Role of Angiotensin II Type 2 Receptor in Blood Pressure Regulation in Obese Rats

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A Dissertation Presented to

The Department of Pharmacological and Pharmaceutical Sciences,

University of Houston

In Partial Fulfillment of

The Requirement for the Degree

Doctor of Philosophy

By

Quaisar Ali

April 2011

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ABSTRACT

Renin angiotensin system (RAS) consists of enzymes, hormones, proteins and peptides. Angiotensin II (Ang II) is an important peptide of RAS. Ang II acts via AT_1 receptor (AT_1R) and AT_2 receptor (AT_2R). While AT_1R is known to cause antinatriuresis and increase in blood pressure, the role of AT_2R in renal function and long-term BP regulation is not well defined. Recently our laboratory showed that AT_2R are upregulated in the kidney of obese rats and selective activation of these receptors stimulates nitric oxide/cGMP pathway, inhibits proximal tubules Na^+/K^+-ATPase (NKA) activity and increases urinary sodium excretion. In light of those findings, we undertook this project to investigate the role of AT_2R in renal function, long-term blood pressure control and interaction with renal AT_1R function in obese Zucker rats, an animal model exhibiting hyperinsulinemia, hyperglycemia and hypertension. Also, we studied the mechanism associated with hyperglycemia induced AT_2R upregulation in proximal tubule cells.

First, we designed experiment to determine whether AT_2R has a protective role in blood pressure increase in obese rats. We treated obese Zucker rats with AT_2R antagonist PD123319 (PD) for two weeks and BP was measured. Treatment with PD significantly increased the blood pressure, which was associated with increased renal renin expression in obese rats. This suggested that AT_2R protect against increase in blood pressure by keeping renal renin expression low. Then, we designed experiments to determine whether chronic AT_2R activation affects

Na-balance and lowers BP in obese rats. We treated lean and obese Zucker rats with AT_2R agonist CGP42112A (CGP) for two weeks. Two weeks treatment caused a decrease in BP by 19 mmHg and in Na-balance in obese but not in lean rats. The plasma renin activity was significantly decreased in both lean and obese CGP-treated rats. The expression of AT_2R, AT_1R, angiotensin converting enzyme (ACE) and renin in the kidney cortex was not affected by the CGP-treatment of obese or lean rats. However, ACE2 expression and activity was significantly increased in CGP-treated obese rats and not in lean rats. These studies suggest that long-term activation of AT_2R decreases BP in obese rats. The reduction in BP by AT_2R agonist treatment may have been contributed by a decrease in Na-balance and an enhanced expression and activity of ACE2 in renal cortex.

In order to determine whether the reduction in BP and decrease in Na-balance might have been contributed by the ability of AT_2R to antagonize renal AT_1R function in CGP-treated obese rats, we again treated the obese Zucker rats with CGP for two weeks. We performed the renal function study after two weeks under anesthesia. We found that CGP-treatment of obese rats caused reduction in Ang II pressor response and blunted the candesartan-induced natriuresis/diuresis in these rats suggesting that chronic activation of AT_2R antagonizes the function of AT_1R.

Earlier studies from our laboratory suggest that AT_2R promote Na-excretion but the contribution of different nephron segments in AT_2R-induced natriuresis is

not known. We investigated the involvement of proximal tubule AT_2R in natriuresis by blocking the two important distal tubule Na-transporters (NaCl cotransporter and ENaC). We found that selective activation of AT_2R with a novel AT_2R agonist C21 promoted natriuresis predominantly via proximal tubules.

We also performed in vitro experiments (HK2 cells) to elucidate the potential signaling mechanism involved in the proximal tubule AT_2R upregulation in diabetes/hyperglycemia. In this experiment, we exposed HK2 cells with high glucose with and without IRF-1 siRNA. High glucose increased AT_2R expression in HK2 cells and is mediated via transcriptional mechanism involving the transcription factor IRF-1.

Collectively, the data suggest that long-term treatment with AT_2R agonist attenuates positive Na-balance, lowers renal renin expression, antagonizes the function of AT_1R and decreases blood pressure in obese Zucker rats. Moreover AT_2R upregulation in response to hyperglycemia may be compensatory mechanism to exert a beneficial role in kidney function. These findings highlight the therapeutic potential of AT_2R for treating obesity/diabetes related hypertension.

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACE2	Angiotensin converting enzyme 2
AM	Amiloride
ANF	Atrial natriuretic factor
Ang II	Angiotensin II
ANOVA	Analysis of variance
AT_1R	Angiotensin II type 1 receptor
AT_2R	Angiotensin II type 2 receptor
BCA	Bicinchoninic acid
BFTZ	Bendroflumethiazide
cGMP	cyclic guanosine monophosphate
CRP-1	C-reactive protein-1
D1R	Dopamine type 1 receptor
DCT	Distal convoluted tubules
DNA	De-oxyribonucleic acid
EDTA	Ethylene-diamine-tetra-acetate
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular-signal-regulated kinases 1/2
FBS	fetal bovine serum

LIST OF ABBREVIATIONS

FE_{Li}	Fractional excretion of lithium
FE_{Na}	Fractional excretion of sodium
FITC-Inulin	Fluoro-Isothiocyanate-Inulin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFR	Glomerular filtration rate
GLUT4	Glucose transporter type 4
GPCR	G-protein coupled receptor
HDL	High density lipoprotein
LDL	Low density lipoprotein
HEPES	4-(2-hydroxyethly)-1-piperazineethsanesulfonic acid
HG	High glucose
HK2	Human kidney-2
HR	Heart rate
HRP	Horse radish peroxidase
IFN-β	Interferon-β
IRF-1	Interferon regulatory factor-1
IRF-2	Interferon regulatory factor-2
i.v.	intravenous
kD	kilo Dalton
KHB	Krebs Henseleit buffer

LIST OF ABBREVIATIONS

L-NAME	Levo-Nitroarginine methyl ester (hydrochloride)
MAP	Mean arterial pressure
MCP-1	Monocyte chemoattractant protein-1
MIF	Migration inhibitory factor
mRNA	messenger Ribonucleic acid
NaCl	Sodium chloride
NKA	Sodium/potassium adenosine triphosphatase
nM	Nanomolar
NO	Nitric oxide
OZR	Obese Zucker rats
PCR	Polymerase chain reaction
PCT	Proximal convoluted tubules
PE	Polyethylene
PI-3K	Phosphoinositol-3-kinase
PMSF	Phenylmethanesulfonyl fluoride
PPARγ	Peroxisome proliferator-activated receptor γ
PRA	Plasma renin activity
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
RAS	Renin angiotensin system
S.E.M	Standard error of the mean

LIST OF ABBREVIATIONS

SDS	Sodium dodecyl sulfate
SHR	Spontaneously hypertensive rats
siRNA	Short interfering ribonucleic acid
SS	Scrambled sequence
STZ	Streptozotocin
TGF	Tubuloglomerular feedback
TNF-α	Tumor necrosis factor-α
UF	Urinary flow
$U_{Na}V$	Urinary sodium volume
UV	Urinary volume
Wt	weight

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1. STATEMENT OF PROBLEM

The renin-angiotensin system (RAS) is a hormonal cascade that acts together to regulate blood pressure (Carey and Siragy, 2003). Angiotensin II (Ang II) is the major octapeptide of RAS and mediates its cellular and physiological actions by acting on AT_1 and AT_2 receptors (de Gasparo et al., 2000). Most of the cellular and physiological actions of Ang II such as cellular growth and proliferation, vasoconstriction, antinatriuresis and increase in blood pressure are mediated via AT_1 receptor (de Gasparo et al., 2000). The functions associated with the AT_2 receptors are less studied, in part, due to its lower expression in adult tissues (de Gasparo et al., 2000; Matsubara et al., 2001). However, AT_2 receptor has been suggested as functional antagonist of AT_1 receptors and thereby opposes the actions of Ang II mediated via AT_1 receptor (Masaki et al., 1998; Akishita et al., 1999). Thus, the activation of AT_2 receptors has been shown to cause vasodilatation, natriuresis and decrease in blood pressure (Akishita et al., 1999).

The AT_2 receptor is involved in blood pressure regulation in various animal models such as the renal wrap hypertension model, AT_2 knock-out mice and diet-induced hypertension (Siragy and Carey., 1999; Siragy et al., 1999; Ichiki et al., 1995; Tamura et al., 2000). The AT_2 receptor null mice develop hypertension associated with an inhibition in pressure natriuresis (Gross et al., 2000). Rats with selective intra-renal reduction of the AT_2 receptors produced by antisense

oligonucleotides exhibit increased blood pressure (Moore et al., 2001). However, these studies involved either genetic manipulations of the AT_2 receptor or blockade of the AT_1 receptor and subsequent infusion of an AT_2 receptor agonist or antagonist to produce acute changes in the arterial blood pressure of these animal models. Although these studies suggest a role for AT_2 receptors in blood pressure regulation, there exists a gap in our understanding the role of AT_2 receptors in long-term blood pressure control.

Obese Zucker rat is a model of insulin resistance and develops hypertension (Kurtz et al., 1989). Our laboratory has demonstrated that hyperglycemia causes increased expression of AT_2 receptors in the kidney of obese Zucker rats (Hakam and Hussain., 2005). The expression of AT_2 receptor is regulated via transcription factors namely, IRF-1 and CEB/Pβ (Matsubara et al., 2001; Kijima et al., 1995; Gendron et al., 1999). Hyperglycemia has been reported to stimulate various transcription factors (Samikkannu et al., 2006). However, cause-effect relationship between hyperglycemia and AT_2 receptor expression and the molecular mechanisms responsible for this association is not known.

After the discovery of the AT_2 receptor in various parts of the kidney, including in proximal tubules, there has been an interest in establishing a link between the renal AT_2 receptor, renal Na-excretion and blood pressure regulation. Earlier, we have reported that activation of renal AT_2 receptors increases urinary Na excretion in obese Zucker rats, in part via inhibiting Na^+/K^+-

ATPase (NKA) activity and stimulating nitric oxide/cGMP pathway in the proximal tubules (Hakam and Hussain, 2006). An impaired pressure natriuresis and increased AT_1 receptor function is believed to be the cause of hypertension in obese Zucker rats and other animal models of obesity (Fujiwara et al., 1999; Granger et al., 1994; Hall., 2003). Since the AT_2 receptors are upregulated in obese rats, it is not known whether pharmacological activation of AT_2 receptors opposes/reduces the function of AT_1 receptors, decreases renal Na-reabsorption and thus lowers blood pressure in the long-term.

In kidney cortex AT_2 receptors are expressed in different parts of nephron including proximal and distal tubules. Stimulation of AT_2 receptors promotes natriuresis by inhibiting NKA pump in the proximal tubules. However, AT_2 receptors and NKA is expressed in distal tubules as well. It is not known whether activation of AT_2 receptors promotes Na-excretion via proximal or distal tubules.

The specific aims of this study were to determine that

1) Long-term selective blockade/activation of AT_2 receptor has a role in blood pressure regulation. 2) Infusion of AT_2 agonist promotes Na-excretion via distal tubules. 3) High glucose up-regulates AT_2 receptor via IRF-1 in proximal tubules epithelial cells.

We used obese Zucker rats as an animal model and HK2 cell lines as a cell culture model to accomplish our specific aims.

2. REVIEW OF LITERATURE

Obesity is defined as having a very high amount of body fat in relation to lean body mass, or body mass index (BMI). Person having BMI of >30 is considered obese. It is one of the most important nutritional disorders worldwide. It has become a global epidemic and is particularly true for the United States, where approximately 300 000 deaths each year are associated with being overweight and obese.

2.1 Prevalence

There is a dramatic increase in the prevalence of obesity in the United States within the last decade. In 1996 no states had obesity prevalence rates above 18%, whereas in 2008, almost all the states had obesity prevalence rates of 29% and 23 states are already in obese category including our state of Texas (Source: Center for Disease Control and Prevention, figure 1). Obesity is associated with an increased risk of hypertension and diabetes. Diabetes/hyperglycemia is a disease in which fasting blood glucose levels are above normal. In 1994 no states had diabetes prevalence of >7 %. However in the year 2008, almost 15 states have diabetes prevalence rate of >9 % (figure 1).

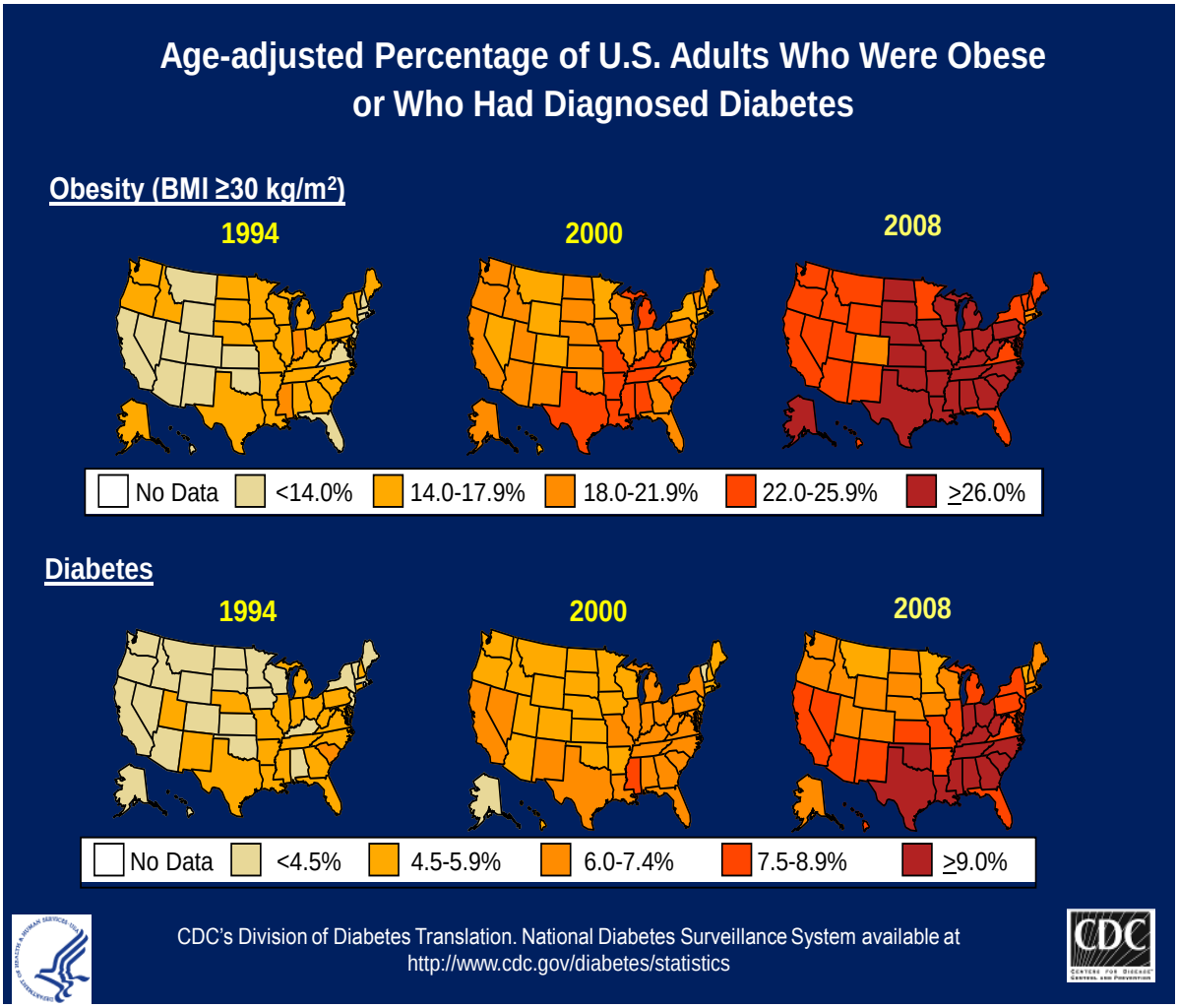


Figure 1: Obesity and diabetes trends among US adults from 1994 till 2008

2.2 Pathological triad of obesity, diabetes and hypertension

Obesity leads to endothelial dysfunction and impairment of renal function that contribute to the maintenance and development of hypertension (Elmarakby et al., 2010). Obesity also increases fat mass in the body. These fat cells release a novel protein called PEDF (pigment epithelium-derived factor). PEDF is released into the bloodstream and causes the muscle and liver to become desensitized to insulin (Famulla et al., 2010). This results in increase in glucose because the pancreas then produces more insulin to counteract these negative effects. This is one of the mechanism by which obesity leads to diabetes. Moreover, adipose tissue also secretes a large number of cytokines in addition to leptin that modulate glucose metabolism and insulin action. These cytokines also induce suppressor of cytokine signaling-3 (SOCS-3), an intracellular signaling molecule that impairs the signaling of both leptin and insulin and are elevated in obesity (Emanuelli et al., 2001). This increased glucose in obesity doubles the risk of mortality in hypertensive patients by affecting renal cellular functions including increase in sodium-glucose transport (Kumar et al., 1998), cellular hypertrophy (Ziyadeh et al., 1994), glycosylation of proteins (Yand et al., 1995), synthesis of transforming growth factor-β (Rocco et al., 1992) and matrix accumulation (Ziyadeh et al., 1990). High glucose also directly affects the cardiovascular functions, vasculature, and neuron damage and also increases sympathetic activation, increased Na absorption which eventually causes

hypertension. This pathological triad of obesity, diabetes and hypertension is becoming an economic burden on USA. According to the Center for Disease Control and Prevention, it's costing more than $187 billion dollar each year to treat obesity associated hypertension. Since renin angiotensin system regulates blood pressure so it is the major therapeutic target to treat hypertension. Although, current available drugs like ACE inhibitors and AT_1 blockers improve renal/cardiovascular functions in hypertensive patients but they remain ineffective in treating obesity/diabetes related hypertension where desirable blood pressure levels is below 120 mmHg. It is very difficult to achieve lower pressure in obese/diabetic patients and requires combination of three or more drugs.

2.3 Mechanism of obesity associated hypertension

There has been a strong positive correlation between weight gain and blood pressure. These obesity related hypertension affects several organs in the body such as heart, vasculature and kidney. The kidney is one such important organ whose function is severely affected by hypertension (Guyton an Coleman, 1999). The mechanism by which obesity causes hypertension can be attributed to the enhanced sympathetic and renin angiotensin system activity, alteration of intrarenal physical forces, and hyperinsulinemia (Hall et al., 2000; Hall et al., 2001). Obesity leads to excessive tubular absorption of Na and alters kidney

function. This leads to increased extracellular blood volume and hence a shift in pressure natriuresis which is believed to be an important mechanism by which obese person develops hypertension (Hall et al., 2003). In obesity, there is also marked increase in glomerular hyperfiltration which leads to increased postglomerular oncotic pressure and this might be another mechanism of enhanced proximal tubule sodium absorption leading to obesity associated hypertension. “This increased sodium reabsorption by some unknown mechanism activates tubuloglomerular feedback mechanism (TGF) which leads to hyperfiltration and hence increased glomerular filtration rate (GFR). As a result of which a deleterious cycle starts resulting in increased flitration fraction, postglomerular oncotic pressure and increased proximal sodium reabsorption which might again lead to an increase in GFR (Hall et al., 1999)”. It has also been postulated that increased renal interstitial pressure due to accumulation of subcapsular fat might lead to tubular compression which further leads to more sodium absorption in the proximal tubules thus raising the blood pressure.

2.4 Obesity associated hypertension

2.4.1 Role of sympathetic nervous system

Sympathetic nervous system (SNS) plays an important role in cardio-renal function. Activation of SNS especially renal sympathetic nerve activity has been linked to the pathogenesis of obesity associated hypertension (Hall et al., 2010).

Activation of SNS is partly mediated by hyperinsulinemia, angiotensin II, melanocortin 4 receptors and adipokines such as leptin, tumor necrosis factor α and interleukin-6. Binding of leptin to its receptors in the brain regions activates neuronal pathways that reduces appetite and increases SNS activity leading to an increase in blood pressure. Mutation in the leptin receptor leads to exaggerated plasma leptin which causes early onset of obesity. Several studies suggest a link between adipose tissue and exaggerated SNS activity in muscles and kidneys of normotensive humans (Hall et al., 2010). Pharmacological blockade of α and β adrenergic receptors lowers the blood pressure in obese subjects by at least 50-60%. Moreover, renal denervation cause natriuresis and decrease in blood pressure. These observations suggest that increased SNS activity contributes to the development of hypertension in obesity.

Hyperinsulinemia in obesity contribute to overactivation of SNS in different tissues including kidney (Rocchini, 1995). However the role of insulin resistance in the development of hypertension is somewhat controversial. There is one study wherein aspirin treatment improved insulin resistance but did not prevent the development of hypertension in high-fat fed dogs.

2.4.2 Role of atrial natriuretic peptide

Renal glomeruli contains peptides called atrial natriuretic factor (ANP) that plays an important role in regulating sodium homeostasis (Chevalier, 1993), fluid balance, vasodilatation and blood pressure. In the kidney, ANP opposes the actions of Ang II via AT_1R and causes natriuresis. Low levels of ANP have been observed in obese people and are suggested as one of the mechanisms for obesity-related hypertension (Wang et al., 2004). The mechanism associated with the decreased ANP levels in obesity is attributed to upregulated natriuretic peptide clearance receptors (NPR-C) which basically removes natriuretic peptides from the circulation (Dessì-Fulgheri et al., 1998). Reduced ANP function on natriuresis has been reported in obese Zucker rats (Baylis et al., 1991).

2.4.3 Role of renin angiotensin system

Renin angiotensin system (RAS) is a very important hormonal regulator of sodium homeostasis in the kidney (Crowley and Coffman, 2007). RAS was believed to be an antinatriuretic but recent data suggest that it has both natriuretic and antinatriuretic components. However, during obesity associated hypertension there is an increased RAS activity (Mathew et al., 2011) which shifts the sodium balance from pronatriuretic to an antinatriuretic direction resulting in increased sodium absorption leading to increase in blood pressure. Moreover increased RAS also elevates plasma aldosterone (Queisser, et al.,

2011) which again leads to an abnormal Na-reabsorption and elevation of arterial pressure. Increased RAS activity has been implicated in the etiology of obesity associated hypertension because blockade of RAS has been implicated as a therapeutic strategy in the management of obesity associated hypertension. Here increased RAS activity is mostly taken in terms of increased renin and/or AT_1R function.

2.5 Overview of renin angiotensin system

According to Guyton's theory, RAS was considered as circulating endocrine system that regulates blood pressure and Na-homeostasis. The discovery of RAS components in different tissues including brain, heart, vasculature, adipose tissue, gonads, pancreas, placenta, and kidney demonstrates the local/tissue production of Ang II (Paul et al., 2006). The tissue RAS plays an important role in normal physiological processes and has been implicated in pathophysiological conditions such as hypertension, congestive heart failure and cardiovascular hypertrophy (Dzau, 2001; Johnston, 1994). The present view of RAS is very complex and is a group of related hormones that act together to regulate blood pressure (Siragy, 2000). When the blood pressure drops for any reasons, special cells in the kidney called juxta-glomerular cells detect those changes and release renin into the blood stream. Renin floats around and converts inactive forms of angiotensinogen into angiotensin I. Angiotensin converting enzyme (ACE)

converts inactive angiotensin I into angiotensin II. Ang I and Ang II are further converted into Ang 1-9 and Ang 1-7 by ACE2. Ang 1-9 gets converted to Ang 1-7 by ACE and acts on Mas receptors. Angiotensin II is the most important peptide of RAS and produces its effect by binding onto AT_1 and AT_2 receptors (de Gasparo, 2000). Ang II via AT_1 receptors causes vasoconstriction, salt and water retention, promotes cell growth, releases aldosterone, activates SNS and all these altogether leads to an increase in blood pressure. The effects of AT_2 receptor activation are the opposite of those mediated through AT_1 receptors (Carey et al., 2000; Gallinat et al., 2000). Ang II via AT_2 receptor promotes vasodilatation and inhibits cellular growth (figure 2). Numerous studies indicate that AT_2 receptor has a potential role in blood pressure and natriuresis (Carey et al., 1999, Hakam and Hussain, 2005, 2006).

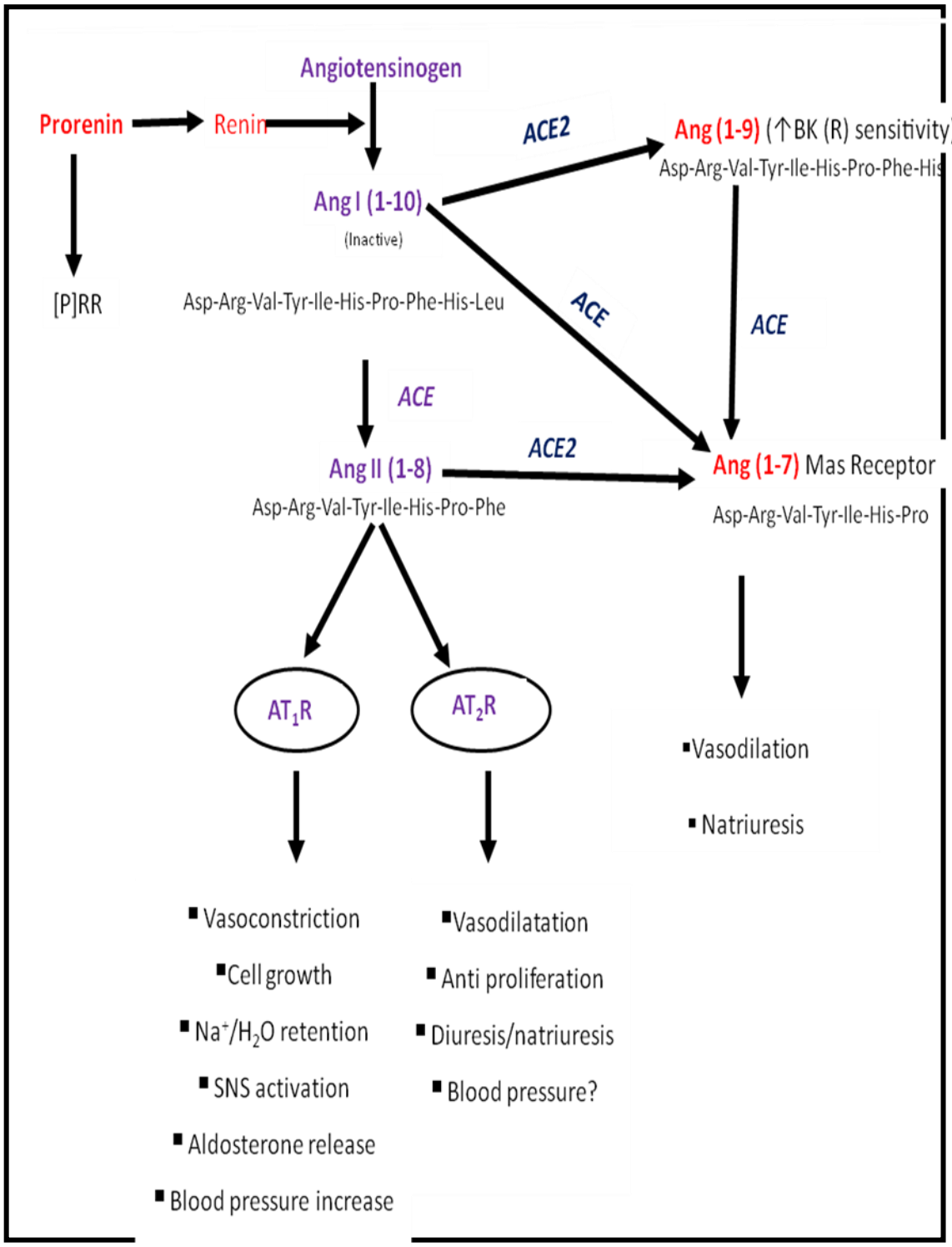


Figure 2: Schematic presentation of renin angiotensin system

2.6 Components of kidney renin angiotensin system

2.6.1 Angiotensinogen (AGT)

AGT is a glycoprotein consisting of 452 aminoacids. AGT is synthesized in several tissues including liver, heart, blood vessels, adipose tissues and kidney. Renin converts this inactive angiotensinogen into angiotensin I. Increased expression of angiotensinogen gene is observed in plasma samples of hypertensive rats. The increased activity of this gene might lead to more Ang II formation and may cause more renal and cardiovascular damage (Ribeiro-Oliveira et al., 2008).

