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Chronic AT2 receptor activation attenuates renal AT1 receptor function and blood pressure in obese Zucker rats

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Abstract

Abnormal regulation of the renin angiotensin system such as enhanced renal AT1R function and reduced ACE2 activity contributes to obesity-related hypertension. Here we tested whether long-term AT2R activation affects renal function in obesity using lean and obese Zucker rats treated with the AT2R agonist CGP42112A for 2-weeks. This caused blood pressure to decrease by 13 mmHg which was associated with increased urinary sodium excretion in the obese rats. Cortical ACE2 expression and activity, the Mas receptor (MasR), and its ligand angiotensin-(1-7) were all increased in CGP-treated obese compared with control rats. Candesartan-induced natriuresis, a measure of AT1R function, was reduced but cortical AT1R expression and angiotensin II levels were similar in CGP-treated obese compared to control rats. Renin and AT2R expression in obese rats was not affected by CGP-treatment. In HK-2 cells *in-vitro*, CGP-treatment caused increased ACE2 activity and MasR levels but decreased AT1R levels and renin activity. Thus, long-term AT2R activation shifts the opposing arms of renin angiotensin system and contributes to natriuresis and blood pressure reduction in obese animals. Our study highlights the importance of AT2R as a target for treating obesity related hypertension.

Keywords

CGP42112A; obesity; RAS; renin. AT₂ receptor

Introduction

Renin angiotensin system (RAS) is comprised of peptide hormones, whose production and biological activities are regulated by multiple enzymes and receptors. Renin is a critical regulator of RAS, it catalyzes the conversion of angiotensinogen into angiotensin I (Ang I),

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which is further converted to Ang II by angiotensin converting enzyme (ACE). ACE2, an isoform of the ACE, converts Ang I and Ang II to Ang-(1-9) and Ang-(1-7), respectively. Ang-(1-9) is further converted to Ang-(1-7) by ACE.¹⁻³

Ang II is a pivotal hormone of RAS and works via activating angiotensin type 1 (AT₁R) and type 2 (AT₂R) receptors.³ Activation of AT₁R promotes cell growth, induces vasoconstriction, antinatriuresis and blood pressure (BP) increase.³ Relative to the AT₁R, AT₂R is less studied,³ but has been shown to produce cellular and physiological responses that are opposite to those of the AT₁R.^{4, 5} Thus, AT₂R activation inhibits cell growth,⁶ promotes cell apoptosis⁷ and differentiation,⁸ contributes to natriuresis,⁹⁻¹² vaso-relaxation¹³ and potentially lowers BP.¹⁴⁻¹⁶ Ang-(1-7) is an important component of RAS and studies suggest that Ang-(1-7) causes vasodilatation and natriuresis by inhibiting Na-transport¹⁷⁻¹⁹ mainly via Mas receptors (MasR).²⁰ However, there is evidence suggesting that Ang-(1-7) has affinity for AT₁R and mediates a biphasic response on fluid absorption²¹ and inhibits cell growth in proximal tubules but induces mesangial cell growth.^{22, 23} Other non ACE2 enzymes such as neprilysin and prolyl-endopeptidase also catalyze the production of Ang-(1-7).²⁴ Overall, ACE/Ang-II/AT₁R and ACE2/Ang-(1-7)/MasR are considered as two arms of the RAS with opposite functions.

Abnormal renal Na excretion is believed to be a major risk factor in obesity-related hypertension.²⁵ Hyperactivity of the RAS, mainly enhanced AT₁R function is suggested to contribute to the excessive renal Na reabsorption and BP increase in obesity.²⁶⁻²⁸ Recently, we have demonstrated that the renal AT₂R expression is increased in obese rats^{9, 16}, which inhibits proximal tubules Na-pump via NO/cGMP pathway and promotes natriuresis in these animals.^{12,29} We also reported that AT₂R in the long-term may have a protective role against BP increase in obese rats.¹⁶ Additionally, there is evidence, including from our laboratory, suggesting that AT₂R exerts anti-oxidative stress and anti-inflammatory activity.³⁰ Both oxidative stress and inflammation, positively regulate each^{31, 32} other and have been implicated in the renal dysfunction and pathogenesis of hypertension via modulating RAS components such as increasing AT₁R function.³¹

The RAS components apart from having independent effects also influence the function and generation of other RAS components and these altogether contribute to the net renal function and BP change. It has been reported that AT₂R is implicated in attenuation of AT₁R-mediated myocyte growth and chronotropic effect³³ and provides negative feedback on renin activity.³⁴ There is evidence showing that Ang II via AT₁R activation decreases ACE2 activity³⁵ and Ang-(1-7) production. Since evidence clearly suggests an enhanced renal AT₁R function, which contributes to excessive renal Na-absorption and obesity-related hypertension,^{9,23,30} it is not known whether chronic AT₂R activation attenuates renal AT₁R function and enhances ACE2-Ang-(1-7) activity/expression in obesity. We hypothesize that AT₂R activation attenuates AT₁R-mediated antinatriuresis and increases cortical ACE2 activity/expression and Ang-(1-7) production in obese rat. We tested this hypothesis by treating obese rats with AT₂R agonist for 2-weeks, followed by measuring renal AT₁R function and the expression of ACE2-Ang-(1-7)-MasR. The changes in these RAS components were correlated with renal Na excretion and BP changes.

Results

General Parameter

Compared with lean, obese rats had greater body weight (lean 295 ± 8 vs obese 473 ± 12 gm) (Table 1) and consistently consumed more food (Fig 2A) and water (Fig 2B) over the treatment period. The CGP-treatment did not affect the food and water intake in either of the rat strains.

