chen et al. 2005)

**C-reactive protein (CRP):** It is an acute phase reactant protein. CRP is mainly produced in liver in response to IL-6. CRP binds to the phospholipids in damaged and apoptotic cells and activates the complement pathway and recruits the phagocytic cells (Hack et al. 1997; Chang et al. 2002). The circulating concentration of CRP is a marker of cardiovascular risk and insulin resistance. In obese subjects CRP level is high and it goes down parallel with weight loss (Pannacciulli et al. 2001; McLaughlin et al. 2002).

**2.6 (7): Interaction between oxidative stress and inflammation:**

In obesity as mentioned above there is an increased production of ROS. The source of ROS can be adipose tissue or immune system. ROS can activate stress sensitive kinases, like c-Jun N-terminal kinase (c-JNK), inhibitor of kappa B kinase (IKK-β), protein kinase C (PKC) isoforms, and mitogen-activated protein kinase (p38-MAPK). These kinases increase the expression of pro-inflammatory mediators like TNF-α, IL-6 and MCP-1. The pro-inflammatory mediators further increase the production of ROS thus forming a vicious cycle.

Activation of IKK-β increases the production of NFκB, a transcription factor. The NFκB family consists of a 50-kDa (p50) protein, which is derived from a 105-kDa precursor, and a 65-kDa (p65) protein (RelA). P50 and p65 can exist as homodimer or heterodimer (Beg and Baldwin 1993); (Lamb and Goldstein

2008). Activation of NFκB involves release of p50 and p65 heterodimer from a cytoplasmic inhibitor protein, IκB, and subsequent translocation of the dimer into the nucleus (Baeuerle and Baltimore 1988). NFκB is involved in the transcription of NAD(P)H oxidase and most of the pro-inflammatory cytokines (Lamb and Goldstein 2008).

**2.6 (8): RAS and inflammation:**

Animal and human studies support the pro-inflammatory action of RAS mainly via AT1 receptors. Enalapril (ACE inhibitor) and losartan (AT1 receptor antagonist) lowers plasma IL-1 and IL-6 levels in patients with stable angina awaiting bypass graft surgery (Schieffer et al. 2004). Candesartan lowers TNF-α, IL-6 and MCP-1 in patients with essential hypertension (Dohi et al. 2003). Ang II via AT1 receptors can activate NFκB, which in turn results in production of various pro-inflammatory mediators (Yamamoto and Gaynor 2001).

The reports about role of AT2 receptors in inflammation are confounding. AT2 receptor inhibition by its antagonist (PD 123319) inhibits nuclear translocation of NFκB suggesting a pro-inflammatory role of AT2 receptors (Ruiz-Ortega et al. 2001; Esteban et al. 2003). However, recent studies using an AT2 receptor agonist (C21) suggest an anti-inflammatory property of AT2 receptors (Rompe et al. 2010; Matavelli et al. 2011; Sabuhi et al. 2011).

## 2.7: Obesity, metabolic syndrome and renal disease:

Obesity, indicated by increased body mass index, is associated with decreased renal function independent of the presence of diabetes and hypertension (Chen et al. 2004; Kurella et al. 2005; Ejerblad et al. 2006; Wahba and Mak 2007). Prospective population studies have shown that patient with metabolic syndrome are at higher risk of developing chronic kidney disease (CKD) (Ejerblad et al. 2006; Wahba and Mak 2007). CKD is a progressive and irreversible loss of kidney function. The final stage of CKD is called end-stage renal disease (ESRD) where kidney is no more functional and the patients need dialysis or kidney transplant.

The increased triglyceride levels and free fatty acid can directly impair renal function by promoting mesangial cell proliferation and production of pro-inflammatory cytokines (Wahba and Mak 2007; Guarnieri et al. 2010). Obesity is also associated with increased inflammation and oxidative stress which can cause renal damage (Guo et al. 2001; Forbes et al. 2008; Qian et al. 2008). TNF-α is implicated in kidney damage in several kidney disease models (Guo et al. 2001; Klahr and Morrissey 2003). The cytokines, especially TNF- α and IL-6, mainly stimulate glomerulosclerosis by promoting extracellular matrix deposition (Klahr and Morrissey 2003; Zhang et al. 2005).

Adipose tissue *per se* is a major source of inflammatory cytokines also known as adipokines (Axelsson and Stenvinkel 2008) which can further increase production of superoxides (Furukawa et al. 2004). Inflammation, increased oxidative stress and adipokines are closely associated with insulin resistance which can further enhance risk of CKD in obese patients (Furukawa et al. 2004; Shoelson et al. 2007).

A progressive decline in renal function, *per se* in obese patients may lead to several metabolic alterations which further add to the incidence and prevalence of metabolic syndrome (Fig 1)

**2.8: Obese Zucker rat model:**

Obese Zucker rats present a mutation in the leptin receptor. The dominant Fa/Fa or Fa/fa is the lean Zucker rat and the recessive fa/fa is the obese fatty (Zucker) rat. Obese Zucker rats develop sever obesity associated with hyperphagia and lower metabolic rates with preferential deposit of energy in adipose tissue (Chua et al. 1996). These animals have dyslipidaemia, mild glucose intolerance, and hyperinsulinaemia. Hyperinsulinemia sets in as early as 3 weeks of age and persists throughout the animals' life. Obese Zucker rats from 12 weeks onwards have significantly higher blood pressure than their lean controls (Kurtz et al. 1989). RAS has been shown to play an important role in

development of hypertension in obese Zucker rats. AT1 receptor function but not expression is increased in these rats. We have recently shown that the expression of renal AT2 receptor is significantly increased in obese rats compared to their lean controls (Hakam and Hussain 2005). They also have increased oxidative stress. The ROS can interact with NO leading to endothelial dysfunction (de Gasparo 2002). Obese Zucker rats also have chronic low-grade inflammation with increased levels of pro-inflammatory mediators (Ecelbarger et al. 2010). We used obese Zucker rats in our study since it is the most representative rat strain to study metabolic syndrome associated with oxidative stress and inflammation.

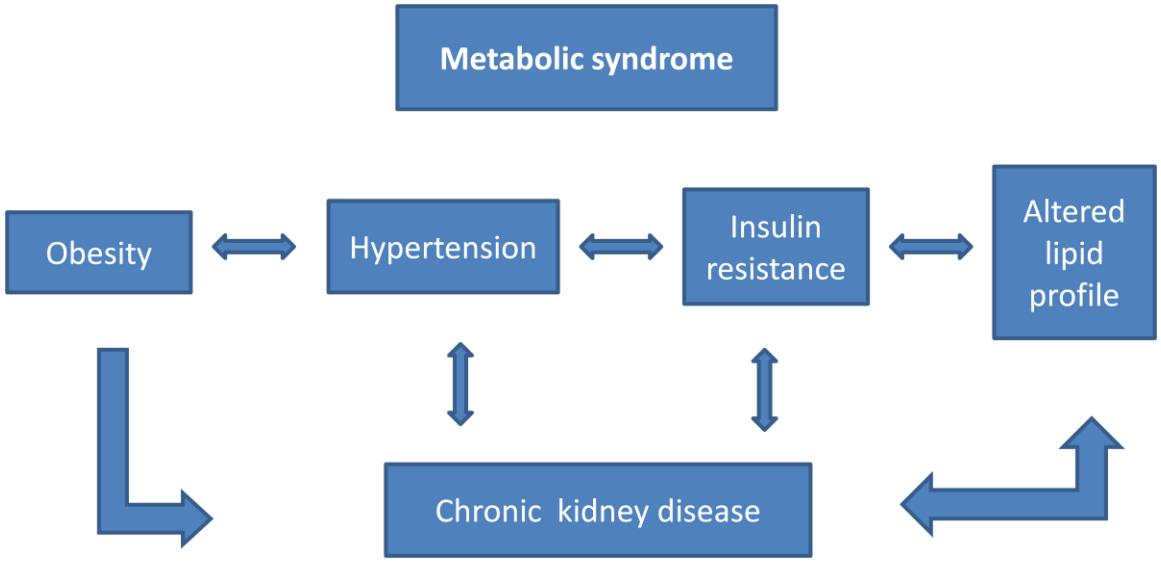


**Fig.1:** Effect of metabolic syndrome on renal function and of renal disease on metabolic syndrome components.

## 3: MATERIALS AND METHODS:

**3.1: Animal models:** Male Sprague-Dawley rats (weighing 250-310 gm), lean and obese Zucker rats (10-11 weeks old) were purchased from Harlan inc. (Indianapolis, IN). The animals were housed in the University of Houston Animal care facility and were maintained on standard rat chow and water *ad libitum*. The Institutional Animal Care and Use Committee approved the experimental protocols.

### 3.2: Evaluation of renal function:

**3.2.1: Surgical procedure*:*** Rats were anesthetized using Inactin® (100mg/kg body weight; i.p). Tracheotomy was performed to facilitate breathing. The left carotid artery was cannulated with PE50 tubing and attached to data acquisition system (PolyView, Grass Ins) via Grass pressure transducer PT300 for blood pressure measurement. The jugular vein was catheterized with PE50 tubing for saline or drug infusion. For collecting urine samples laparotomy was performed via midline abdominal incision, the left ureter was isolated and catheterized with PE-10 tubing connected to tygon® tubing for urine collection. At the completion of the surgery, normal saline (1% body weight, ml/hr) was infused continuously through the jugular vein throughout the experimental period to maintain a stable urine output and to prevent rats from dehydration.

### 3.2.2: Experimental protocol for evaluation of the effects of various drugs on renal function in SD, lean and obese Zucker rats:

After one hour of stabilization period, urine was collected at 30-minutes intervals. The first two collections were considered as basal. In the **first protocol** AT1 receptor antagonist candesartan (100 µg/kg, bolus dose; $K_d$= 51pM; $IC_{50}$= 12nM; $t_{1/2}$ =152min) or AT2 receptor agonist CGP-42112 (1 µg/kg/min, continuous infusion; $K_d$=0.1nM; $IC_{50}$= 6.3µM; $t_{1/2}$= 30 min) was given through the jugular vein. After the drug delivery two collections of urine were obtained at 30-minute intervals each. In the **second protocol** after basal urine collection NAD(P)H oxidase inhibitor apocynin (3.5 µg/kg/min; $t_{1/2}$=60min) was infused through the jugular vein. Two collection of urine were obtained. At the end of second collection of apocynin, candesartan or CGP-42112A was given along with apocynin and urine was collected for another two 30 min intervals. The different protocols used in the experiment are shown in figure 2. Blood pressure and heart rate were recorded throughout the experimental period.

### 3.2.3: Measurement of plasma and urinary sodium and calculation of kidney function parameters:

Urine was collected throughout the 30-minute periods of all kidney function experiments. Blood sample was collected for plasma at the end of

every 30 min urine collection period. Plasma was separated by centrifuging blood samples at 1500 xg for 15 min at 4 $^{o}$C. Urine and plasma samples were stored at –20 $^{o}$C until analyzed for sodium and creatinine. Sodium concentration in the urine and plasma was measured using a flame photometer 480 (Ciba Corning Diagnostics, Norwood, MA). Creatinine Assay Kit (BioVisioin, Mountain View, CA) was used for measuring creatinine. Creatinine clearance ($CL_{creatinine}$), a measure of glomerular filtration rate (GFR) and fraction excretion of sodium ($FE_{Na}$), a measure of natriuresis was calculated from the following formules:

$U_{Na}V$ ($\mu$mol/min) = urine flow ($\mu$l/min) X urinary sodium concentration ($\mu$mol/ $\mu$l)

$$CL_{creatinine} = \text{GFR (ml/min)} = \frac{\text{urine flow (ml/min) X urine creatinine (mg/dl)}}{\text{Plasma creatinine concentration (mg/dl)}}$$

$$FE_{Na}\ (\%) = \frac{\text{urinary sodium excretion } (U_{Na}V;\ \mu\text{mol/min}) \text{ X } 100}{\text{Plasma Na concentration } (\mu\text{mol/ } \mu\text{l}) \text{ X GFR}}$$

[$U_{Na}V$ = Urinary sodium excretion; GFR = Glomerular filtration rate;

$FE_{Na}$ = Fractional excretion of sodium]

### 3.3: Total urinary Nitrite/Nitrates in SD rats:

Total nitrite/nitrate in the urine samples was determined as a measurement of NO production at baseline and during infusion of apocynin, CGP-42112A alone or in combination. Total nitrite/nitrate was measured using an enzymatic kit (R&D Systems, Minneapolis, MN). The values are represented as nmol/min. A set of standards (3.25-100 µM) was assayed in duplicate along with the samples. Urine was diluted ten fold and a volume of 50 µl per well was used for the assay. Reconstituted NADH reagent and nitrate reductase (25 µl of each) were added to all wells. Plate was incubated for 30 min at 37$^{o}$C followed by addition of 50 µl each of Griess reagent I and II. Plate was incubated again for 10 min at room temperature. Optical density was determined using a microplate reader at 540 nm. The background was subtracted from each reading and the average optical density was calculated. The data were processed using GraphPad Prism-4 software.

### 3.4: Urinary cyclic guanosine 3' , 5'-monophosphate (cGMP) levels in SD rats:

Total cGMP level in urine was determined by using an ELISA kit (R&D Systems, Minneapolis, MN). Urine that was collected during the functional study was diluted 100 folds according to the manufacturer recommendation and assayed in duplicate. Standards/samples 100µl of each were added to appropriate wells. cGMP conjugate, 50µl and 50µl of primary antibody was added to each well and the plate was incubated at room temperature for 3 hours. After incubation content of each well was aspirated and washed 4 times with the wash buffer. Substrate solution (200µl) was added and the plate was incubated for 30min. Stop solution (50µl) was added and the optical density was determined using a microplate reader at wavelength 450nm. The data were processed using GraphPad Prism and the concentration was extrapolated from the standard curve then the 100-fold dilution was accounted for. The final concentration was multiplied by the UF to calculate the concentration per minute.

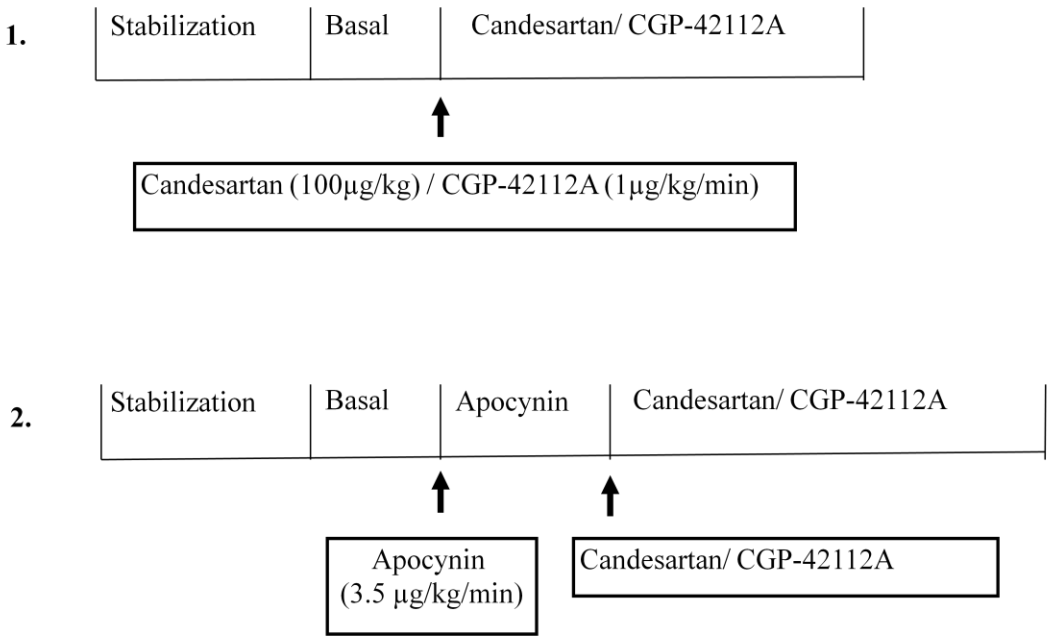


**Fig. 2:** Renal function experimental protocols.

### 3.5: Chronic CGP-42112A treatment in lean and obese Zucker rats:

#### 3.5.1: Surgical procedure:

The lean and obese rats were divided into vehicle and AT2 receptor agonist (CGP-42112A)-treated groups. Vehicle (saline) or CGP-42112A (1µg/kg/min) separately was continuously infused for 2 weeks by implanting Alzet osmotic pumps (Model 2ML-2, Alza, Palo Alto, CA) subcutaneously. The dose and length of CGP-42112A treatment were determined based on previous studies from our and other laboratories (Whitebread et al. 1991; Carey et al. 2001; Siddiqui et al. 2009). Both *in vitro* and *in vivo* studies suggest that various responses such as renal natriuresis with CGP can be blocked by PD suggesting the specificity of agonist (Kambayashi et al. 1994; Carey et al. 2001; Hakam and Hussain 2006). After the treatment period, blood from carotid artery and kidneys were collected under anesthesia. The blood was centrifuged at 1500g at $4^{o}$C to obtain plasma. The kidneys were de-capsulated, rinsed with cold PBS to remove blood, sectioned sagittally with razor blade and cortices separated. All samples were stored at $-80^{o}$C until further analyses.

### 3.5.2: Enzyme-linked immuno sorbent assays (ELISA):

**3.5.2.1: MCP-1:** MCP-1 ELISA kit was obtained form Immuno-Biological Laboratories Co., Ltd. (Japan). Appropriate blank, test samples (25 fold diluted) and series of diluted standards (100µl each) were added to the wells. The plate was incubated for 1 hour at 37$^{o}$C after covering it with plate lid. The wells were washed with the wash buffer seven times and pat dried. HRP conjugated Anti-Rat MCP-1 Mouse IgG (100 µl) was added to all wells and incubated for 30 min at 37$^{o}$C. After incubation plate was washed nine times with the provided wash buffer. Tetra Methyl Benzidine (TMB, a coloring agent, 100 µl) was added to all wells and the plate was incubated for 30 minutes at room temperature in the dark. At the end of incubation period the liquid in the wells turn blue. Stop solution (1N $H_2SO_4$) was added to all wells which turn the liquid into yellow color. The optical density was measured at 450nm using a micro-plate reader.