2.6.2 Renin and (pro) renin receptor [P] RR

Renin is a key enzyme of RAS and is produced from the juxtaglomerular apparatus of the kidney. Renin is considered as a rate limiting enzyme in Ang II production as it converts inactive angiotensinogen into angiotensin I. In addition to enzymatic action, renin and pro-renin also acts as ligands for two receptors leading to cellular responses. The first is the mannose-6-phosphate (M6P) receptor which binds and internalizes both renin and prorenin and hence is called a clearance receptor. The second receptor is the specific (pro) renin receptor ([P]RR), the activation of which initiates downstream signaling cascades (Nguyen, 2007). [P]RR is made up of 350 amino acids and consists of single transmembrane domain. Since prorenin is an inactive form of renin, it undergoes

proteolytic and non proteolytic activation which leads to the increased activity of the receptor. The binding of this active form of renin to [P]RR decreases its activation energy and leads to the phosphorylation of mitogen-activated protein kinases (MAP kinase p44/42 and extracellular regulated kinases 1/2 (ERK1/2). The phosphorylation of these kinases leads to an increase in plasminogen activator inhibitor 1 and enhanced expression of transforming growth factor (TGF) β1. This result in synthesis of fibronectin and collagen 1 which is important in regulating actin filament dynamics, maintenance of cell structure, growth, movement and cell death (Nguyen, 2007). Several studies suggest that overexpression of [P]RR leads to increased blood pressure and aldosterone secretion. As a result of which prorenin receptor inhibitors like allikskerin are used as a therapeutic target to treat high blood pressure (Nguyen, 2007).

2.6.3 Angiotensin II

Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) is the most important hormone of RAS and produces its effect by binding onto AT_1 and AT_2 receptor (Wolf, 2003). Most of the actions of Ang II are mediated via AT_1 receptors because these are abundant as compared to AT_2 receptor. Ang II via AT_1 receptor increases peripheral vascular resistance and increased blood pressure.

2.6.4 ACE/ACE2

ACE is an important enzyme of RAS as it converts Ang I to Ang II. ACE also converts Ang 1-7 into smaller angiotensin fragments thereby reversing the vasodilatory effect of Ang 1-7. ACE is considered a pro-hypertensive enzyme because it generates Ang II and also inhibits the peptides such as bradykinin, responsible for vasodilatation (Ribeiro-Oliveira et al., 2008; Fleming et al., 2006). ACE2 is a recently discovered enzyme which has 42% structural resemblance to ACE but implicated in reducing the actions of ACE. ACE2 converts Ang II to Ang 1-7 which acts on Mas receptor and causes vasodilation and natriuresis (Burns, 2007). This enzyme also converts Ang I to Ang 1-9, however the affinity for ACE2 for Ang II is 400-fold higher than Ang I. Since ACE and ACE2 leads to the generation of peptides which has nearly opposite function, a novel concept has been proposed wherein imbalance between ACE/ACE2 could result in different functions. For example increased ACE activity concomitant with reduced ACE2 activity would lead to generation of peptides which would cause more vasoconstriction and vice versa. This balance of ACE/ACE2 in the regulation of different components of RAS is novel target to treat hypertension and renal damage (Iwai and Horiuchi, 2010).

2.6.5 Mas receptor

Ang 1-7 (Asp-Arg-Val-Tyr-Ile-His-Pro) is formed from Ang II by the action of ACE2 and is the agonist for Mas receptor. The association of Ang 1-7, ACE2 and Mas receptor forms a separate branch of renin-angiotensin system called the ACE2/Ang 1-7/Mas axis (Ferrario, 2010). The physiological effects mediated by Ang 1-7 is opposite to that of Ang II acting via AT_1 receptor. Ang 1-7 by acting on Mas receptor causes vasodilatation and antiproliferation. Several studies suggest that ACE2/Ang 1-7/Mas axis interacts with the AT_1 and AT_2 receptor stimulation. For example, it has been shown that stimulation of mas receptor inhibits the AT_1 mediated regulation of ERK1/2 activity which was reversed by Mas receptor antagonist (Iwai and Horiuchi, 2010, Burrell et al., 2004). However, the interaction between AT_2 receptor mediated signaling cascade and the ACE2/Ang 1-7/Mas axis is still not known.

2.6.6 Angiotensin III

Ang III (Arg-Val-Tyr-Ile-His-Pro-Phe) is formed from Ang II by the action of aminopeptidase A. The physiological effects of Ang III are similar to that of Ang II but are less potent. Infusion of Ang III is known to increase BP and intracerebroventricular injection of Ang III is known to increase thirst, vasopressin release and hypertension in animal models (Ribeiro-Oliveira et al., 2008). Recent studies suggest that Ang III might be the preferred agonist for AT_2

receptor. Infusion of Ang III produces natriuresis via AT_2 receptor in AT_1 blocked rats (Padia et al., 2008).

2.6.7 Angiotensin IV/AT4 receptor

The receptor for Ang IV (Val-Tyr-Ile-His-Pro-Phe) is known as insulin-regulated aminopeptidase (IRAP) or AT4 receptor. IRAP/AT4 is a zinc-bound metalloenzyme attached to the transmembrane domain and their translocation to the cytosol is regulated by insulin. It has a molecular mass of 165 kDa and is made up of 1025 amino acid (Albiston et al., 2001). Since IRAP is an endopeptidase, it cleaves substrates at the N-terminal of cysteine and leucine amino acids. Ang IV produces its effect by inhibiting activity of IRAP/AT4. This might be one of the mechanism by which Ang IV binds to AT4/IRAP and reduce the cleavage of important peptides and prolong their actions (Ribeiro-Oliveira et al., 2008). IRAP/AT4 receptor has a role in maintaining homeostasis during pregnancy by cleaving and inactivating Ang III, oxytocin and vasopressin. The expression of IRAP/AT4 receptor is seen in heart, muscles, liver, spleen, colon and kidney. In kidney these receptors are restricted to proximal tubules, glomerulus, thick ascending loop and collecting ducts (Handa et al., 1998). The physiological function of IRAP/AT4 receptor is believed to be similar to that of AT_2 receptor in the sense that they can antagonize the function of AT_1 receptor

by regulating blood flow and promoting Na-excretion (de Gasparo, 2000, Hamilton, et al., 2001).

2.6.8 AT_1 receptor

AT_1 receptor belongs to the family of G-protein coupled receptors. Human AT_1 receptor is made up of 359 amino acids and has almost 95% homology with bovine and rodent AT_1 receptors (Curnow et al., 1992, Furuta et al., 1992). It has an extracellular N-terminus followed by a seven transmembrane domain which is connected by three extracellular and intracellular loops linked to the C-terminus. Ang II binds to the extracellular loop and to the transmembrane domain. Receptor internalization, desensitization and phosphorylation of AT_1 receptors are linked to the C-terminus of AT_1 receptor (Guo et al., 2001; Thomas et al., 1999; Griendling et al., 1993). Majority of the action of Ang II such as Na-retention, increase in blood pressure, sympathetic activation and aldosterone release are known to be mediated via AT_1 receptor because they are more in numbers as compared to AT_2 receptors or other angiotensin receptors. The AT_1 receptors are expressed in most of the tissue including lung, heart, liver, vascular smooth muscles and kidney. Within the kidney they are found abundantly in the glomerulus, renal tubules and efferent arterioles (Burson et al., 1994; Matsusaka et al., 1997; Inagami et al., 1999; Thekkumakara et al., 1998). In kidney, stimulation of AT_1 receptors recruits various Na transporters like

Na^+/H^+exchanger (NHE) to the brush bordered membrane, NKA and Na^+/HCO_3^- (NBC) on the basolateral membrane of the proximal tubules and leads to Na and water absorption (Brown and Douglas, 1983; Greger et al., 2000). Studies suggest that effect of Ang II on the sodium transporters are biphasic. That means that at low concentration, Ang II stimulates the Na-transporters whereas at higher concentration the Na-transporters are inhibited (Bharatula et al., 1998; Houillier et al., 1996; Aperia et al., 1994). The activation of AT_1 receptors initiates a cascade of signaling events which are mediated via G-protein dependent and G-protein independent intracellular second messengers. Ang II on activation of G-protein coupled AT_1 receptors affects several downstream molecules like adenylyl cyclase, phospholipase A2, phospholipase C (Ruiz et al., 1995; Schelling et al., 1994; Becker et al., 1997) and produces its cellular effects as shown in figure 3.

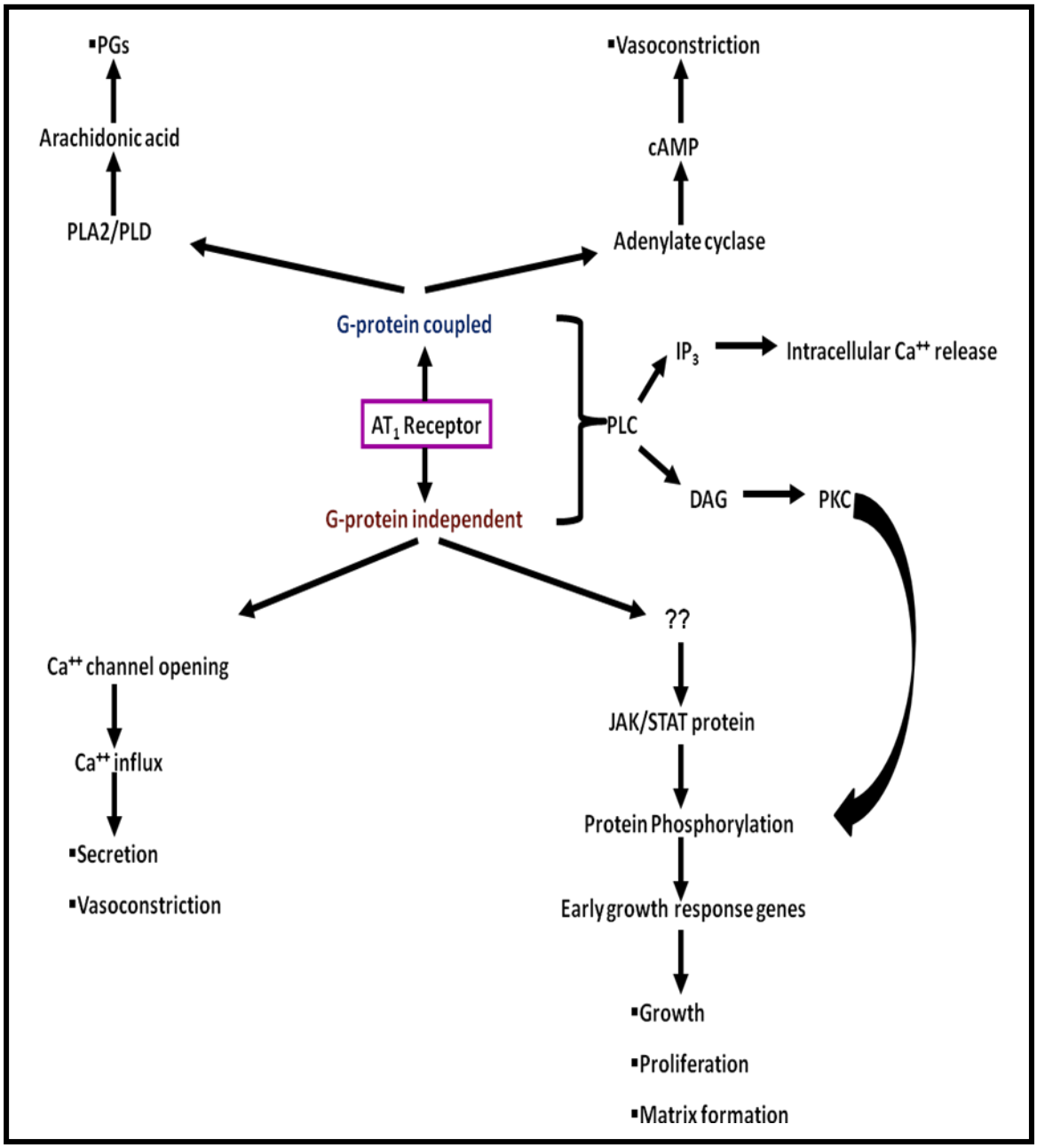


Figure 3: Signaling mechanism of AT_1 receptor

2.6.9 AT_2 receptor

2.6.9.1 General characteristics

AT_2 receptor belongs to the family of G-protein coupled receptor with a molecular mass of 41,000 kDa (de Gasparo and Siragy, 1999). The gene which codes for AT_2 receptor is present on the X chromosome and has 34% resemblance with the protein sequence of AT_1 receptor (Mukoyama et al., 1993; Kambayashi et al., 1993). There are five potential N-glycosylation sites on the extracellular surface of AT_2 receptor (de Gasparo, 2000).

2.6.9.2 Signal transduction

Although, AT_2 receptor belongs to the family of G-protein coupled receptor (GPCR), but the entire signaling cascade through G-protein coupling is not known (Horiuchi et al., 1999). It is suggested that the third loop of this 7-TM receptor is involved in the downstream signaling cascade. Some evidence of AT_2 signaling comes from studies in COS-7 cell line and neuronal cell line where it has been shown that agonist occupied AT_2 receptor stimulates Giα (Hayashida et al., 1996; Zhang and Pratt, 1996). This receptor is different as compared to other GPCR as these agonist occupied receptor does not undergo desensitization. The reason is still not known but it is speculated that the third intracellular loop is short and does not provide enough binding sites for phosphorylation. Stimulation of AT_2 receptor leads to an increase in phosphotyrosine phosphatase activity (figure 4)

and inhibition of MAP kinase (p42/p44) or ERK1/2 (Widdop et al, 2003; Berry et al, 2001; Carey et al., 2000). AT_2 receptor stimulation also leads to increase in bradykinin production which via NO/cGMP pathway causes vasodilation (Siragy, 2000). In our laboratory, we have demonstrated that in proximal tubules of obese Zucker rats, acute activation of AT_2 receptor inhibits NKA via NO/cGMP pathway and promotes Na excretion (Hakam and Hussain, 2005).

2.6.9.3 Expression

The expression of the AT_2 receptor is observed in several organs like heart, brain, vasculature, testes and kidney. Within the kidneys, proximal tubules, distal tubules, afferent and efferent arterioles express AT_2 receptor (Clauser et al., 1996; Pieruzzi et al., 1995). The AT_2 receptor is widely expressed during embryonic stage and gradually decreases after birth (Grady et al., 1991; de Gasparo et al, 2000). AT_2 receptor has drawn limited attention mainly due to its low expression (Zhuo et al., 1992). However, AT_2 receptors are overexpressed in various pathophysiological and experimental conditions like obesity/diabetes, nephrectomy, atherosclerosis, cardiac overload and myocardial infarction. We believe that these overexpressed receptors in these pathophysiological conditions might have a protective role in disease conditions.

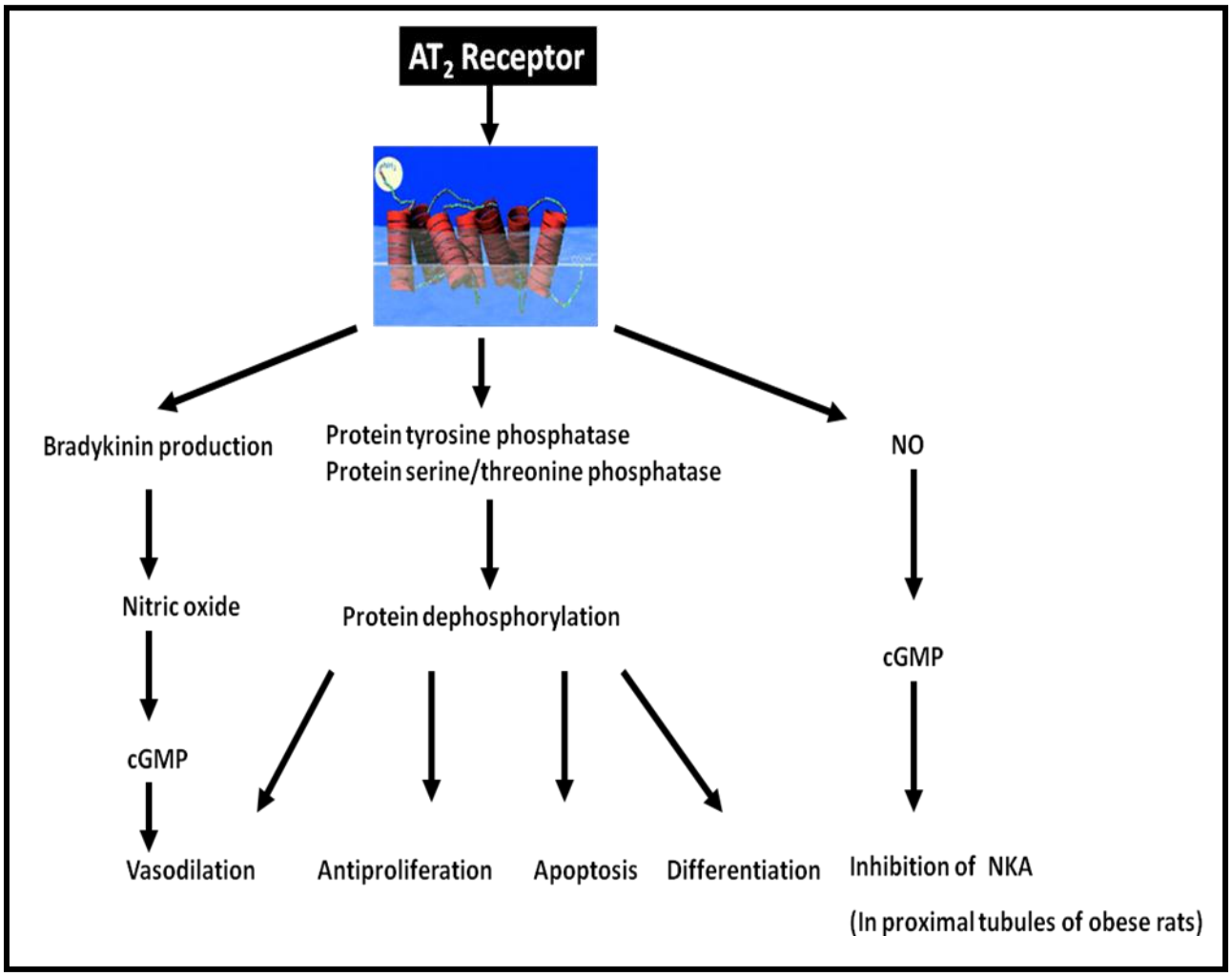


Figure 4: Signaling mechanism of AT_2 receptor

2.7 Physiological function

2.7.1 AT_2 receptor opposes AT_1 receptor function

AT_2 receptors have been shown to produce cellular and physiological responses that are opposite to that produced by AT_1 receptor. For example AT_2 receptor mediates cellular differentiation and apoptosis in various cells/tissues like vascular smooth muscle cells, endothelilal cells whereas AT_1 receptor causes cellular hypertrophy and growth (Millat et al., 1999). While Ang II via AT_2 receptor causes vasodilation via NO/cGMP pathway whereas AT_1 receptor causes vasoconstriction (Carey et al., 2000). The mechanism by which AT_2 receptor opposes the action of AT_1 receptor is not clear. However, there are some studies which suggest that AT_2 receptor binds directly to the AT_1 receptor and antagonizes its function and this antagonism was linked to the heterodimerization of these receptors in transfected foetal fibroblast and myometrium of pregnanat women (Said Abdalla et al., 2001). Ang II via activation of AT_1 receptor is known to stimulate NKA causing anti-natriuresis while activation of AT_2 receptors inhibits NKA and causes sodium excretion (Hakam and Hussain, 2005, 2006; Padia et al., 2006). Further, AT_1 receptor stimulates NKA activity by reducing cellular cAMP contents (Inagami and Ichiki, 1999) whereas AT_2 receptor increases cGMP generation which via a cGMP-dependent pathway inhibits NKA activity in the proximal tubules of obese rats (Hakam and Hussain, 2006). Since cGMP is a known inhibitor of

phosphodiesterase-3 (PDE-3), an enzyme that degrades cAMP, AT_2 receptor, by increasing cGMP could be inhibiting PDE-3, preventing cAMP reduction and thereby reversing AT_1-mediated NKA stimulation (Dousa, 1999).

2.7.2 AT_2 receptor and Na-excretion

Studies on the role of AT_2 receptors on renal sodium transport are limited. In-vitro studies suggest that AT_2 receptor mediates inhibition of sodium transport in the proximal tubules of rabbit (Haithcock et al., 1999). AT_2 receptor knock-out mice show antinatriuretic hypersensitivity to Ang II and a shift in pressure natriuresis curve. Pressure natriuresis is the mechanism by which renal function is linked to long-term blood pressure regulation. However it is difficult to predict whether the effects on sodium excretion are due to absence of AT_2 receptor activation or due to enhanced AT_1 receptor activity (Siragy et al., 1999). AT_2 receptors are over expressed in the proximal tubules of obese Zucker rats. Activation of AT_2 receptors inhibit NKA activity in the proximal tubules and promote natriuresis (Hakam and Hussain 2005). Infusion of this AT_2 receptor agonist does not affect the glomerular filtration rate (GFR) or mean arterial pressure, suggesting that the changes in natriuresis may be linked to the changes in tubular sodium transport (Hakam and Hussain, 2006). It is known that acute activation of renal AT_2 receptors promote natriuresis/diuresis but whether the

long-term AT_2 receptor activation modulates the tubular sodium transport, leading to a decrease in sodium balance is not known.

2.7.3 AT_2 receptor and blood pressure

The long-term regulation of blood pressure is linked to the ability of kidneys to excrete sufficient sodium to maintain normal sodium balance and blood volume (Navar, 1997). The AT_2 receptor is involved in the production of cGMP, NO and prostaglandin $F_{2\alpha}$ thereby playing an important role in renal function, vasodilatation and blood pressure regulation (Murphy et al., 1991; Sasaki et al., 1991). Data from our laboratory and elsewhere suggest that AT_2 receptor plays a protective role against increase in blood pressure by promoting sodium excretion. The argument that increase in sodium excretion due to AT_2 receptor activation may shift the blood pressure can be supported by selective inhibition/disrupting AT_2 receptor gene in the kidney. AT_2 receptor disrupted mice have increased blood pressure compared to the wild type control and there is sustained hypersensitivity of blood pressure and sodium excretion to Ang II (Siragy et al., 1999). In conscious rats, direct stimulation of AT_2 receptor with its agonist CGP42112A in the presence of AT_1 blocker lowers the arterial pressure. The studies so far have been focused on the acute stimulation of AT_2 receptor by CGP42112A in spontaneously hypertensive rat **(**SHR), normotensive or Sprague Dawley (SD) rats (Widdop et al, 1999). There is no study done to look at chronic

activation of AT_2 receptor and its role in long-term blood pressure regulation in obese rats.

2.8 Current therapeutic target

Obesity associated hypertension and other renal-cardiovascular diseases are the leading causes of death in the United States. It leads to cardiovascular diseases including renal ischemia and its dysfunction. In obesity associated hypertension the regulatory function of kidney is severely disrupted resulting in irregular sodium excretion and retention. While the known therapeutic target such as ACE, renin inhibitors and AT_1 receptor blockers have been effective in treating various forms of hypertension, these targets are often not sufficient to achieve blood pressure goals in obesity/diabetes related hypertension. Recently, discovery of AT_2 receptors in adult renal tissues has offered the potential for a novel approach in improving renal function and decreasing high blood pressure. An increase in AT_2 receptor expression has recently been reported in diabetic human kidney and animal models (Sergio et al., 2003; Hakam and Hussain, 2005). The selective activation of AT_2 receptor leads to a greater increase in renal sodium excretion in hyperglycemic animals compared to the normal animals (Hakam and Hussain, 2006). Since excessive retention of sodium is a factor for developing hypertension in obesity and diabetes, AT_2 receptor may therefore be a potential candidate for treating hypertension in obese individuals.

Understanding the role of AT_2 receptor in renal function and blood pressure control will provide a new potential target to treat obesity/diabetes related hypertension.

2.9 Obese Zucker rat: Model

Obese Zucker rats are a genetic model of obesity. These rats are obese due to the decreased function of leptin gene ($Lepr^{fa}$) which controls hunger. As a result of which the metabolic profile of these rats is severely disrupted and becomes hyperlipidemic, hyperphagic, exhibit elevated levels of free radicals leading to low metabolic rate and body temperature. These rats are markedly obese by the end of 5 weeks (Stern et al., 1997). These rats develop glucose intolerance at very early age followed by insulin resistance and become slightly hypertensive due to excessive tubular Na-absorption in the kidney.