Hemodynamic and Renal Functions—As shown in table 1, treatment with CGP for 2-weeks decreased the BP in conscious obese animals by 13 mm Hg without affecting the heart rate. Since we have reported earlier that CGP42112A treatment did not change the BP of lean Zucker rats,³⁰ we did not measure BP in lean rats in this study. However, we measured BP in obese rats to ensure whether the BP changes reported earlier under anesthesia³⁰ are reproducible in conscious animals. Renal blood flow was significantly higher in obese compared to lean rats and was not affected by CGP-treatment. The kidney weight of obese rats was higher compared to that of lean rats and was also not affected by CGP-treatment. Obese rats excreted higher amount of total urinary nitrates/nitrites compared to lean rats. Consistent with previous studies,³⁶ excretion of urinary nitrates/nitrites, as measured on days 2 and 14 of the treatment period was greater in obese rats compared to lean rats. CGP-treatment had no effect on nitrates/nitrites excretion in lean rats but caused an increase in obese rats only during the initial (measured at 2 day) treatment period. The daily urinary volume (UV) in obese rats was greater than that of lean rats (Fig 2C). However, the daily urinary Na excretion was significantly increased in obese CGP-treated rats during early days of the treatment and mostly stayed high through remaining treatment period (Fig 2D). The GFR as measured by FITC-inulin clearance on days 6, 9 and 14 in obese rats was elevated when compared to that of lean rats. Treatment with CGP for 2-weeks did not cause any change in GFR in either lean or obese rat (Fig 2E).

RAS components

AT₁R and AT₂R: Western blot showed presence of AT₂R as two bands (44 and 39 kDa), which is likely due to various degrees of glycosylation.⁹ Earlier we reported that deglycosylation of AT₂R protein shifted all bands to one band, which was displaced by the AT₂R-blocking peptide.⁹ Also we have validated the antibody using AT₂R knock-down (using siRNA) kidney samples (unpublished data). Analysis of AT₂R bands revealed that the AT₂R expression was significantly elevated in obese compared to lean rats, as reported earlier.¹⁶ The CGP-treatment caused a modest but insignificant increase in the AT₂R expression in obese rat (Fig 3A). Analysis of AT₁R bands at 43 kDa suggests that the AT₁R expression was similar in lean and obese rats and CGP-treatment did not cause any change in its expression (Fig 3B).

Renin and ACE: Analysis of the renin band at 41 kDa revealed that the renin expression in obese rats was significantly lower by approximately 45% compared to lean rats and was not affected by CGP-treatment (Fig 4A). Analysis of the ACE bands at 170 kDa revealed that the ACE expression in obese rats was significantly higher compared to lean rats. The CGP-treatment of lean and obese rats did not affect the ACE expression (Fig 4B).

ACE2 and Mas receptor: Western blot demonstrates the presence of ACE2 as approximately 90 kDa 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 (Fig 5A), but it had no effect in lean rats. ACE2 activity in the kidney cortex of obese rats was lower than in lean rats, and CGP-treatment significantly increased this activity in obese rats (Fig 5B). Changes in ACE2 protein expression and activity were not parallel which could be due to the unavailability of newly synthesized ACE2 protein post-translates into active protein. Analysis of the Mas receptor bands at 47 kDa revealed that the CGP-treatment increased Mas receptor expression in both lean and obese rats (Fig 5C).

Ang II and Ang-(1-7) peptides: The LC/MS quantification of angiotensin peptides revealed that the levels of Ang II and Ang-(1-7) were similar in control lean and obese rats. CGP-treatment caused no changes in Ang II levels but a 2-fold increase in Ang-(1-7) peptide levels in the kidney cortex of obese rats (Fig 6 A-B).

Effect of AT₁R antagonist candesartan-induced diuresis and natriuresis: To study the AT₁R function on diuresis and natriuresis in obese rats we infused candesartan (100 µg/kg, i.v. bolus) under anesthesia. As shown in figure 7(A-B), infusion of candesartan in control obese rats increased diuresis and natriuresis, which were significantly attenuated in obese CGP-treated rats. Administration of AT₁R antagonist candesartan did not affect the BP or heart rate in either of the groups (Fig 7C-D). Previously this dose of candesartan had been reported ineffective for BP in obese rats.^{37, 9}

Effect of AT₂R agonist and antagonist on RAS components: To study the effect of AT₂R agonist and antagonist on RAS components under controlled non-pathological conditions, we treated HK-2 cells with CGP42112A (10 nM), PD123319 (1 µM) alone or in combination. Treatment with CGP caused an increase in the ACE2 activity and MasR expression, while it caused a decrease in the renin activity and AT₁R expression (Fig 8 A-D). These changes were blocked by simultaneous treatment with PD. Treatment with PD alone did not affect the activity/expression levels of these RAS components (Fig 8 A-D).