**3.5.2.2: CRP:** The level of CRP in plasma was determined by using ELISA from Alpha Diagnostics Intl (San Antonio, TX). The plate provided was pre-coated with anti-rat CRP antibody. Before addition of samples the wells were soaked in 200-300µl wash buffer for 30 minutes. Appropriate blank, standards, and samples (200 fold dilution with assay buffer) were

added to appropriate wells in 96-well plate. The plate was incubated for one hour at room temperature. After incubation the wells were washed with wash buffer four times and pat dried. Anti-rat CRP-HRP conjugate (100 µl, secondary antibody) were added to each well. The plate was incubated for 30 min at room temperature and washed five times with wash buffer. HRP substrate TMB (3,3,5,5-tetramethylbenzidine) was added in plate and incubated for 15 min in dark. The reaction for CRP was terminated with stop solution (100µl). The yellow color developed was read at 450nm using a micro-plate reader.

**3.5.2.3: Superoxide dismutase (SOD) activity in plasma and kidney cortex:** SOD activity was determined using kit based assay from Cayman chemical company (Ann Arbor, Michigan). The plasma samples were diluted 1:5 with sample buffer. The kidney cortex samples were homogenized in 5-10 ml of 20mM HEPES buffer (1mM EGTA, 210mM mannitol and 70mM sucrose per gram tissue, pH 7.2). After homogenization the cortex samples were centrifuged at 1,500 x g for 5 min at 4$^{o}$C and the supernatant was kept for the assay. Series of diluted standards and samples (10 µl) were added to appropriate wells along with 200 µl of diluted radical detector. To initiate the reaction, 20 µl of xanthine

oxidase was added to each well. The plate was incubated for 20 min at room temperature. The absorbance was determined at 440-460 nm using a plate reader.

### 3.5.3: Thiobarbituric acid reactive substance (TBARS) assay for malondialdehyde (MDA):

The kidney cortex samples were homogenized in ice cold 1.5% KCl for the assay. The homogenate was centrifuged at 12000g for 5min and the supernatant was used for measurement of TBARS. A stock buffer was made with 15% tri chloro acetic acid (TCA), 0.375% thio-barbituric acid (TBA) and 0.25N hydrochloric acid (HCl). A 1ml aliquot of the kidney cortex supernatant (protein conc. 2mg/ml) was added to 2ml of stock solution. The reaction mixture was heated for 15 min in boiling water and then cooled. The pink color developed was extracted with isobutanol. Absorbance of the isobutanol extract was measured spectrophotometrically at 535nm and quantified using the molar extinction coefficient of MDA ($1.56 \times 10^5$ $M^{-1}$ $cm^{-1}$) and expressed as nmoles MDA/mg of protein.

### 3.5.4: Western blotting:

The protein levels of cytokines (IL-1β, IL-6, TNF-$\alpha$), superoxide radical producing NADPH-gp$^{91phox}$, p-47$^{phox}$, Hemeoxygenase-1 (HO-1) and antioxidant (Cu/Zn-SOD, Mn-SOD and Ec-SOD) enzymes were determined by standard western blotting technique. Briefly, kidney cortices were homogenized in a buffer containing (in mM) Tris 50, EDTA 10, PMSF 1, cocktail of protease inhibitors (aprotinin, calpain inhibitors, leupeptin, pepstatin and trypsin inhibitor) and protein concentrations were determined by BCA method using a kit (Pierce, Rockford, IL). Equal amounts of protein (20µg) from various rat groups were subjected to SDS-PAGE and were transferred onto nitrocellulose membrane (blot). The blots were blocked using 5% milk in PBS with 0.1% tween-20 for an hour at room temperature followed by overnight incubation with primary antibodies for IL-1β, IL-6, TNF-$\alpha$, HO-1, Cu/Zn-SOD, Mn-SOD and Ec-SOD separately at 4$^{o}$C. Appropriate HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were used to detect protein bands using ECL system. The protein bands were analyzed by Fluorchem 8800 (Alpha Innotech Imaging System, San Leandro, CA) for the densitometric analysis. β-actin was used as protein loading control.

### 3.6: Chemicals:

Candesartan was a generous gift from AstraZeneca (Wilmington, Del), CGP-42112A was custom synthesized (21$^{st}$ Century Biochemicals, MA). ELISA kits for CRP and MCP-1 were purchased from Alpha Diagnostics Intl (San Antonio, TX) and Immuno-Biological Laboratories Co., Ltd. (Japan), respectively. SOD activity kit was bought from Cayman chemical company (Ann Arbor, Michigan). Enhanced chemiluminescence substrates were obtained from Alpha Diagnostics Intl (San Antonio, TX). Creatinine assay kit was purchased from BioVision (Mountain View, CA). The NO/Nitrite/Nitrate and cGMP assay kit were obtained from R&D systems (Minneapolis,MN). Apocynin and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO). The primary antibodies used and their sources are listed in table-1.

### 3.7: Statistical analysis:

Results are presented as mean ± SE with an n= 4-10 in each experiment. The data were subjected to statistical analyses using GraphPad Prism 4, San Diego, CA. To analyze variations between group's student *t*-test and one-way ANOVA with post hoc (Newman-Keuls) tests was used. A value of $P<0.05$ was considered statistically significant.

| Antibody | Source | Dilution | Company |
|---|---|---|---|
| HO-1 | Mouse monoclonal | Dilution 1:1000 | Assay Design |
| TNF-α | Rabbit Polyclonal | Dilution 1:1000 | Cell Signaling |
| IL-6 | Rabbit Polyclonal | Dilution 1:1000 | Invitrogen |
| IL-1β | Mouse Monoclonal | Dilution 1:750 | Santa Cruz |
| Gp-91 | Mouse Monoclonal | Dilution 1:1000 | BD biosciences |
| P-47 | Rabbit polyclonal | Dilution 1:1000 | Cell signaling |
| Cu/zn SOD | Rabbit Polyclonal | Dilution 1:1000 | Stressgen |
| Mn-SOD | Rabbit Polyclonal | Dilution 1:1000 | Chemicon |
| Ec-SOD | Rabbit polyclonal | Dilution 1:1000 | Chemicon |
| β-Actin | Mouse Monoclonal | Dilution 1:1000 | Santa Cruz |

**Table 1:** List of antibodies

## 4: EFFECT OF NAD(P)H OXIDASE INHBITION ON AT1 RECEPTOR ANTAGONIST (CANDESARTAN) AND AT2 RECEPTOR AGONIST (CGP-42112A)-MEDIATED NATRIURESIS:

### 4.1: Results:

#### 4.1.1: Effect of apocynin on candesartan-induced diuresis/natriuresis in SD rats:

As shown in fig.3 and 4, a bolus dose of the AT1 receptor antagonist candesartan (100µg/kg), produced significant increase in UF (53.08 µl/30min), $U_{Na}V$ (0.3985 µmole/min) and $FE_{Na}$% (2.673) over the basal. Infusion of apocynin (3.5µg/Kg/min), an NAD(P)H OXIDASE inhibitor, only modestly increased UF (27.77 µl/30min), $U_{Na}V$ (0.1916 µmole/min) and $FE_{Na}$% (1.648), however, the increases are not significant over the basal (Fig.5 and 6). Pre infusion of apocynin did not change the net natriuretic/diuretic response to candesartan. Apocynin and candesartan together had an additive effect on UF (82.07 µl/30min), $U_{Na}V$ (0.7443 µmole/min) and $FE_{Na}$% (4.313) over the basal (Fig. 5 and 6).

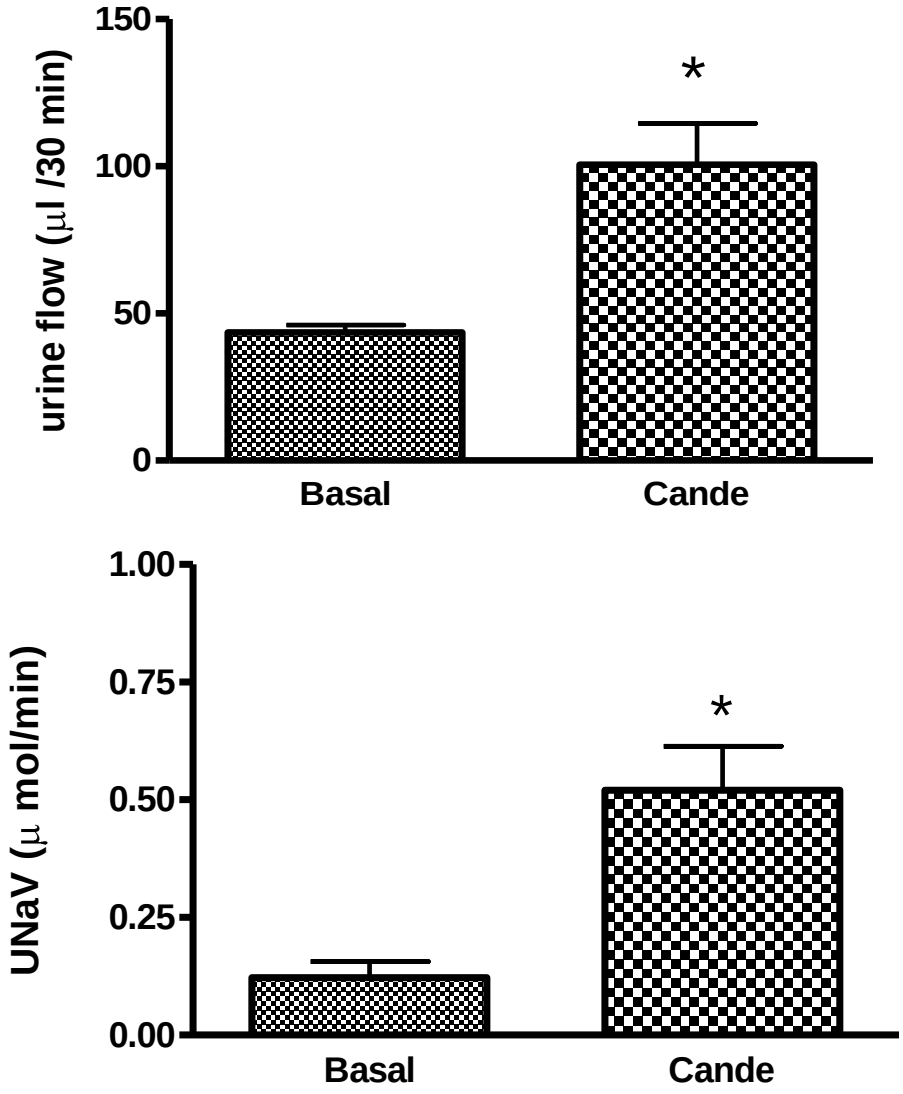


**Fig.3:** *Candesartan-induced diuresis and natriuresis in SD rats.* The dose of candesartan was 100µg/kg; i.v bolus. The values in the bar graph are represented as mean ± SEM. Student-t test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 5-10). * = significantly different from basal.

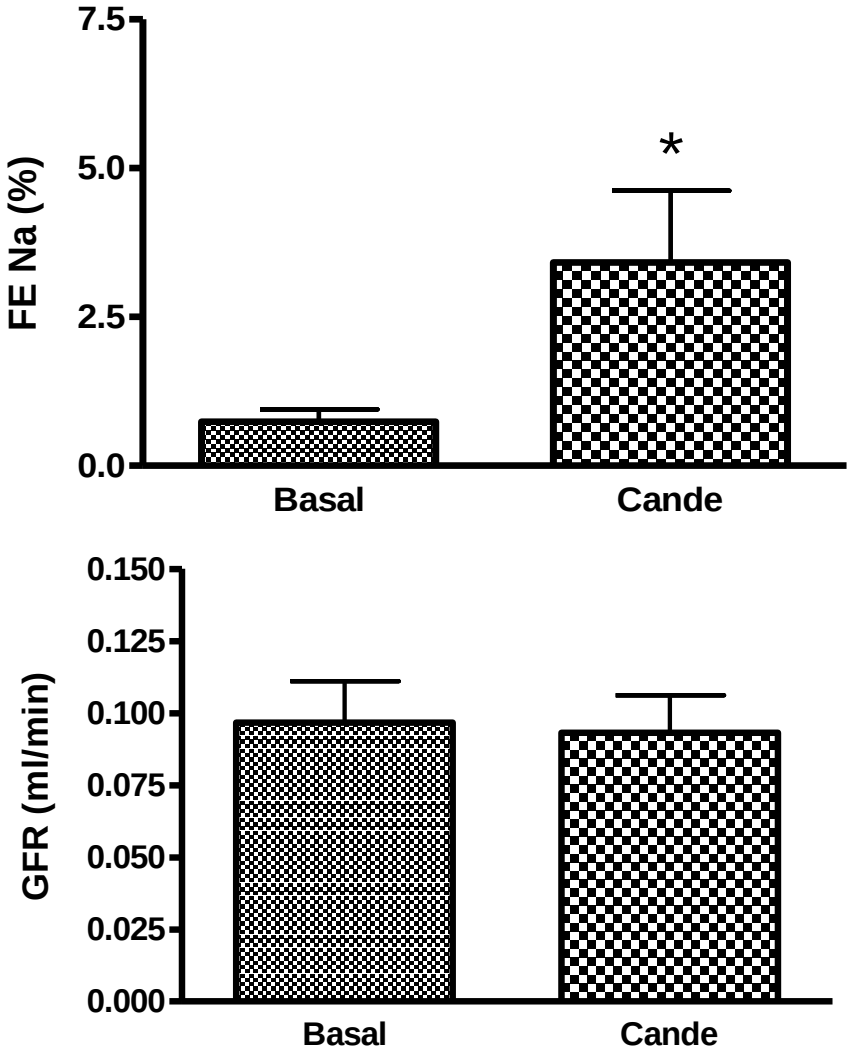


**Fig.4 :** *Candesartan-induced change in FE Na and GFR in SD rats.* The dose of candesartan was 100µg/kg; i.v bolus. The values in the bar graph are represented as mean ± SEM. Student-t test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 5-10). * = significantly different from basal.

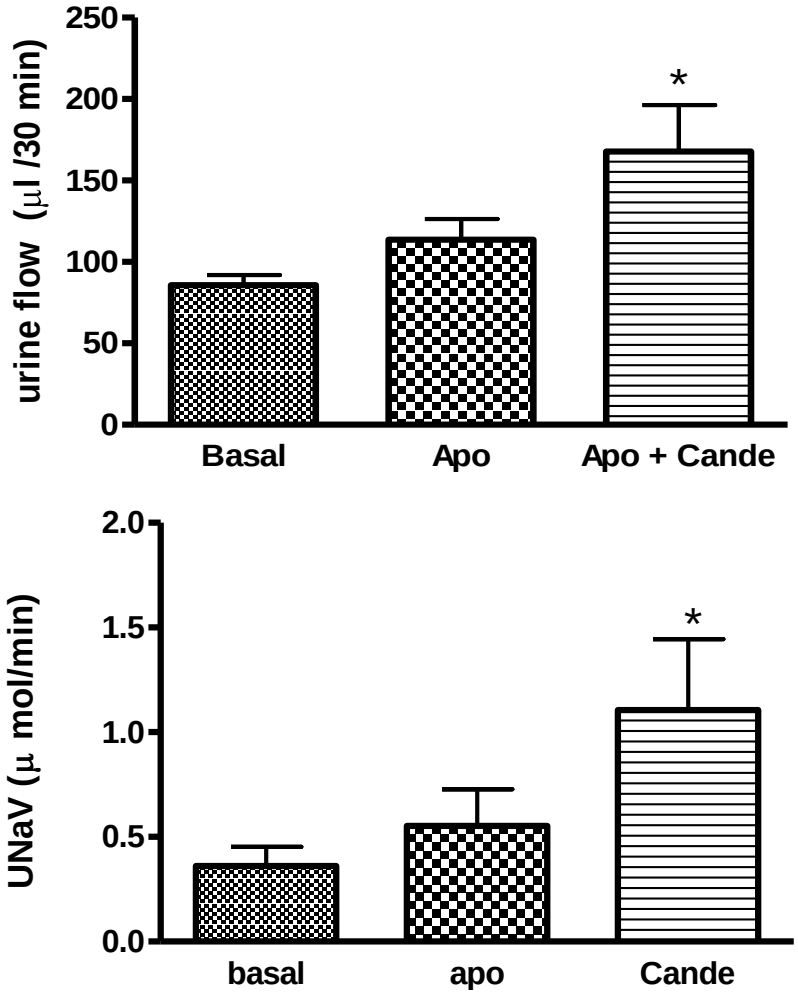


**Fig.5:** *Effect of pre-treatment of apocynin on candesartan-induced diuresis and natriuresis in SD rats.* Apocynin was given at a rate of 3.5µg/kg/min; i.v infusion and the dose of candesartan was 100µg/kg; i.v bolus. The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Kules multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 5-10). * = significantly different from basal.