The levels of various hormones including renin, angiotensin II, cortisol and leptin are severely altered which activates the SNS (Hall et al., 2001; Clapham et al., 1997) and RAS and contributes to hypertension in these rats. Studies indicate that RAS mainly AT_1 receptor is hyperactive in obese Zucker rats and leads to an increase in blood pressure due to a shift in pressure natriuresis (Alonso-Galicia et al., 1996). Moreover, we have shown in our laboratory that these obese Zucker rats have upregulated AT_2 receptors in the kidney cortex which might be a compensatory mechanism to protect against increase in BP in these rats. Obese

rats at the age group of 12-13 weeks shows increased glomerular filtration rate (GFR), vasodilatation and filtered sodium load (Hall et al., 1996; Rochini et al., 19870 which suggest that vasoconstriction does not contribute to blood pressure increase in these rats. We have used this model in our study because they truly depict and resemble the metabolic, renal/cardiovascular and several other features with humans who are predisposed to obesity.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

PD123319 and candesartan was a generous gift from Pfizer Inc and Astrazeneca. CGP42112A and C21 was custom synthesized from 21st Century Biochemicals, MA and SPS-Alfachem. siRNA IRF-1 (h), control siRNA, siRNA transfection reagent containing lipofectamine were purchased from Santa Cruz, CA. AT_2 receptor antibody was also custom raised from EZBiolab and the peptide sequence was CSQKPSDKHLDAIP. Human kidney proximal tubule epithelial (HK2) cells were purchased from ATCC Chicago, IL. Keratinocyte-serum free media (K-SFM) and fetal bovine serum (FBS) were purchased from Invitrogen Corporation, NY. HRP-coupled anti-IgG and enhanced chemiluminescence substrates were obtained from Alpha Diagnostics Intl, San Antonio, TX. Other chemicals used in the study were purchased from Sigma-Aldrich, St. Louis, MO.

3.1.2 Antibodies

PRIMARY

Antibody	Source	Dilution	Cat #	Company
IRF-1	Rabbit Polyclonal	Dilution 1:500	sc-640	Santa Cruz
IRF-2	Rabbit Polyclonal	Dilution 1:750	sc-498	Santa Cruz
AT_1	Goat Polyclonal	Dilution 1: 250	sc-1173	Santa Cruz
AT_2	Rabbit Polyclonal	Dilution 1:200	sc-9040	Santa Cruz
AT_2	Rabbit Polyclonal	Dilution 1:500	custom	EZ Biolabs
Renin	Goat Polyclonal	Dilution 1:200	sc-27320	Santa Cruz
ACE	Rabbit Polyclonal	Dilution 1:250	sc-12187	Santa Cruz
ACE2	Goat Polyclonal	Dilution 1:250	sc-20998	Santa Cruz
β-Actin	Mouse Monoclonal	Dilution 1:1000	sc-47778	Santa Cruz
GAPDH	Mouse Monoclonal	Dilution 1:1000	ab9484	Abcam

SECONDARY

Antibody	Source	Dilution	Cat #	Company
IRF-1	Anti-Rabbit	Dilution 1: 1000	20320	α-Diagnostics
IRF-2	Anti-Rabbit	Dilution 1: 1000	20320	α-Diagnostics
AT1	Donkey Anti-Goat	Dilution 1:1000	sc-2020	Santa Cruz
AT2	Anti-Rabbit	Dilution 1:500	sc -2301	Santa Cruz
AT2	Anti-Rabbit	Dilution 1:1000	sc-2301	Santa Cruz
Renin	Donkey Anti-Goat	Dilution 1:1000	sc-2020	Santa Cruz
ACE	Anti-Rabbit	Diltuion 1:1000	20320	α-Diagnostics
ACE2	Donkey Anti-Goat	Dilution 1:!000	sc-2301	Santa Cruz
β-Actin	Anti-mouse IgG	Diluion 1:2000	40320	α-Diagnostics
GAPDH	Anti-mouse IgG	Dilution 1:2000	4030	Abcam

Table 1: List of antibodies

3.2 Animals

Male obese and lean Zucker rats (10-11 weeks of age) were purchased from Harlan, Indianapolis (IN). Animals were housed in the University of Houston animal care facility. Normal rat chow and water were provided ad libitum The Institutional Animal Use and Care Committee approved the animal experimental protocols.

3.3 Blood Pressure, renal function in chronically-treated animals

3.3.1 Drug Treatment

3.3.2 PD123319-Treatment: For PD 123319 (PD) treatment, the obese rats were divided into 2-sub groups (N=6-7 per group) i.e., obese-control rat group and obese-PD rat group. Obese-control group was treated with normal saline as vehicle, and obese-PD group was treated with the AT_2R antagonist PD123319 (30 µg/kg/min) for 2 weeks using Alzet osmotic pumps, implanted subcutaneously (model 2ML-2, Alza, Palo Alto, CA). Lean Zucker rats (N=6) served as normal control.

3.3.3 CGP42112A-Treatment: For CGP42112A (CGP) treatment, lean and obese rats were divided into 2-sub groups (N=7-12 per group). The lean-control and obese-control groups were treated with normal saline as vehicle and the lean-CGP and obese-CGP groups were treated with CGP (1 µg/kg/min) for two weeks

using Alzet osmotic pumps (Model 2ML-2, Alza, Palo Alto, CA) implanted subcutaneously. Another micro-osmotic pump (Alzet, model 1002) filled with FITC-inulin was placed at the same time in the peritoneal cavity for the measurement of glomerular filtration rate (GFR) in these rats. After the animals gained consciousness, they were placed singly in metabolic cages. Daily 24-hour food and water intake was recorded over 2-weeks period.

3.4 Blood pressure and heart rate measurements

On day 15 of the PD/CGP-treatment, blood pressure was monitored under anesthesia. Rats were anesthetized using Inactin (150 mg/kg i.p.). After tracheotomy right carotid artery was cannulated with PE-50 (Intramedic Inc, NJ) and attached to data acquisition system (PolyView, Grass Instruments) via Grass pressure transducer PT300. The jugular vein was cannulated with PE-50 for saline infusion. After 30-45 min of stabilization period, blood pressure and heart rate were recorded. At the end of the blood pressure measurements, blood was collected from the carotid artery and the kidneys were removed. Blood was centrifuged at 1500 rpm for 15 minutes to obtain the plasma. Kidneys were decapsulated and cortices were removed and stored frozen at –80°C for measuring the cortical expression of RAS components.

In a separate set of experiments, blood pressure also was measured by tail-cuff method using CODA system (Kent Scientific, CT, USA) in obese rats treated with

CGP42112A alone and with the AT_2 receptor antagonist PD123319. The CODA tail-cuff blood pressure system utilizes volume pressure recording (VPR) sensor technology to measure the rat tail blood pressure. VPR recording is validated and provides 99% correlation with telemetry and direct blood pressure measurements. The blood pressure in these animals was measured for three consecutive days to ensure the reproducibility of the results.

3.5 Plasma renin activity (PRA)

The PRA was assayed by radioimmunoassay (GammaCoat ^{125}I-PRA Radioimmunoassay Kit, cat # CA-1533, DiaSorin, Stillwater, MN) as per the manufacturer's instructions. The plasma samples were subjected to the Ang I generation reaction in tubes coated with rabbit anti-Ang I. After the reaction was terminated, the tubes were washed and decanted. The ^{125}I radioactivity in the tubes was counted using gamma counter (LKB Wallace Model 1282).

3.6 Plasma insulin and blood glucose

After two weeks of treatment, plasma insulin was determined by using radioimmunoassay kits (Linco Research, St. Charles, MO). Blood from 6-hour fasting rats was drawn from the tail vein and glucose was measured using a glucometer (Breeze 2, Mishawaka, IN).

3.7 Evaluation of renal functions

Chronic studies

3.7.1 *Measurement of Na in plasma, urine and feces and calculation of renal function in lean and obese Zucker rats*

After implantation of osmotic pumps in lean and obese Zucker rats, 24-hour urine, 24-hour feces were collected for two weeks. Urine samples were diluted (1:10) and analyzed for Na by flame photometer (Parmer, Model 2655-10 Vernon Hills, Illinois) or AAnalyst 500 atomic absorption spectrometer (Perkin Elmer, Waltham, MA). Feces were dissolved in 6M HCl and diluted (1:10) and analysed for Na by flame photometer. For measurement of GFR, we used FITC-inulin clearance method. FITC-inulin is an inert compound which is freely filtered through the glomerular filtration barrier and is neither secreted nor absorbed by the tubules and hence used as a marker to measure GFR.

Preparation of FITC-inulin: FITC-inulin (5%) was dissolved in 0.9% normal saline by heating the solution in boiling water. The unbound inulin was dialyzed in normal saline using 1,000-Da cutoff dialysis membrane. Rats were anesthetized and one microosmotic pump (Alzet, model 1002) was filled with FITC-inulin and placed in the peritoneal cavity through a midline incision. The implantation of FITC-inulin pump was placed simultaneously along with the CGP42112A-filled osmotic pump subcutaneously in the rats. After implanting the osmotic pumps

the rats were placed in metabolic cage. The urine was collected over 24 hr and blood from tail vein was collected at the end of 24 hr on day 6, 9 and 14. The plasma and urine samples were buffered with 10 µl HEPES and fluorescence was measured using fluorimeter (Cytofluor Series 4000, Applied Biosystem) with 485-nm excitation and 538-nm emission. The GFR was calculated by using the following formula

$$\text{GFR (µl/min)} = \frac{\text{Urinary fluorescence counts/min}}{\text{Plasma fluorescence counts/µl}}$$

After 2-weeks of CGP42112A-treatment, urinary microalbumin in all the four animal groups was determined using enzyme immunoassay (EIA) kit (SPI-BIO Bertin Pharma, MI).

3.8 To determine the effect of AT_2 receptor activation on AT_1 receptor function in obese rats

3.8.1 Drug treatment

Obese Zucker rats (male 10-11 weeks old) were divided into 2 groups: (i) obese-vehicle control and (ii) obese-CGP. After two weeks of treatment with CGP, we measured the following under anaesthesia.

3.8.2 Blood pressure change in response to pressor dose of Ang II and natriuretic response to AT_1 receptor antagonist (candesartan)

After chronic treatment with AT_2 receptor agonist CGP, we measured renal function in obese rats. After surgery the carotid artery was cannulated for blood pressure measurement; jugular vein was cannulated and PD was infused continuously throughout the experiment.

Protocol 1: Basal urine was collected twice for thirty minutes by cannulating the ureter. After that, bolus dose of pressor Ang II (200 ng/kg/min, Cachofeiro et al., 1990) was infused via jugular vein and change in blood pressure was recorded. After 1-hr of Ang II infusion, AT_1 antagonist (candesartan) was infused through jugular vein and urine was collected twice for thirty minutes. Urine was measured and analyzed for Na using flame photometer or AAnalyst 500 atomic absorption spectrometer (Perkin Elmer, Waltham, MA). The schematic representation of protocol used in the study is shown in figure 5.

3.9 To determine that proximal tubules is the major site of AT_2 receptor action

Acute studies

3.9.1 Surgical procedures

On the day of experiment, we anaesthetized control lean and obese Zucker rats with inactin (150 mg/kg body wt, i.p.). After tracheotomy, the right carotid artery was cannulated with PE 50 and attached to data acquisition system

(PolyView, Grass Ins) via Grass pressure transducer PT300 for blood pressure measurement. The jugular vein was cannulated with PE 50 for saline/drug infusion. After opening the abdominal cavity, the ureter was catheterized with PE 10 for urine collection. After surgery, continuous infusion of saline (1% of body wt per hour) was administered through jugular vein till the end of the experiment.

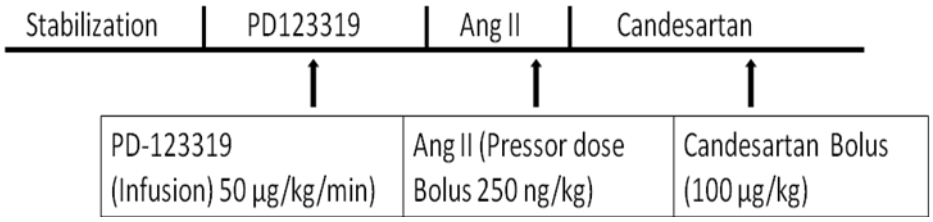
3.9.2 Protocols for drug infusion and urinary/blood collection

Protocol 2: After 40 minutes of stabilization period, two basal urine samples were collected at 30 minutes intervals. In this set of experiments, we used a novel non-peptide AT_2 receptor agonist C21 (1µg/kg/min and 5µg/kg/min) which was infused through jugular vein and two urine samples were collected again at 30 minutes intervals. The schematic representation of protocol used in the study is shown in figure 5.

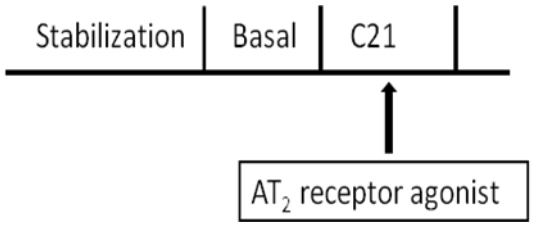
Protocol 3: To determine whether C21 was acting via AT_2R and to determine the role of proximal vs distal tubule in AT_2R-mediated natriuresis, we infused AT_2R antagonist PD (50 µg/kg/min) or bolus dose of distal tubules Na-channel blockers amiloride 5mg/kg body wt. (AM) and bendroflumethiazide 12 mg/kg body wt. (BFTZ)] followed by C21. Two urine samples were collected at 30 minutes interval before and after administration of drugs. At the end of the experiment, blood was drawn from the carotid artery for the measurements of sodium,

lithium and creatinine. The schematic representation of protocol used in the study is shown in figure 5.

Protocol 1



Protocol 2



Protocol 3

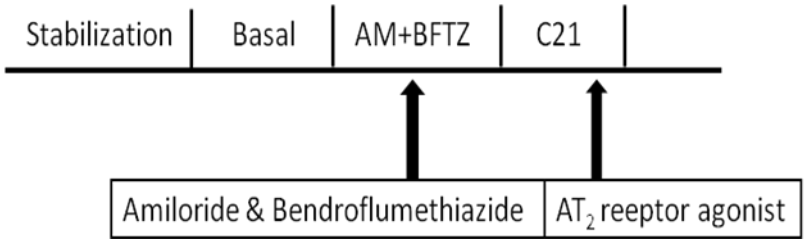


Fig 5: Renal function protocols

3.9.3 Measurement of sodium in plasma and urine of obese Zucker rats

Urinary and plasma sodium concentrations were measured using AAnalyst 500 atomic absorption spectrometer (Perkin Elmer, Waltham, MA). Plasma and urinary creatinine was measured by creatinine assay kit (BioVision, Cat# K625) for the estimation of glomerular filtration rate.

Calculations of renal function parameters

1) Urine flow rate (UF) was calculated as:

$$\textbf{UF (µl/min)} = \frac{\text{Urinary volume (UV)}}{\text{30 min}}$$

2) The urinary sodium excretion rate ($U_{Na}V$ µmol/min) was computed as:

$$\textbf{U}_{\textbf{Na}}\textbf{V} = \text{UF (µl/min) X urinary Na concentration (mg/dl) (µmol/µl)}$$

3) The glomerular filtration rate (GFR µl/min) was calculated based on creatinine clearance.

$$\textbf{CL}_{\textbf{creatinine}}\textbf{= GFR, µl/min} = \frac{\text{UF (µl/min) X Urine creatinine concentration (mg/dl)}}{\text{Plasma creatinine concentration (mg/dl)}}$$

4) The fraction of sodium excreted in urine (FE_{Na} %) was calculated as:

$$\textbf{Fraction of sodium excretion, (FE}_{\textbf{Na}}\textbf{ \%)} = \frac{U_{Na}V \text{ (µmol/min)}}{\text{Plasma Na (mg/dL)}}$$

Figure 6: Formulas used to calculate renal functions

3.10 Western blotting

Western blotting was performed in kidney cortex, proximal tubules and human kidney epithelial cells (HK-2).

3.10.1 In kidney cortex

The expression of various RAS components (AT_2, AT_1, renin, ACE and ACE2) in the kidney cortex of various rat groups was determined by western blotting. For this purpose, the kidney cortices were homogenized in the buffer containing (in mM) Tris 50, EDTA 10, PMSF 1, cocktail of protease inhibitors (aprotinin, calpain inhibitors, leupeptin, pepstatin and trypsin inhibitor). Proteins in the homogenates were determined by BCA method using a kit (Pierce, Rockford, IL). Equal amounts of protein, 30 µg for AT_1 receptor, 60 µg for AT_2 receptors, 30 µg for renin, 60 µg for ACE and 65 µg for ACE2 from various rat groups were subjected to SDS-PAGE and electroblotting onto immobilon P (blot). The blot was incubated with primary polyclonal antibodies for the AT_1 receptor, AT_2 receptor, renin, ACE and ACE2. Following the incubation with the primary antibodies, the blots were incubated with HRP-conjugated anti-rabbit IgGs. The signal was detected by ECL system, recorded and analysed by Fluorchem 8800 (Alpha Innotech Imaging System, San Leandro, CA) for the densitometry of the bands. For loading control, the blots were stripped, and re-probed with either β-actin antibody or GAPDH antibody.

3.10.2 In Proximal tubules

Preparation of proximal tubules from lean and obese Zucker rats

Male obese and lean Zucker rats (12 weeks of age) were anaesthetized with inactin (100 mg/kg body wt). After midline incision, kidneys were removed and kept in Krebs Henselet buffer containing calcium chloride and potassium phosphate (KHB A). The proximal tubules were isolated using the percoll density gradient centrifugation method. The kidney cortex was minced and digested with collagenase type IV in KHB A pH 7.4 with constant oxygenation until a uniform suspension is formed. The suspension was filtered through a nylon 105 um sieve and centrifuged at 100 X g for 2 minute. The pellet was suspended and washed two times in Krebs Henselet buffer containing potassium phosphate with no calcium chloride (KHB B). The final pellet suspension in KHB B was mixed thoroughly with 40% percoll and centrifuged at 14 000 g for 5 min. The pellet was suspended in Krebs Henselet buffer containing only calcium chloride (KHB C) buffer and protein in the proximal tubules were determined by BCA method using a kit (Pierce, Rockford, IL). 60 µg of the protein samples were subjected to western blotting for AT_2 receptor, IRF-1 and IRF-2 using respective protein specific polyclonal antibodies.

3.10.3 In HK-2 cells

The HK2 cells were washed with cold phosphate buffered saline and lysed in 0.3 ml of lysis buffer containing 0.5M Tris base (pH 6.8), 1% SDS, 1mM EDTA, 1mM PMSF and protease inhibitor (aprotinin, calpain inhibitors, leupeptin, pepstatin and trypsin inhibitor). The cell lysates were used for protein estimation by BCA method using a kit. Equal amounts (60 μg for AT_2, IRF-1 and IRF-2) of proteins from HK2 cells dissolved in Laemmlli buffer were separated in 10% gel and transferred onto immobilon P (blot).

3.12 Urinary cyclic guanosine 3', 5'-monophosphate (cGMP) measurement in lean and obese Zucker rats

3', 5'-monophosphate (cGMP) was measured in urine by using ELISA kit (R&D Systems, Minneapolis, MN). Urine samples were diluted 10-fold in calibrator diluent provided by the manufacturer and was assayed in duplicate. Non-specific binding and the background were substracted from each reading and the average optical density was calculated. The concentration of cGMP was calculated based on the mean absorbance from the standard curve. The concentration so obtained was multiplied by the dilution factor.

3.13 Urinary nitrate/nitrite measurement in lean and obese Zucker rats

Total nitrate/nitrite in urine from lean and obese Zucker rats was measured by using ELISA kit (R&D Systems, Mineapolis, MN). The samples were diluted 5-fold in reaction diluents supplied by the manufacturer. A set of standards ranging from 200 µmol/L to 3.12 µmol/L was assayed simultaneously along with the samples. The standard curve was constructed by plotting the mean absorbance for each standards using GraphPad Prism software. The values were represented as µmol/L.

3.14 ACE2 activity in the kidney cortex of lean and obese Zucker rats

ACE2 activity in kidney cortex was measured by fluorescence resonance energy transfer (FRET) using SensoLyte 390 ACE2 activity assay kit, CA. Briefly, the kidney cortex was homogenized in assay buffer and incubated on ice for 15 min. The homogenate was centrifuged for 10 min at 20,000 X g at 4°C and supernatant was collected. 50 µL of ACE2 substrate solution was added into each well and the reaction was incubated for 30 min. At the end of incubation fluorescence intensity was measured at excitation/emission =330/390 nm.

3.15 In-vitro

3.15.1 Cell culture

HK2 cells were cultured using K-SFM supplemented with 5% fetal bovine serum (FBS), epidermal growth factor (EGF) and bovine pituitary extract (BPE). The cells used in the experiment were between passages 4-12. Cells were seeded at 1 X 10^6 in 100 X 20 mm culture dish.

3.15.2 Treatment with glucose

At ~60% confluency, the HK-2 cells were treated with normal (5mM) and high glucose (25mM) for 24 hrs. In another set of experiments, the cells also were treated with equimolar glucose+sorbitol (20mM sorbitol + 5mM glucose) to examine the role of hyperosmolarity per se on AT_2 receptor expression. After treatment with glucose or sorbitol, the cell were lysed and processed for western blotting for AT_2 receptor as described in section 3.10.3.

3.15.3 siRNA transfection of HK2 cells

The transfection of HK2 cells with siRNA iRF1 was performed as per manufacturer's instructions. Briefly, the cells at 50% confluency were incubated with serum free medium for 3-4 hours before transfection. Thereafter, the transfection was performed using lipofectamine as transfection reagent. The cells were incubated with the siRNA IRF-1 at different concentrations (0.01-0.1

μM). After 6 hrs of incubation, the existing media was replaced with a fresh media and the cells were incubated for additional 24 and 48 hrs. The significant knock-down of IRF-1 was achieved at 0.1 μM siRNA at 48 hrs, the regimen used in subsequent experiments for testing the effect of glucose on AT_2 receptor expression. After siRNA transfection (0.1 mM, 48 hrs), the cells were incubated with normal and high glucose for 24 hours. Cells treated with transfecting agent alone served as control and also incubated with normal and high glucose. We also used control siRNA (Santa Cruz, CA Cat # 37007) which had a scrambled sequence that does not lead to degradation of any known cellular mRNA.

3.15.4 qRT-PCR

Quantitative RT-PCR (qRT-PCR) was performed both in HK-2 cells and in rat proximal tubules by using the SYBR green detection method. After treating HK-2 cells with normal and high glucose, RNA was isolated by RNeasy mini kit (Qiagen, Valencia, CA). The RNA (50 ng) was reverse-transcribed using random hexamers and multi-scribe reverse transcriptase in a two-step RT-PCR reaction, following the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Primers for PCR was designed to span intron/exon junctions (Primer Express; Applied Biosystems, Foster City, CA) to minimize amplifications of residual genomic DNA. Quantitative RT-PCR (qPCR) was performed using an ABI Prism 7300 sequence detector. Thermal cycling conditions included pre-incubation at 50°C for 2 min,

DNA polymerase activation at 95°C for 1min, and 40 PCR cycles for 15 s at 95°C and for 1 min at 60°C. The transcript for each gene was calculated at cycle threshold values (C_T) at which each fluorescent signal was first detected above background; these were determined using the ABI Prism 7300 SDS software (version 1.4) (Applied Biosystem). mRNA levels was normalized to that of the housekeeping gene 18s rRNA to control for input RNA and expressed as arbitrary units. The primer used for amplification of AT_2 receptor in HK2 cells is given below. The results obtained were compared between normal and high glucose treated cells. The protocol used for qRT-PCR experiments in kidney cortex of lean and obese Zucker rats was similar to that of HK2 cells but the primer used for amplifying is given below.

GENE	**Forward**	**Reverse**
HK2 Cells	3'CCCGTGACCAAGTCCTGAA'5	5'GCAAATGATGAAGGCCAGAA3'
GAPDH	3'CAAGGCTGTGGGCAAGGT5'	5' GGAAGGCCATGCCAGTGA3'
Tissue	3'GCTGTTGTGTTGGCATTCA5'	5'ATCCAAGAAGGTCAGAACATGGA3'
GAPDH	3'GTAGTCGCCGTGCCTACCAT5'	5' TCCGGAATCGAACCCTGAT3'

3.16 Statistical analysis

Results are expressed as mean±SEM. The data were subjected to statistical analyses using GraphPad Prism 4, San Diego, CA. Student's t-test and one-way analysis of variance (ANOVA) followed by post hoc tests (Newman-Keuls test) were performed to determine the significance of differences between groups. A value of $p<0.05$ was considered statistically significant.

4. PROTECTIVE ROLE OF AT_2 RECEPTORS IN BLOOD PRESSURE REGULATION IN OBESE ZUCKER RATS

4.1. RESULTS

4.1.1 General and renal parameters in obese Zucker rats

As shown in table 2 compared with lean Zucker rats, obese Zucker rats had greater body weight and consumed more food and water. The kidney weight of obese rats was higher comparing with those from lean rats. The PD treatment did not affect the food intake in obese animals. Plasma glucose was greater in obese than in lean rats and the PD-treatment modestly, but statistically insignificant, increased fasting blood glucose in obese rats.

The urinary volume (UV) and urinary Na ($U_{Na}V$) excretion over 24-hour period in obese rats was greater than in lean rats. The PD treatment caused a significant increase in UV, but had no effect on $U_{Na}V$ in obese rats. It should be noted that the extent of increase in UV was similar to the extent of increase in water-intake in PD-treated obese rats.

Table 2: General and renal parameters in lean and obese Zucker rats treated with PD123319 for 2-weeks

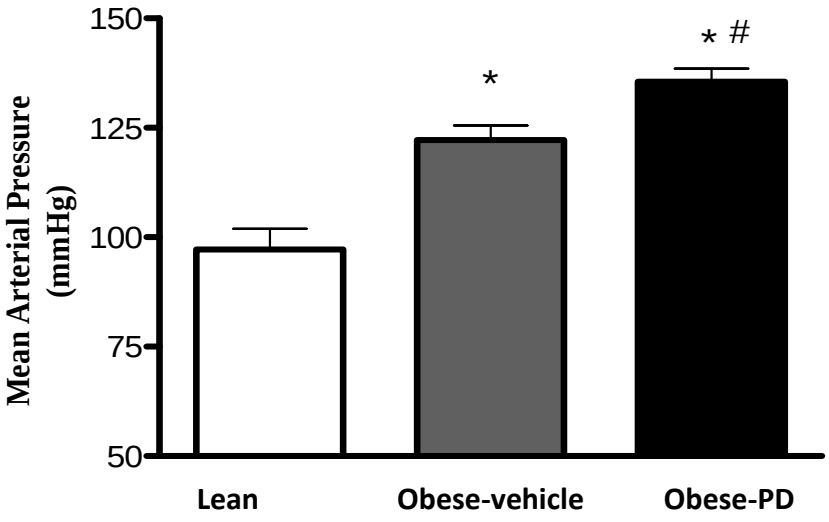
Parameter/ Rat group	Lean	Obese (Vehicle)	Obese (PD-123319)
Food, g/day	23±1	37±2*	35±1*
Water, ml/day	26±3	52±2*	61±3*†
Body wt, g	300±9	562±8*	580±8*
Kidney wt, g	2.9±0.1	3.5±0.1*	3.5±0.1*
Urine volume, ml/day	18±1	28±3*	39±5*
$U_{Na}V$, mmole/day	2.7±0.4	4.6±0.4*	4.9±0.7*
Fasting glucose, mg/dl	104±7	123±5*	130±5*

*statistically significant from lean Zucker rats, †significantly different from obese vehicle

4.1.2 Effects of PD123319 on blood pressure and heart rate

Compared to lean rats, obese Zucker rats exhibit higher systolic and diastolic blood pressure (lean: 114±5/88±5 vs obese: 135±5/105±6 mmHg, $p<0.05$). The 2 week treatment of obese Zucker rats with PD caused a significant increase by 13 mmHg in mean arterial blood pressure (MAP) (figure 7A). The heart rates were similar in lean and obese rats and were not affected by the PD-treatment (control obese: 389±12 bpm vs. PD-obese 391±7 bpm) (figure 7B).