Discussion

The novel findings of this study are that AT₂R agonist treatment caused changes in two arms of the RAS, namely decrease in AT₁R function and increase in the levels of ACE2-Ang-(1-7)-MasR axis, and that these changes likely are directly related to AT₂R activation as suggested by the *in-vitro* studies. These two arms of RAS have been implicated in exerting opposing effects on natriuresis and blood pressure.^{17, 38-40} The enhanced AT₁R function is one of the mechanisms responsible for enhanced Na reabsorption and BP increase in obese rats.^{9, 26, 28} In this study, decreased candesartan-induced natriuresis in CGP-treated obese rats suggests a reduced AT₁R function. The AT₁R activation has been shown to down-regulate ACE2 expression and activity.³⁵ Therefore it is likely that the reduced ACE2 activity in obese rats was due to the higher AT₁R function, which is blunted by AT₂R treatment leading ACE2 activity bounce back to the normal level. These findings were also supported by cell culture studies, wherein we found that CGP-treatment of HK-2

cells significantly increased ACE-2 activity while lowering the AT₁ receptor expression. The reduced expression of renal ACE2 in obesity is consistent with previous studies.⁴¹

The ACE2 is an important regulatory enzyme which converts Ang II to Ang-(1-7). Analysis of angiotensin peptides revealed that treating obese rats with CGP led to an increased formation of Ang-(1-7) in the kidney cortex. This increase in the levels of Ang-(1-7) could have been due to increased activity of ACE2 leading to a shift in the metabolism of Ang II by converting Ang II to Ang-(1-7), or less efficiently through conversion of Ang I to Ang-(1-9). Surprisingly, we found no significant changes in the Ang II levels in obese CGP-treated rats. Ang-(1-7) binds to MasR and exerts beneficial effects such as vasodilatation and opposes action of Ang II mediated via AT₁R.^{20, 42-44} Ang-(1-7) in the kidney also has been implicated in natriuresis.⁴⁵ Ang-(1-7) upregulates central nitric oxide synthase in spontaneously hypertensive rats and this increase is suggested as a compensatory and protective mechanism against hypertension.⁴⁶ Interestingly, although ACE2 activity was significantly lower in obese rats but Ang-(1-7) was similar in both lean and obese rats. Furthermore, the enhanced activity of ACE2 in CGP-treated obese rats was equal to the lean rats, but the Ang-(1-7) in CGP-treated obese rats was un-proportionally high. These un-parallel changes in ACE2 activity and Ang-(1-7) levels suggest that a non-ACE pathway may be involved in Ang-(1-7) production. Neprilysin and prolyl-endopeptidase are abundantly expressed in the proximal tubules and catalyze Ang I to Ang-(1-7).²⁴ Recently, proangiotensin 12 [Ang-(1-12)] has been identified and is believed to be a precursor to the formation of Ang II and Ang-(1-7) via non-renin dependent pathway.⁴⁷ Although renin is the critical regulator of RAS pathway, these observations also suggest the importance of formation of Ang II and Ang-(1-7) from non-renin/ACE2 dependent sources. In pathological conditions like those in obese rats and in the present study, Ang-(1-7) production could also have been contributed via non-renin/ACE2 enzymatic pathways. Role of AT₂R in regulation of non-renin/ACE2 pathways needs to be investigated in obese kidney.

AT₂R is believed to be a functional antagonist of the AT₁R functions.^{4, 5, 48} As indicated above, our renal function study revealed that candesartan produced marked increase in Na and urine excretion in obese rats which was significantly blunted in CGP-treated obese rats and may have contributed to the enhanced natriuresis and BP reduction. The reduced AT₁R function is not related to the reduction of AT₁R expression or its ligand Ang II in obese rats treated with CGP, as both the cortical AT₁R expression and Ang II levels were similar in control and CGP-treated obese rats. Our earlier studies using western blotting, RT-PCR⁴⁷ and ligand binding⁹ also showed no difference in the AT₁R expression in the lean and obese rats. On the other hand, Xu et al.,⁴⁹ have reported an increased AT₁R expression in obese rat kidney. Also, we have reported in one study using western blotting a modest increase in the cortical AT₁R expression in obese rats compared to lean rats.¹⁶ The basis of discrepancy is not clear; however, the batch and source of animals and the antibody used could be reasons of such discrepancy. Overall, we believe that there is either no difference or a modest increase in AT₁R expression in obese rats compared to lean rats. However, enhanced renal AT₁R function has been reported from multiple laboratories, including ours.^{9,23,30} We speculate a post-receptor signaling that contributes to enhanced AT₁R function in obese animals. The AT₂R agonist treatment via a post-receptor cross-talk may have caused an attenuation in AT₁R expression and function as supported by a recent study, where AT₂R is

shown to decrease AT₁R function in renal proximal tubules cells from Wistar-Kyoto rats via cGMP pathway.⁵⁰ Consistent to these findings, we also observed that selective stimulation of AT₂R in HK-2 cell lines decreased AT₁R expression. Considering all findings together, it appears that AT₂R agonist treatment lowered AT₁R function, which in turn allowed ACE2-Ang-(1-7) to increase. However, molecular mechanisms are yet to be determined as to how AT₂R attenuates renal AT₁R function in obese rats, and if AT₂R directly regulates ACE2 expression/activity and other alternate pathways leading to enhanced Ang-(1-7) levels.

Most of the effects on RAS components, kidney function or BP associated with AT₂R agonist treatment were observed only in obese rats; whereas MasR expression in response to AT₂R agonist treatment was increased in both the lean as well as obese rat kidneys. These selective changes in RAS could plausibly be attributed to the two sets of AT₂R signaling in proximal tubules of obese and lean rats.²⁹ We have shown that the proximal tubule AT₂Rs stimulate cGMP/NO only in obese rats, but are linked to cAMP inhibition to similar extent in both the lean and obese rats.²⁹ Therefore, it is possible that AT₂R via cGMP decreases AT₁R function, which in turn allows ACE2 expression to go up in obese rats. MasR regulation by AT₂R agonist may be linked to the AT₂R's ability to affect cAMP in both of the rat strains.