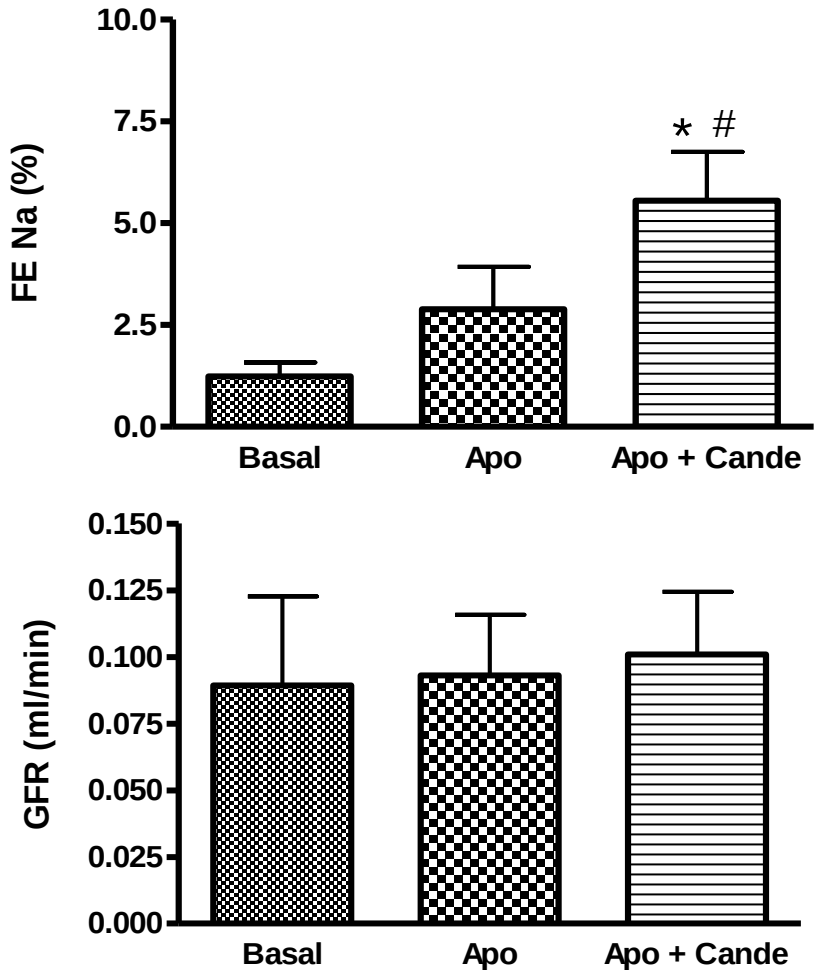


**Fig.6:** *Change in FENa and GFR with infusion of apocynin alone or in combination with candesartan in SD rats.* Apocynin was given at a rate of 3.5μg/kg/min; i.v infusion and the dose of candesartan was 100μg/kg; i.v bolus. The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Kules multiple comparison post hoc test was used to compare the data within the group. The value of $p < 0.05$ was considered significant. (n= 5-10). * = significantly different from basal; # = significantly different from apocynin.

### 4.1.2: Effect of apocynin on candesartan-induced diuresis/natriuresis in lean Zucker rats:

A bolus dose of the AT1 receptor antagonist candesartan (100µg/kg), caused modest increase in UF (32.70 µl/30min), $U_{Na}V$ (0.3985 µmole/min) and $FE_{Na}$% (1.144) over the basal (Fig. 7 and 8). Infusion of apocynin (3.5µg/Kg/min) alone modestly increased UF (26.62 µl/30min), $U_{Na}V$ (0.1245 µmole/min) and $FE_{Na}$% (0.655), however, the increases are not significant over the basal (Fig.9 and 10). Pre infusion of apocynin in lean rats did not change the net natriuretic/diuretic response to candesartan as seen in SD rats. Apocynin and candesartan together had an additive effect on UF (57.36 µl/30min), $U_{Na}V$ (0.4688 µmole/min) and $FE_{Na}$% (1.900) over the basal (Fig.9 and 10).

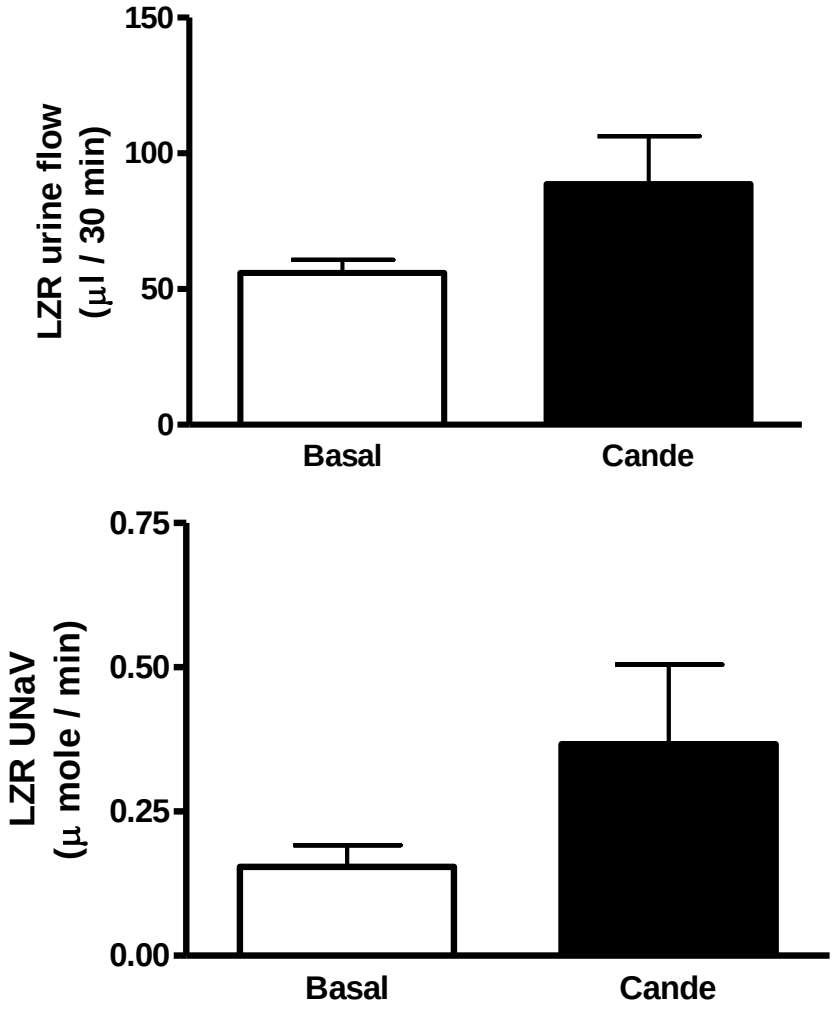


**Fig.7:** *Candesartan-induced diuresis and natriuresis in lean Zucker rats.* The dose of candesartan was 100µg/kg; i.v bolus. The values in the bar graph are represented as mean ± SEM. Student-t test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 6).

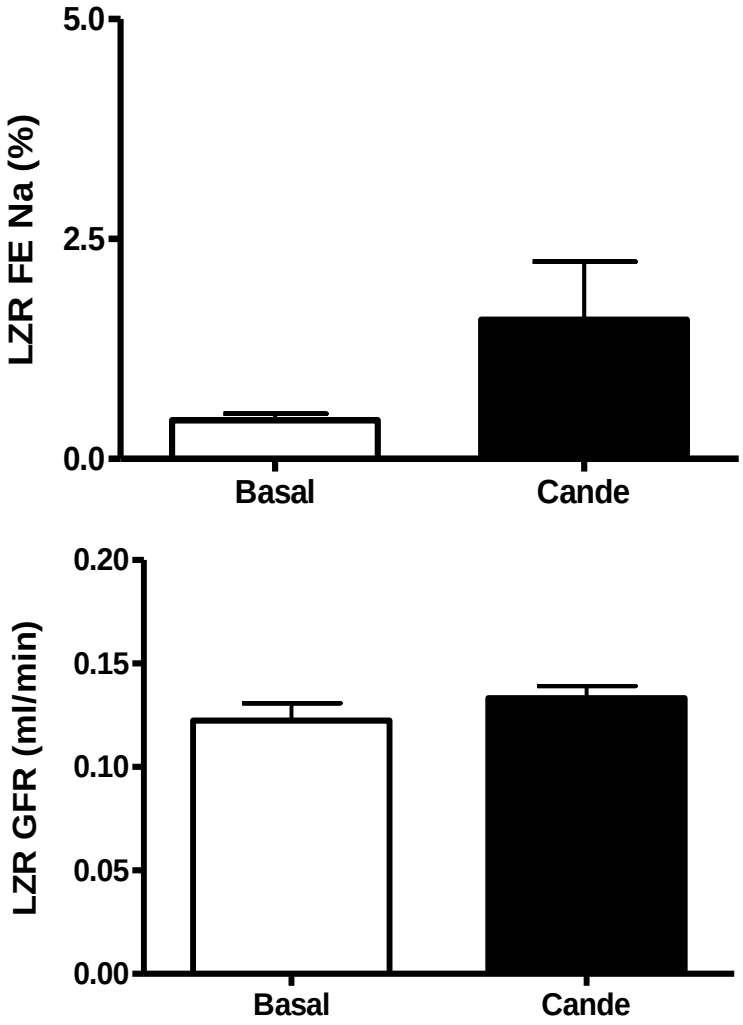


**Fig.8:** *Candesartan-induced change in FE Na and GFR in lean Zucker rats.* The dose of candesartan was 100µg/kg; i.v bolus. The values in the bar graph are represented as mean ± SEM. Student-t test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 4-6). * = significantly different from basal.

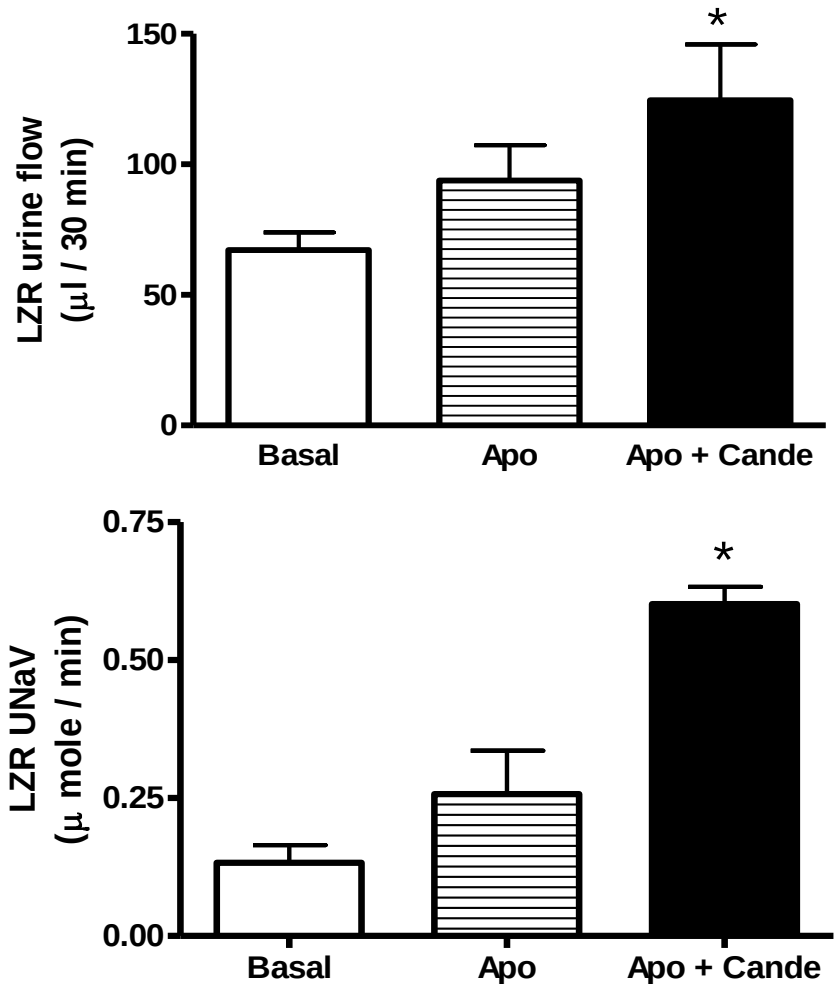


**Fig.9:** *Effect of pre-treatment of apocynin on candesartan-induced diuresis and natriuresis in lean Zucker rats.* Apocynin was given at a rate of 3.5µg/kg/min; i.v infusion and the dose of candesartan was 100µg/kg; i.v bolus. The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Kules multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 6). * = significantly different from basal.

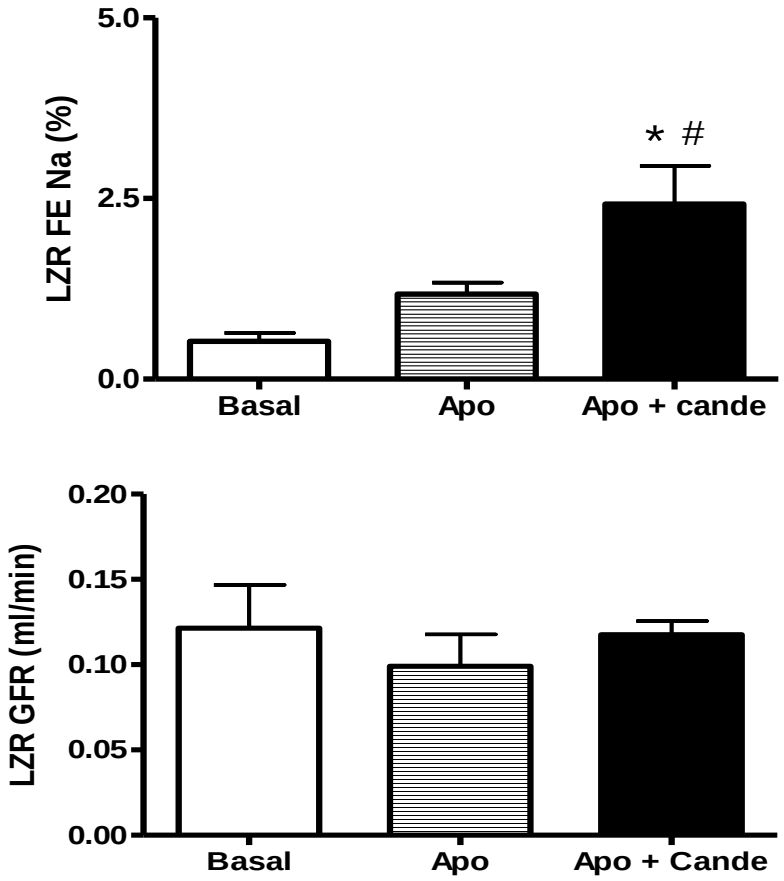


**Fig.10:** *Change in FENa and GFR with infusion of apocynin alone or in combination with candesartan in lean Zucker rats.* Apocynin was given at a rate of 3.5μg/kg/min; i.v infusion and the dose of candesartan was 100μg/kg; i.v bolus. The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Kules multiple comparison post hoc test was used to compare the data with in the group. The value of $p < 0.05$ was considered significant. (n= 4-6). * = significantly different from basal; # = significantly different from apocynin.

### 4.1.3: Effect of apocynin on candesartan-induced diuresis/natriuresis in obese Zucker rats:

The obese Zucker rats had higher basal UF (81.56 Vs. 55.93) and $U_{Na}V$ (0.2307 Vs.0.1539) when compared to their lean controls. A bolus dose of candesartan (100µg/kg), significantly increased UF (91.04 µl/30min), $U_{Na}V$ (0.6256) µmole/min) and $FE_{Na}$% (2.362) over the basal (Fig.11 and 12). Infusion of apocynin (3.5µg/Kg/min) alone had no effect on UF (11.80 µl/30min), $U_{Na}V$ (0.0277 µmole/min) and $FE_{Na}$% (0.453), over the basal (Fig. 13 and 14). Pre infusion of apocynin significantly inhibited the response of candesartan on UF (-23.20 µl/30min), $U_{Na}V$ (-0.1762 µmole/min) and $FE_{Na}$% (-0.6274) over the basal (Fig. 13 and 14).

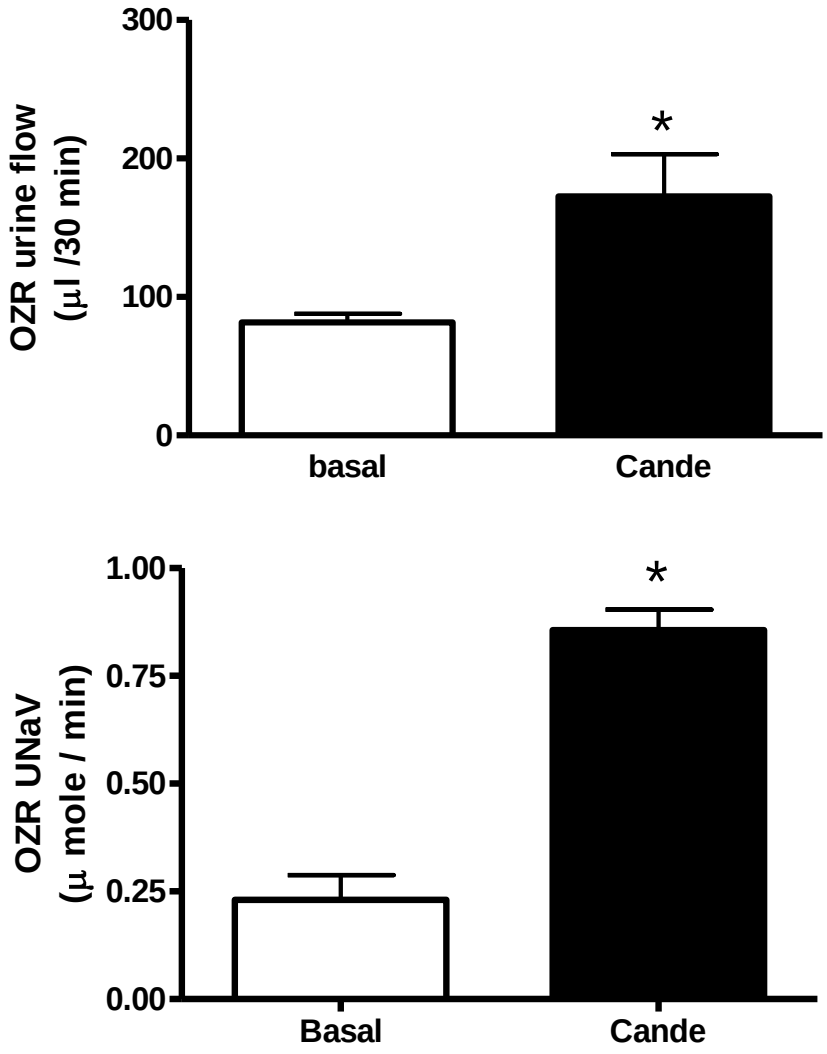


**Fig.11:** *Candesartan-induced diuresis and natriuresis in Obese Zucker rats.* The dose of candesartan was 100µg/kg; i.v bolus. The values in the bar graph are represented as mean ± SEM. Student-t test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 6).