A



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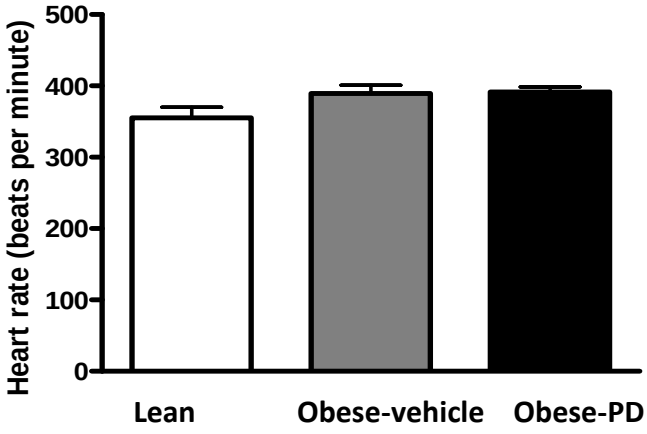


Fig. 7(A) Mean arterial blood pressure and **(B)** heart rate of lean, obese vehicle and obese Zucker rats treated with PD for two weeks. *significantly different compared with lean rats, #compared with obese control. Values are represented as mean ± SEM; One-way ANOVA followed by Neuman- Keuls test, $p<0.05$, N=5-6 rats in each group.

4.1.3 Effect of PD123319 on the expression of AT_1 *and* AT_2 *receptor in the kidney cortex*

Western blot shows the presence of AT_2 receptor in multiple bands (44 and 39 kDa) in the renal cortex. These multiple bands of the AT_2 receptor are likely due to various degree of glycosylation, as reported earlier (Hakam and Hussain, 2005). Densitometric analysis of the AT_2 bands revealed that the cortical AT_2 receptor expression was significantly elevated in obese compared with lean rats. The PD-treatment did not affect either the AT_2 receptor (figure 8A) or the AT_1 receptor (figure 8B) expression in the kidney cortex of obese rats. The cortical AT_1 receptor expression was modestly but significantly greater in obese rat compared to lean Zucker rats (figure 8B).

4.1.4 Effect of PD123319 on plasma rennin activity (PRA) and the expression of renin in the kidney cortex

The PRA in lean and obese control rats was similar. However, treatment with PD123319 for two weeks increased the PRA by 40% in obese Zucker rats (figure 9A). Western blot demonstrates the presence of renin as approximately 41 kDa band in the renal cortex. Densitometric analysis of the bands suggests that the cortical renin expression in obese rats was significantly lower compared with lean rats which are consistent with earlier study (Harker et al., 1993). The PD-treatment of obese rats caused a significant increase in the cortical renin expression (figure 9B).

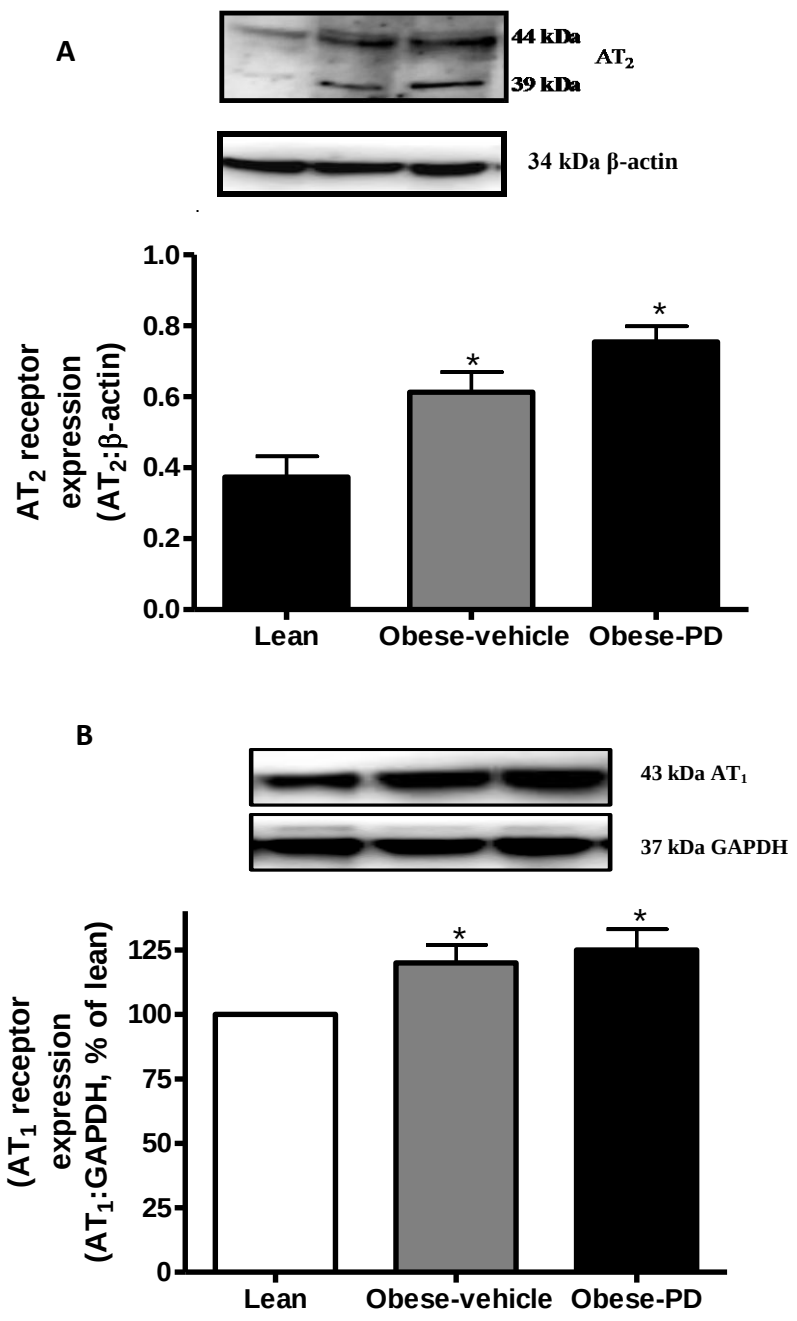


Fig. 8(A) AT_2 receptor expression and **(B)** AT_1 receptor expression in the kidney cortex of lean, obese vehicle and PD-treated obese rats. Upper panel: Representative western blots for AT_2 and AT_1 receptor expression. Lower panel: Bar graphs represent the densities of AT_2 and AT_1 receptor bands normalized with β-actin and GAPDH respectively as loading controls. *significantly different from lean rats. Values are represented as mean ± SEM; One way ANOVA, $p<0.05$ N=5 in each group.

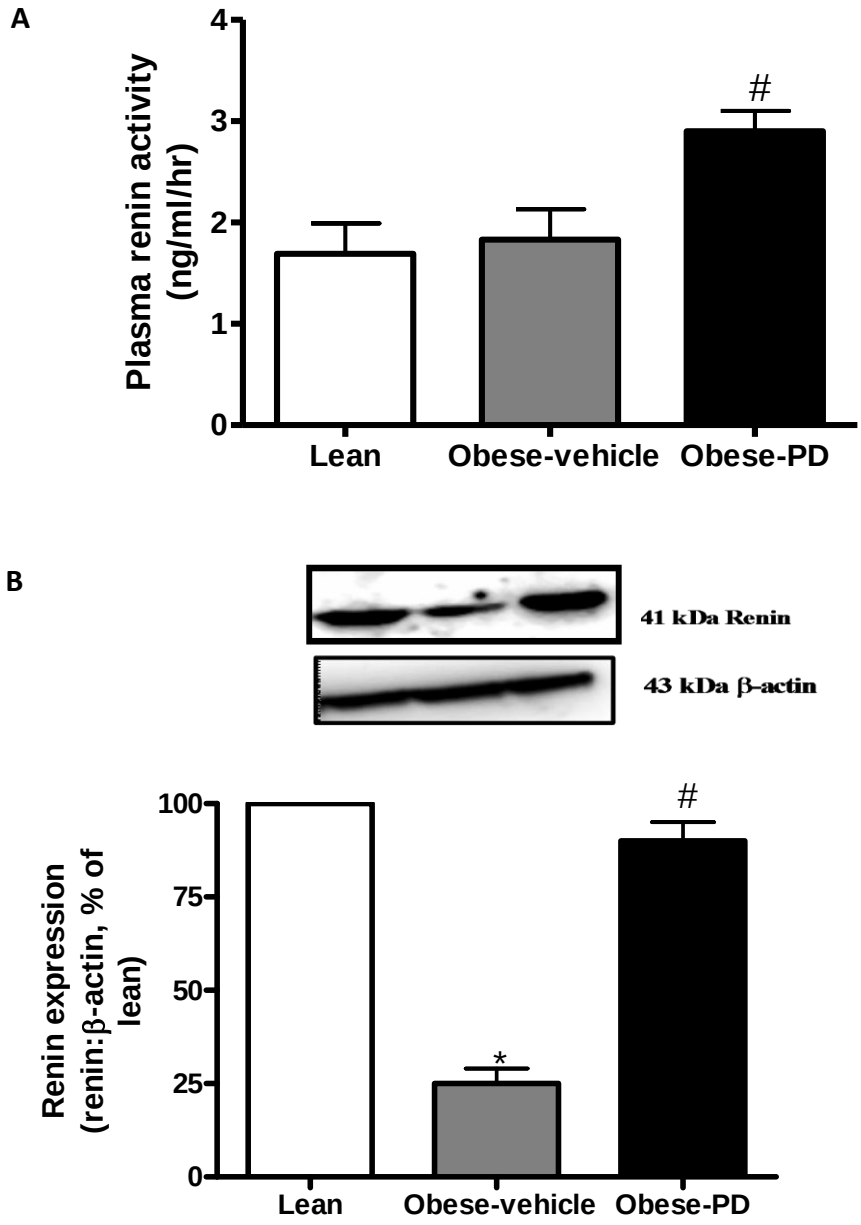


Fig 9 (A) Plasma renin activity and **(B)** expression of renin in the kidney cortex of lean, obese vehicle and PD-treated obese Zucker rats. **(B)** Upper panel: representative western blot of renin and β-actin. Lower panel: Bar graphs with values of renin band density normalized with β-actin as loading control. *compared with lean rats, #compared with obese vehicle. Values are represented as mean ± SEM; One way ANOVA, $p<0.05$, N=5 in each group.

4.2 Discussion

In the present study, we demonstrate the protective role of the AT_2 receptor in long-term blood pressure regulation in obese Zucker rats. Treatment of obese Zucker rats with the AT_2 receptor antagonist PD for 2-weeks caused a significant elevation in blood pressure. PD did not affect the expression of the AT_2 receptor or the AT_1 receptor in the kidney cortex. However, the PD-treatment of obese rats caused an increase in the PRA and the kidney renin expression.

Earlier we have shown that the AT_2 receptor expression in the proximal tubules and renal cortical membranes is increased in obese compared with lean Zucker rats (Hakam and Hussain, 2005). A selective activation of the AT_2 receptors promotes natriuresis via tubular mechanism, possibly by inhibiting NKA activity (Hakam and Hussain, 2005, 2006). Obese Zucker rat is a model of insulin resistance that exhibits hyperglycemia and high blood pressure (Kurtz et al., 1989). Impaired renal function and consequently, abnormal renal Na handling is believed to be a factor that contributes to the development of hypertension in this animal model (Alonso-Galacia et al., 1996; Fujiwara et al., 1999). Based on the enhanced renal AT_2 receptor expression and natriuretic function in obese rats, we predicted a compensatory and protective role of the AT_2 receptor against blood pressure increase in obese Zucker rats. Earlier studies using a genetic manipulation of the AT_2 receptor, including selective renal AT_2 receptor knockout approach (Moore et al., 2001) have suggested the role of the AT_2

receptor in blood pressure regulation. In our study, we used a direct pharmacological approach to test the role of AT_2 receptor in the long-term blood pressure control in a disease animal model. The 2-weeks treatment with AT_2 receptor antagonist clearly elevated systolic and diastolic blood pressure in obese Zucker rats, suggesting a protective effect.

Since treatment with PD was systemic, the precise mechanism responsible for BP increase by PD-treatment is not known. It could be the global blockade of AT_2 receptors as well as the un-opposed action of AT_1 receptors in the central and peripheral organs/tissues that contributed to the BP increase by PD-treatment. The BP increase in PD-treated rats was also associated with water intake and proportional urinary excretion. Although the increase in water intake in the present study may be related to the blockade of the central AT_2 receptor, the role of the central AT_2 receptors in thirst is not clear. Some studies have suggested that AT_2 receptors are not involved in thirst regulation (Beresford et al., 1992; Weisinger et al., 1997; Cooney et al., 1993) while other studies reported that AT_2 receptor may have a role in thirst in response to water-deprivation (Abrao et al., 2004; Lee et al., 1996; Widdop et al., 1994; Hein et al., 1995). Higher blood pressure could be responsible for increase in diuresis in the PD-treated obese rats. Another plausible explanation of the increase in water-intake and urinary volume could be related to a modest increase in blood glucose by PD-treatment of obese rats, which already are hyperglycemic

compared with lean rats. While the increase in fasting blood glucose did not reach statistical significance in PD-treated obese rats compared with control obese, the non-fasting blood glucose (measured on day 15 of the treatment) was significantly higher 160±10 mg/dl in PD-treated compared with 136±4 mg/dl in control obese rats. The positive relationship among hyperglycemia, water consumption and polyuria is well known. However, interesting observation is that the blockade of AT_2 receptors contributed to hyperglycemia in obese rats. This could be due to enhanced action of AT_1 receptors and/or inaction of AT_2 receptors affecting insulin sensitivity in PD-treated rats. There is evidence implicating AT_1 receptors in the development of insulin resistance in obese rat models and in humans (Cefalu, 2001; Toblli et al., 2008). On the other hand, AT_2 receptors have been shown to stimulate peroxisome-proliferation-activator receptor-γ (PPARγ) in PC12 cells (Zhao et al., 2005). The PPARγ is a transcription factor known to enhance insulin sensitivity (Kim et al., 2008). If AT_2 receptors are linked to PPARγ stimulation in insulin-dependent tissues, such as muscles and adipose tissue, the AT_2 receptor antagonism potentially would affect insulin-sensitivity. Therefore, it is possible that blocking of the AT_2 receptor and un-opposed action of the AT_1 receptors by PD-treatment contributed to insulin resistance further elevating blood glucose, which in turn induces thirst and increase in the urinary excretion. This notion warrants further systematic study. Earlier, it has been shown that AT_2 receptor knock-out in mice causes a shift in

pressure-natriuresis and an increase in blood pressure (Gross et al., 2000). Whether BP elevation by long-term pharmacological blockade of the AT_2 receptor, as seen in our present study, is a consequence of a shift in pressure natriuresis is yet to be investigated. We found that PD-treatment caused a modest increase in PRA but a profound increase in kidney renin expression. Higher Ang II in the circulation and in the kidney and subsequent un-opposed function of AT_1 receptor could have a differential role in the regulating blood pressure in obese animals. However, the enhanced contractile response mediated by AT_1 receptor in obese rats might have been compensated by higher endothelial AT_1 receptor and eNOS expression as shown in earlier studies (Siddiuqi and Hussain., 2007). Therefore, it is unlikely that the vascular AT_1 receptor might have contributed to the blood pressure increase in PD-treated obese rats. Higher kidney renin expression and unopposed action of tubular AT_1 receptor function might have contributed to the BP increase in PD-treated rats. This notion is consistent with other studies suggesting abnormal tubular handling of Na as the cause of hypertension in this and other animal model of obesity. The AT_2 receptors have been implicated in inhibiting the renin synthesis (Siragy et al., 2005). Increase in the cortical renin expression in PD-treated obese rats is a remarkable observation suggesting that the AT_2 receptor maintains kidney renin expression at lower level, and provides a protective and compensatory role in limiting blood pressure increase in obese rats. Since the dose of PD used in the

present study, when given acutely does not affect BP (Hakam and Hussain., 2005) it further supports the notion that long-term blocking of the AT_2 receptor may reset the blood pressure raising mechanisms. Since this study does not include experimental protocols to determine the renal function parameters such as glomerular filtration rate (GFR), blood flow and fraction of sodium excretion (FE_{Na}), a definite role of the kidneys in the blood pressure increase by PD-treatment of obese rats cannot established. Some investigators have reported a decrease in the renal expression of AT_2 receptors in diabetic animal models (Bonnet et al., 2002, Wehbi et al., 2001). Contrary to these reports, we found enhanced renal AT_2 receptor expression as reported earlier and in the present study (Hakam and Hussain., 2006; Hakam et al., 2006). Our findings are supported by other reports showing that AT_2 receptor expression is increased in human diabetic kidney (Mezzano et al., 2003), and in diabetic rat aorta (Lee et al., 2008). Reasons for the discrepancy in AT_2 receptor expression could be due to the difference in renal preparations, strain of rats and the methods of AT_2 receptor measurements. In our studies, we measured AT_2 receptor expression by western blotting in the isolated proximal tubules, and purified basolateral and brush-border membranes prepared from the kidneys of either Zucker rats or streptozotocin (STZ)-treated Sprague Dawley rats. Additionally, the increase in AT_2 receptor expression was supported by the enhanced AT_2 receptor functions, in terms of NKA inhibition in the proximal tubules, urinary sodium excretion and

vascular tone in these animals models (Hakam and Hussain., 2005; Hakam et al., 2006; Lee et al., 2008). On the other hand, Wehbi et al measured AT_2 receptors by western blotting in glomeruli and by immunostaining in the kidney from STZ-treated rats. Bonnet et al measured AT_2 receptors by RT-PCR, immunostaining and autoradiography in STZ-treated spontaneously hypertensive rats and Wistar-Kyoto rats. These investigators did not extend the studies to demonstrate whether reduction in AT_2 receptor expression was associated with a reduction in AT_2 receptor-mediated functions in their animal models.

Protective effects of the AT_2 receptor against various patho-physiological conditions have been documented. For example, treatment with AT_2 receptor antagonist caused an increase in blood pressure in the renal-wrap hypertensive rat (Siragy and Carey., 1999). In another study, cardiac-specific over-expression of AT_2 receptor provides protection against AT_1 receptor-mediated pressure and chronotropic effects (Masaki et al., 1998). The AT_2 receptor provides protection against L-NAME-induced cardiac hypertrophy and fibrosis (Masaki et al., 1998). In brief, our study demonstrates that long-term blockade of the AT_2 receptor results in an increase in blood pressure in obese Zucker rats, suggesting a protective and possibly compensatory role of AT_2 receptor in blood pressure increase in this animal model. This AT_2 receptor role could be important in light of the reports showing that the various hormone-receptors regulating renal sodium homeostasis are impaired. For example, natriuretic function of dopamine

D1 receptors (Marwaha and Lokhandwala, 2003; Hussain et al., 1999) and ANP (Zeigler and Patel, 1991) is reduced, while antinatriuretic function of Ang II AT_1 receptor is enhanced in obese Zucker rats (Hakam and Hussain, 2005; Tallam and Jandhyala, 2001). However, the mechanism of the protective role of the renal AT_2 receptor in blood pressure increase remains to be determined.

5. CHRONIC TREATMENT WITH AT_2 RECEPTOR AGONIST CGP42112A LOWERS BLOOD PRESSURE IN OBESE ZUCKER RATS

5 .1 Results

5.1.1 General parameters

As shown in table 3, compared with lean rats, obese Zucker rats had greater body weight (lean 295±8 vs obese 473±12 gm) and consumed more food (lean 25±1 vs obese 46±3 gm) and water (lean 39±2 vs obese 55±1 ml). The CGP-treatment did not affect the food and water intake in either of the animal types. The kidney weight of obese rats was higher compared with those from lean rats (lean 2.2±0.1 vs obese 2.8±0.1 gm) and was not affected by CGP-treatment. Fasting plasma glucose levels were higher in obese than in lean rats (lean 98±4 vs obese 115±3 mg/dl). Interestingly, CGP-treatment increased fasting blood glucose by 28 mg/dl in obese rats but had no effect in lean rats. Plasma insulin in obese rats was also greater than in lean rats (lean 1.0±0.4 vs obese 9±3 ng/ml). CGP-treatment did not change the levels of plasma insulin in either lean or obese rats.

Table 3: General parameters, blood chemistry and kidney parameters in control and CGP-treated lean and obese Zucker rats

Parameters/Rat groups	LCT	LCGP	OCT	OCGP
Food, g/day	25±1	26±0.5	46±3*	49±1*
Water, ml/day	39±2	40±2	55±1*	53±2*
Change in body wt, g	18±2	13±3	67±3*	50±4*#
UV, ml/day	10±1	10±1	17±3*	19±2*
Kidney wt, g	2.2±0.1	23±0.1	2.8±0.1 *	2.7±0.1*
Fasting blood glucose, mg/dl	98±4	90±4	115±3*	143±8*#
Plasma Insulin, ng/ml	1±0.4	1±0.4	9± 3*	8±1*
Microalbumin, ng/ml	63±17	74±12	229±98*	200±68*
HDL, mg/dl	28±2	28±3	54±10*	54±6.*
LDL, mg/dl	<50	<50	193±22*	213±20*

LCT- lean control, LCGP- lean treated with CGP, OCT- obese control, OCGP- obese rats treated with CGPA; *- compared with lean control;#-compared with obese control.

5.1.2 Effect of CGP42112A on blood pressure and heart rate

As shown in figure 10, we measured blood pressure in conscious and anesthetized animals. Compared to lean rats, obese Zucker rats exhibit higher mean arterial pressure (MAP) under anesthesia (MAP, lean 95±5 vs obese: 131±3 mmHg, $p<0.05$). The 2-week treatment of obese Zucker rats with CGP caused a significant decrease by 19 mm Hg in the MAP (figure 10A) but did not cause any change in the MAP of lean rats. Blood pressure measured in conscious animals was lower as compared to that under anesthesia. However, compared to obese control rats, treatment with CGP for two weeks significantly decreased MAP in conscious obese rats by 9 mm Hg (figure 10B).

As shown in figure 11, heart rates were similar in lean and obese rats and were not affected by CGP-treatment

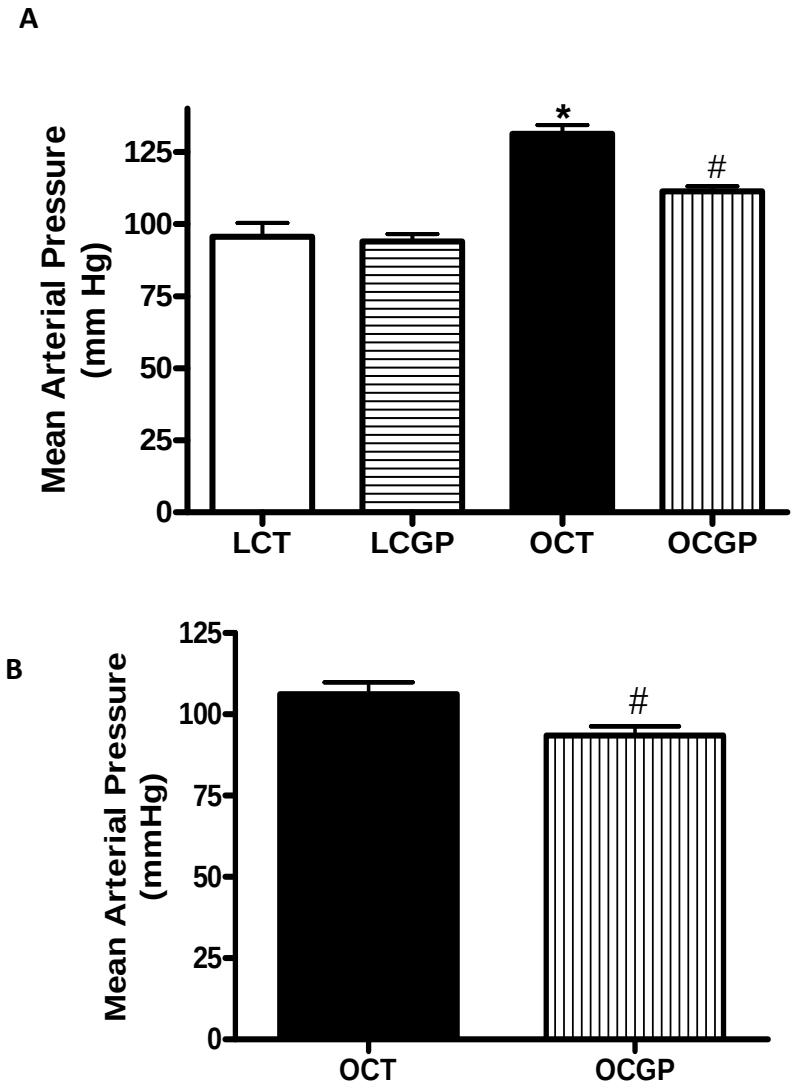
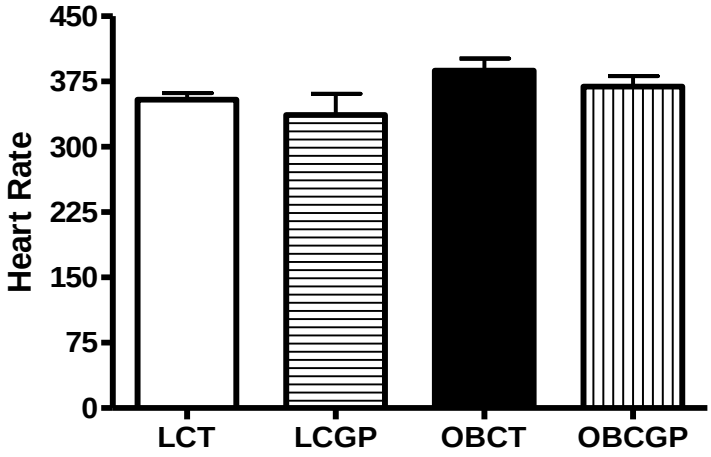


Fig 10 (A) Mean arterial blood pressure under anesthesia of control and CGP-treated lean and obese Zucker rats and **(B)** Mean arterial blood pressure in conscious control, CGP+PD and CGP-treated obese rats. Values are represented as mean ± SEM; *significantly different compared with lean rats, #significantly different compared with obese control, One-way ANOVA followed by Neuman-Keuls test, $p<0.05$; N=7-12 rats in each group). (LCT- lean control, LCGP-lean treated with CGP, OCT-obese control, OCGP-obese treated with CGP).