The AT₂R activation has been reported to exert a negative feedback on renin production.³⁴ We have shown that renal renin expression was lower in obese compared to lean rats.⁵¹ However, in the present study we found that despite renin expression being lower in obese rats, Ang II levels were similar in lean and obese rats. This could be due to the fact that ACE expression in the kidney is significantly increased in obese rats, thus converting Ang I to more Ang II and thereby compensating for reduced renin expression. Moreover, it is likely that proangiotensinogen (Ang 1-12) is converted to Ang II which is independent of renin. Contrary to our results, Sharma et al.,⁵² reported an increase in renal Ang II levels in obese compared to lean rats. The basis of this discrepancy is not clear; although it must be noted that different methods (HPLC/ELISA vs LC/MS) were used in these studies. The AT₂R antagonist PD treatment caused an increased cortical expression of renin in obese rats¹⁶ suggesting a role of AT₂R regulating renin in obesity. In the present study, treatment of HK-2 cells with CGP decreased renin expression. These findings suggest that similar to AT₁R, AT₂R also negatively regulates renin levels. In light of these reports, we expected that AT₂R agonist treatment will lower the renal renin expression in obese rats but we observed no changes. Since the renal renin expression in obese rat kidney is already low, it is likely that renin could not be further reduced by the AT₂R agonist treatment in obese rats. Nevertheless, the role of AT₂R in keeping low renin in obese rat kidney highlights the importance of AT₂R as a multifaceted regulator of the kidney RAS.

Obesity is reported to be associated with higher NO generation,⁵³ which could be a compensatory mechanism that contributes to vasodilatation in obesity.⁵⁴ Since AT₂R coupled with NO pathway is functional in obese and not in lean rats, AT₂R activation by CGP was expected to cause an increase in urinary nitrates in response to CGP-treatment. However, this increase was not sustained throughout the 14 day treatment period. Although AT₂R is classified as a G-protein coupled receptor (GPCR), unlike most GPCRs the AT₂R does not undergo endocytosis in the presence of the agonist,^{55, 56} and thereby could be

resistant to agonist-induced cellular degradation. This notion is supported by our observation that there was no reduction in AT₂R expression in CGP-treated rats. Whether AT₂Rs are desensitized without leading to endocytosis and degradation upon long-term agonist exposure, and thereby could not produce the sustained increase in nitrates over the agonist treatment period is not known. We have earlier demonstrated that AT₂R inhibits Na-pump via NO/cGMP pathway.^{29, 57} The increased natriuresis observed in obese CGP-treated rats could be the direct effect of AT₂R on Na-pump via NO pathway. Pharmacological activation of AT₂R reduced BP in conscious animals which were measured by tail-cuff method. This decrease in BP is in agreement with our earlier studies,³⁰ whereby arterial pressure was measured in anesthetized animals. It is likely that increased urinary Na excretion shifted the BP to a lower level in AT₂R agonist-treated obese rats. This is further supported by our observation that there were no changes in GFR or renal blood flow, which were already higher in obese rats compared to lean rats. Since obesity is associated with enhanced GFR and vasodilatation,^{25, 58} our findings support the notion that obesity-related hypertension is caused by excessive renal Na reabsorption and not by changes in GFR and/or vascular tone. Consistent with this notion, AT₁R antagonist losartan treatment has been shown to reduce renal Na reabsorption and BP without causing any changes in GFR and renal blood flow in obese rats.²⁶

Numerous studies linked abnormal renal sodium handling (excessive Na reabsorption) with the pathogenesis of hypertension in obesity.²⁵ Since obesity is also associated with low grade inflammation⁵⁹ and oxidative stress⁶⁰ as well as enhanced renal AT₁R functions²⁸, which are inter-regulatory in a positive manner,⁶⁰ all of them are likely to contribute to Na reabsorption and blood pressure increase in obesity. The anti-natriuresis and blood pressure increase in obesity may be further contributed by a reduced ACE2 expression^{41, 61} and consequently reduced levels of Ang (1-7),⁴¹ which recently has been implicated in promoting natriuresis^{17, 45, 62, 63} and blood pressure regulation.⁶⁴⁻⁶⁶ We have earlier reported that the AT₂R agonist CGP treatment in obese rats caused a reduction in oxidative stress and inflammation.³⁰ Advancing our understanding of the AT₂R functions, present study shows that lesser known arm of the RAS, ACE2-Ang-(1-7) levels were increased in response to AT₂R agonist treatment in obese rats. Role of these parameters as independent regulators of renal Na handling and blood pressure increase in obesity has not been tested, and it may be difficult due to their inter-regulation. However, it is reasonable to suggest that the changes in these parameters including reduced AT₁R function and enhanced ACE2-Ang-(1-7)/MasR expression might have contributed to the AT₂R agonist-induced changes in Na excretion and blood pressure in obese rats. These studies provide a new dimension to the AT₂R function and amplification of the net response. To ascertain the role of ACE2-Ang-(1-7)/MasR axis in renal Na handling and lowering blood pressure in obesity warrants a further investigation.

Materials and Methods

Chronic Study

Male lean and obese Zucker rats (10-11 weeks) were purchased from Harlan, Indianapolis, IN. The detailed protocol is provided in the supplemental file.

Hemodynamic parameters

BP was measured by tail-cuff method using CODA system which provides 99% correlation with telemetry and direct BP measurements.^{67, 68} The detailed protocol is provided in the supplemental file.

Renal functions

The urine samples were analyzed for Na and urinary Na volume ($U_{Na}V$) mmol/day was calculated. GFR was measured as described by Qi *et al.*⁶⁹ The detailed protocol is provided in the supplemental file.

RAS Components

The detailed protocol is provided in the supplemental file.

LC/MS Quantification of angiotensin peptides

The angiotensin peptides were analyzed as described in our publication.⁶¹ The detailed protocol is provided in the supplemental file.