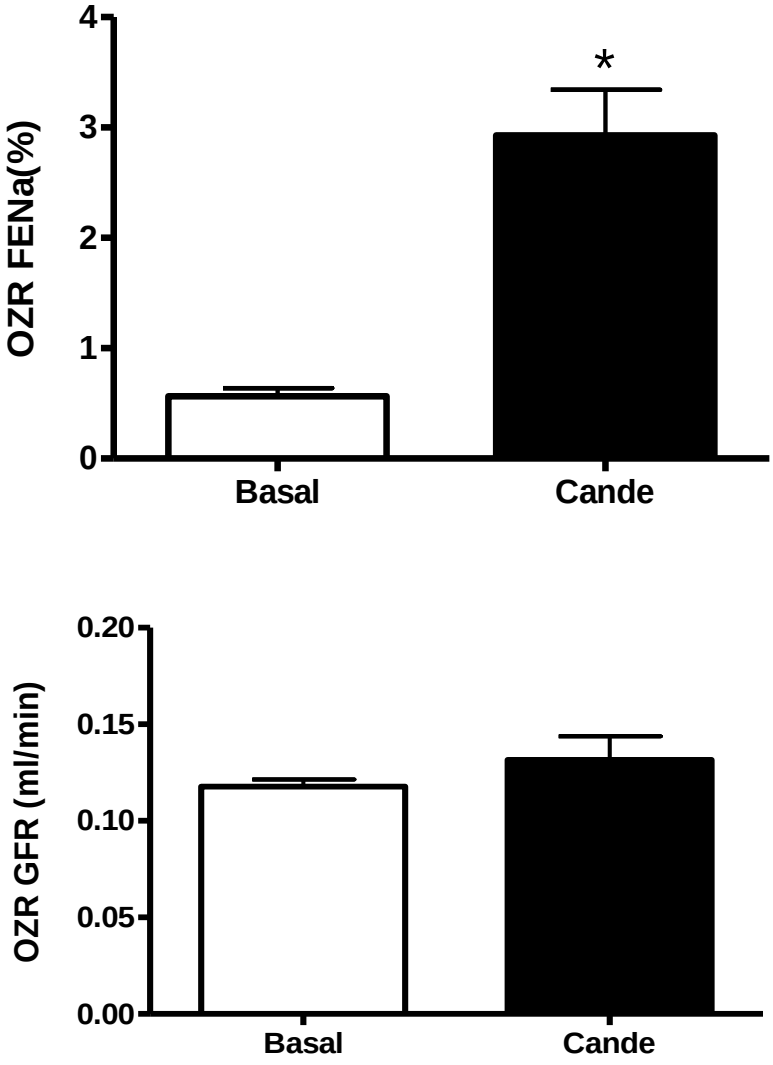


**Fig.12:** *Candesartan-induced change in FE Na and GFR in obese Zucker rats.* The dose of candesartan was 100µg/kg; i.v bolus. The values in the bar graph are represented as mean ± SEM. Student-t test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 4-6). * = significantly different from basal.

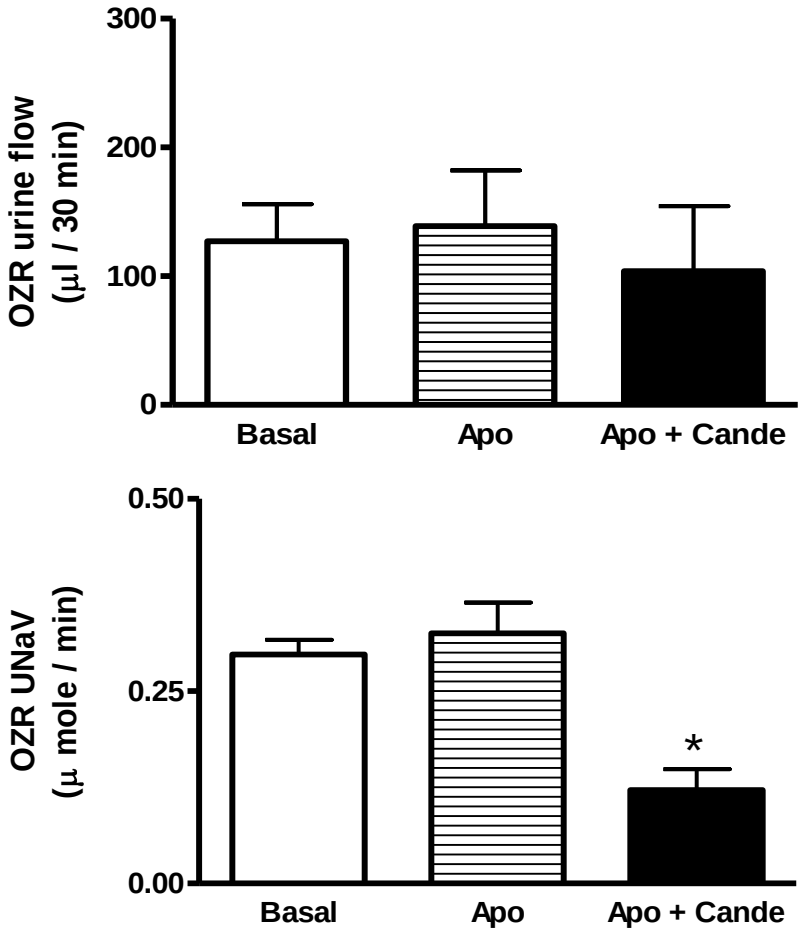


**Fig.13:** *Effect of pre-treatment of apocynin on candesartan-induced diuresis and natriuresis in obese Zucker rats.* Apocynin was given at a rate of 3.5µg/kg/min; i.v infusion and the dose of candesartan was 100µg/kg; i.v bolus. The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Kules multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 6). * = significantly different from basal and apocynin.

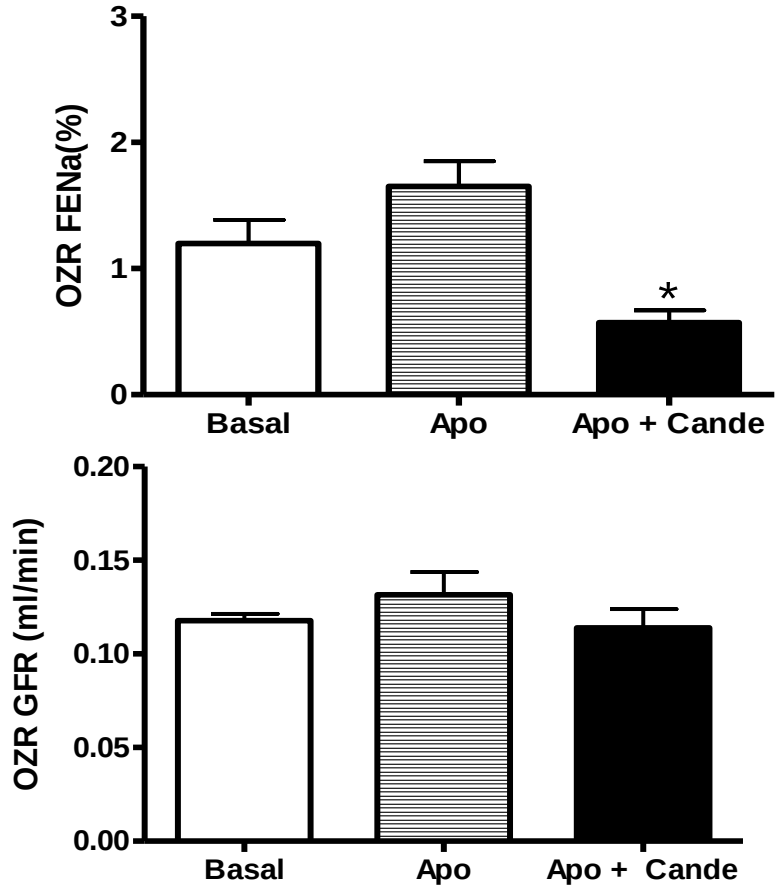


**Fig.14:** *Change in FENa and GFR with infusion of apocynin alone or in combination with candesartan in obese Zucker rats.* Apocynin was given at a rate of 3.5μg/kg/min; i.v infusion and the dose of candesartan was 100μg/kg; i.v bolus. The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Kules multiple comparison post hoc test was used to compare the data with in the group. The value of $p < 0.05$ was considered significant. (n= 4-6). * = significantly different from apocynin.

### 4.1.4: Effect of apocynin on CGP-42112A-induced diuresis/natriuresis in SD rats:

Intravenous infusion of CGP-42112A (1µg/Kg/min), an AT2 receptor agonist, produced moderate increase in UF (29.97 µl/30min), $U_{Na}V$ (0.1537 µmole/min) and $FE_{Na}$% (1.449) over the basal, however it was not statistically significant (Fig. 15 and 16). As seen already, infusion of apocynin (3.5µg/Kg/min) also did not cause significant increase in UF (16.90 µl/30min), $U_{Na}V$ (0.1475 µmole/min) and $FE_{Na}$%(1.880) over the basal (Fig. 17 and 18). However, pre infusion of apocynin significantly increased the net diuretic/natriuretic effect of CGP-42112A. Apocynin and CGP-42112A together had synergistic effect on UF (72.87 µl/30min), $U_{Na}V$ (1.142 µmole/min) and $FE_{Na}$% (7.862) over the basal, apocynin alone and CGP-42112A alone in SD rats (Fig.17 and 18).

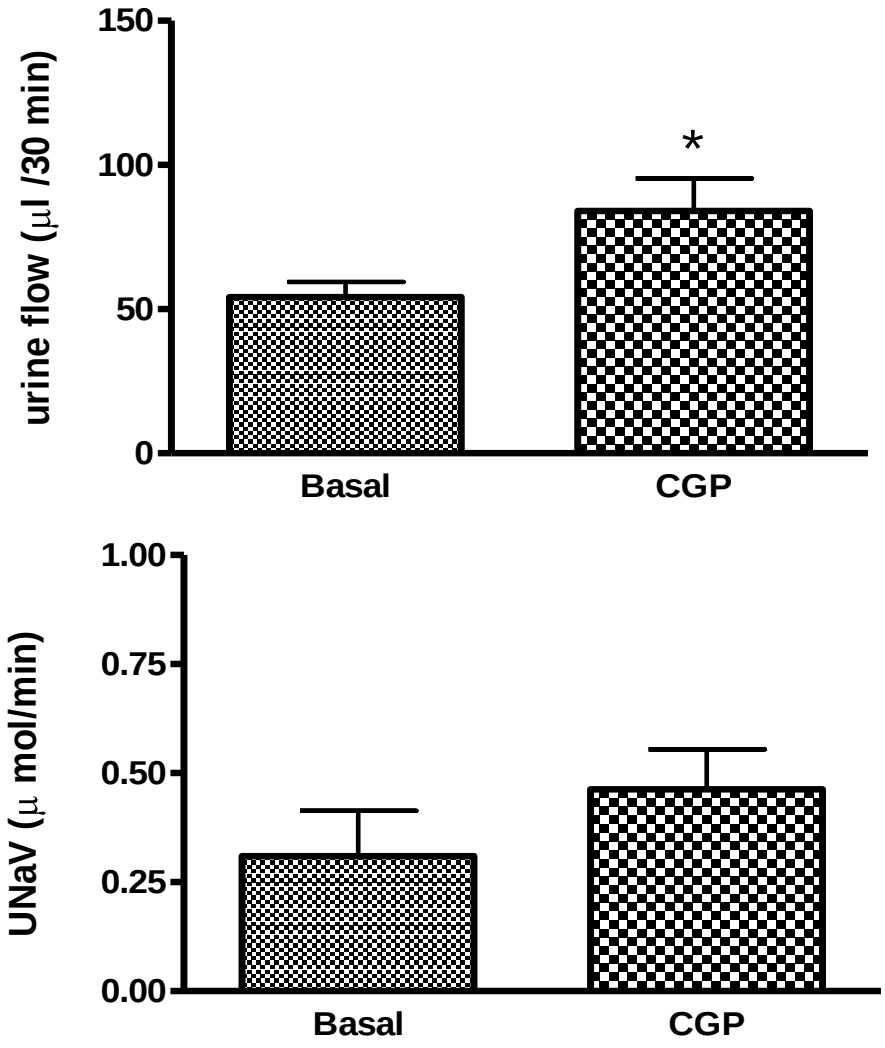


**Fig.15:** *CGP-42112A-induced diuresis and natriuresis in SD rats.* i.v infusion of CGP-42112A was given at a rate of 1 μg/kg/min. The values in the bar graph are represented as mean ± SEM. Student's - t test was used to compare the data within the group. The value of $p < 0.05$ was considered significant. (n= 5-9). * = significantly different from basal.

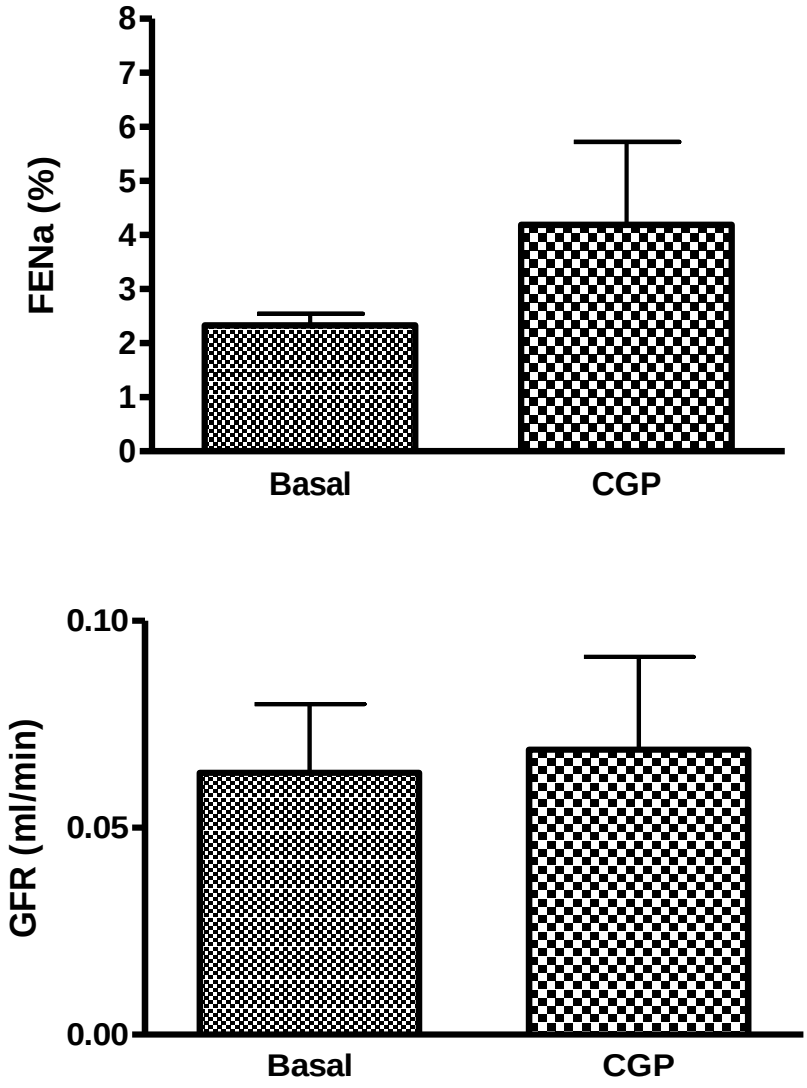


**Fig.16:** *CGP-42112A-induced change in FENa and GFR in SD rats.* i.v infusion of CGP-42112A was given at a rate of 1 μg/kg/min. The values in the bar graph are represented as mean ± SEM. Student's-t test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 5-9).

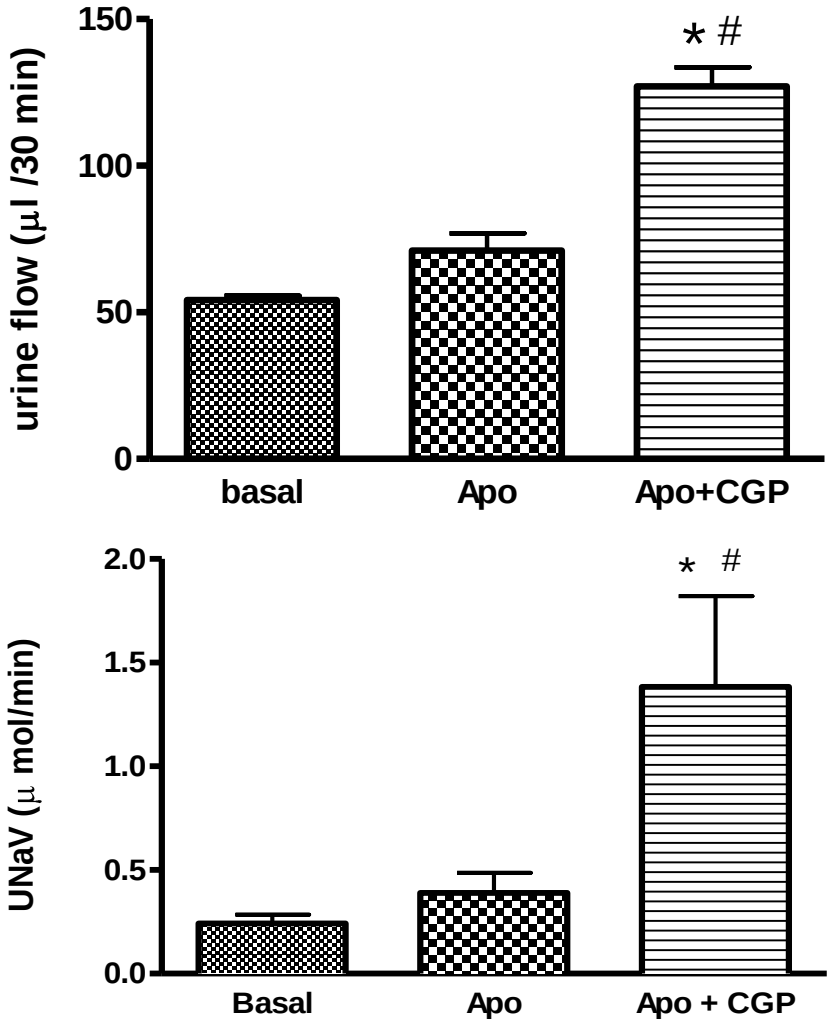


**Fig.17:** *Effect of pre-treatment of apocynin on CGP-42112A-induced diuresis and natriuresis in SD rats.* i.v infusion of CGP-42112A and apocynin were given at a rate of 1 µg/kg/min and 3.5µg/kg/min respectively. The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Kules multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 5-9). * = significantly different from basal; # = significantly different from apocynin.