A



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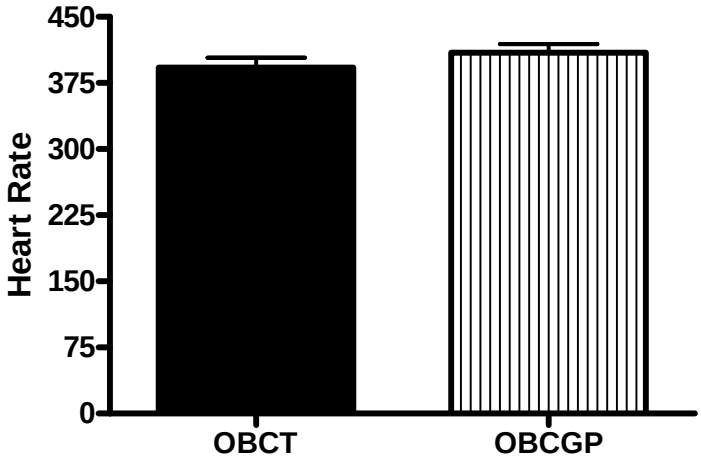

Fig 11 (A) Heart rate under anesthesia of control and CGP-treated lean and obese Zucker rats and **(B)** Heart rate in conscious control, CGP+PD and CGP-treated obese rats. Values are represented as mean ± SEM; N=7-12 rats in each group). (LCT- lean control, LCGP-lean treated with CGP, OCT-obese control, OCGP-obese treated with CGP).

5.1.3 Effect of CGP42112A on renal functions:

The urinary volume (UV) over 24-hour period in obese rats was greater than in lean rats (lean 10±1 vs obese 17±3 ml) (table 3). GFR in conscious lean and obese Zucker rats was measured by FITC-Inulin clearance method. Compared to lean rats, obese Zucker rats had elevated GFR (lean 389±12 vs obese 736±59 µl/min). Treatment with CGP for two weeks did not cause any significant change in GFR in either lean or obese rats (figure 12).

The Na-balance was determined by calculating the difference between Na consumed via food and Na excreted in urine and feces every 24 hours. The data were presented as cumulative over 2-weeks treatment. Lean rats had slightly positive Na-balance. However, obese control rats had significantly higher positive Na-balance compared to their lean control group. Interestingly, the Na-balance in obese rats is significantly decreased by CGP-treatment and was comparable to the control lean rats. The Na-balance in lean rats was not affected by CGP- treatment (figure 13).

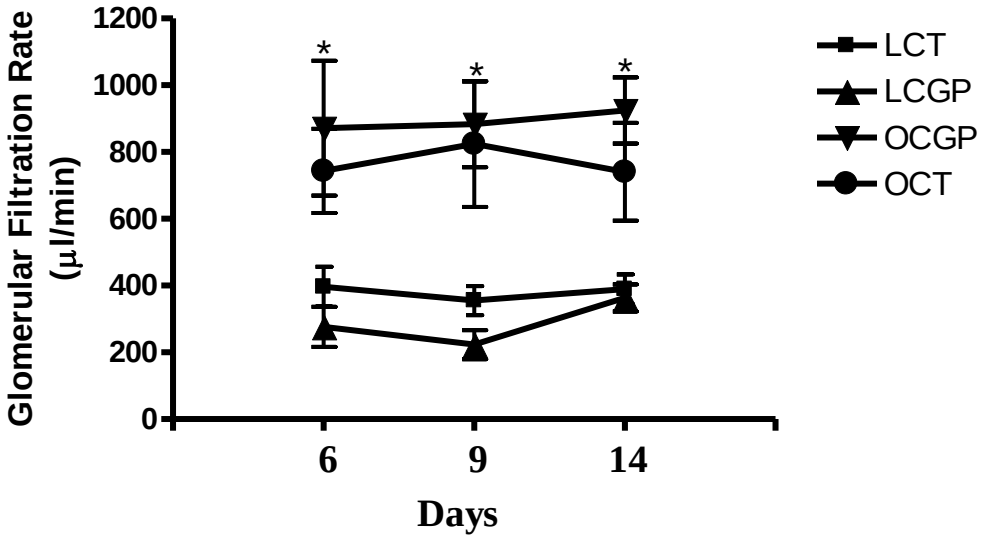

Fig 12) Glomerular filtration rate (GFR) measured using FITC-inulin clearance method in conscious control and CGP-treated lean and obese Zucker rats. Values are represented as mean ± SEM; *significantly different compared with lean control rats. One-way ANOVA followed by Neuman-Keuls test, $p<0.05$; N=7-12 in each group. (LCT- lean control, LCGP-lean treated with CGP, OCT-obese control, OCGP-obese treated with CGP).

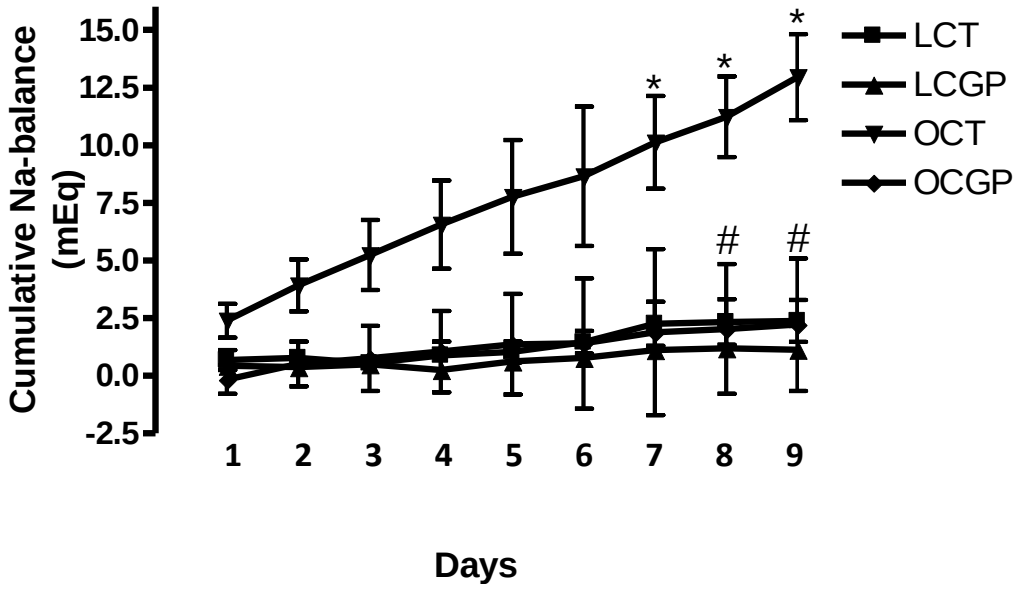

Fig 13) Cumulative Na-balance (Na consumed- Na excreted) over 2-weeks in control and CGP-treated lean and obese Zucker rats. *significantly different compared with lean rats, #significantly different compared with obese control rats. Values are represented as mean ± SEM; One-way ANOVA followed by Neuman-Keuls test, $p<0.05$; N=7-12 in each group. (LCT- lean control, LCGP-lean treated with CGP4, OCT-obese control, OCGP-obese treated with CGP).

5.1.4 Effect of CGP42112A on early and late urinary nitrates/nitrites

Total urinary nitrates/nitrites in obese Zucker rats were significantly higher in obese Zucker rats as compared to lean control rats. Treatment with CGP for two-weeks (late urinary nitrates/nitrites) did not change these levels after two weeks of treatment in either obese or lean Zucker rats (figure 14B). However, total nitrates/nitrites measured after two days of treatment (early nitrates/nitrites) was significantly higher in obese Zucker rats and again no change was observed in lean rats (figure 14A).

5.1.5 Effect of CGP42112A on RAS components

Plasma and renal renin

The PRA in lean and obese control rats was similar. However, treatment with CGP for two weeks reduced the PRA by 59% in both lean and obese Zucker rats (figure 15A). Cortical renin expression was measured by western blotting, which demonstrates the presence of renin as approximately 41 kDa. Densitometric analysis of the bands revealed that the cortical renin expression in obese rats was significantly lower by approximately 45% compared with lean rats, and was not affected by CGP-treatment (figure 15B).

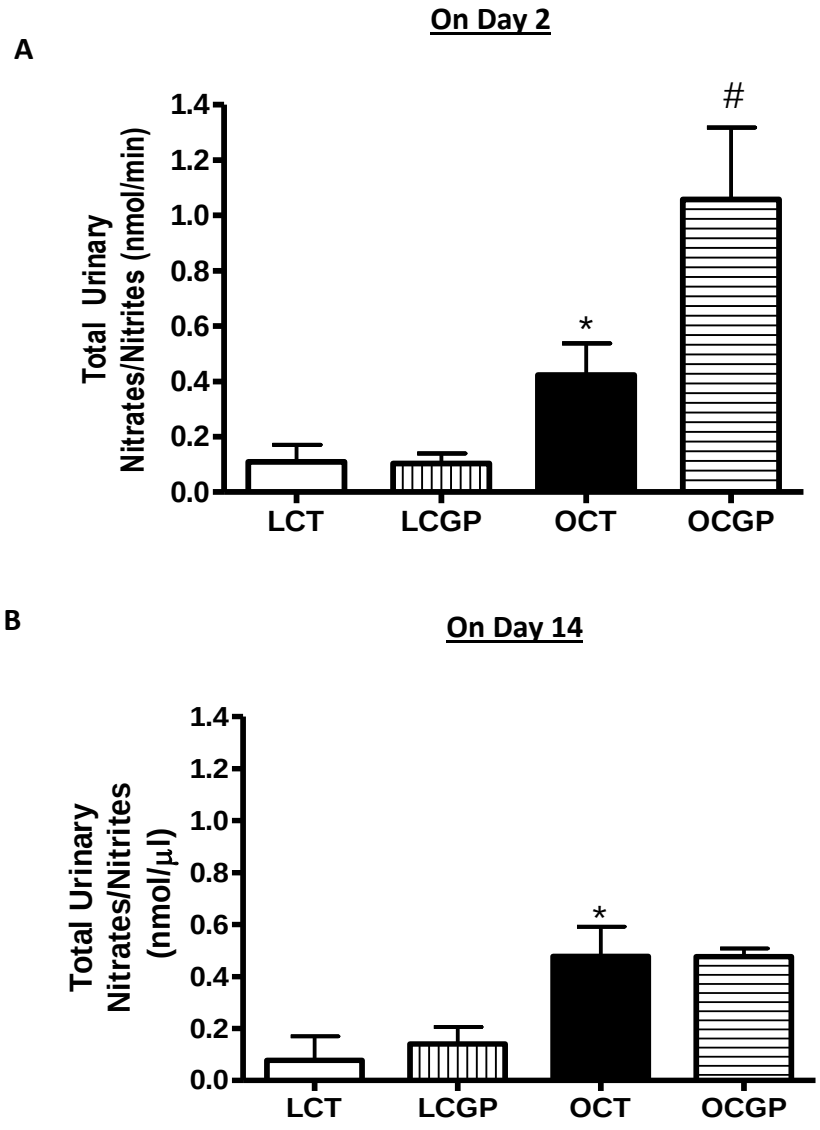

Fig 14) Total urinary nitrites/nitrates during **(A**) early (day 2) and **(B)** late (day 14) stage of CGP42112A treatment of lean and obese Zucker rats. *significantly different compared with lean rats, #significantly different compared with obese control rats. Values are represented as mean ± SEM; One-way ANOVA followed by Neuman-Keuls test, $p<0.05$; N=7-12 in each group. (LCT- lean control, LCGP-lean treated with CGP, OCT-obese control, OCGP-obese treated with CGP).

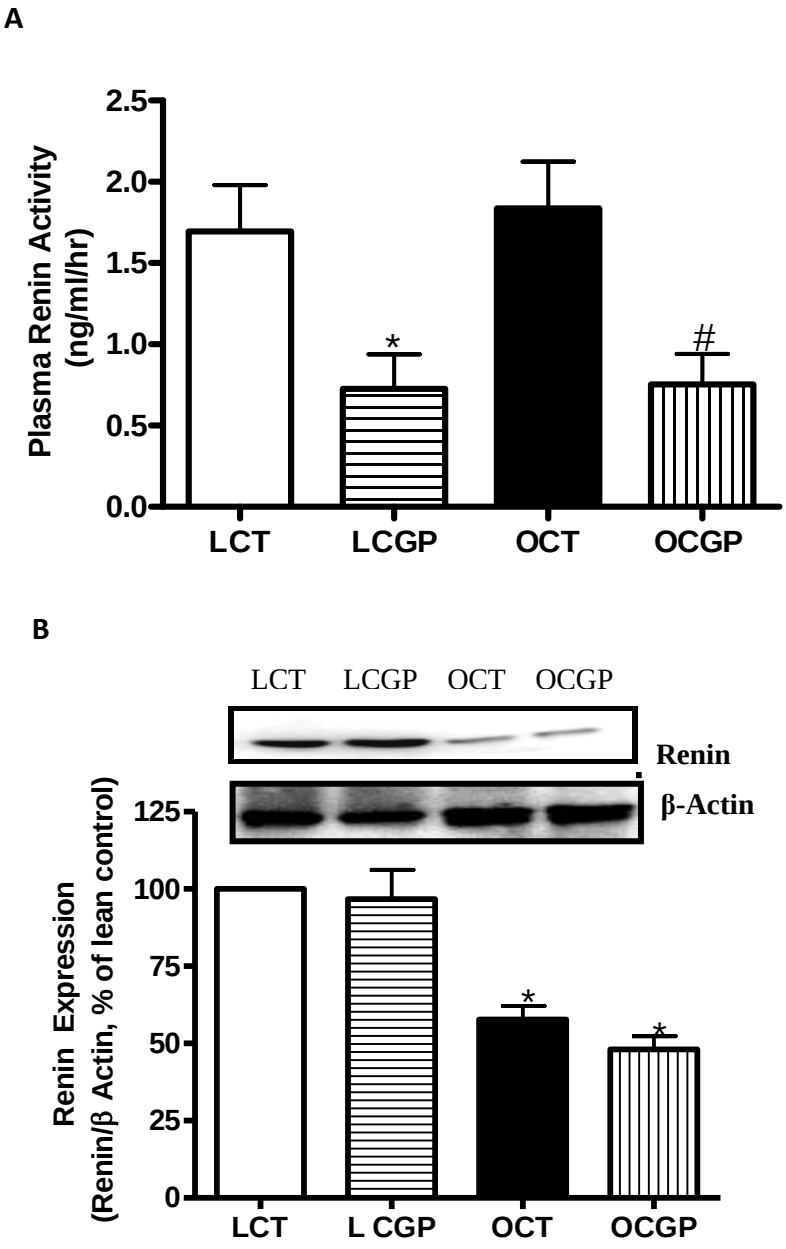

Fig 15) (A) Plasma renin activity (PRA) and (**B)** expression of renin in the kidney cortex of control and CGP-treated lean and obese Zucker rats. **(B)** Upper panel: Representative western blots for renin with loading control β-actin. Bar graphs: represent the ratio of density of renin and β-Actin. *significantly different compared with lean rats, #compared with obese control rats. Values are represented as mean ± SEM; One-way ANOVA followed by Neuman-Keuls test, $p<0.05$; N=7-12 in each group. (LCT- lean control, LCGP-lean treated with CGP, OCT-obese control, OCGP-obese treated with CGP).

AT_1 and AT_2 receptor

Western blot shows presence of AT_2 receptor as two bands (44 and 39 KDa) in the renal cortex. These two bands of AT_2 receptor are likely due to various degree of glycosylation, as shown earlier (Hakam and Hussain, 2005). Densitometric analysis of AT_2 bands revealed that cortical AT_2 receptor expression was significantly elevated in obese compared with lean rats, as reported earlier (Siddiqui et al., 2009). The CGP-treatment caused modest, but non-significant increase in cortical AT_2 receptor expression in obese rat (figure 16A). The AT_1 receptor antibody detected AT_1 receptor as approximately 43 kDa band in the renal cortex. Densitometric analysis of the bands suggests that cortical AT_1 expression was similar in lean and obese rats and CGP-treatment did not cause any change in its expression (figure 16B).

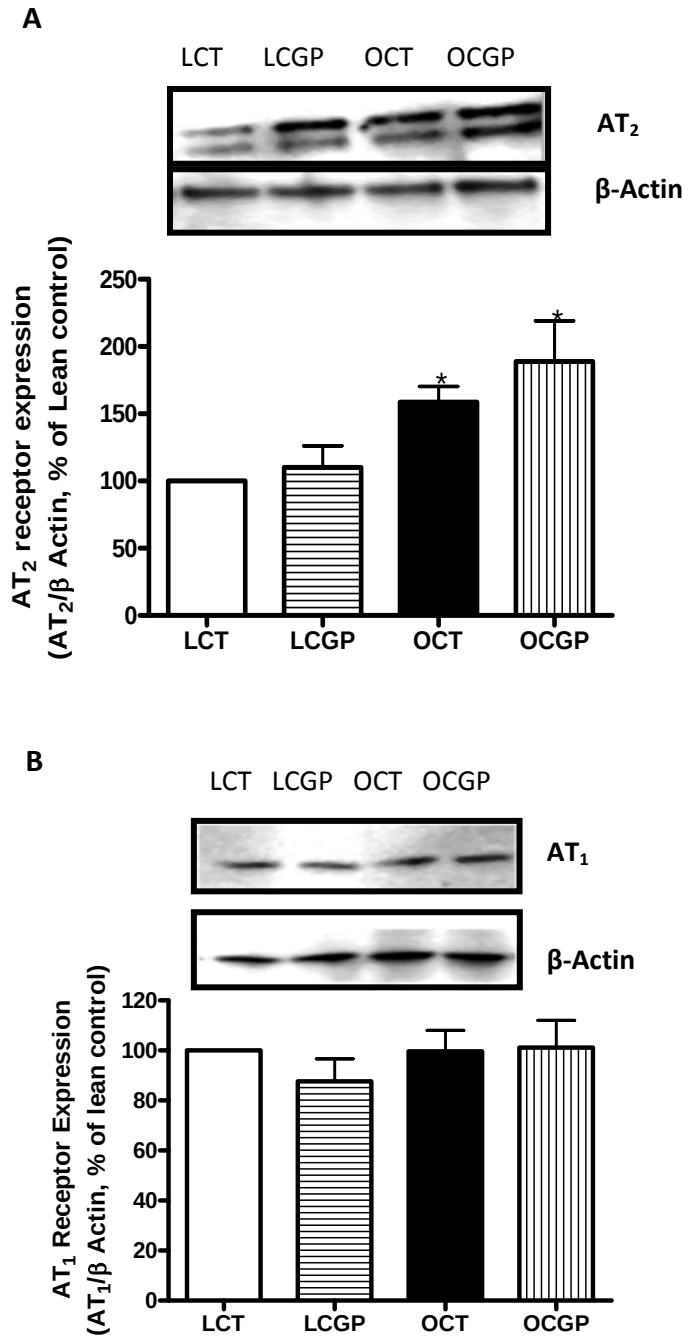

Fig 16) Expression of **(A)** AT_2 receptor and **(B)** AT_1 receptor in the kidney cortex of control and CGP-treated lean and obese Zucker rats. Upper panel: Representative western blots for proteins with loading control β-actin. Bar graphs: represent the ratios of densities of respective protein bands and β-Actin i.e. AT_2/β-Actin, AT_1/β-Actin, *significantly different compared with lean rats, Values are represented as mean±SEM; One-way ANOVA followed by Neuman-Keuls test, $p<0.05$; N=7-12 in each group). (LCT- lean control, LCGP-lean treated with CGP, OCT-obese control, OCGP-obese treated with CGP).

ACE and ACE2

Western blot demonstrates the presence of ACE as approximately 170 kDa band in the renal cortex. Densitometric analysis of the bands revealed that cortical ACE expression in obese rats was significantly higher compared with lean rats. The CGP-treatment of lean and obese rats did not cause any change in the cortical ACE expression (figure 17A). Western blot demonstrates the presence of ACE2 as approximately 90 kDa band, which is significantly less expressed in obese rats compared with lean rats. The CGP-treatment of obese rats caused approximately 3-fold increase in the ACE2 expression as compared to obese control rats (figure 17B), but had no effect in lean rats.

ACE2 activity: Similar to expression of ACE2 in kidney cortex, we observed that obese control rats had significantly lower ACE2 activity in kidney cortex and treating with CGP increased the activity in obese but not in lean rats (figure 17C).

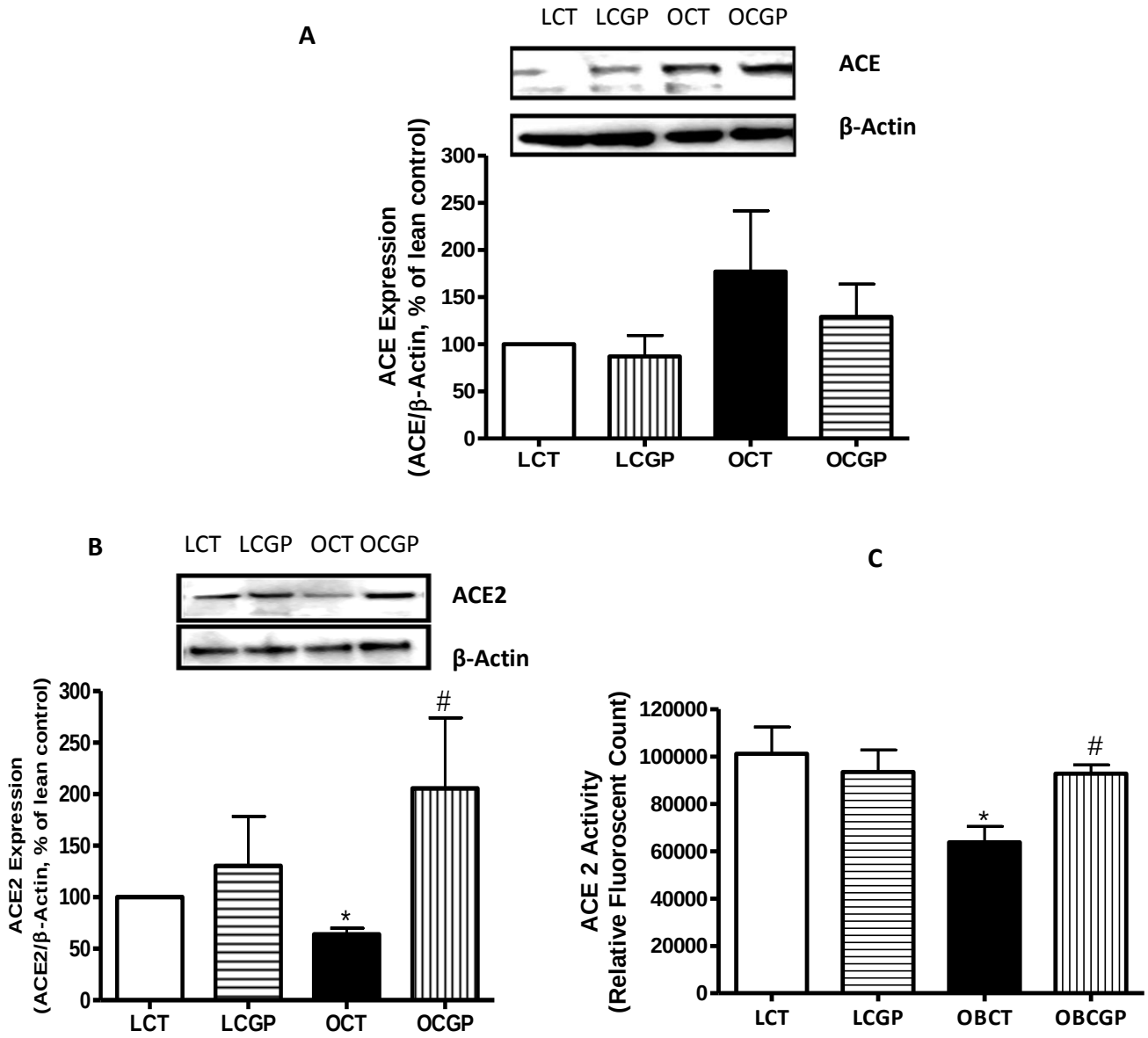

Fig 17) Expression of **(A)** ACE, **(B)** ACE2 and **(C)** ACE2 activity in the kidney cortex of control and CGP-treated lean and obese Zucker rats. Upper panel: Representative western blots for respective proteins with loading control β-actin. Bar graphs: represent the ratios of densities of respective protein bands and β-Actin i.e. ACE/β-Actin and ACE2/β-Actin. *significantly different compared with lean rats, #compared with obese control rats. Values are represented as mean±SEM; One-way ANOVA followed by Neuman-Keuls test, $p<0.05$; N=7-12 in each group. (LCT- lean control, LCGP-lean treated with CGP, OCT-obese control, OCGP-obese treated with CGP).

5.1.6 Effect of CGP42112A on mRNA levels of AT_1, AT_2 and renin

qRT-PCR analysis in the kidney cortex of lean and obese Zucker rats demonstrates increased levels of AT_1, AT_2 and renin mRNA in CGP-treated obese rats as compared to their lean controls (Fig 18). Treatment with CGP significantly decreased the AT_2 and renin mRNA levels as compared to obese control rats. Contrary to the effects seen in obese CGP-treated rats, mRNA levels of AT_1, AT_2 and renin was significantly increased in lean CGP-treated rats. Treatment with CGP did not affect the AT_1 mRNA levels of obese CGP-treated rats as compared to the obese controls rats.