Acute Study Candesartan-induced natriuresis as AT₁R function in CGP-treated obese rats

This protocol is represented in figure 1 and the details are provided in the supplemental file

Cell culture

HK2 cells expresses RAS components^{70, 71} and they were cultured as described earlier.⁷⁰ The detailed protocol is provided in the supplemental file.

Chemicals

The details are provided in the supplemental file

Statistical analysis

Data are presented as mean±SEM and were analyzed using GraphPad Prism 4 and subjected to one-way ANOVA with Newman-Keuls post hoc test. N= 5 to 7 in each group, as detailed in figure legends. A *p* value of less than 0.05 was considered statistically significant.

An expanded version of the methods is given here as supplemental file

Chronic Study

All the animal experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Houston. The lean and obese-control groups (N=5-7 per group) were treated with normal saline as vehicle and the lean and obese-CGP42112A (CGP) groups were treated with CGP (1 µg/kg/min) for 2-weeks using Alzet osmotic pumps (Model 2ML-2, CA) implanted subcutaneously. In one set of treatment groups, another micro-osmotic pump (Alzet, model 1002) filled with FITC-inulin (5%) was placed at the same time in the peritoneal cavity for the measurement of glomerular filtration rate (GFR) in these rats. The animals were placed singly in metabolic cages. Normal rat chow and water

were provided ad libitum. Daily 24-hour food and water intake and urine volume were recorded during the treatment period.

Hemodynamic parameters

BP was measured by tail-cuff method using CODA system which is clinically validated and provides 99% correlation with telemetry and direct BP measurements. The CODA tail-cuff blood pressure system utilizes Volume Pressure Recording (VPR) sensor technology to measure the rat tail blood pressure. At the end of the blood pressure measurements, the kidneys were removed and stored frozen at -80°C for measuring the cortical expression of RAS components.

Renal functions

The urine samples were analyzed for Na by the AAnalyst 400 atomic absorption spectrometer (Perkin Elmer, Waltham, MA) and urinary Na volume (UNaV) mmol/day was calculated. To measure GFR, blood (80 μl) from tail vein on multiple days (days 6, 9 and 14) during drug treatment was collected. The FITC-inulin fluorescent count in plasma samples and in urine collected on the same day was determined using spectrofluorometer (Cytofluor Series 4000, Applied Biosystem). The GFR was calculated using the formula $\text{GFR} = (\text{Urinary fluorescence counts}/24\text{h}) / (\text{Plasma fluorescence counts}/\mu\text{l})$, as described by *Qi et al.*¹ On the last of the treatment (day 14), renal blood flow was determined under anesthesia using a transit time blood flowmeter (Model T402, Transonic Ststems Inc, NY, USA). Briefly, a midline incision was made in the abdominal region and an ultrasonic flow probe was carefully placed around the left renal artery. The blood volume flow (ml/minute) was recorded and analyzed by using Powerlab 4/35 data acquisition system (PL3504, ADI Instruments, Colorado Springs, CO, USA). Total nitrates/nitrites excreted at two time points (day 3 as early and day 14 as late) of the treatment were measured using EIA kits (R&D System, Minneapolis, MN).

Western blotting

The expression of AT_1R , AT_2R , and renin, ACE, ACE2 and MasR in the kidney cortex was determined by Western blotting and ACE2 activity was measured by Sensolyte 390 ACE2 activity kit (Anaspec Inc, CA) using spectrofluorometer (Cytofluor Series 4000, Applied Biosystem). For western blotting, 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, 65 μg for ACE2 and 40 μg for Mas receptor) from various rat groups were subjected to SDS-PAGE and were transferred onto immobilon P (blot). The blots were incubated with primary polyclonal antibodies for the AT_1 receptor, AT_2 receptor, renin, ACE, ACE2 and Mas receptor. Following the incubation with the primary antibodies, the blots were incubated with appropriate HRP-conjugated anti rabbit/anti-goat IgGs. The signal was detected by ECL system, recorded and analyzed by Fluorchem 8800 (Alpha Innotech Imaging System,

San Leandro, CA) for the densitometric analysis. For loading control, the blots were stripped, and re-probed with β -actin mouse monoclonal antibody.

Determination of angiotensin peptides

To determine angiotensin peptide levels quantitatively in the kidney cortex, tissue was homogenized in lysis buffer (10 mM Tris, pH 7.4) with and without protease inhibitors. Since the presence or absence of protease inhibitors did not affect the Ang II and Ang-(1-7) levels measured in the tissues, we presented data for only with protease inhibitors. After homogenization the samples were centrifuged for 15 min at 4°C at 1,600g, and resulting supernatant was loaded onto an C18-E (55 μ m, 70A) cartridge, equilibrated with 60% acetonitrile, 1% TFA and 39% distilled water. After sample application C18-E cartridge was washed twice with 3ml of 1% TFA. The column was eluted with the equilibrium buffer and collected in a 15 ml tube. The eluent was dried under vacuum centrifuge, and reconstituted in 80% acetonitrile and 0.1 % formic acid prior to LC/MS analysis. Protein samples were analyzed using an Ultra Performance LC Systems (ACQUITY, Waters Corp., Milford, MA, USA) coupled with a quadrupole time-of-flight mass spectrometer (Q-TOF Premier, Waters) with electrospray ionization (ESI) in both ESI⁻-MS and ESI⁺-MS/MS (SetMass without fragmentation) mode operated by the Masslynx software (V4.1) as discussed in our earlier publication by *Samuel et al.*, 2012.