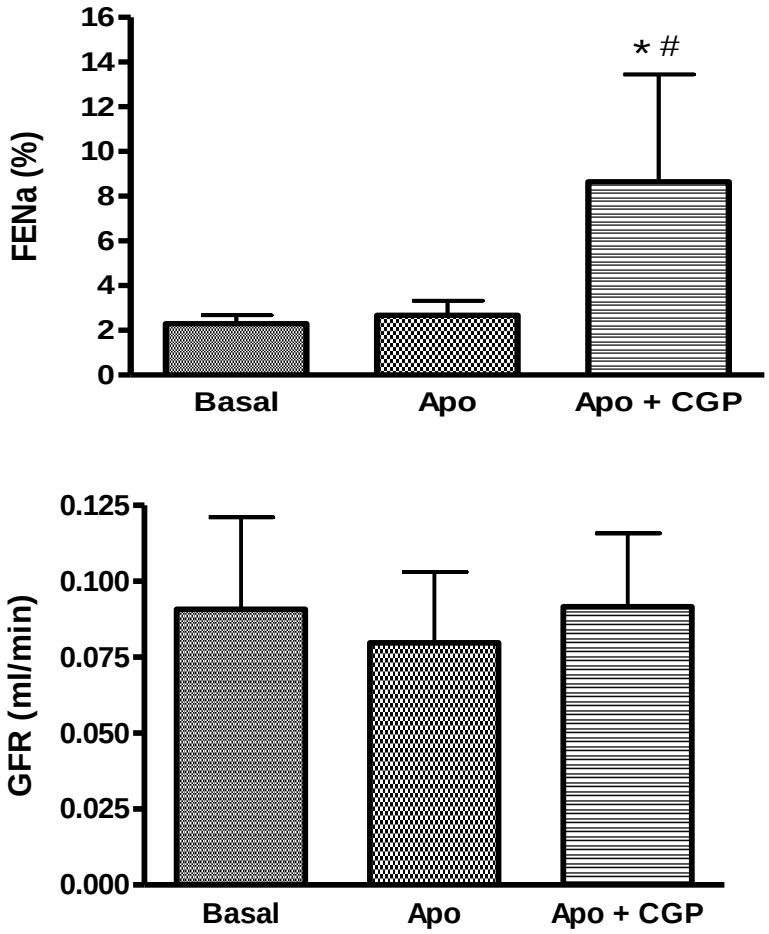


**Fig.18:** *Change in FENa and GFR with infusion of apocynin alone or in combination with CGP-42112A in SD rats.* i.v infusion of CGP-42112A and apocynin were given at a rate of 1 μg/kg/min and 3.5μg/kg/min respectively. The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Kules multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 5-9). * = significantly different from basal; # = significantly different from apocynin.

### 4.1.5: Change in urinary nitrates and cGMP levels with infusion of apocynin alone or in combination with CGP-42112A:

Since AT2 receptor has been shown to be linked to NO/cGMP pathway leading to natriuresis/diuresis. We measured urinary nitrates and cGMP levels. The level of nitrates (nmole/min) increased modestly with apocynin (0.21) and CGP-42112A (0.51) alone however; it was not significant over the basal. A combination of apocynin and CGP-42112A produced significant increase in nitrates (0.73) levels over the basal (Fig.19A). Similarly, there was a significant increase (0.4304, µmol/min) in the cGMP level over the basal (0.5442) when apocynin and CGP-42112A were given in combination (Fig.19B). Apocynin (0.08626) and CGP-42112A (0.1958) alone did not produce significant increase in urinary cGMP level over the basal.

### 4.1.6: Blood pressure and GFR:

There were no significant changes in the GFR (Fig. 3-18) with administration of candesartan, CGP-42112A and/or apocynin over the basal in SD rats or in lean and obese Zucker rats. Also administration of these drugs had no effect on blood pressure (Table 1). These data along with the changes in $FE_{Na}$ suggest the tubular effect of the drugs.

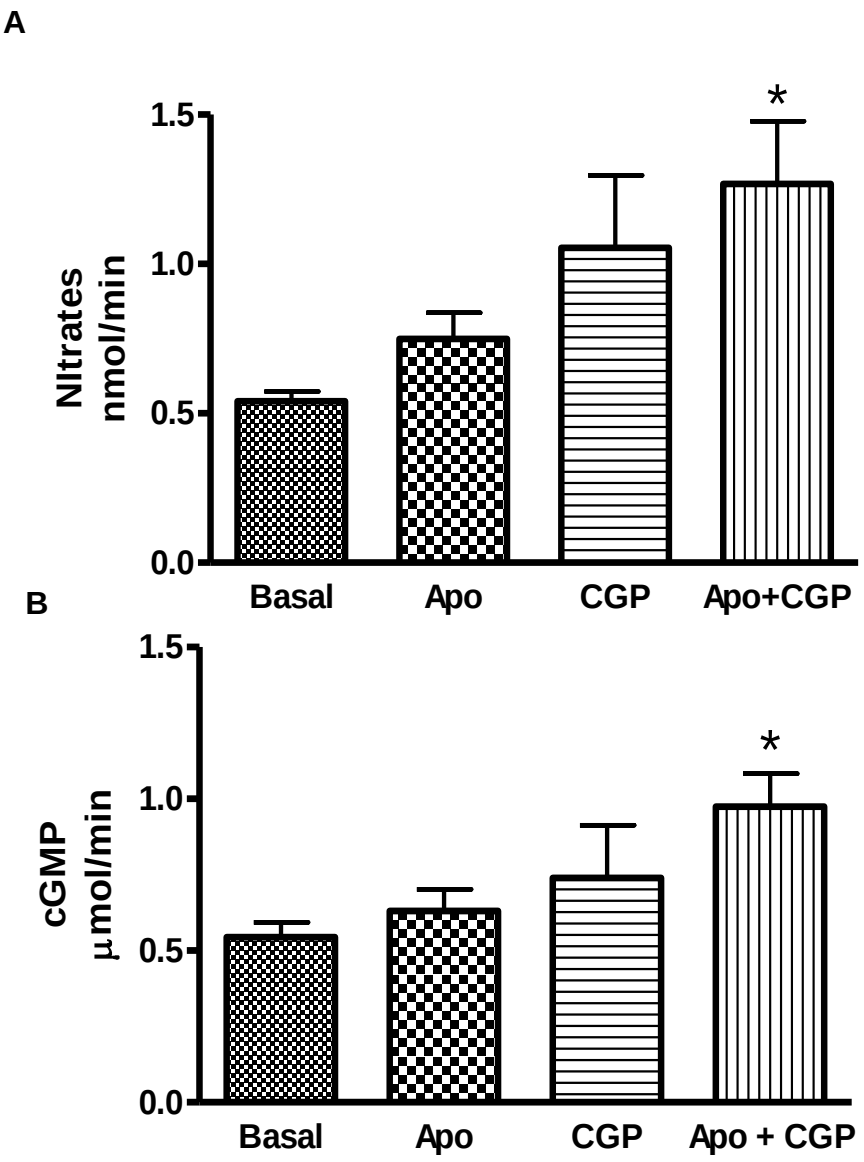


**Fig.19:** *Total nitrates (A) and cGMP (B) measured in urine samples.* The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Kules multiple comparison post hoc test was used to compare the data with in the group. The value of $p < 0.05$ was considered significant. n= 4-10. * = significantly different from basal.

| | Basal | Cande | CGP |
|---|---|---|---|
| SD | 86 ± 4.1 | 80 ± 3.6 | - |
| SD | 78 ± 1.8 | - | 69 ± 3.7 |
| LZR | 87 ± 3.8 | 80 ± 4.6 | - |
| OZR | 105 ± 2.7 | 97 ± 1.9 | - |

| | Basal | Apo | Apo + Cande | Apo + CGP |
|---|---|---|---|---|
| SD | 85 ± 1.7 | 82 ± 1.3 | 76 ± 3.1 | - |
| LZR | 97 ± 2.5 | 97 ± 5.0 | 95 ± 3.9 | - |
| OZR | 102 ± 4.4 | 102 ± 3.4 | 92 ± 2.5 | - |
| SD | 85 ± 4.5 | 75 ± 2.1 | - | 69 ± 3.6 |

**Table 2:** Change in mean arterial blood pressure (MAP; mmHg) with infusion of candesartan, CGP-42112A and apocynin alone or in combination in SD, lean Zucker *and obese Zucker rats.* The dose of candesartan was 100µg/kg; i.v bolus, CGP-42112A and apocynin were given as i.v infusion at a rate of 1 µg/kg/min and 3.5µg/kg/min respectively. The values are represented as mean ± SEM. One-way ANOVA with Newman-Kules multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant.

## 4.2: Discussion:

The main new findings of this study are that the natriuretic function of AT2 receptors is masked by NAD(P)H oxidase by decreasing the availability of nitric oxide (NO) as potential mechanism and there may not be a cross talk between NAD(P)H oxidase and AT1 receptors function on natriuresis and diuresis in SD and lean Zucker rats.

Oxidative stress is mainly defined as an imbalance between rate of generation of reactive oxygen species (ROS) and the rate at which ROS are scavenged by the superoxide dismutase (SOD) enzymes (Thannickal and Fanburg 2000; Chabrashvili et al. 2003). Although, it is agreed upon that increased ROS is deleterious (Banday et al. 2005; Drechsel and Patel 2008), a basal level of ROS has been suggested to play an important role in normal functioning of the cells (Chakraborti et al. 1998; Griendling et al. 2000).

Growing bodies of evidence support the role of RAS in development of oxidative stress (Rajagopalan et al. 1996; Haas et al. 1999; Ortiz et al. 2001). Prolonged AngII-infusion has been shown to increase renal cortical NAD(P)H oxidase activity (Rajagopalan et al. 1996; Chabrashvili et al. 2003). AngII infusion develops a pressor response and mimics the pathological condition of AngII-mediated hypertension

(Rajagopalan et al. 1996; Chabrashvili et al. 2003). However, in our study we investigated the role of NAD(P)H oxidase inhibition on AT1 and AT2 receptor-mediated natriuresis and diuresis in normal SD rats. AT1 and AT2 receptors are reported to be expressed in the various kidney parts, including in the proximal tubules, which is the site of major Na-reabsorption (Miyata et al. 1999; Hakam and Hussain 2005; Shah and Hussain 2006).

In the present study, inhibition of AT1 receptors by candesartan caused significant natriuresis in SD rats. In lean rats there was a moderate effect of candesartan on natriuresis; however, in obese rats the effect of candesartan on natriuresis was more pronounced. Inhibition of NAD(P)H oxidase alone produced certain degree of natriuresis/diuresis indicating its independent role on Na and fluid homeostasis in SD and lean rats. Studies have supported the role of NAD(P)H oxidase in natriuresis and attributed this effect as a response to change in GFR with release of NO (Lopez et al. 2003). However, in our study we did not observe any significant increase in NO with apocynin infusion, and also the GFR remained unchanged. The difference between our study and earlier (Lopez et al. 2003) could possibly be due to the low dose of apocynin, used by us. In obese rats apocynin failed to show any effect on diuresis and natriuresis.

The response of candesartan on natriuresis did not change by NAD(P)H oxidase inhibitor in SD and lean rats. However, in obese rats pre-infusion of apocynin to our surprise completely inhibited the effect of candesartan. Studies have supported that during pathological conditions the activation of AT1 receptors increase NAD(P)H oxidase activity (Chabrashvili et al. 2003; Gill and Wilcox 2006; Manrique et al. 2009) however, we did not observe such effect in obese rat, which is a animal model of metabolic syndrome. In SD and lean rats, had there been any relation between AT1 receptors and NAD(P)H oxidase, the natriuretic response of NAD(P)H oxidase alone should have been comparable to that of candesartan. This suggests that under normal physiological conditions, NAD(P)H oxidase may not be a part of AT1 receptor signaling leading to antinatriuresis. The effect of apocynin in inhibiting the effect of candesartan induced natriuresis in obese rats was unexpected and the reason behind this response is unclear.

In the second set of experiments, we sought to study if there is any interaction between the AT2 receptors and NAD(P)H oxidase in terms of natriuresis in SD rats. Selective activation of AT2 receptors by CGP-42112A caused natriuresis. However, the natriuresis produced by CGP-42112A was not significant over the basal. The difference in natriuretic response can be attributed to low AT2 receptor density when compared to

AT1 receptors in the kidney (de Gasparo et al. 2000; Kaschina and Unger 2003). Inhibition of NAD(P)H oxidase alone again produced moderate increase in natriuresis as seen in earlier groups.

Selective activation of the AT2 receptor by the agonist CGP-42112A while NAD(P)H oxidase was inhibited, to our surprise, produced tremendous increase in UF, $U_{Na}V$ and $FE_{Na}$% over the basal. The mechanism behind this increase in renal function by a combination of CGP-42112A and apocynin, right now is not clear. However, it can be speculated that there is certain interaction between the downstream signaling molecules which amplify the net urinary Na output.

The superoxide radicals are reported to quickly quench the nitric oxide (NO) molecules and convert them to peroxynitrite ($ONOO^-$) (Sachse and Wolf 2007; Nistala et al. 2008; Paravicini and Touyz 2008). The NO is known to inhibit various Na-transporters in the proximal tubules with a potential to regulate urinary Na excretion (Liang and Knox 1999; Liang and Knox 2000). Several studies including from our laboratory have shown that that CGP-42112A via AT2 receptors activate NO/cGMP pathway leading to increase in urinary Na-excretion (Siragy and Carey 1997; Millatt et al. 1999; Carey et al. 2000; Hakam and Hussain 2006; Zhuo and Li 2007; Gwathmey et al. 2009). Considering these findings, we

measured the total nitrate (as an index of NO levels) and cGMP excreted in urine, in response to apocynin and CGP-41221A infusion alone or in combination, as a potential mechanism of enhanced AT2 receptor-mediated natriuresis in presence of NAD(P)H oxidase inhibitor apocynin. The total nitrate and cGMP significantly increased over the basal when apocynin and CGP-42212A were administered together. However, the increase in total nitrate and cGMP levels was not synergistic as we observed with Na. Although reasons are not clear why we did not see similar response with nitrate/cGMP, a plausible explanation for this could be that systemic administration of these drugs to some extent could have caused NO/cGMP release from the vasculature which might have masked the tubular NO/cGMP release affecting the net urinary nitrate and cGMP excretion pattern. On the other hand, the changes in Na-excretion in response to drugs are tubular effect as suggested by increase in $FE_{Na}$ % without any changes in GFR or blood pressure. Also, certain threshold increase in NO-cGMP could have been enough to synergistically amplify the AT2 receptor signal leading to inhibition in Na-transport and promoting natriuresis.

Considering these studies, it may be proposed that in pathological conditions where there is increased oxidative stress, lesser availability of NO will decrease AT2 receptor-mediated natriuresis leaving

AT1 receptor-mediated antinatriuresis unopposed. This might increase Na-retention shifting pressure natriuresis contributing to hypertension. On the other hand inhibition or lowering of NAD(P)H oxidase activity can lead to beneficial effects of AT2 receptors like cell proliferation, vasorelaxation and natriuresis.

In summary, we found that NAD(P)H oxidase inhibitor causes synergistic effect on AT2 receptor-mediated natriuresis/dieresis in SD rats, suggesting a potential interaction between AT2 receptor and NAD(P)H oxidase. Such interaction appears to be at the level of reactions between NO and superoxide reducing NO availability and thereby regulating renal tubular Na-reabsorption. To our knowledge this the first report suggesting a potential role of NAD(P)H oxidase via reducing AT2 receptor function in tubular Na-reabsorption/excretion and blood pressure control.

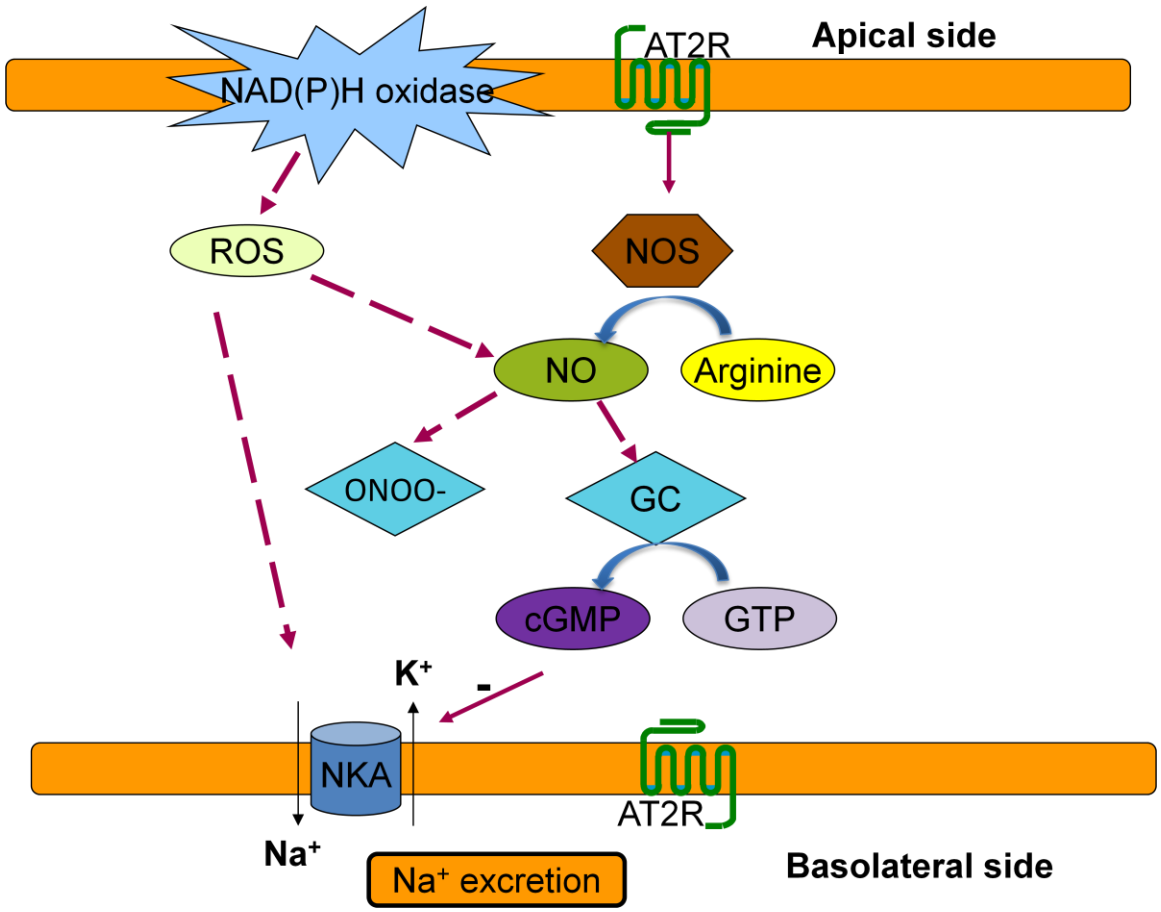


**Fig. 20:** Interaction between superoxide radicals and AT2 receptor.

## 5: EFFECT OF CHRONIC AT2 RECEPTOR AGONIST (CGP-42112A) TREATMENT ON OXIDATIVE STRESS AND INFLAMMATION IN LEAN AND OBESE ZUCKER RATS:

### 5.1: RESULTS:

#### 5.1.1: Effect of chronic CGP-42112A treatment on inflammatory markers in plasma and kidney cortex:

##### 5.1.1.1: Plasma CRP levels:

Obese control rats had significantly higher plasma CRP (1.2 ± .07 mg/ml) levels when compared to the lean control rats (0.8 ± 0.2 mg/ml). Chronic treatment with CGP-42112A for 2 weeks significantly decreased the plasma CRP (0.6 ± 0.2 mg/ml) levels in obese rats (Fig.21). The trend of increase in CRP levels seen in lean CGP-42112A rats was subjected to power analysis, which suggests that a sample size of 12 for one-tailed analysis will show statistical significance.