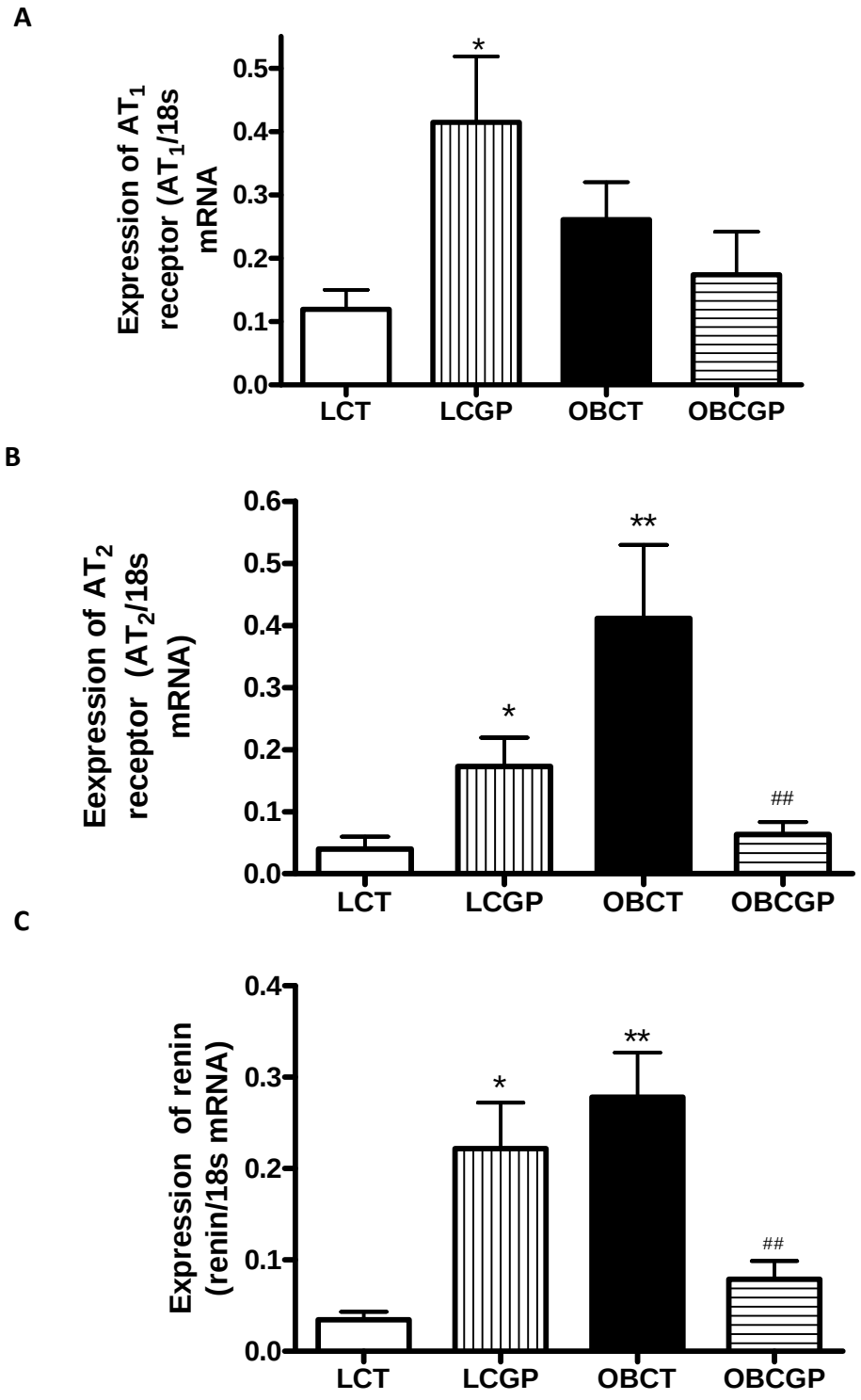

Fig 18) (A) AT_1 receptor, **(B)** AT_2 receptor and **(C)** renin mRNA expression measured by qRT-PCR in kidney cortex of control and CGP-treated lean and obese Zucker rats. **significantly different from lean control rats, ## compared to obese control rats. Values are presented as mean ± SEM; One way ANOVA followed by Neuman-keuls test, $p<0.05$, n= 7-12. (LCT- lean control, LCGP-lean treated with CGP42112A, OCT-obese control, OCGP-obese treated with CGP42112A).

5.2 Discussion

The most notable finding of this set of studies is that treatment of obese Zucker rats with AT_2 receptor agonist CGP42112A for 2-weeks causes a significant decrease in blood pressure under conscious and anesthetized state, which is associated with a decrease in Na-balance and enhanced expression and activity of ACE2 in renal cortex.

Obesity and diabetes are major risk factors contributing to the pathogenesis and sustenance of hypertension. Kidney dysfunction and defective pressure-natriuresis leading to increased renal Na reabsorption is one of the mechanisms that contribute to increased blood pressure in obesity/diabetes, including in obese Zucker rats (Tallam and Jandhyala, 2001). This increased Na reabsorption activates tubuloglomerular feedback mechanism (TGF) which leads to hyperfiltration and hence increased GFR. Our laboratory has reported that the renal expression of AT_2 receptor is increased in obese Zucker rats (Hakam and Hussain, 2005). Studies discussed in the previous section demonstrated a preventive role of AT_2 receptor in blood pressure increase in obese rat (Siddiqui et al., 2010). In the present study, for the first time we demonstrate that chronic treatment with AT_2 receptor agonist causes significant blood pressure reduction in obese rats. In other model system, such as spontaneously hypertensive rats (SHR), it has been reported that stimulation of AT_2 receptors lowers blood pressure **(**Bosnyak et al., 2010**).** However, Steckeling et al, reported that stimulation of AT_2 receptor did not cause any change in blood pressure in normotensive, healthy C57BL/6 mice or diabetic

SHR (Steckelings et al., 2010). Data from our laboratory suggests that AT_2 receptors are not only preventive against BP increase, but pharmacological activation of AT_2 receptors may have therapeutic role in treating obesity/diabetic related hypertension. One of the likely mechanisms for this role of AT_2 receptor could be related to the ability of AT_2 receptor to promote urinary Na excretion. This notion is supported by our data showing a reduction in the positive Na-balance in AT_2 receptor agonist-treated obese rats. Considerable numbers of studies indicate a role of AT_2 receptors in promoting urinary Na excretion (Padia et al., 2010; Carey et al., 2008) including participation of AT_2 receptors in pressure natriuresis (Gross et al., 2000). We have shown that AT_2 receptor agonist infusion promotes natriuresis by inhibiting NKA activity in obese rats, but not in lean rats (Hakam and Hussain. Thus it is reasonable mechanism to suggest that AT_2 receptor agonist treatment inhibited tubular Na transport, increased Na excretion and thereby decreased the positive Na-balance. Since enhanced renal Na reabsorption is a major mechanism of hypertension in obese rats (Alonso-Galicia, 1996) the long-term reduction in Na reabsorption in response to AT_2 receptor agonist might have led to a blood pressure reduction in obese rats. The tubular role of AT_2 receptor is partly supported by the observation that AT_2 receptor agonist treatment had no effect on GFR measured in conscious animals.

Interestingly, AT_2 receptor agonist treatment of obese rats altered the activity and expression of other RAS components, notably remarkable increase in the renal

expression and activity of ACE2, which might have contributed to AT_2 receptor agonist-mediated increase in Na excretion and thereby blood pressure reduction in obese rats. The ACE and ACE2 are important RAS regulatory enzymes (Mizuiri, 2008). The ACE converts Ang I to Ang II and ACE2 converts Ang I to Ang (1-9) and Ang II to Ang (1-7). Ang (1-9) and Ang (1-7) opposes the vasoconstrictive, proliferative and profibrotic actions of Ang II mediated via AT_1 receptors (Ferrario, 2010) In the present study we found that ACE expression was higher whereas ACE2 expression was significantly lower in kidney cortex of obese compared with lean Zucker rats. The CGP-treatment did not affect the expression of ACE, but increased ACE2 activity and expression in obese rats only. Such an increase in ACE2 activity and expression in AT_2 receptor agonist-treated obese rats might have shifted the metabolism of Ang II by converting more Ang II to Ang (1-7) and Ang I to Ang (1-9) reducing Ang II availability for acting on AT_1 receptors. Ang (1-7) in the kidney, acting via Mas receptor has been implicated in natriuresis and diuresis via an increase in prostaglandin production. Therefore, Ang (1-7) acting via MAS receptor might have contributed to natriuresis and vasodilatation leading to a decrease in blood pressure in obese rats. However, the metabolism of angiotensin peptides and their role in AT_2 receptor-mediated natriuresis and blood pressure need systematic investigation.

Selective changes in the renin expression or activity in the kidney and plasma, respectively, in response to AT_2 receptor agonist treatment were other interesting observations in this study. The PRA reduction was observed in both lean and obese rats,

but the blood pressure was reduced only in obese, not in lean rats. Simple inference may be drawn in that PRA alone without altering in renal function may not be sufficient to affect long-term blood pressure in lean rats. However, the implications of changes in PRA may be more complex in light of the type of changes in various RAS components, as we observed in obese rat kidney. Thus it is imperative to be cautious in extrapolating the PRA data to the renal RAS activity and functions

It is known that other hormone systems also participate in obesity-related kidney dysfunction and hypertension. For example, atrial natriuretic factor (ANF) and D1 receptor-mediated natriuresis is reduced in obese rats (Wang et al., 2004; Banday et al., 2004) whereas sympathetic activity is greater (Morgan et al., 1995), which shifts balance towards higher anti-natriuretic response of the kidney affecting blood pressure. Since AT_2 receptor is a functional antagonist of AT_1 receptor, which is positively connected to sympathetic activity, it is likely that the treatment of obese Zucker rats with AT_2 receptor agonist might have functionally antagonized the action of AT_1 receptor, which in turn reduced sympathetic activation. However, this notion warrants further study to establish a link between role of renal AT_2 receptors and other receptors in regulating Na excretion and blood pressure.

While AT_2 receptor agonist treatment produced desirable response on blood pressure and kidney function, we observed reduced insulin sensitivity in obese rats treated with AT_2 receptor agonist. Although initial studies suggests that AT_2 receptor is an activator

of PPARγ, a transcription factor whose activation is required to improve insulin sensitivity (Morgan et al., 1995), recent animal studies demonstrated an inverse relationship between AT_2 receptor and insulin resistance. Deletion of AT_2 receptor gene improves insulin sensitivity and prevents high fat diet induced insulin resistance (Yvan-Charvet et al., 2005). This study corroborates our finding that agonist activation of AT_2 receptors might have contributed to insulin resistance, further elevating blood glucose levels. There are some studies, which also demonstrate a negative cross-talk between AT_2 receptor and insulin receptor signaling. The AT_2 receptor inhibits the phosphoinositol-3-kinase (PI-3K) activity, which is responsible for insulin-induced translocation of GLUT4 to the plasma membrane for facilitating glucose transport (Cui et al., 2002; Tobbli et al., 2004). Treatment with CGP might have further reduced the translocation of GLUT4 proteins making it more insulin resistant causing increase in blood glucose. The increase in hyperglycemia in response to AT_2 receptor agonist treatment may not be unique to CGP, because several antihypertensive drugs including thiazide diuretics exhibit increased incidence of hyperglycemia (Banday et al., 2004). Interestingly, we observed that fasting blood glucose is raised in CGP-treated obese rats, but fasting plasma insulin remained unaltered. This could be either insulin produced by obese Zucker rats is already at its maximum capacity or CGP has affected pancreas's ability to secrete more insulin in response to increase in blood glucose in CGP-treated animals.

In summary, this is the first report showing that chronic treatment with AT_2 receptor agonist caused blood pressure reduction in obese rats. The reduction in blood pressure could have been resulted from a direct action of AT_2 receptor on renal Na-transport causing a decrease in Na-balance. Additional mechanisms responsible for decrease in Na-balance and blood pressure reduction could be related to the enhanced activity and expression of cortical ACE2 and reduced PRA in obese rats treated with AT_2 receptor agonist. Thus direct pharmacological activation of AT_2 receptor may have long-term effect on renal physiology and blood pressure control, by affecting renal Na-transport directly and by resetting the expression and function of RAS components.

6. FUNCTIONAL ANTAGONISM OF AT_1 RECEPTOR BY AT_2 RECEPTOR

6.1 Results

6.1.1 *Effect of AT_1 antagonist on blood pressure and heart rates of control and CGP42112A-treated obese rats*

As reported earlier in figure 10, treatment with CGP for two weeks significantly decreased the blood pressure in obese Zucker rats under conscious condition as well as under anesthesia. No change was observed in heart rate after two weeks of treatment with CGP in obese Zucker rats (figure 11). Administration of AT_1 receptor antagonist (Candesartan, 100 µg/kg, i.v. bolus) did not affect either the blood pressure or the heart rate in either of the groups (figure 19).

6.1.2 *Effect of AT_1 antagonist on diuresis in control and CGP42112A -treated obese rats*

To study the AT_1 receptor function on diuresis in obese rats, we infused candesartan (100 µg/kg, i.v. bolus). Infusion of candesartan in control obese rats increased the diuresis and natriuresis which was significantly reduced in obese CGP-treated rats. Since this dose of candesartan did not affect either the blood pressure or the heart rate, it suggests that only kidney and not systemic vasculature was affected by the treatment. This is why we study the diuresis in these rats (figure 20).

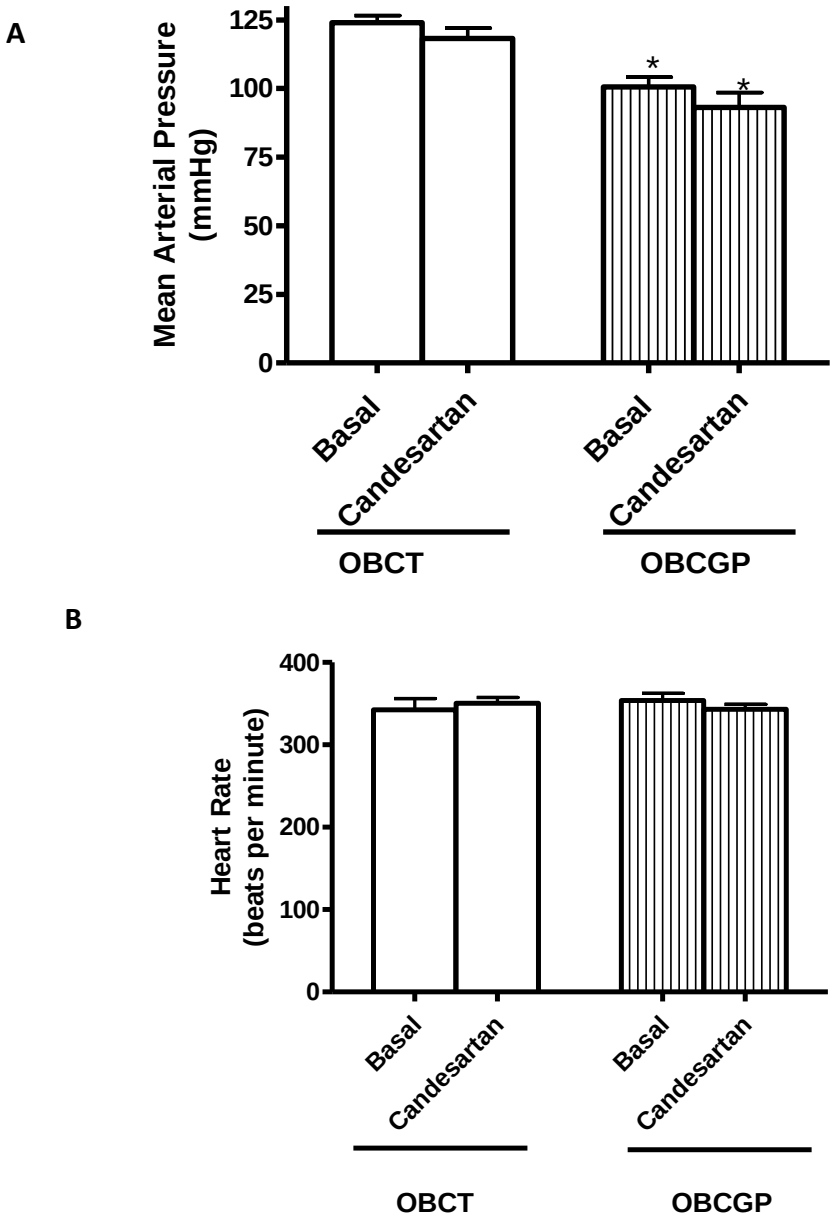

Fig 19) Effect of Candesartan (100 µg/kg bolus) on **(A)** Mean arterial blood pressure and **(B)** heart rate of control and CGP-treated obese Zucker rats. *significantly different compared with obese control groups. Values are represented as mean ± SEM; One-way ANOVA followed by Neuman- Keuls test, $p<0.05$, n=5-6 rats in each group). (OCT-obese control, OCGP-obese treated with CGP42112A).

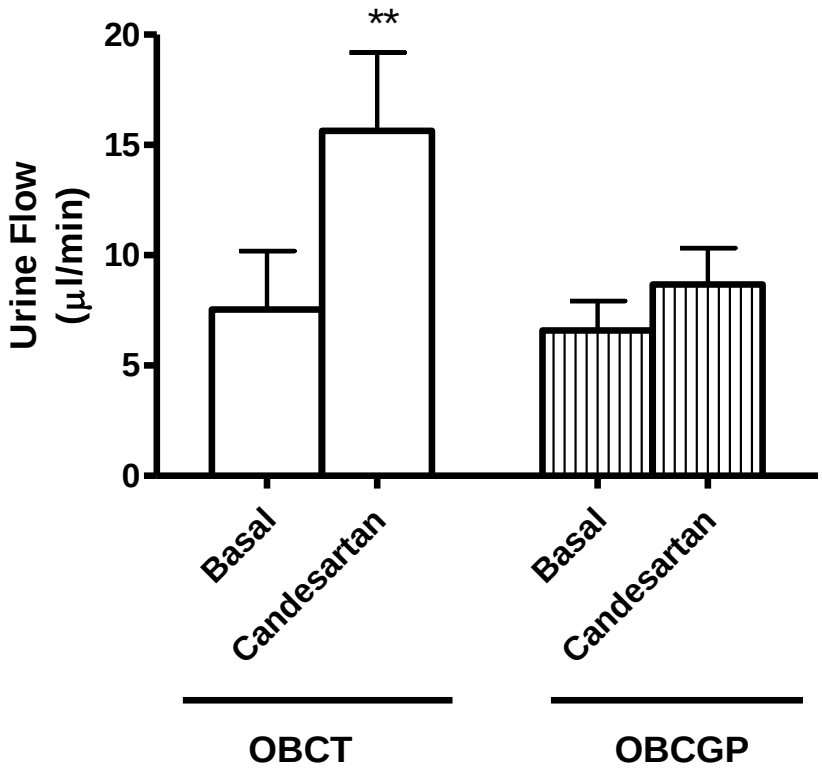

Fig 20) Effect of candesartan (100 µg/kg bolus) on urine flow in control and CGP-treated obese Zucker rats. **significantly different compared with obese basal. Values are represented as mean ± SEM; One-way ANOVA followed by Neuman- Keuls test, $p<0.05$, n=5-6 rats in each group). (OCT-obese control, OCGP-obese treated with CGP).

6.3 *Ang II pressor response in control and CGP42112A-treated obese rats*

In order to examine whether chronic treatment with AT_2 receptor agonist may have reduced AT_1 receptor function, we used exogenous Ang II pressor dose (200 ng/kg/min). The pressor response was studied after AT_2 receptor in all rats groups have been blocked by infusing AT_2 receptor antagonist. Since control and CGP-treated groups differed in various degree of AT_2 receptor function, blocking AT_2 receptor in all groups allowed us to study AT_1 receptor function in more uniform pharmacological functions. Ang II infusion caused increase in BP but pressor response to Ang II was not significant in CGP-treated groups compared to basal. The changes in systolic blood pressure (peak over basal) was measured in obese control and CGP-treated rats. Ang II pressor response was reduced in obese CGP-treated rats (Fig 21) compared with control obese.

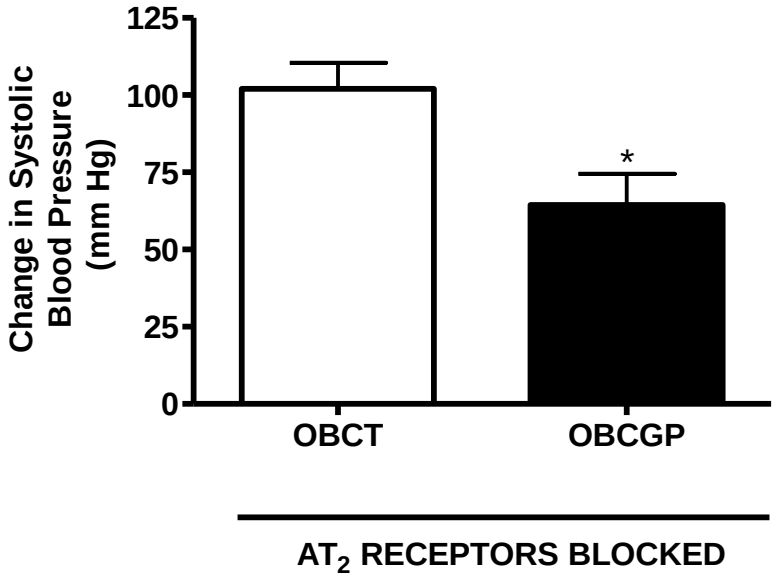

Fig 21) Ang II (200 ng/kg/min) pressor response in control and CGP-treated obese Zucker rats. Values are represented as mean ± SEM. *significantly different as compared to obese control rats. Student's t-test, $p<0.05$, N= 4-5). (OCT-obese control, OCGP-obese treated with CGP42112A).

6.2 Discussion

As we have presented the studies in previous section that long-term activation of AT_2 receptor affects blood pressure and Na balance in obese rats. In the present study we found that this reduction in blood pressure and Na balance is contributed by the ability of AT_2 receptor to antagonize the function of AT_1 receptor.

Several studies suggest that AT_2 receptor acts as a functional antagonist of AT_1 receptor (Masaki et al., 1998; Akashika et al., 1999; Horiuchi et al., 1999). It means that activation of AT_2 receptor reduces the AT_1 receptor mediated functions such as natriuresis and diuresis. We therefore performed renal function experiments in obese Zucker rats to determine if the decrease in blood pressure and sodium balance might have been contributed by the ability of AT_2 receptor to antagonize the renal AT_1 receptor function. This notion was tested by determining AT_1 receptor function in chronically CGP-treated obese rats. After treating obese Zucker rats for two weeks with AT_2 receptor agonist CGP and performing renal function study, we concluded that CGP-treatment not only decreased the Ang II pressor response but also blunted the candesartan induced diuresis in these rats.

Apart from some exceptions, several studies suggest that AT_2 receptor counteracts AT_1 receptor function. The mechanism by which AT_2 receptor opposes the function of AT_1 receptor has been linked to heterodimerization of these receptors. Said Abdallah et al

reported that this antagonism was due to the direct binding of the AT_2 receptor to the AT_1 receptor in fetal fibroblasts and human myometrium and was independent of endogenous Ang II. We have earlier reported that AT_2 receptors are upregulated in the obese Zucker rats (Hakam and Hussain, 2005) so it might be possible that it might be a compensatory mechanism for these upregulated receptors to allow the tissue RAS to modulate AT_1R activity independent of Ang II. Recently, Eguchi proposed a novel mechanism of AT_2 antagonism of AT_1 receptor in vascular smooth muschle cells (VSMCs). He demonstrated that stimulation of AT_2 receptor inhibits Rho/Rho-kinase involving Src homology 2 domain–containing protein-tyrosine phosphatase 1(SHP-1), casein kinase II (CK2) and Ste20-related kinase (SLK) and therefore inhibits VSMC hypertrophy and migration (Eguchi et al., 2009). However, there is no study published so far to determine the in-vivo functional interaction between renal AT_1 and AT_2 receptors.

The present study suggests that AT_2 receptor affects blood pressure and Na balance independently and also via antagonizing the function of AT_1 receptor; the precise mechanism that is responsible for this antagonism remains yet to be investigated.

The unpublished data from our laboratory showed that activation of AT_2 receptor abolished Ang II-mediated stimulation of NKA pump in the proximal tubules of obese rats. Further studies revealed that this AT_2 receptor antagonism of AT_1 receptor stimulation of NKA pump was sensitive to guanylate cyclase and phosphodiesterase-3

(PDE3) inhibition. Thus these in-vitro studies revealed that AT_2 receptor via stimulation of cGMP and PDE3 pathway affect AT_1 receptor function.

In the present study, after treating obese rats with CGP for two weeks, we measured renal function and this was performed under anesthesia after blocking AT_2 receptors and this was done by constantly infusing AT_2 receptor antagonist PD until the termination of the experiment. This was done so that we can exclusively study AT_1 receptor function in control and chronically CGP-treated obese rats. Candesartan is an AT_1 receptor antagonist and natriuresis produced after blocking AT_1 receptor reflects the basal Na excretion. Earlier we have demonstrated that candesartan produces marked increase in sodium and urine excretion in obese as compared to lean rats. After infusion of PD we administered bolus dose of candesartan which too resulted in marked diuresis without affecting the blood pressure or renal hemodynamics in obese rats. However, diuresis produced by candesartan was significantly blunted in obese CGP-treated rats. These data suggest that CGP treatment for two weeks not only opposes the AT_1 receptor but also decreases AT_1 receptor function, as we blocked all the AT_2 receptors with PD123319. Moreover, either AT_1 receptor function is significantly increased which results in increased Na absorption or Ang II levels are greatly enhanced in obese Zucker rats and treatment with CGP42112A significantly reduced the AT_1 receptor function.

Obese Zucker rats are hypertensive and this is attributed to their increased sensitivity to the pressor dose of Ang II. In this study, bolus intravenous pressor dose of exogenous Ang II significantly enhanced the blood pressure in obese control and CGP-treated rats. This suggests that these rats have exaggerated vascular response to Ang II. However the rise in blood pressure in obese CGP-treated rats was significantly reduced as compared to obese control rats. This reduction in blood pressure in response to exogenous Ang II, despite the fact that the same concentration of Ang II was infused in both sets of rats demonstrate that AT_2 receptor has the ability to oppose the AT_1 receptor function which resulted in reduction in Ang II pressor response in CGP-treated obese rats. Several studies suggest that the increase in blood pressure in response to Ang II could be possible because of loss of baroreflex, since obese rats exhibit impairment in these reflexes. However, obese rats demonstrated increased pressor response to Ang II even after blocking of ganglions. These studies suggest that the vasculatures of these obese rats are hyperresponsive to exogenous Ang II (Zemel et al., 1992).

In summary, we found that chronic AT_2 receptor agonist treatment reduced AT_1 receptor-mediated natriuresis and diuresis and also attenuated Ang II mediated pressor response in obese rats. Thus long-term activation of AT_2 receptor may not only produce its direct affect on urinary sodium excretion, but potentially reduces AT_1 receptor function, amplifying the overall response on natriuresis and thus on long-term control of blood pressure.

7. NOVEL ANGIOTENSIN II TYPE 2 RECEPTOR AGONIST C21 PROMOTES NATRIURESIS PREDOMINANTLY VIA PROXIMAL TUBULES IN OBESE RATS.

7.1 Results

7.1.1 Effect of C21 on natriuresis and diuresis in obese rats

In the initial set of experiments, we determined the effect of AT_2 receptor agonist C21 (1 and 5 µg/kg/body wt, i.v) on natriuresis and diuresis in obese rats. We did not use lean rats in any of the experiments because previous data from our laboratory suggest that stimulation of AT_2 receptor in lean rats does not promote natriuresis (Hakam and Hussain, 2006). Infusion of C21 dose-dependently caused an increased UF and $U_{Na}V$ over basal, being highly significantly greater at 5 µg/kg/body wt dose (figure.22), which was used in subsequent set of experiments. In order to demonstrate that C21 induced Na-excretion is mediated via AT_2R, we infused the AT_2R antagonist PD123319 (50 µg/kg body wt, i.v.) prior C21. While PD123319 alone did not affect the UF and $U_{Na}V$ as compared to basal values, it completely abolished the increase in UF and $U_{Na}V$ in response to C21, suggesting the involvement of AT_2R (figure 23).