Candesartan-induced natriuresis as AT₁R function in CGP-treated obese rats

After tracheotomy the carotid artery, jugular vein and ureter were cannulated for BP measurement, drug infusion and urine collection, respectively. For studying the AT₁R function in response to candesartan, the AT₂R were blocked by infusing AT₂R antagonist PD (50 μ g/kg/min) in both the control and CGP-treated obese rats. Basal urine was collected twice for 30 min each time. After that, the AT₁R antagonist candesartan (100 μ g/kg bolus) was infused and urine was again collected twice for thirty minutes each time (figure1).

Cell Culture

We performed in-vitro experiments using HK2 cells which is human kidney proximal tubule cell line and routinely used in our laboratory and expresses all the components of RAS.² HK2 cells were cultured using K-SFM supplemented with 5% FBS, epidermal growth factor (EGF) and bovine pituitary extract (BPE). The cells used in the experiment were between passages 5-9. Cells were seeded at 1×10^6 in 100 \times 20 mm culture dish. At ~65% confluency, the HK-2 cells were treated with CGP42212A (10nM), PD 123319 (1 μ M) and CGP (10nM) + PD (1 μ M) for 24 hrs. After treatment with the drugs, the cell were lysed and processed for ACE2 and renin activity and western blotting for AT₁R and MasR. ACE2 and renin activity was measured using Varioskan plate reader, Thermo Scientific, IL.

Chemicals

CGP was synthesized (21st Century Biochem, MA). PD was a gift from Pfizer Inc and candesartan was a gift from AstraZeneca. Antibody for MasR (Alomone Labs Ltd) and for renin, ACE, ACE2, AT₁ and β -actin were purchased from Santa Cruz, CA. AT₂R antibody

was custom raised (EZ Biolab). K-SFM was purchased from Life Technologies, Grand Island, NY.

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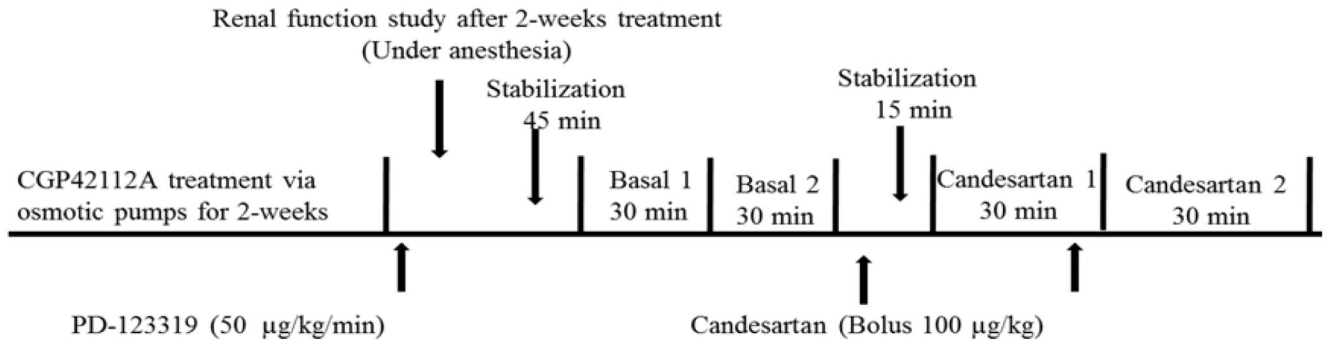


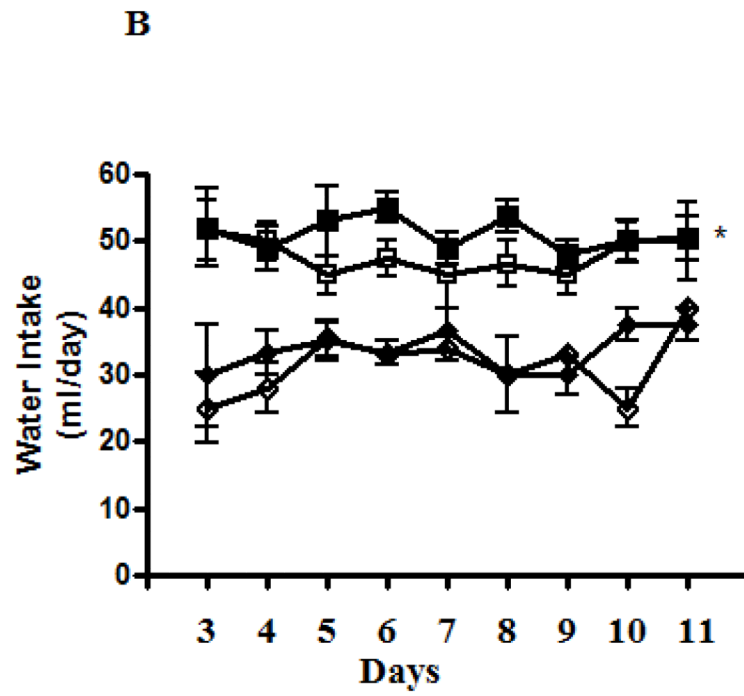
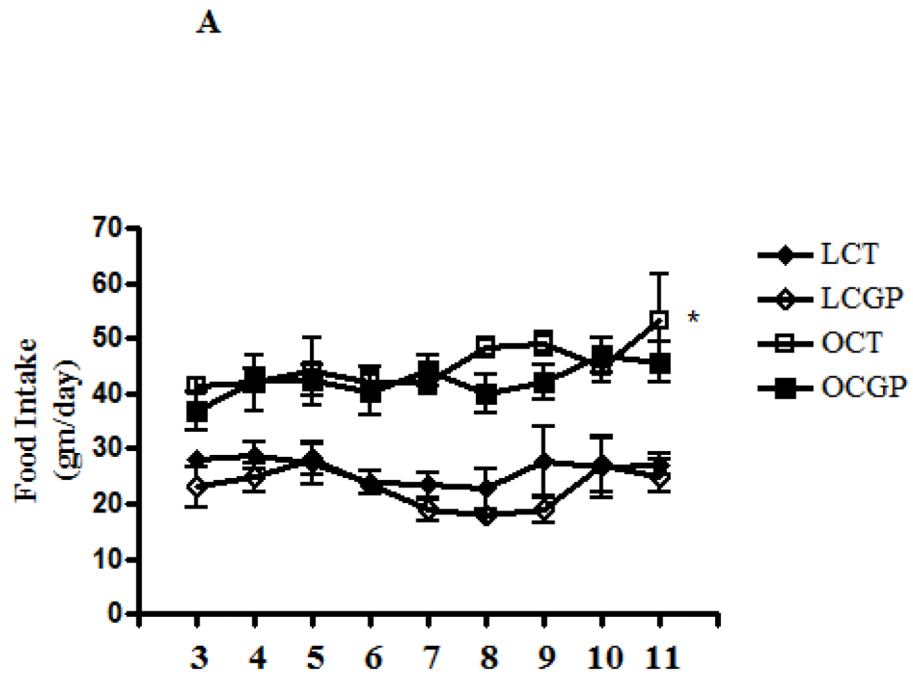
Fig. 1.
Schematic representation of the protocol used in the renal function study.