##### 5.1.1.2: Plasma MCP-1:

Obese control rats had significantly higher plasma MCP-1(4.9 ± 1.6 ng/ml) levels when compared to the lean control rats (0.3 ± 0.2 ng/ml).

Chronic treatment with CGP-42112A for 2 weeks significantly decreased the plasma MCP-1 (1.9 ± 0.8 ng/ml) levels in obese rats but had no significant effect in lean rats (Fig.22).

#### 5.1.1.3: Plasma and Kidney IL-6:

The plasma expression of IL-6 was higher in obese control than in lean control rats, which decreased with CGP-42112A treatment in obese rats (Fig.23). Conversely, CGP-42112A treatment in lean rats caused insignificant increase in the plasma expression of IL-6 (Fig.23). Furthermore, the kidney cortical expression of IL-6 in control lean and obese Zucker rats was similar, which did not change with CGP-42112A treatment in these rats (Fig.24).

#### 5.1.1.4: Plasma and Kidney TNF-α:

The plasma and kidney cortical expression of TNF-α was significantly higher in control obese compared to lean rats, which decreased with CGP-42112A treatment in obese rats. However, CGP-42112A treatment in lean rats significantly increased the plasma expression of TNF-α (Fig.25 and 26).

**Plasma IL-1 β:**

The plasma and kidney cortical expression of TNF-α was significantly higher in control obese compared to lean rats, which decreased with CGP-42112A treatment in obese rats. However, CGP-42112A treatment in lean rats significantly increased the plasma expression of TNF-α (Fig.27).

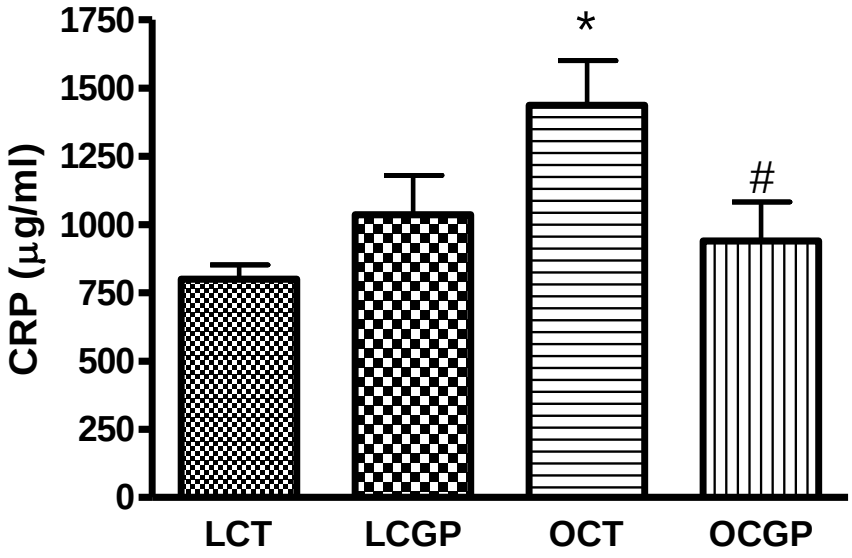


**Fig.21:** *Effect of 2-week CGP-42112A treatment on plasma levels of CRP in lean and obese Zucker rats.* The infusion rate of CGP-42112A was 1µg/kg/min via subcutaneous osmotic pumps. The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 4-6). * = significantly different from lean vehicle treated rats and/or lean CGP-42112A treated rats. # = significantly different from obese vehicle treated rats.

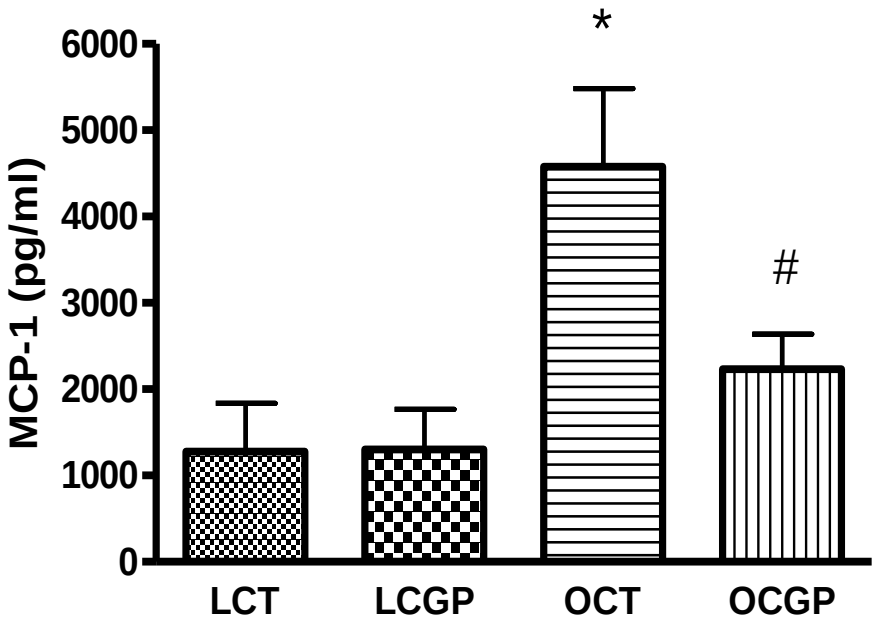


**Fig.22:** *Effect of 2-week CGP-42112A treatment on plasma levels of MCP-1 in lean and obese Zucker rats.* The infusion rate of CGP-42112A was 1µg/kg/min via subcutaneous osmotic pumps. The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 4-6). * = significantly different from lean vehicle treated rats and/or lean CGP-42112A treated rats. # = significantly different from obese vehicle treated rats.

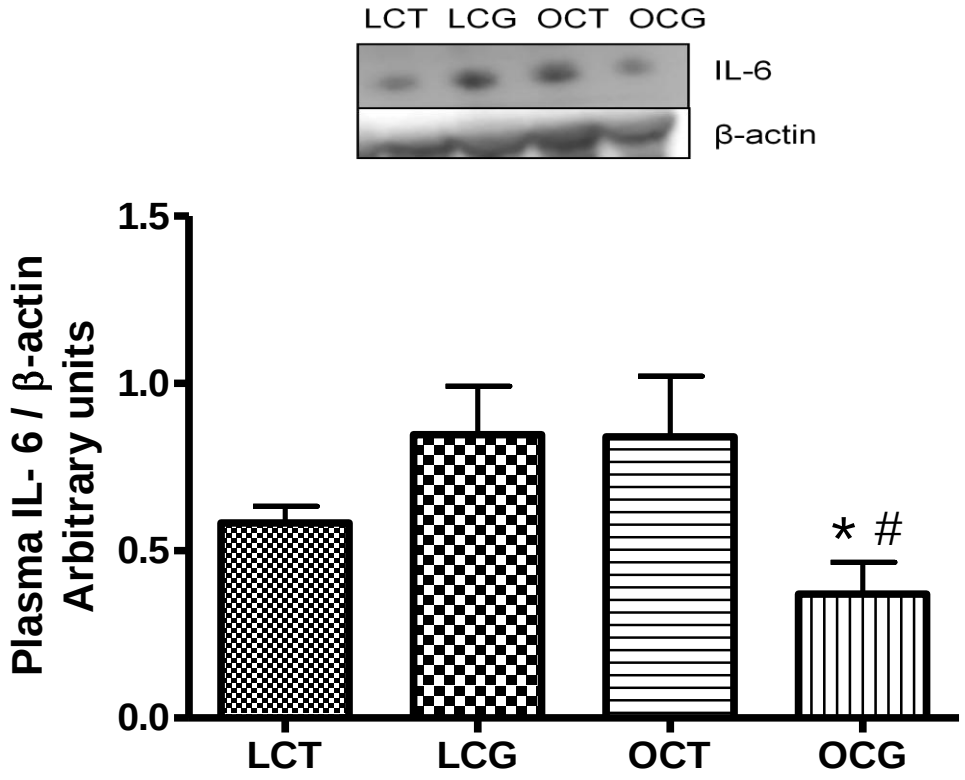


**Fig.23:** *Effect of 2-week CGP-42112A treatment on expression of IL-6 (24kDa) in plasma of lean and obese Zucker rats.* The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 5-7). * = significantly different from lean CGP-42112A treated rats; # = significantly different from obese vehicle treated rats.

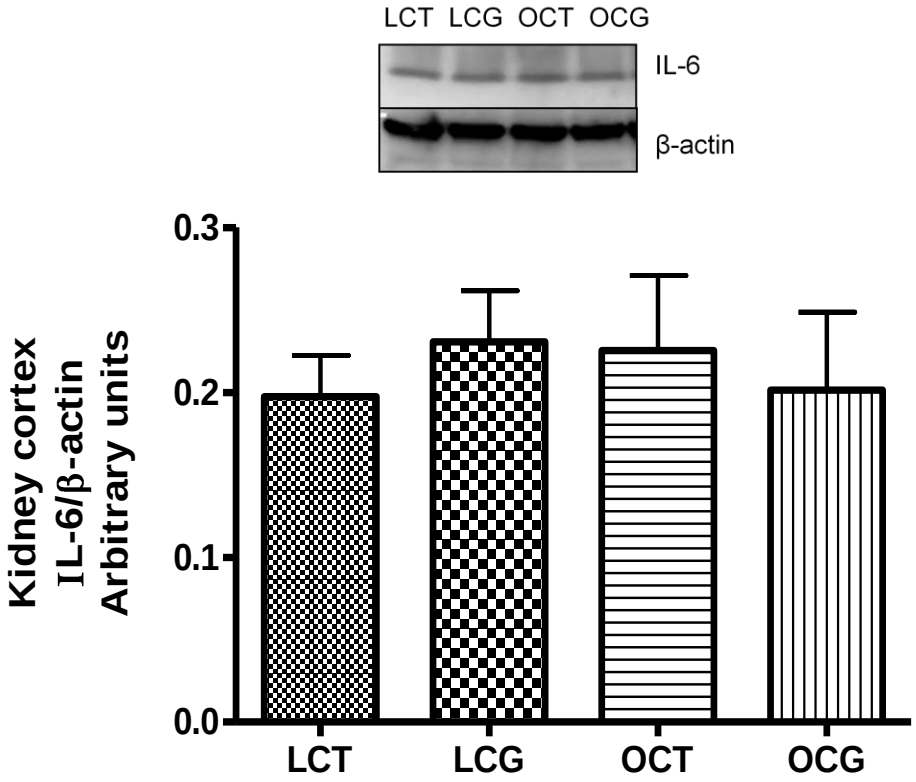


**Fig.24:** *Effect of 2-week CGP-42112A treatment on expression of IL-6 (24kDa) in kidney cortex of lean and obese Zucker rats.* The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 5-7).

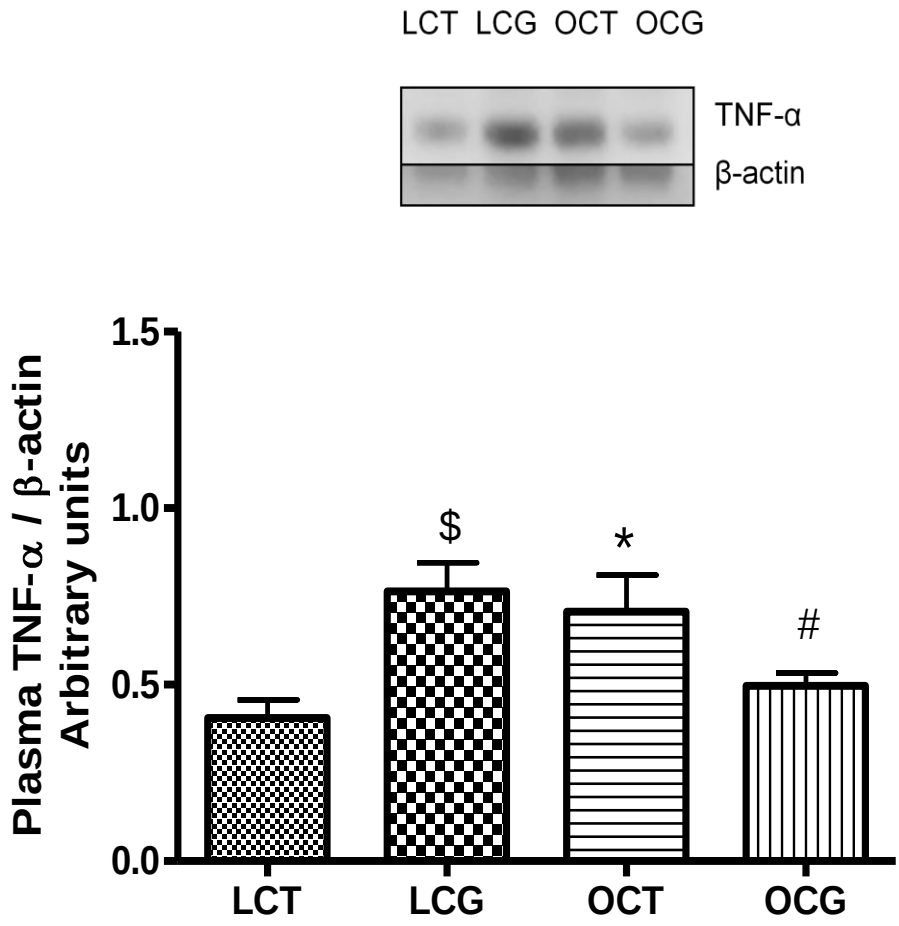


**Fig.25:** *Effect of 2-week CGP-42112A treatment on expression of TNF-α (25kDa) in plasma of lean and obese Zucker rats.* The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 5-7). $ and * = significantly different from lean vehicle-treated rats. # = significantly different from obese vehicle-treated rats and lean CGP-treated rats.

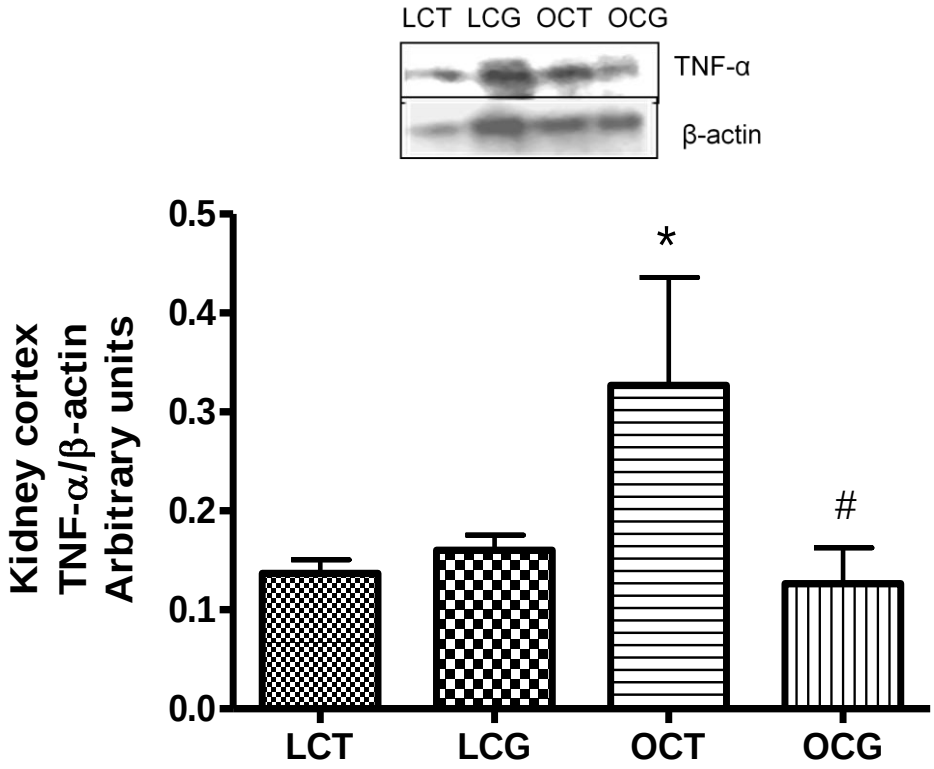


**Fig.26:** *Effect of 2-week CGP-42112A treatment on expression of TNF-α (25kDa) in kidney cortex of lean and obese Zucker rats.* The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 5-7). * = significantly different from lean vehicle-treated rats. # = significantly different from obese vehicle-treated rats.