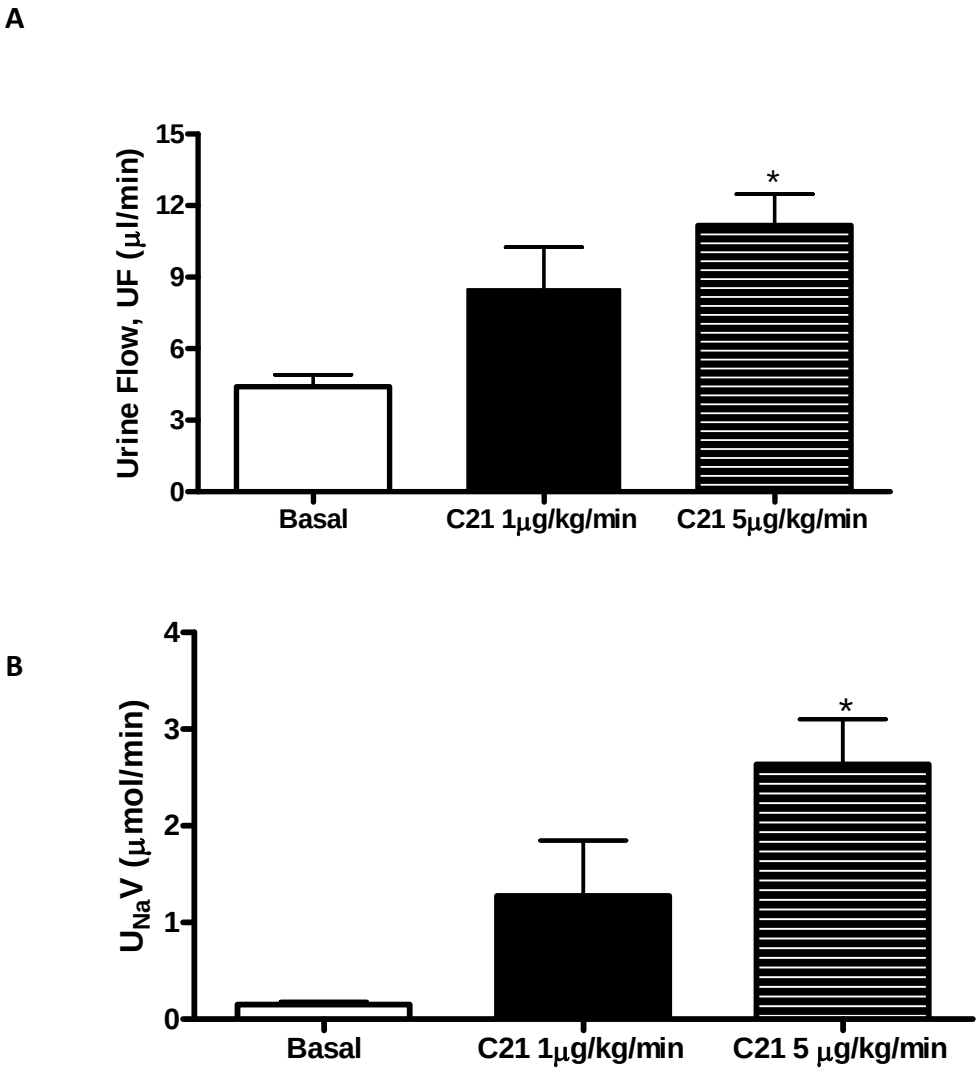

Fig. 22) Effect of C21 (1 and 5µg/kg per minute) on **(A**) UF and **(B**) $U_{Na}V$ in obese Zucker rats. *significantly different from basal, Values are are represented as mean ± SEM; One-way ANOVA followed by Neuman-Keuls test, $p<0.05$; N=5-6 in each group

7.1.2 Effect of C21 on the contribution of proximal and distal tubules in mediating natriuresis in obese rats

In this set of experiment, we determined the role of proximal vs distal tubules in AT_2R-medaited natriuresis and diuresis in obese rats. As shown in figure 26, compared to basal urine, blocking of epithelium Na-channels (ENaC) and Na^+/Cl^- co-transporters (NCC) in the distal tubules with AM and BFTZ significantly increased the UF (Basal: 0.27±.04 AM+BFTZ: 1.4±.4 µmol/min and and $U_{Na}V$ (Basal: 3.9±.6 AM+BFTZ:8.9±.8 µmol/min). Treatment with C21 increased UF and $U_{Na}V$ to approximately 4 times as compared to basal. Interestingly, C21 infusion along with AM+BFTZ caused further increase in both UF and $U_{Na}V$ (figure 24). Mean arterial pressure and heart rate remained unchanged after infusion of drugs (figure 25).

7.1.3 FE_{Na} and GFR

FE_{Na} was significantly increased after treatment with AM+BFTZ (Basal: 3.0±.9% vs AM+BFTZ: 8.1±1.4 %) as well with C21. However C21 treatment further increased the FE_{Na} over FE_{Na}-induced by AM+BFTZ (Figure 24C). Glomerular filtration rate was similar and was not changed after treatment with drugs (Basal, 175±27 µl/min, AM+BFTZ, 192±57 µl/min, C21 167±14 µl/min) (figure 27).

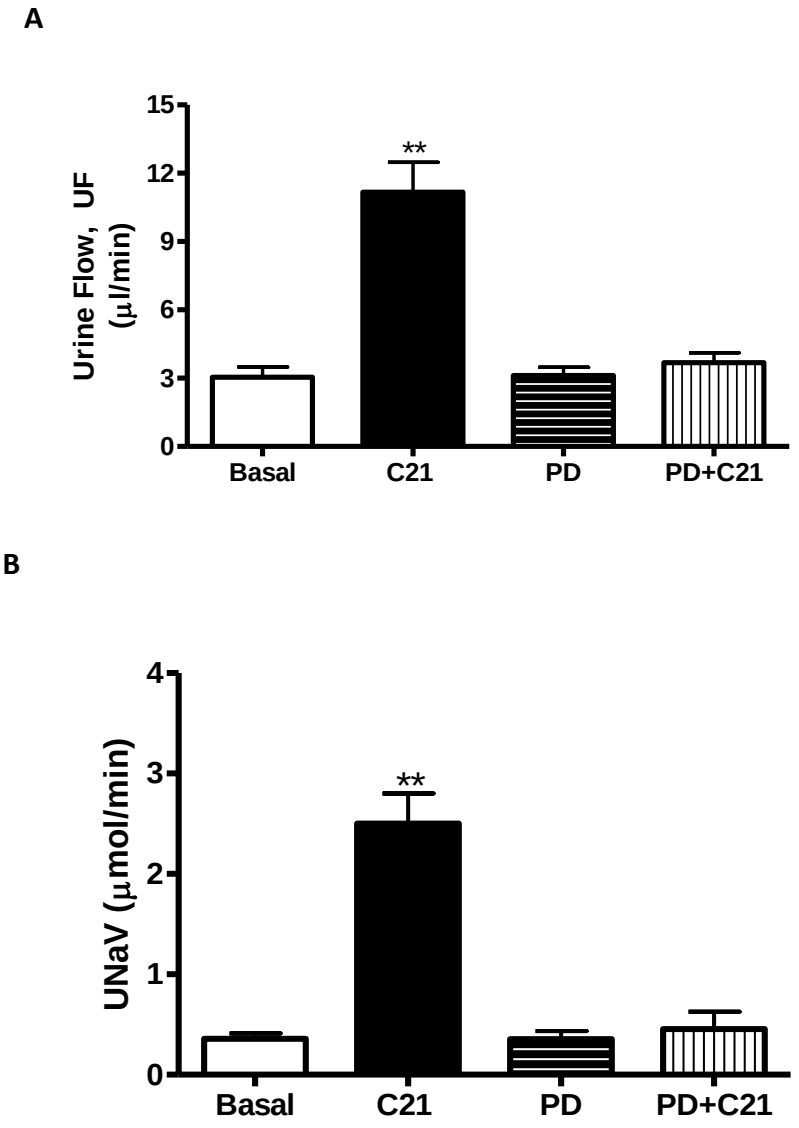

Fig. 23) Effect of PD123319 (50 µg/kg per minute) on **(A)** UF and **(B)** $U_{Na}V$ in obese Zucker rats. **significantly different from basal, Values are are represented as mean ± SEM; One-way ANOVA followed by Neuman-Keuls test, $p<0.05$; N=3 in each group.

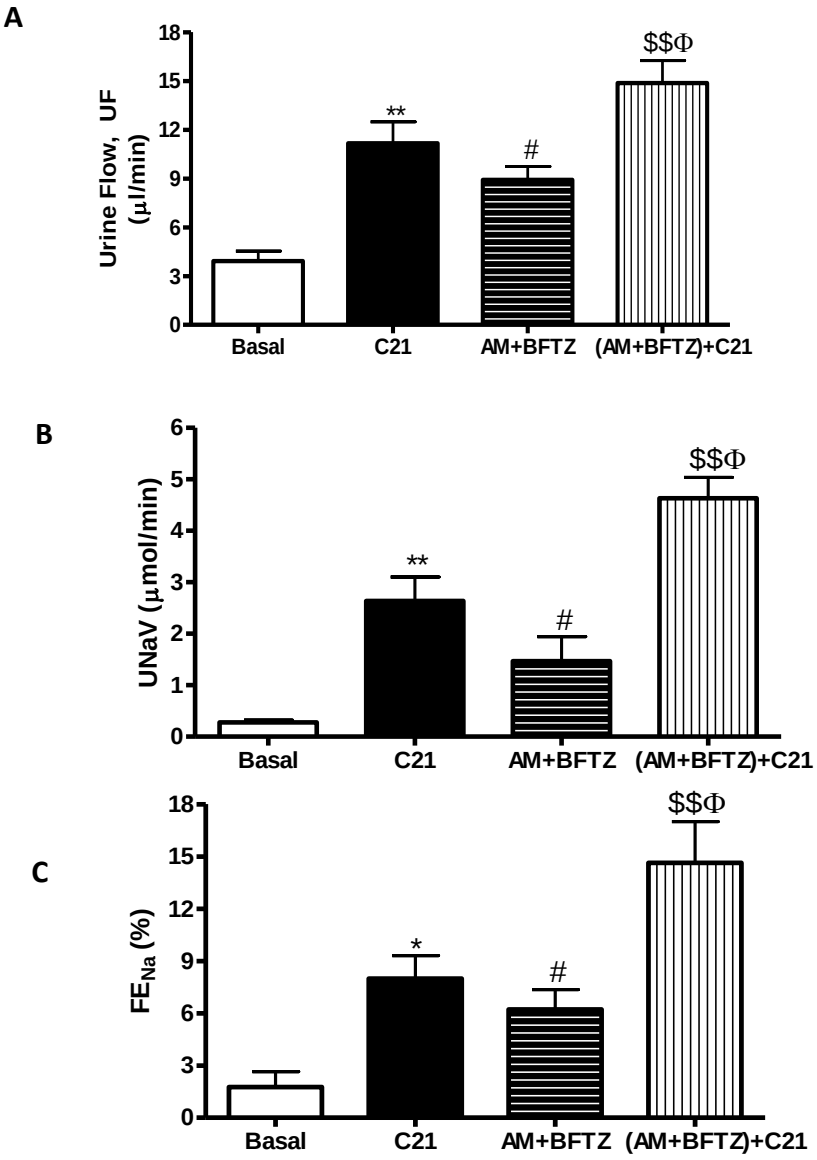

Fig.24) Effect of AM+BFTZ and C21 on **(A)** UF, **(B)** $U_{Na}V$ and **(C)** FE_{Na} in obese Zucker rats. #significantly different from basal, $$Φ significantly different from basal and C21. Values are represented as mean ± SEM; One-way ANOVA followed by Neuman-Keuls test, **significantly different from basal, $p<0.05$; N=7-10 rats.

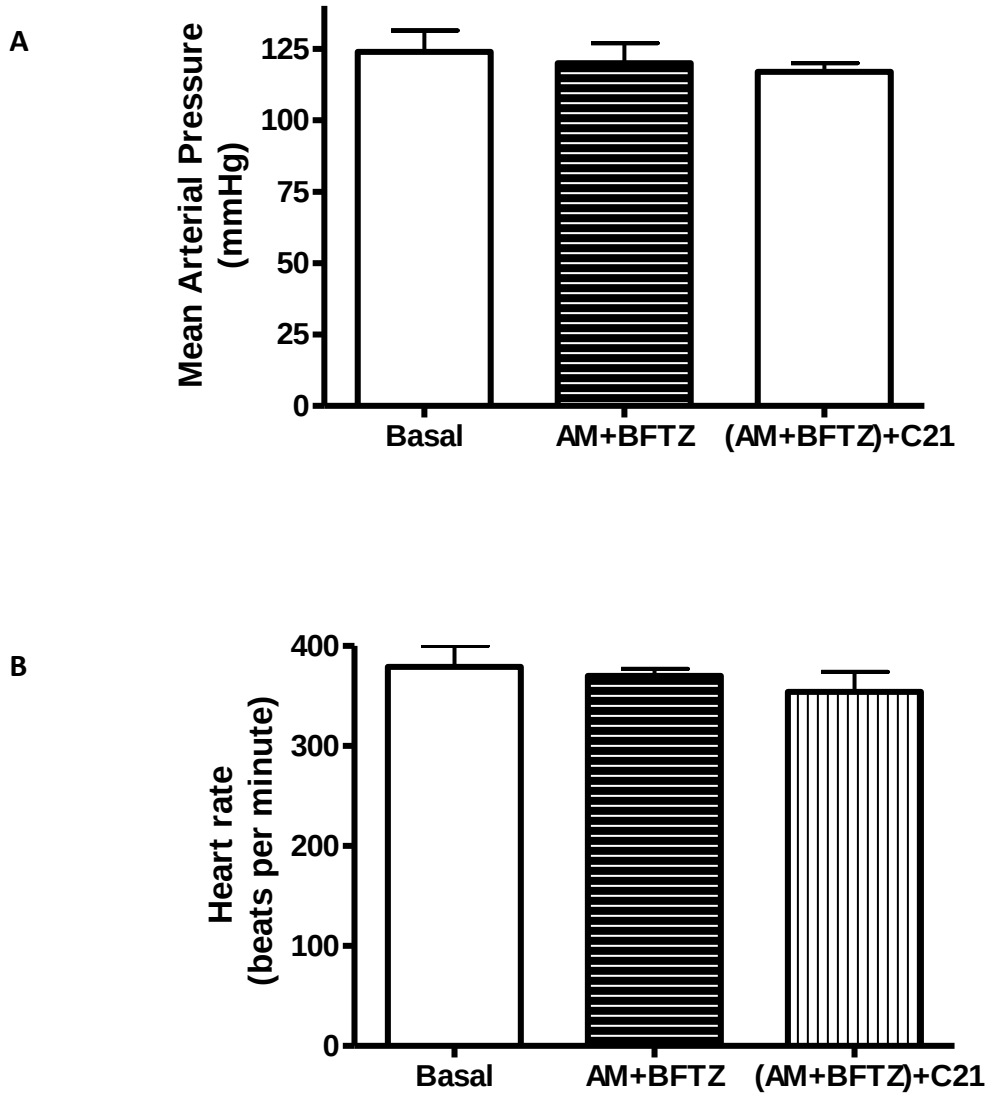

Fig. 25) Effect of AM+BFTZ and C21 on **(A)** mean arterial pressure and **(B)** heart rate in obese Zucker rats. Values are represented as mean ± SEM; One-way ANOVA followed by Neuman-Keuls test, $p<0.05$; N=7-10 in each group.

7.1.5 Effect of AM+BFTZ and C21 on urinary nitrates/nitrites and cGMP in obese Zucker rat

Total urinary nitrates/nitrites and cGMP levels in urine did not change after treating with AM+BFTZ in obese Zucker. C21 treatment significantly increased the total nitrates/nitrites and cGMP levels to approximately 3-fold as compared to basal as well as over AM+BFTZ infusion (figure 26).

7.1.4 Calculation for C21 mediated Na-excretion by proximal and distal tubule

The contribution of proximal tubules was determined by comparing the difference between $U_{Na}V$ after infusion of AM+BFTZ followed by C21 infusion and blockade of distal tubules ($U_{Na}V_{AM+BFTZ+C21}$-$U_{Na}V_{AM+BFTZ}$) alone in OZR. Distal tubular effect of C21 was determined by finding the difference between $U_{Na}V$ after infusion of C21 alone and contribution of proximal tubules ($U_{Na}V_{C21}$-$U_{Na}V_{proximal}$). As shown in figure 28 acute stimulation of AT_2 receptor by C21 led to exaggerated Na-excretion which was almost 2.5 times greater than distal tubules suggesting AT_2 induced Na-excretion predominantly via proximal tubules (proximal 2.7±.5 µmol/min vs distal tubules 0.4±.2 µmol/min).

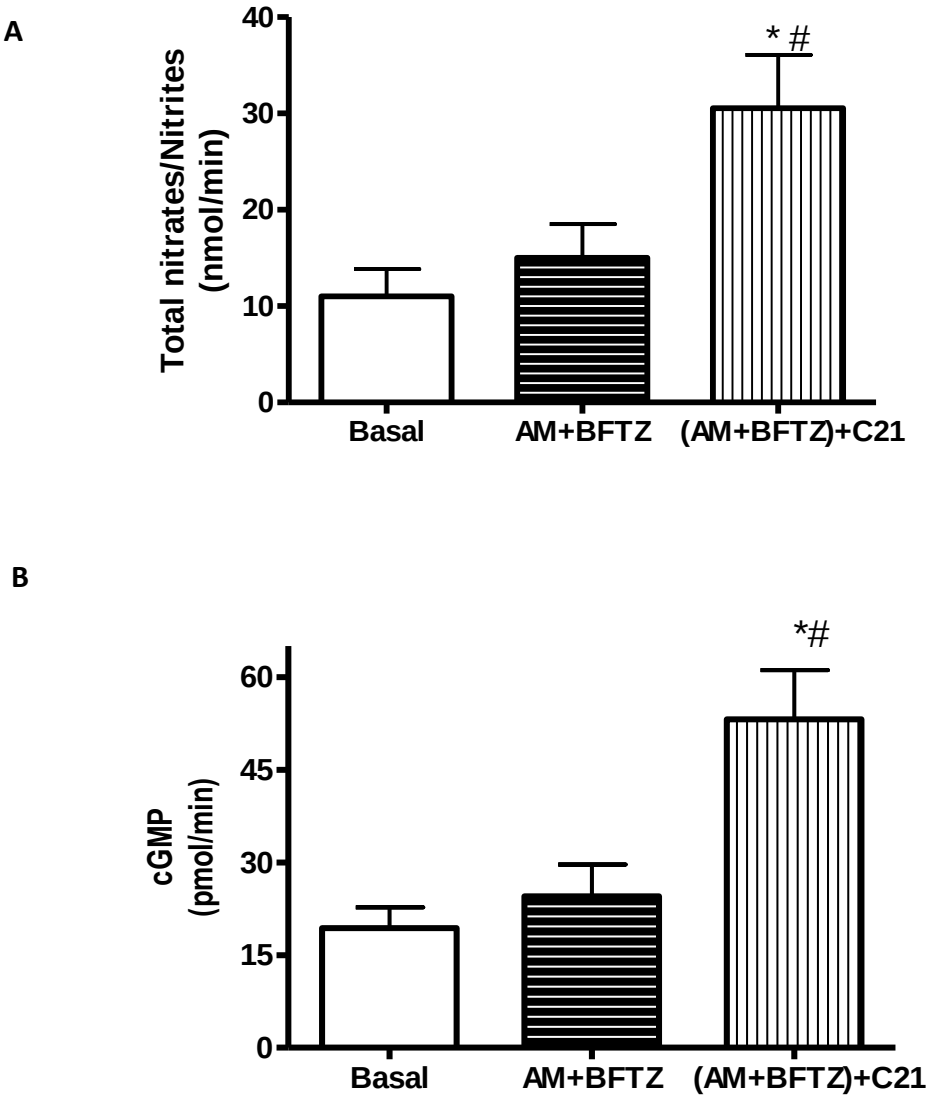

Fig. 26) Effect of AM+BFTZ and C21 on **(A)** urinary nitrates/nitrites and **(B)** cGMP in obese Zucker rats. *significantly different from basal, #significantly different from AM+BFTZ. Values are represented as mean ± SEM; One-way ANOVA followed by Neuman-Keuls test, $p<0.05$; N=7-10 in each group.

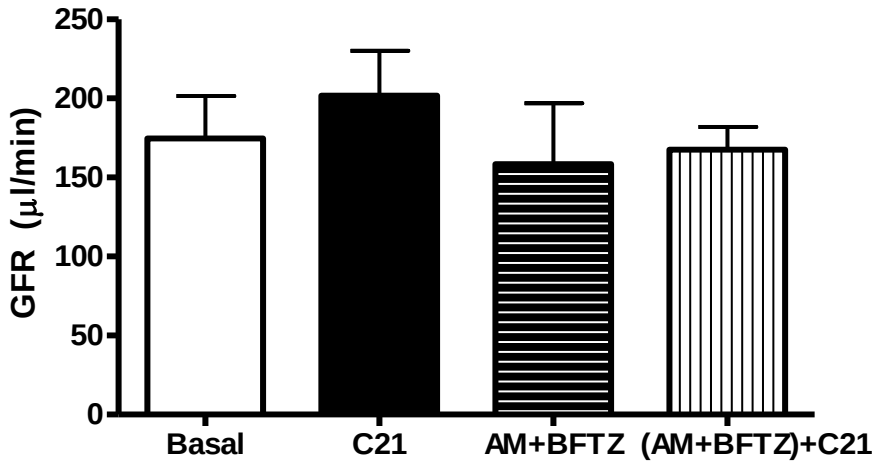

Fig. 27) Effect of AM+BFTZ and C21 on glomerular filtration rate (GFR) measured using creatinine clearance method in obese Zucker rats. Values are represented as mean ± SEM; One-way ANOVA followed by Neuman-Keuls test, $p<0.05$; N=7-10 in each group.

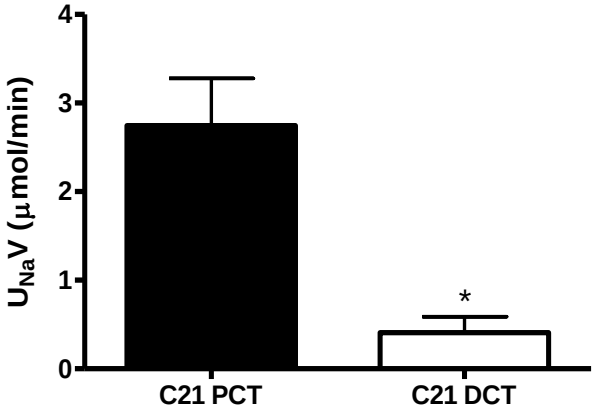

Fig. 28) Effect of C21 on the contribution of proximal and distal tubules to $U_{Na}V$ in obese Zucker rats. *significantly different from C21 proximal. Values are are represented as mean ± SEM; Student t test, $p<0.05$; N=7-10 in each group.

7.2 Discussion

In the present study, for the first time we demonstrate that selective activation of AT_2 receptors by a novel AT_2 receptor agonist C21 promotes Na-excretion predominantly via proximal tubules. The natriuretic response to C21 involves NO/cGMP pathway.

The long-term regulation of blood pressure is linked to the ability of kidney to excrete sodium i.e pressure natriuresis (Guyton, 1991). Several experimental studies suggest that weight gain associated with obesity increases blood pressure (Hall, 2000, Hall et al., 2002). Kidney is severely impaired in obesity associated hypertension and results in impairment of pressure natriuresis. This results in the inability of kidney to effectively excrete Na and therefore it leads to excessive Na absorption. GFR and renal blood flow is increased significantly in response to high tubular Na absorption (Hall, 2003). Earlier we have reported that AT_1 receptor function is increased in obese rats and therefore cause more Na retention in these rats (Shah and Hussain, 2006). Obese Zucker rats are an animal model of obesity and closely resemble the human metabolic syndrome. Recently, we also demonstrated that AT_2 receptors are upregulated in the kidney cortex of obese rats and promotes Na excretion (Hakam and Hussain, 2005). Kidney cortex is composed of proximal tubules, loop of Henle, distal tubules and collecting ducts. Several studies suggest that AT_2 receptors are expressed in the different regions of the kidney cortex including proximal and distal tubules (Zimpelmann and Burns, 2001, Tejara et al., 2004). But the contribution of different nephron segments especially proximal and distal

tubules in AT_2R-induced natriuresis is not known. Although, proximal tubule is the major site of Na reabsorption (Féraille and Doucet, 2001) but distal tubules is also of particular importance in regulating Na for the proper maintenance of Na balance, osmolality of the fluid and blood pressure. In this study we investigated the involvement of proximal tubule AT_2 receptors in natriuresis by blocking the two important distal tubule Na-transporters (sodium chloride cotransporter and epithelial sodium channel. AM+BFTZ are Na channel blockers and belongs to the class of diuretics. Infusion of these drug caused significant diuresis/natriuresis. Selective infusion of AT_2 receptor agonist C21 after administration of AM+BFTZ caused further increase in diuresis/natriuresis but did not change either the blood pressure or the heart rate in any of the treatment groups, suggesting that renal hemodynamic are not affected during drug infusion. Consistently, treatment with these drugs did not change the GFR suggesting a tubular effect of the drugs. Blocking the distal tubules Na-transporters by AM+BFTZ, fractional sodium excretion rates (FE_{Na}) increased from 1% to 6% and 15% after infusion of C21 in the presence of AM+BFTZ, thus demonstrating the tubular effect and reflecting the Na-excretion as a result of change in sodium transporters in the tubules. Blocking Na channels in distal tubules with AM+BFTZ led to natriuresis which is an indicator of Na delivery to the distal tubules. In the present study, this sodium delivery to the distal tubules increased after C21 treatment. This increase in the Na delivery is an indicator of

decreased Na reabsorption at the earlier tubular segments (proximal tubules and loop of Henle).

Here, we could not delineate the contribution of proximal tubules and loop of Henle in natriuresis which is a limitation of the present study. However we believe that it's the proximal tubules which are contributing to natriuresis because majority of the filtrate is reabsorbed in the proximal tubules and not loop of Henle. More importantly, literature available so far does not suggest the expression of AT_2 receptors in the loop of Henle.