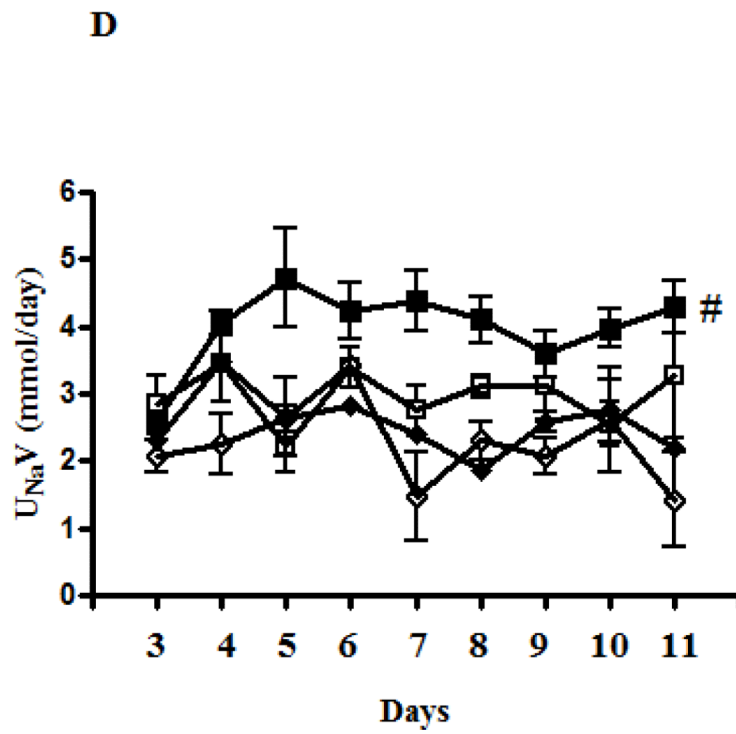
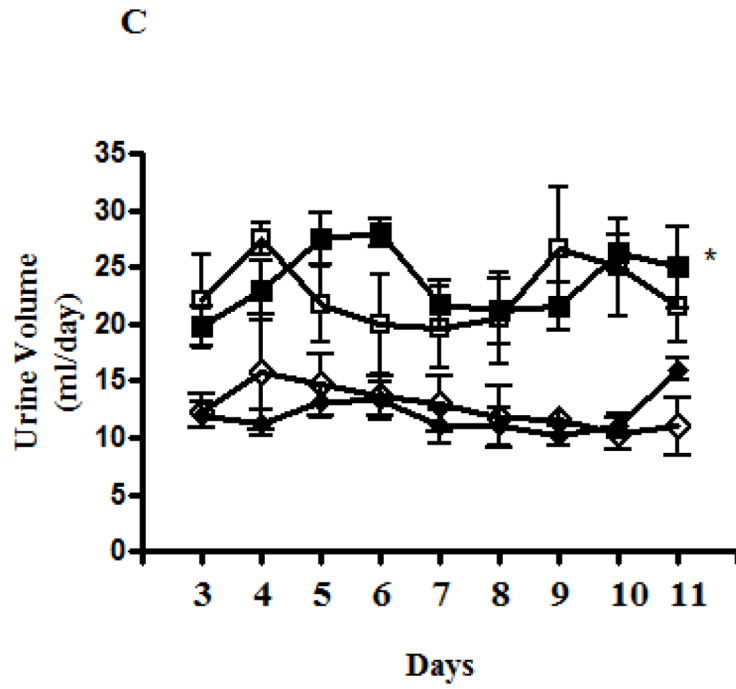
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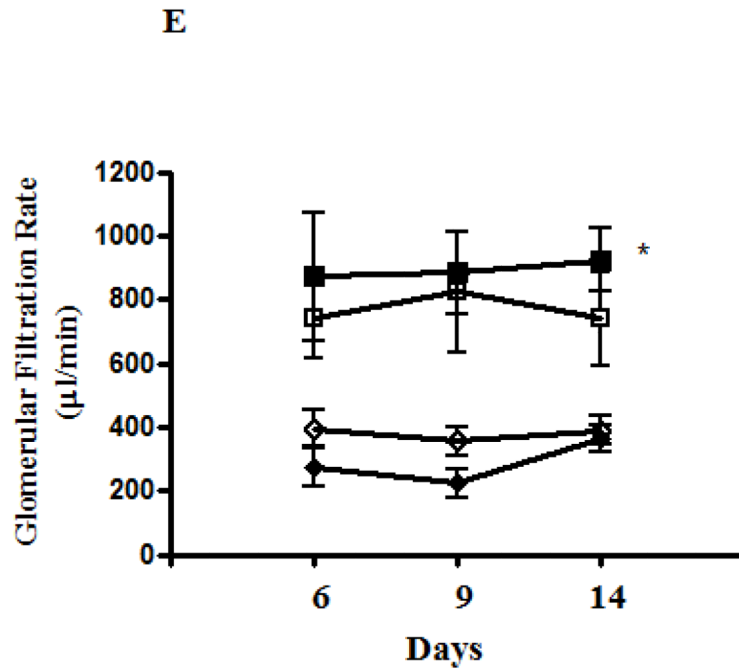