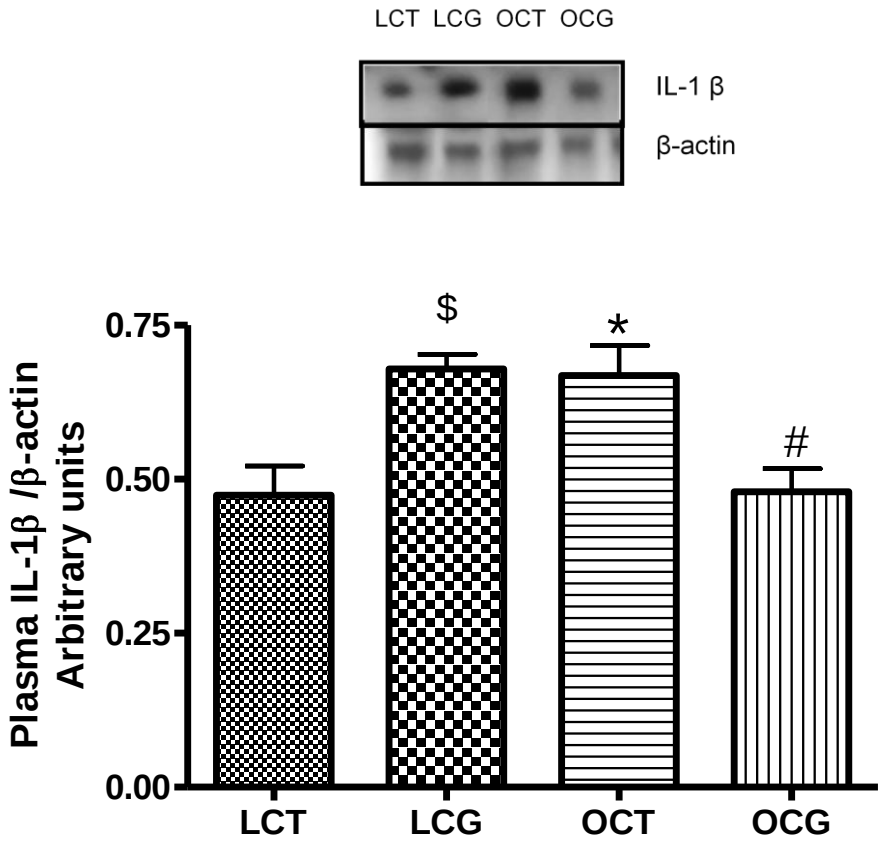


**Fig.27:** *Effect of 2-week CGP-42112A treatment on expression of IL-1β (24kDa) in plasma of lean and obese Zucker rats.* The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 5-7). $ and * = significantly different from lean vehicle-treated rats. # = significantly different from obese vehicle-treated rats and lean CGP-treated rats.

### 5.1.2: Effect of chronic CGP-42112A treatment on oxidative stress markers in plasma and kidney cortex:

#### 5.1.2.1: Plasma SOD activity:

Plasma total superoxide dismutase (SOD) activity was significantly lower (Fig.28) in obese control (3.8 ± 1.2 U/ml) than in lean control rats (8.8 ± 0.7 U/ml), which with CGP-42112A treatment significantly increased in obese rats (8.5 ± 0.6 U/ml). CGP-42112A treatment caused no change in the plasma SOD activity of lean rats.

#### 5.1.2.2: Kidney SOD activity and expression:

In the kidney cortex, there was no change in total SOD activity in all the four treatment groups (Fig.29A). Also, there was no change in the expression of different SOD iso-forms (Cu/Zn-SOD, Mn-SOD and Ec-SOD) in control or CGP-42112A treated lean and obese rats (Fig.29B, 30A and 30B).

### 5.1.2.3: Plasma HO-1:

HO-1 is an inducible enzyme and its expression goes up due to stress conditions like oxidative stress. The plasma expression of heamoxygenase-1 (HO-1; 32kDa) was significantly higher in obese when compared to lean rats (Fig.31), which significantly decreased with CGP-42112A treatment for 2 weeks in obese rats and there was no change in lean rats.

### 5.1.2.4: Expression of kidney NAD(P)H oxidase subunits:

The NAD(P)H oxidase (major superoxide producing enzyme in kidney) membranal subunit gp-91$^{phox}$ (58kDa) and cytosolic subunit p-47$^{phox}$ expression were increased in obese control rats when compared to the lean control rats (Fig.32 A and B). Chronic CGP-42112A significantly decreased the expression of gp-91$^{phox}$ in obese rats. However, CGP-42112A treatment in lean rats significantly increased the expression of gp-91$^{phox}$ (Fig.33).

### 5.1.2.5: Kidney MDA levels:

Kidney cortex MDA level was moderately high (Fig.34) in obese control (0.4599 ± 0.03911) than in lean control rats (0.3910 ± 0.01869), which with CGP-42112A treatment decreased to the level of lean in obese rats (0.3291 ± 0.02778). CGP-42112A treatment caused no change in the MDA levels of lean rats.

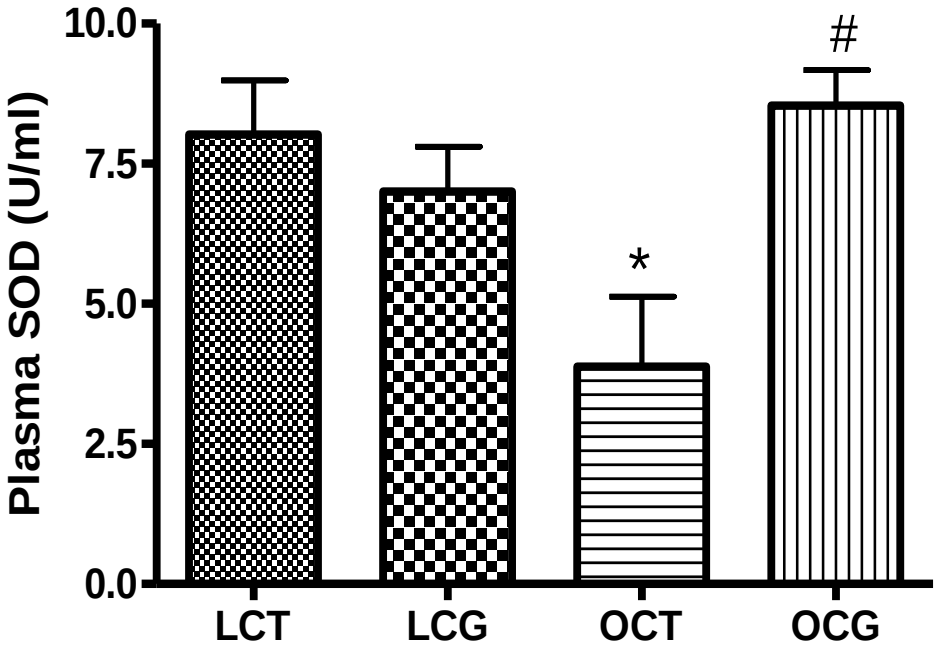


**Fig.28:** *Effect of 2-week CGP-42112A treatment on total plasma SOD activity in lean and obese Zucker rats.* The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 5-8). * = significantly different from lean vehicle-treated and/or lean CGP-42112A treated rats. # = significantly different from obese vehicle-treated rats.

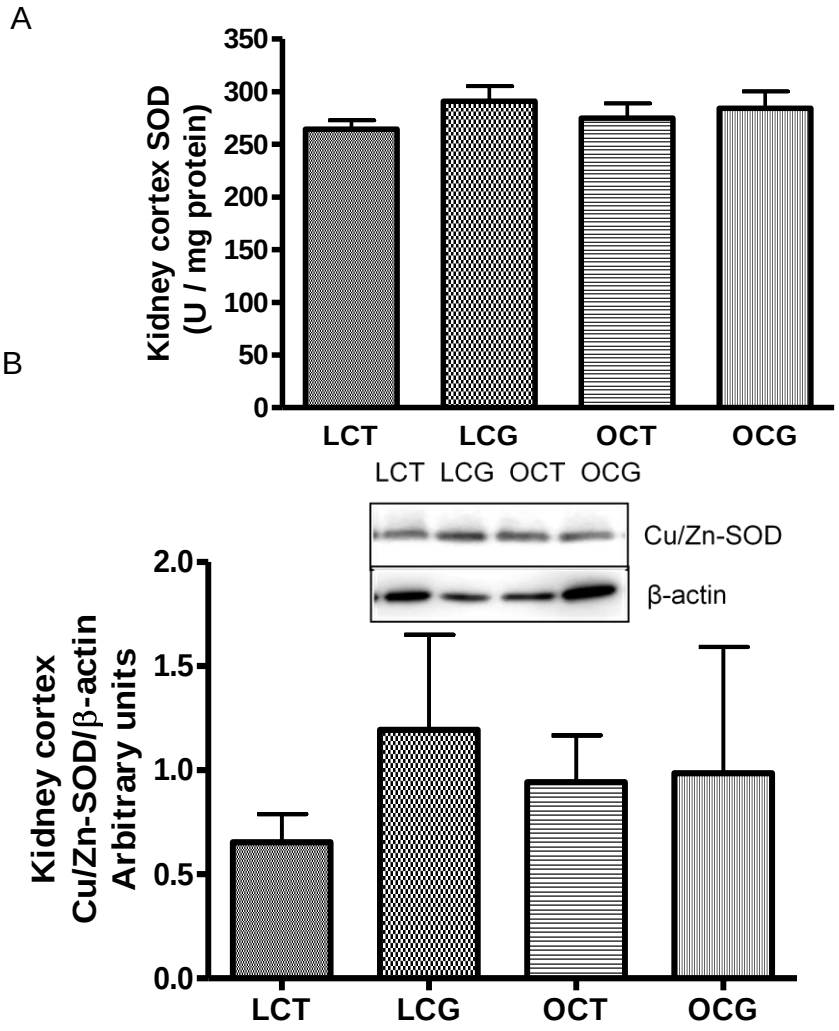


**Fig.29:** *Effect of 2-week CGP-42112A treatment on SOD activity (A) and expression of Cu/Zn-SOD (B) in kidney cortex of lean and obese Zucker rats.* The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 4-5).

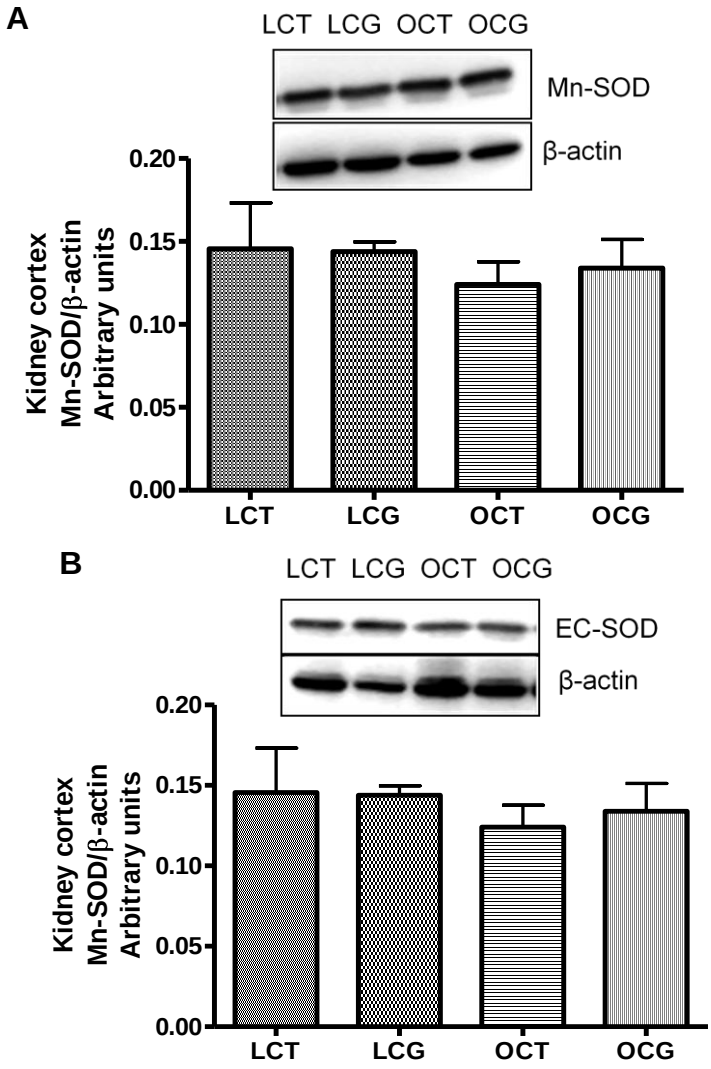


**Fig.30***: Effect of 2-week CGP-42112A treatment on expression of Mn-SOD (A) and Ec-SOD (B) in kidney cortex of lean and obese Zucker rats.* The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 4-5).

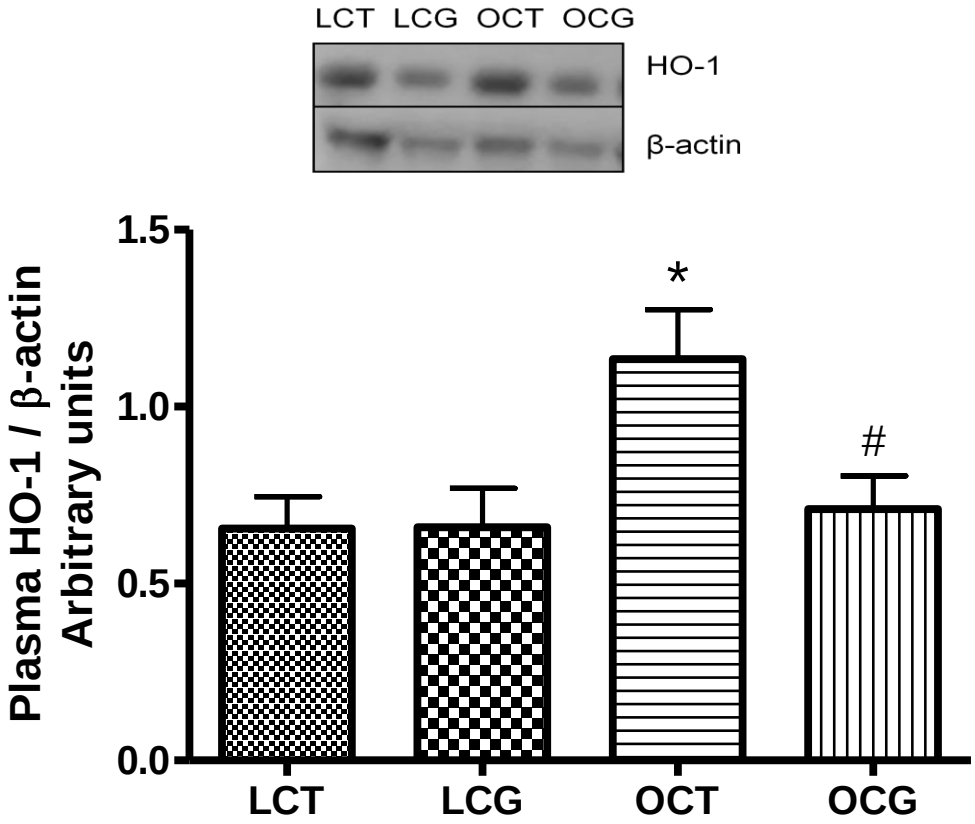


**Fig.31:** *Effect of 2-week CGP-42112A treatment on expression of 32kDa plasma HO-1 in lean and obese Zucker rats.* The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 5-8). * = significantly different from lean vehicle-treated and/or lean CGP-42112A treated rats. # = significantly different from obese vehicle-treated rats.

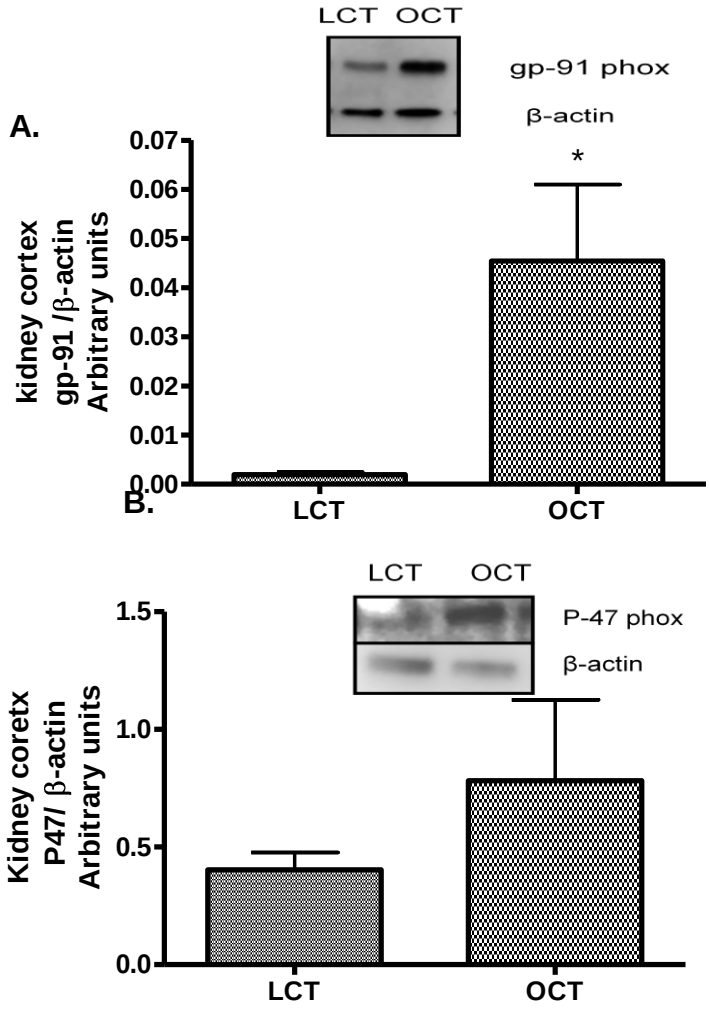


**Fig.32:** *Expression of gp-91$^{phox}$ (A) and p-47 $^{phox}$ (B) in kidney cortex of lean and obese Zucker rats.* The values in the bar graph are represented as mean ± SEM. Student-t test was used to compare the variations between groups. The value of $p < 0.05$ was considered significant. (n= 3-4). * = significantly different from lean control rats

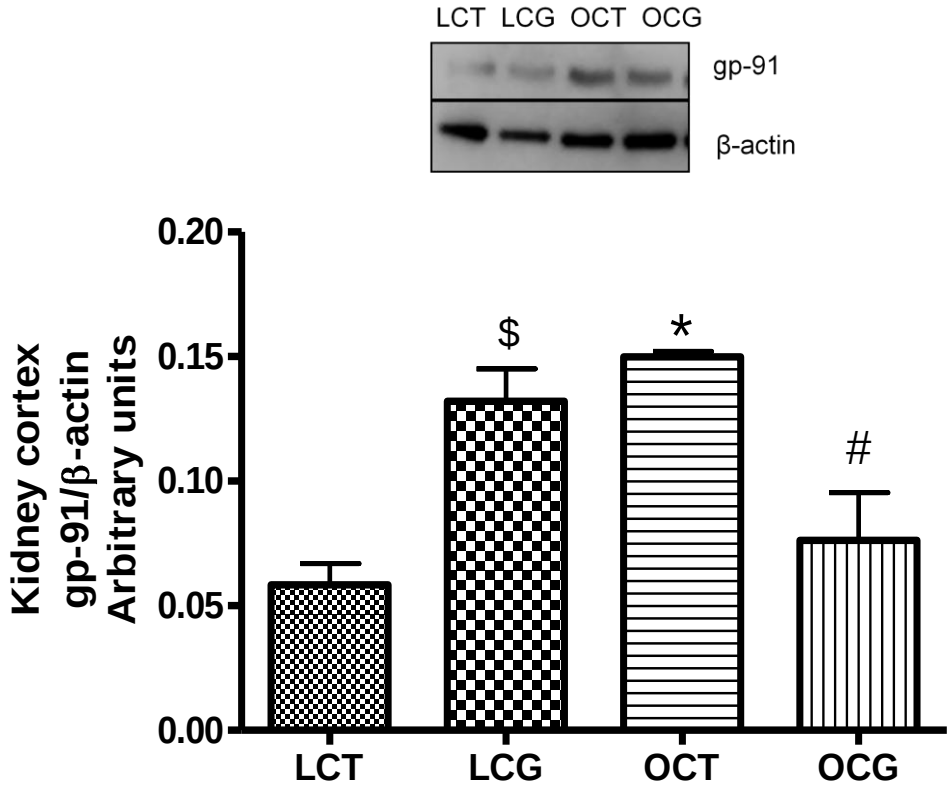


**Fig.33:** *Effect of 2-week CGP-42112A treatment on expression of gp-91$^{phox}$ in kidney cortex of lean and obese Zucker rats.* The values in the bar graphs are represented as mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison post hoc test was used to compare the data between the groups. The value of $p < 0.05$ was considered significant. (n= 4-7). $ and * = significantly different from lean vehicle-treated; # = significantly different from obese vehicle-treated rats and lean CGP-42112A treated rats.