AT_2 receptor has a role in vasodilatation and natriuresis and this is linked via bradykinin (BK)/NO/cGMP pathway. However NO and cGMP can be produced in the kidney directly by the stimulation of AT_2 receptors in the proximal tubules. (Abadir and Siragy, 2003, Hakam and Hussain, 2006). NO and cGMP produced as a result of direct AT_2 receptor stimulation causes natriuresis by inhibiting Na-transporters in the renal tubules. Our laboratory has earlier demonstrated that AT_2 receptors are significantly increased in brush bordered membrane (BBM) and basolateral membrane (BLM) in kidney cortex of STZ induced diabetic rats. These upregulated AT_2 receptors lead to increased urinary cGMP levels which were abolished by infusing AT_2 receptor antagonist PD. This suggests that cGMP is an important signaling mediator in response to AT_2 receptor activation. NO generated as a byproduct of AT_2 receptor stimulation also acts as a natriuretic factor and directly inhibits Na transport in the proximal tubules and collecting ducts of the kidney. Infusion of C21 after blocking the distal tubule Na transporters produced

significant increase in urinary NO/cGMP which demonstrates that C21 promotes natriuresis/diuresis by NO/cGMP pathway.

Obesity associated hypertension is linked to increased SNS activity and dopaminergic system activity which ultimately leads to an increase in blood pressure (Hall et al., 2003; Banday et al., 2003). Apart from defective dopaminergic system and increased sympathetic activity in obesity, RAS is overactive and leads to enhanced Na-reabsorption in the proximal tubules. Some studies suggest that although excessive stimulation of proximal tubules might leads to less Na-excretion and hypertension (Hall et al., 2000), but distal tubules might also play an important role in sodium regulation. The evidence for the role of distal tubules Na-transporters in hypertension comes from genetic studies wherein single-point mutation in hypertensive individuals has overactive epithelium Na-channels and Na^+/Cl^- co-transporters in the distal tubules (Zhao and Navar, 2009). Several drugs including diuretics are considered first line of therapy for hypertensive individuals because they primarily act on distal tubules which are the site for the fine regulation of Na. However, opposite to other antihypertensive drugs, we found that selective activation of AT_2 receptors does not promote natriuresis via distal tubules. Here we have only calculated the Na-excretion via proximal tubules and distal tubules but we did not have data to see how much exactly the sodium absorption was occurring in these two nephron segments in obese Zucker rats.

In summary, we found that the selective activation of AT_2 receptor by a novel AT_2 receptor agonist C21 in obese rats promoted natriuresis predominantly via proximal tubules. C21 stimulated the production of urinary NO/cGMP. C21 is a novel non-peptide and has the potential to become antihypertensive drug. Using pharmacological interventions we found out that as opposed to other class of antihypertensive drugs, C21-mediated natriuresis is mainly based on proximal tubules, with minor effects in the distal parts of the nephron.

8. HIGH GLUCOSE UP-REGULATES ANGIOTENSIN II SUBTYPE 2 RECEPTORS VIA INTERFERON REGULATORY FACTOR-1 IN PROXIMAL TUBULE EPITHELIAL CELLS

8.1 Results

8.1.1 *AT_2 receptor and IRF-1 expression in HK2 cells and obese Zucker rat proximal tubules:* Western blotting demonstrates the presence of IRF-1 as approximately 52 kDa band and AT_2 receptor as 45 kDa band in HK2 cell lysates as well as in the proximal tubules. Densitometric analysis of IRF-1 and AT_2 receptor bands revealed that 24 hr incubation of HK2 cells with high glucose (25 mM) caused an increase in the AT_2 receptor and the IRF-1 expression, as compared to control HK2 cells incubated with normal (5 mM) glucose (figure 29A & 32). Sorbitol (20 mM+5 mM glucose) treatment of HK2 cells for 24 hr had no significant effect on AT_2 receptor expression (figure 29C) suggesting that the change in expression of the AT_2 receptor protein during high glucose incubation was not due to hyper-osmolarity of the medium. Similarly, densitometric analysis revealed that the IRF-1 expression as well as the AT_2 receptor expression in the proximal tubules was significantly elevated in obese compared with lean Zucker rats (figure 30C & 30A).

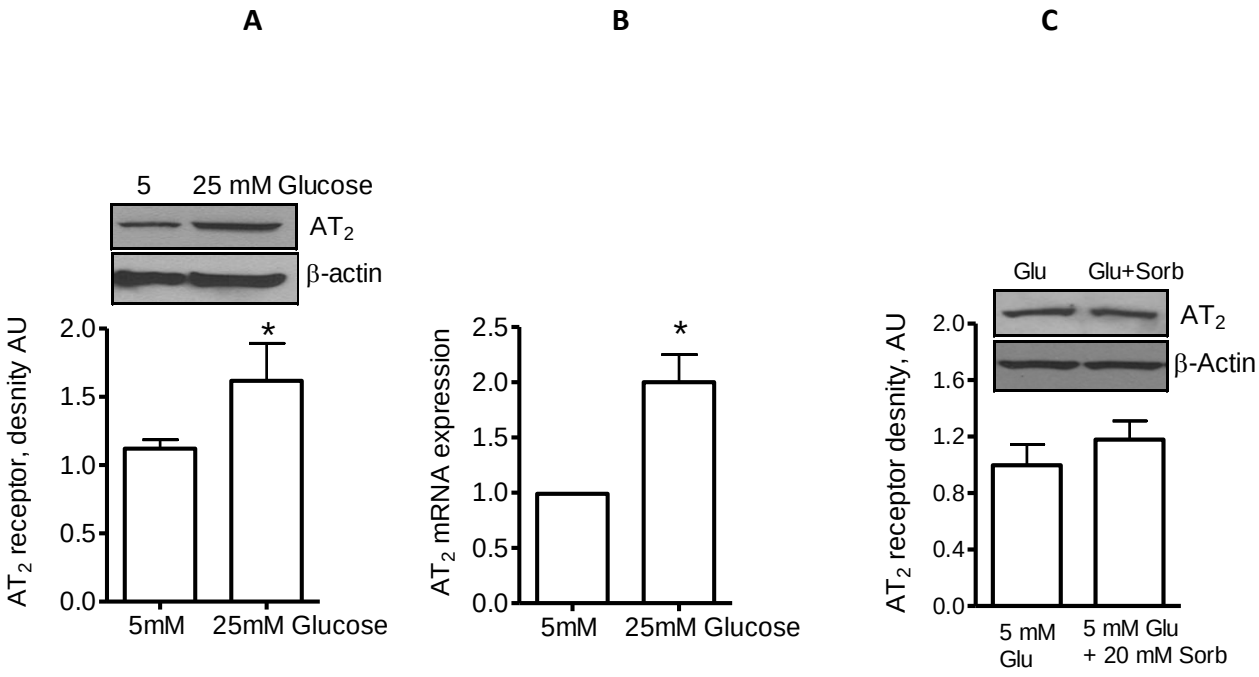

Fig 29) HK2 cells: (A) AT_2 receptor expression in HK2 cells treated for 24 hrs with normal (5mM) and high glucose (25 mM). Upper panel: representative Western blots. Lower panel: bar graph of band density of AT_2 receptor normalized with β-actin. **(B)** AT_2 mRNA expression measured by qRT-PCR in HK2 cells treated for 24 hrs with normal (5 mM) and high (25 mM) glucose. **(C)** AT_2 receptor expression in HK2 cells treated for 24 hrs with normal glucose (5mM) and sorbitol (sorbitol 20 mM + glucose 5mM). Upper panel: representative Western blot. Lower panel: bar graph of band density of AT_2 receptor normalized with β-actin. Values are represented as mean ± SEM, *significantly different from 5mM glucose. (Student's t test, $p<0.05$ n=4 in each group).

qRT-PCR analysis also revealed a significant increase in AT_2 mRNA levels in both the high glucose-treated cells as compared to control HK2 cells (figure 29B) and the proximal tubules of obese Zucker rats as compared to their lean controls (figure 30B).

8.1.2 Effect of IRF-1 knockdown on glucose-induced upregulation of AT_2 receptor:

To study the role of IRF-1 in high glucose-induced AT_2 receptor upregulation, we optimized the conditions to knock-down IRF-1 using IRF-1 siRNA (10 nM and 100 nM) for 24 and 48 hrs (figure 31). Treatment with siRNA (100nM) for 48 hrs down regulated IRF-1 protein expression by 50% in HK2 cells. Figure 32 shows that IRF-1 siRNA did not affect the expression of IRF-2, suggesting the specificity of the siRNA. The reduction in IRF-1 expression was associated with the reduction in AT_2 expression in HK2 cells. However, incubation of 100 nM siRNA-treated cells with high glucose restored the expression of both the IRF-1 and the AT_2 receptors (figure 32). In another set of experiments, we used higher siRNA concentration (500 nM) in order to prevent the effect of high glucose on IRF-1 expression. Higher siRNA concentration was able to maintain lower IRF-1 expression even in the presence of high glucose. This lowering of IRF-1 expression abolished glucose-induced AT_2 receptor upregulation (figure 33). The data clearly suggest that glucose induces an IRF-1 dependent upregulation of AT_2 receptor.

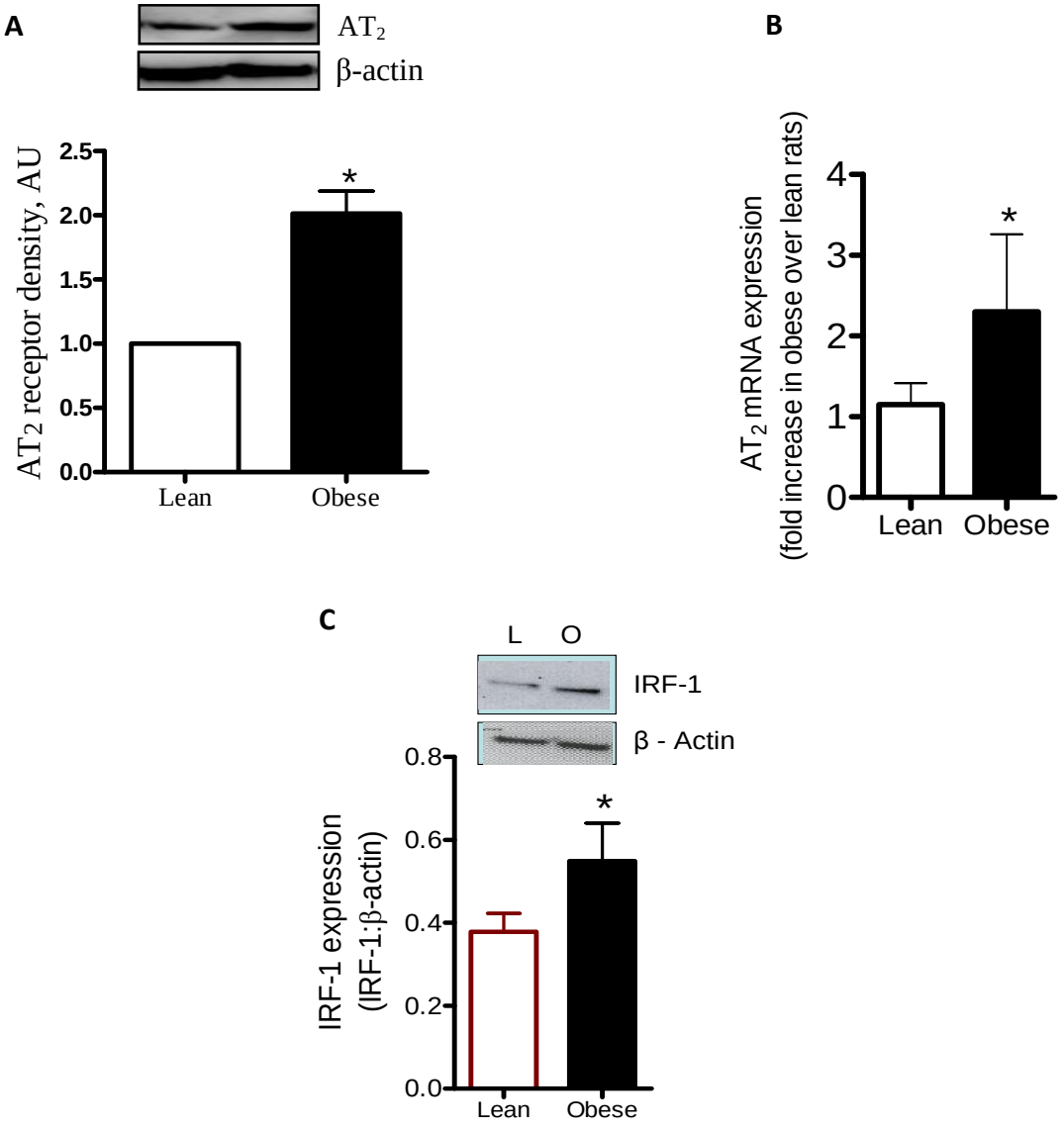

Fig 30) Proximal tubules: (A) AT_2 receptor expression in the proximal tubules of lean and obese Zucker rats. Upper panel: representative western blot. Lower panel: bar graph of band density of AT_2 receptor expressed as percent of lean. **(B)** AT_2 receptor mRNA expression measured by qRT-PCR in proximal tubules of lean and obese Zucker rats. (**C)** Expression of IRF-1 in proximal tubules of lean and obese Zucker rats. Upper panel: representative Western blot. Lower panel: bar graph of band density of IRF-1 normalized with β-actin. Values are represented as mean ± SEM, *significantly different from lean rats. (Student's t test, $p<0.05$ n=4 in each group).

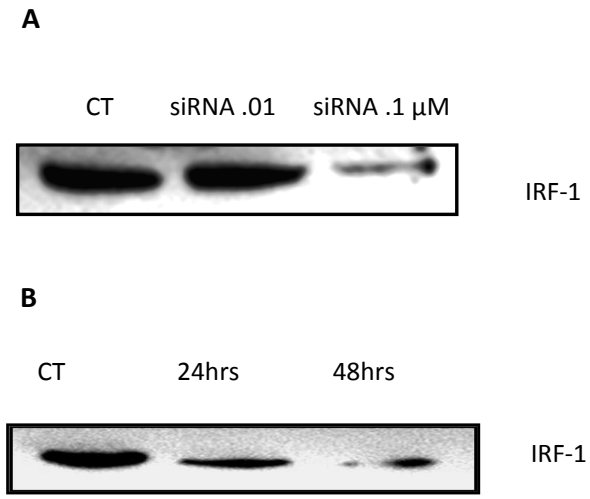

Fig 31) Concentration and time course study of siRNA transfection: IRF-1 expression in HK2 cells transfected with (A) different concentrations of siRNA IRF-1 (10 and 100 nM) for 48 hrs and (B) 100 nM siRNA IRF-1 for 24 and 48 hrs.

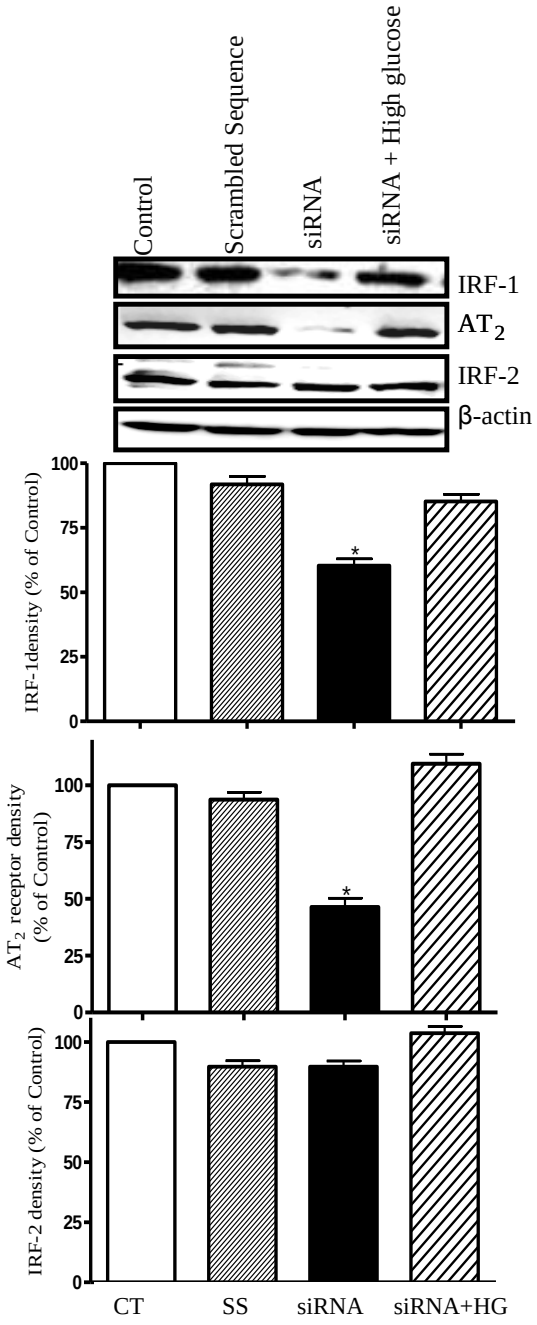

Fig 32) Effect of glucose (25 mM) on the expression of AT_2 receptor and IRF-1 in HK2 cells transfected with 100 nM IRF-1 siRNA. Upper Panels: Western blots of IRF-1, IRF-2, AT_2 receptor and β-actin. Lower panels: bar graphs of band density of IRF-1, IRF-2, AT_2 normalized with β-actin. *significantly different from control HK2 cells. Values are represented as mean ± SEM; One way ANOVA followed by Neuman-keuls test, $p<0.05$, n= 3). (CT- Control, SS-Scrambled Sequence, HG-High Glucose)

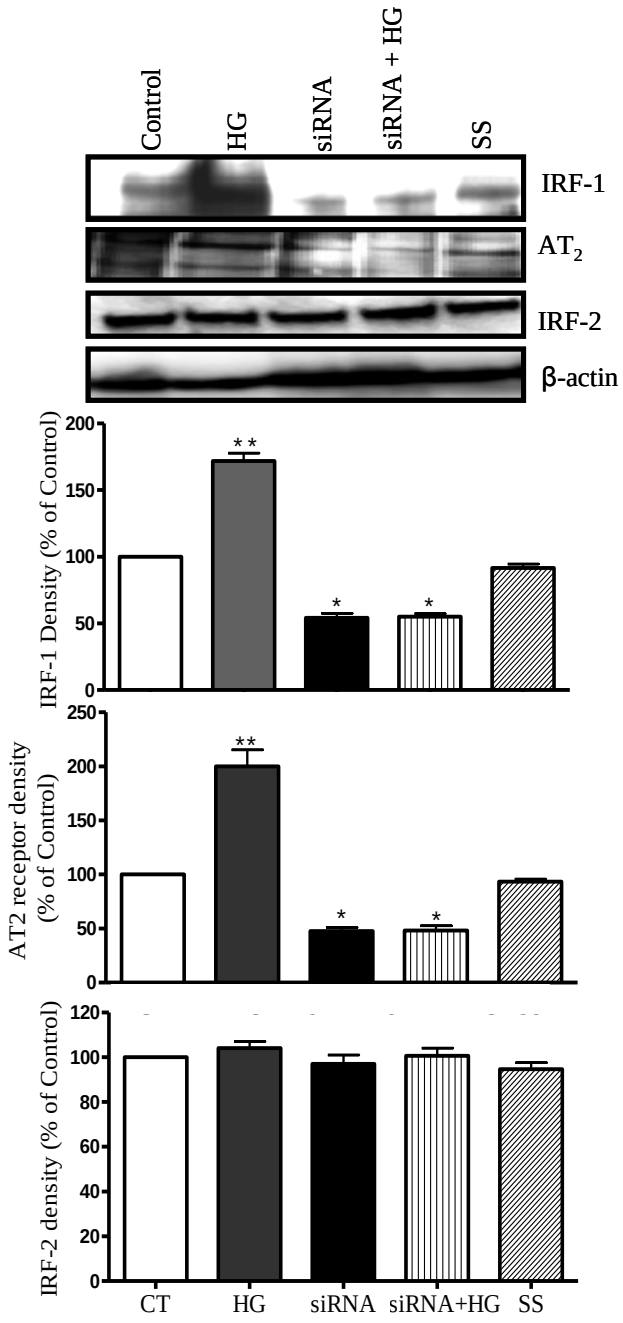

Fig 33) Effect of glucose (25 mM) on the expression of AT_2 receptor and IRF-1 in HK2 cells transfected with 500 nM IRF-1 siRNA. Upper Panels: Western blots of IRF-1, IRF-2, AT_2 receptor and β-actin. Lower panels: bar graphs of band density of IRF-1, IRF-2, AT_2 normalized with β-actin. **, *significantly different from control HK2 cells. Values are represented as mean ± SEM; One way ANOVA followed by Neuman-keuls test, $p<0.05$, n=3. (CT-Control, HG-High Glucose, SS-Scrambled sequence).

8.2 Discussion

As discussed before, we observed that AT_2 receptors are upregulated in obese Zucker rats and this upregulated AT_2 receptor has a protective role in blood pressure. In this study we determined the possible mechanism of AT_2 receptors upregulation observed in obese rats. The present study directly demonstrates that high glucose elicits up regulation of AT_2 receptors in the proximal tubule epithelial cells and this process is mediated via an increase in the expression of transcription factor IRF-1.

Earlier, we have reported increase in the AT_2 receptor expression in the proximal tubules of obese Zucker rats as well as in the streptozotocin-induced diabetic rats (Hakam and Hussain, 2005; Hakam et al., 2006). This upregulation of AT_2 receptor potentially promotes renal sodium excretion and protects against blood pressure increase in these animals (Siddiqui et al., 2009). The incubation of HK2 cells with high glucose concentration suggests that glucose has a positive regulatory role in AT_2 receptor expression. Hyperglycemia is known to activate various stress kinases leading to hypertrophy and tissue damage, including kidney damage (Satriano., 2007). The increase in AT_2 receptor expression in response to high glucose could be a compensatory mechanism to improve kidney function and protect against blood pressure increase in diabetic animals. In pursuit of understanding the mechanism of glucose-induced AT_2 receptor expression, we made 2 observations, one- increase in AT_2 receptor mRNA along with AT_2 receptor protein, and two-increase in IRF-1 expression in

the proximal tubules of obese Zucker rats suggesting a potential association between the increases in the AT_2 receptor and the IRF-1. This notion is supported by the IRF-1 knock-down studies. However, high glucose reverted the knock-down of IRF-1 expression by siRNA (100 nM), which was associated with simultaneous reversal in the reduction of AT_2 receptor expression. This suggested that high glucose is a potent stimulus, which overcame effectiveness of 100 nM siRNA on IRF-1 expression and hence on AT_2 receptor expression. In order to maintain IRF-1 knock-down in the presence of high glucose, we used higher concentration of siRNA (500 nM). With this strategy, we were able to keep the low expression of IRF-1, and high gluocse was unable to increase AT_2 receptor expression, suggesting a role of IRF-1.

How does glucose regulates IRF-1 expression is not clear. However, p38 pathway may be a plausible mechanism responsible for high glucose-induced IRF-1 up-regulation/activation leading to AT_2 receptor transcription. Activation of p38 has been implicated in the cellular response to hyperglycemia. High levels of glucose stimulate p38 in proximal tubular cells and other kidney cells such as mesangial cells (Zhang et al., 2000). Also, enhanced p38 activation is reported in the proximal tubules of obese Zucker rats (Banday et al., 2007). Moreover, the activated p38 pathway causes activation of a number of transcription factors, including IRF-1 (Cheong et al., 1998; Faure et al., 1999; Seymour et al., 2006; Horiuchi et al., 1995; Sanchez-Nino et al., 2009; Giovanna et al., 2002). Since IRF-1 regulates AT_2 receptor transcription, it might be possible that glucose-

induced AT_2R up-regulation involves IRF-1 via p38 pathway. Glucose is known to stimulate various inflammatory signals leading to tissue injury, including kidney (Satriano., 2007). For example high glucose concentrations activate TNF-α (tumor necrosis factor-α) and migration inhibitory factor (MIF) which leads to phosphorylation of p38 and ERK1/2 pathway (Zhang et al., 2000). The increase in AT_2 receptor expression in response to high glucose appears to be beneficial, in terms of renal function and protection against blood pressure increase (Siddiqui et al., 2009). However, it is not clear whether increase in the IRF-1 expression in response to hyperglycemia has other cellular and physiological consequences. IRF-1 is known to act as a transcriptional activator and is regulated by different cytokines such as IFN-α, IFN-γ and mediates cell growth, transformation and apoptosis (Sanchez-Nino et al., 2009; Tadatsug et al., 2001). IRF-1 is dramatically upregulated in certain path-physiological conditions (Taniguchi et al., 2001). If increase in the IRF-1 expression and hence in AT_2 receptor expression and function are beneficial to the diabetic kidney and blood pressure control, these molecules provide novel targets to treat kidney disease and hypertension. Studies for understanding the role of selective activation of AT_2 receptor in long-term blood pressure control are underway.

In summary, we found that AT_2 receptor and IRF-1 expression is increased in proximal tubules of obese Zucker rats and in HK2 cells treated with high glucose concentration. The siRNA knock-down studies suggest that glucose is a potent stimulator of IRF-1

expression, which mediates increase in AT_2 receptor expression in response to high glucose. While there is reasonable physiological relevance of AT_2 receptor, the role of IRF-1 in renal physiology and blood pressure control needs to be investigated.

SUMMARY AND CONCLUSION

Summary

1. We demonstrate that blocking AT_2 receptor by PD123319 significantly increased blood pressure in obese rats with a significant increase in renal renin expression. However no change was observed in the expression of AT_2 or AT_1 receptor in the kidney cortex of obese PD-treated rats as compared to control rats.
2. On the other hand, treating lean and obese rats with AT_2 agonist CGP42112A significantly decreased blood pressure in obese. There was no change in blood pressure in lean rats after CGP-treatment. This decrease in blood pressure in response to CGP42112A is likely caused by decrease in positive Na balance. The CGP42112A treatment significantly increased ACE2 expression and activity in the kidney cortex of obese rats.
3. Chronic activation of AT_2 receptor by CGP-treatment did not affect the AT_1 receptor expression, but chronic treatment of obese rats reduced the Ang II-mediated pressor response and blunted the candesartan mediated natriuresis as compared to control rats suggesting that AT_2 receptor has reduced AT_1 receptor function.
4. Novel AT2 receptor non-peptide agonist C21 caused significant natriuresis/diuresis in obese, not in lean rats. Further pharmacological interventions revealed that C21-mediated natriuresis is mainly based on

proximal tubules, with minor effects in the distal parts of the nephron. Also, we found that C21 stimulated the production of urinary NO/cGMP.

5. We found that high glucose elicits up regulation of AT_2 receptors in the proximal tubule epithelial cells and this process is mediated via an increase in the expression of transcription factor IRF-1.

Conclusion

AT_2 receptor has a protective role in blood pressure by regulating RAS components at multiple levels in obese Zucker rats. Selective activation of AT_2 receptors in obese rats counteracts the AT_1 receptor functions, increases ACE2 expression/activity and promotes natriuresis mainly at proximal tubules. We therefore conclude that AT_2 receptors can serve as a potential therapeutic target to improve kidney function and treat obesity associated hypertension.

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