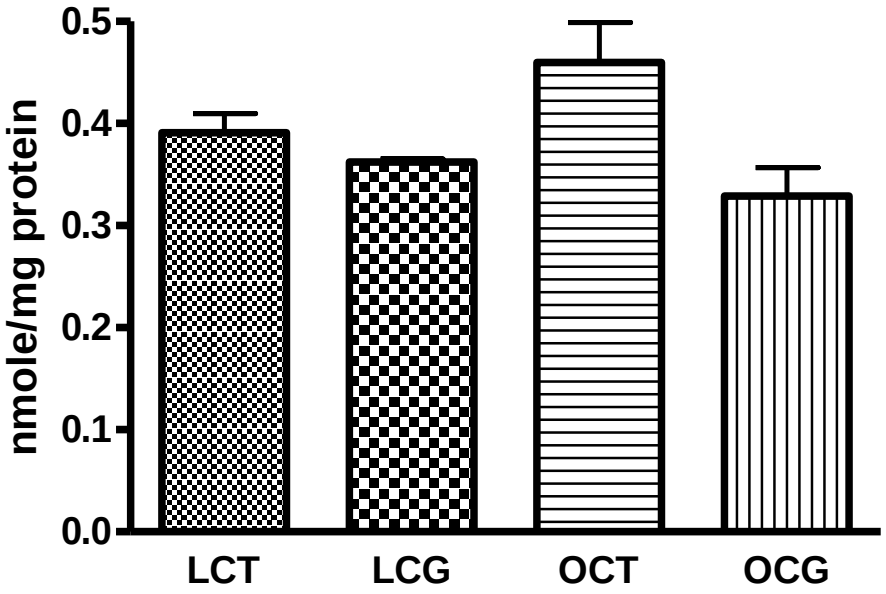


**Fig.34:** *Effect of 2-week CGP-42112A treatment on kidney cortex MDA levels in lean and obese Zucker rats.* The values in the bar graph are represented as mean ± SEM. One-way ANOVA with Newman-Keuls multiple comparison post hoc test was used to compare the data between the groups (n=3-5). The value of $p < 0.05$ was considered significant.

## 5.2: Discussion:

In the present study we clearly demonstrated that both inflammation and oxidative stress with chronic (2-weeks) administration of AT2 receptor agonist (CGP-42112A) decrease in obese but increase in lean rats indicating its opposing effects in normal and patho-physiological situations.

Metabolic syndrome is a term given to a group of abnormalities such as obesity, hyperglycemia, hyperinsulinemia, dyslipidemia and high blood pressure, which further increases the risk of overt diabetes mellitus and renal/cardiovascular diseases (Elmarakby and Imig 2010; Mathieu et al. 2010). Obesity is one of the most common nutritional disorders and is associated with chronic low-grade inflammation and oxidative stress independent of hypertension (Van Guilder et al. 2006). Studies suggest that increased inflammation and oxidative stress are tightly regulated by the elevated renin angiotensin system (RAS) activity in obese animals and hence can contribute to development or maintenance of hypertension (Suzuki et al. 2003; Sachse and Wolf 2007; Kaneto et al. 2010). The main effector peptide of RAS is angiotensin II (AngII) which works via activation of AT1 and AT2 receptors (Bader and Ganten 2008). The pro-inflammatory and pro-oxidative actions of AT1 receptors are well

established (Wassmann and Nickenig 2006; Skultetyova et al. 2007); however, the reports about role of AT2 receptor in inflammation and oxidative stress are sparse and quite ambiguous (Rompe et al. 2010; Steckelings et al. 2010). Therefore, the present study was designed to determine the effects of chronic AT2 receptor activation on inflammatory and oxidative stress markers in obese Zucker rats, a rodent model of metabolic syndrome (Bray 1977), and compared these markers to their lean counterparts.

Obesity-related inflammation is associated with increase in circulating markers of inflammation such as CRP, cytokines [(TNF)-$\alpha$, interleukins] and chemokines (MCP-1) (Lind 2003; Fujiwara and Kobayashi 2005; Kaneto et al. 2010; Mathieu et al. 2010). Although they serve as independent markers of inflammation, on the molecular level they are intimately intertwined and regulate each other's expression and function (Tzanavari et al. 2010). In the present study, the plasma levels of CRP, MCP-1, IL-1β and TNF-α were significantly high in obese Zucker rats. There was a modest increase in the plasma level of IL-6 in obese rats. Previous studies have reported a significant increase in the IL-6 levels during inflammation (Vaziri et al. 2005; Nishimatsu et al. 2008). This slight difference in observation can be attributed to the method of IL-6

determination used in the present study (western blot Vs ELISA/RT-PCR). Chronic AT2 receptor agonist treatment significantly reduced the levels of all the markers of inflammation measured in this study in obese Zucker rats, suggesting an anti-inflammatory function of AT2 receptors in obese rats.

HO-1 is an inducible enzyme and is elevated during oxidative stress (Ndisang 2010), and thus HO-1 measurement serves as an indirect measure of oxidative stress. We found that obese Zucker rats have significantly higher HO-1 expression in plasma and chronic AT2 receptor activation significantly reduced HO-1 expression in obese rats. Lower plasma SOD activity in obese rat indicates that these rats have poor anti-oxidant defense system and chronic AT2 receptor activation is able to rescue this defect. Although, there are other oxidative stress markers available, however, measurement of these two enzymes (HO-1, SOD) in the present study clearly demonstrates anti-oxidant property of AT2 receptors in obese Zucker rats.

Numerous studies indicate that AT2 receptors expression is increased during pathological conditions such as hyperglycemia, cardiovascular disease and renal failure (Nio et al. 1995; Hakam and Hussain 2005; Ali et al. 2010). Recently, we have shown that in obese

Zucker rats, the renal AT2 receptors expression is increased and play important role in the regulation of Na-excretion (Hakam and Hussain 2005; Hakam and Hussain 2006). We sought to examine whether AT2 receptor activation in kidney apart from Na-excretion has any effect in inflammation and oxidative stress as seen in plasma. We measured inflammatory and oxidative stress markers in renal cortex. TNF-$\alpha$ showed similar trend as in plasma; however, the expression of IL-6 was not changed. Kidney cortex MDA level was moderately increased in obese rats which were brought to lean levels with chronic AT2 receptor activation. The total SOD activity and expression of all the SOD iso-forms in the kidney cortex were not significantly different in any of the treatment groups; however, the NAD(P)H oxidase membrane-bound subunit gp-91$^{phox}$ and p-47$^{phox}$ expression were increased in obese rats. Chronic AT2 receptor activation decreased the expression of gp-91$^{phox}$ in obese rats.

Although, these results are difficult to explain, a plausible explanation would be that the renal damage at this age (11 weeks) in obese rat was in the initial stages where we found an increase in TNF-α, MDA gp-91$^{phox}$ and p-47$^{phox}$ but the inflammation was not at a stage where we can observe a plethora of inflammatory and oxidative stress markers. There are studies reporting increased inflammatory cytokines and

oxidative stress markers in the kidneys of obese rats, however, these studies included older (18 weeks) obese rats as opposed to 11-weeks of age used by us (Vaziri et al. 2005; Nishimatsu et al. 2008; Ecelbarger et al. 2010). One of the limitations of our study is that we have not measured the complete array of pro-inflammatory cytokines. There are studies which support the notion that not all pro-inflammatory cytokines are increased during inflammation (Ecelbarger et al. 2010). Apart from this the other possible reasons could be the use of renal cortex in our study rather than the whole kidney homogenate or use of western blotting technique rather than ELISA or RT-PCR. It is also worth mentioning that increased circulating levels of plasma inflammatory markers can be an index of multiple organ inflammation like adipocytes, liver, spleen rather than just kidney in obese rats.

One of the major and most unexpected findings of our study was increased expression/levels of some of the inflammatory and oxidative stress markers in AT2 agonist-treated lean rats. The expression of TNF-$\alpha$ and IL-1β in plasma and gp-91$^{phox}$ in kidney cortex was significantly increased. There was a trend of increase, though not significant, in plasma level/expression of CRP and IL-6. Total SOD activity in plasma was also less when compared to the lean control rats. This

unanticipated outcome of AT2 receptor activation in lean rats might be a clue to understand why AT2 receptors are expressed less during normal physiological condition compared to the AT1 receptors. However, pro-inflammatory role of AT2 receptors (Ruiz-Ortega et al. 2001; Esteban et al. 2003) have been described in normal rats that is in agreement with our findings in lean rats. Although, further comprehensive study is required to understand the underlying mechanism for the opposing role, a simple explanation can be attributed to the different signaling pathways of AT2 receptor in lean and obese Zucker rats. It is known that AT2 receptors activation leads to increase in the levels of NO/cGMP as well as decrease in the accumulation of cAMP (Siragy and Carey 1997; Hakam and Hussain 2006; Ali et al. 2010). We recently reported that selective activation of AT2 receptors in the kidney increases NO/cGMP levels in obese but not in lean rats and causes similar reduction in cAMP accumulation in both the rats (Hakam and Hussain 2006; Ali et al. 2010). The increase in cellular cAMP accumulation has been suggested to decrease oxidative stress (Bokoch et al. 2009) and suppresses immune responses (Serezani et al. 2008; Ernst et al. 2010). Hence, it is plausible that AT2 receptor-mediated reduction in cAMP levels without having any effect on NO/cGMP in lean rats (Hakam and Hussain 2006), leads to increase in oxidative and inflammatory stresses in lean rats. Whereas, in

obese rats, AT2 receptors by mediating NO/cGMP accumulation (14) produces antioxidant and anti-inflammatory responses and cancels out modest effects mediated via cAMP. Furthermore, we found that increases in oxidative and inflammatory stresses were independent of blood pressure change in lean rats. This suggests that early stage oxidative stress and inflammation can exist independent of hypertension. Whether or not late stage oxidative stress and inflammation produced by beyond 2-week CGP-42112A treatment in lean rats result in hypertensive phenotype is not known.

The molecular mechanism(s) relating to the anti-inflammatory and/or anti-oxidative property of AT2 receptors in obese rats is not clear. However, from the present study it can be speculated that activation of AT2 receptors lowers oxidative stress by increasing the SOD activity and decreasing the expression of superoxide radical producing enzyme, NADPH oxidase ($gp\text{-}91^{phox}$). The anti-inflammatory property, depending on very recent studies can be attributed to inhibitory role of AT2 receptors on nuclear translocation of NFκ-B (Wu et al. 2004; Rompe et al. 2010), a well known transcription factor regulating most of the pro-inflammatory cytokines (Razani and Cheng 2010).

To summarize, the present study underscores the anti-inflammatory and anti-oxidative properties of AT2 receptors in obese Zucker rats. Our findings suggest that CGP-42112A-induced activation of AT2 receptors has contradictory effects on inflammation and oxidative stress in normal and patho-physiological situations.

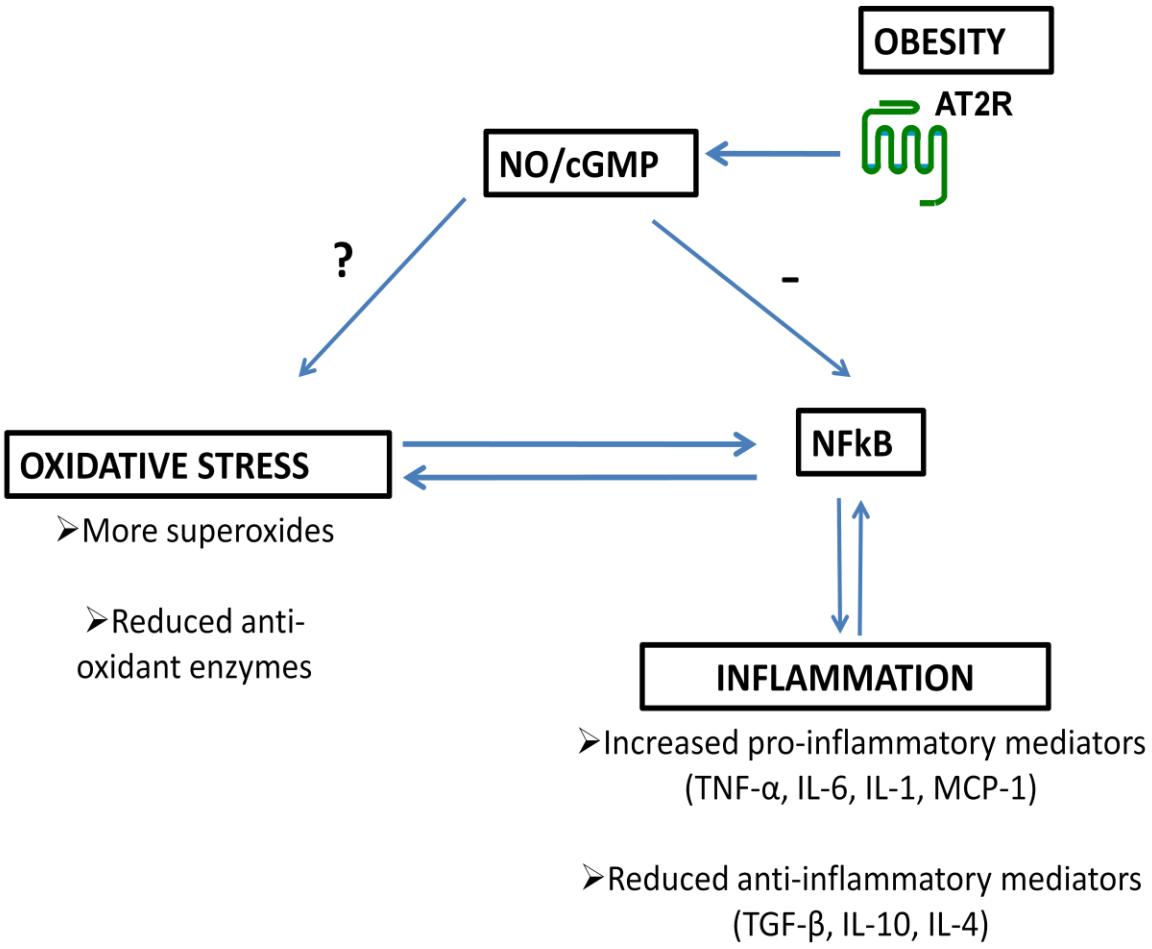


**Fig. 35:** Inhibition of oxidative stress and inflammation via activation of AT2 receptors in obese Zucker rats.

## 6: Summary and conclusion:

### Summary:

- Under normal physiological conditions there is no interaction between AT1 receptors and NAD(P)H oxidase in terms of natriuresis in SD and lean rats (Section 4.1.1 and 4.1.2).
- NAD(P)H oxidase has a role in natriuresis/diuresis independent of AT1 reptors in SD and lean rats (Section 4.1.1 and 4.1.2).
- NAD(P)H generated superoxide inhibits AT2 receptor function by reducing nitric oxide availability (Section 4.1.4).
- Chronic selective activation AT2 receptor can lower expression of inflammatory markers and mediators in plasma and kidney of obese Zucker rats (Section 5.1.1).
- Selective AT2 receptor activation for 2-weeks can lower systemic as well as renal markers of oxidative stress in obese Zucker rats (Section 5.1.2).
- Selective AT2 receptor activation may increase oxidative stress and inflammation in lean Zucker rats (Section 5.1.1 and 5.1.2).

**Conclusion:**

Form the present study we conclude that in normal physiological condition superoxides generated by NAD(P)H oxidase inhibit AT2 receptor function by lowering NO availability. On the other hand, chronic selective activation of AT2 receptors in obese Zucker rats lowers oxidative stress and inflammation. We therefore conclude that AT2 receptors may serve as a potential therapeutic target to improve oxidative stress and inflammation-related pathological conditions like hypertension, diabetes, atherosclerosis, and chronic kidney/end-stage renal disease.

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