Fig.2. (A) Food (B) water intake (C) urinary volume (D) urinary sodium excretion ($U_{Na}V$) and (E) glomerular filtration rate (GFR) measured using FITC-inulin clearance method in conscious control and CGP-treated lean and obese Zucker rats. *significantly different compared with lean control rats, #significantly different compared with obese control rats. Values are represented as mean \pm SEM; One way ANOVA followed by Neuman-Keuls test, $p < 0.05$; $N = 5-7$ in each group). (LCT- lean control, LCGP-lean treated with CGP42112A, OCT-obese control, OCGP-obese treated with CGP42112A).

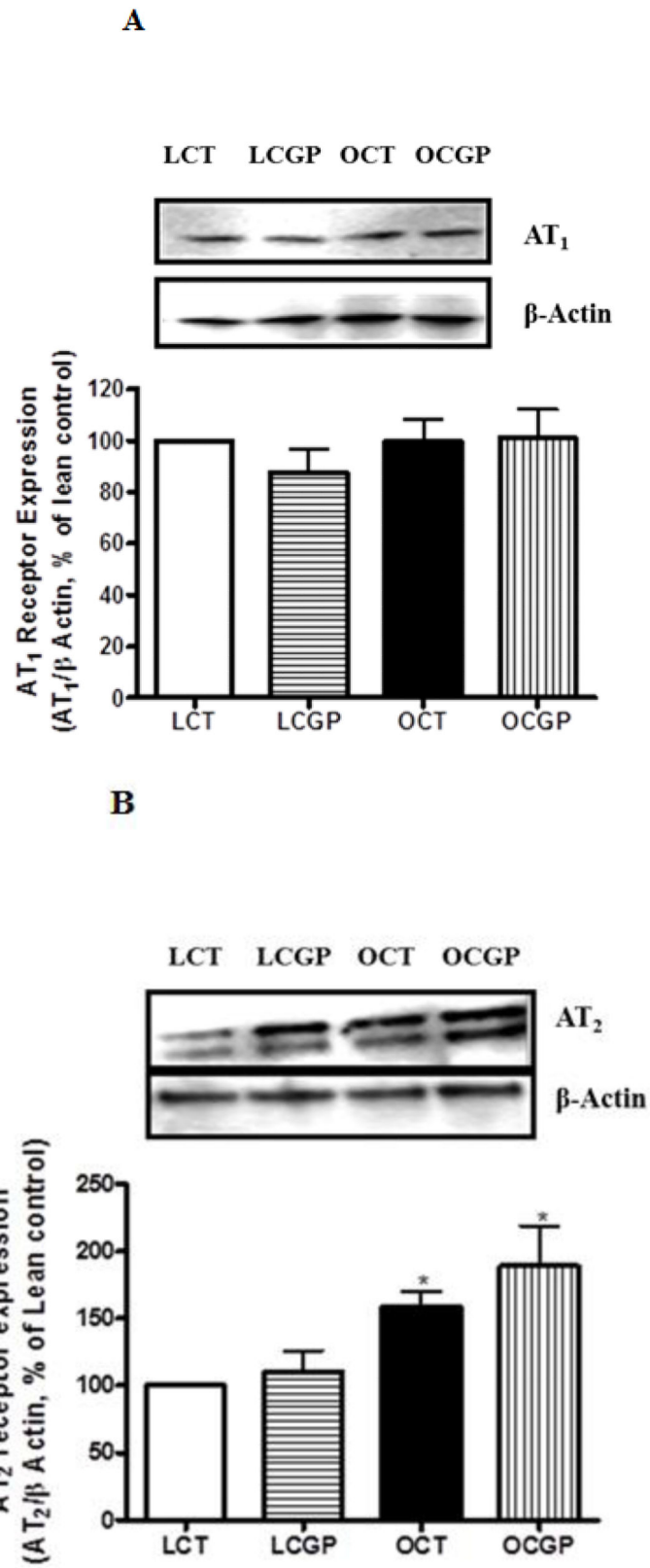


Fig. 3.

Expression of (A) AT₁R and (B) AT₂R in the kidney cortex of control and CGP-treated lean and obese Zucker rats. Upper panels: Representative Western blots for respective proteins with loading control β -actin. Bar graphs: represent the ratios of densities of the respective protein bands and β -Actin i.e. AT₁/ β -Actin, AT₂/ β -Actin *significantly different compared with lean control rats. Values are represented as mean \pm SEM; One-way ANOVA followed by Neuman-Keuls test, $p < 0.05$; N=5-7 in each group). (LCT- lean control, LCGP-lean treated with CGP42112A, OCT-obese control, OCGP-obese treated with CGP42112A).

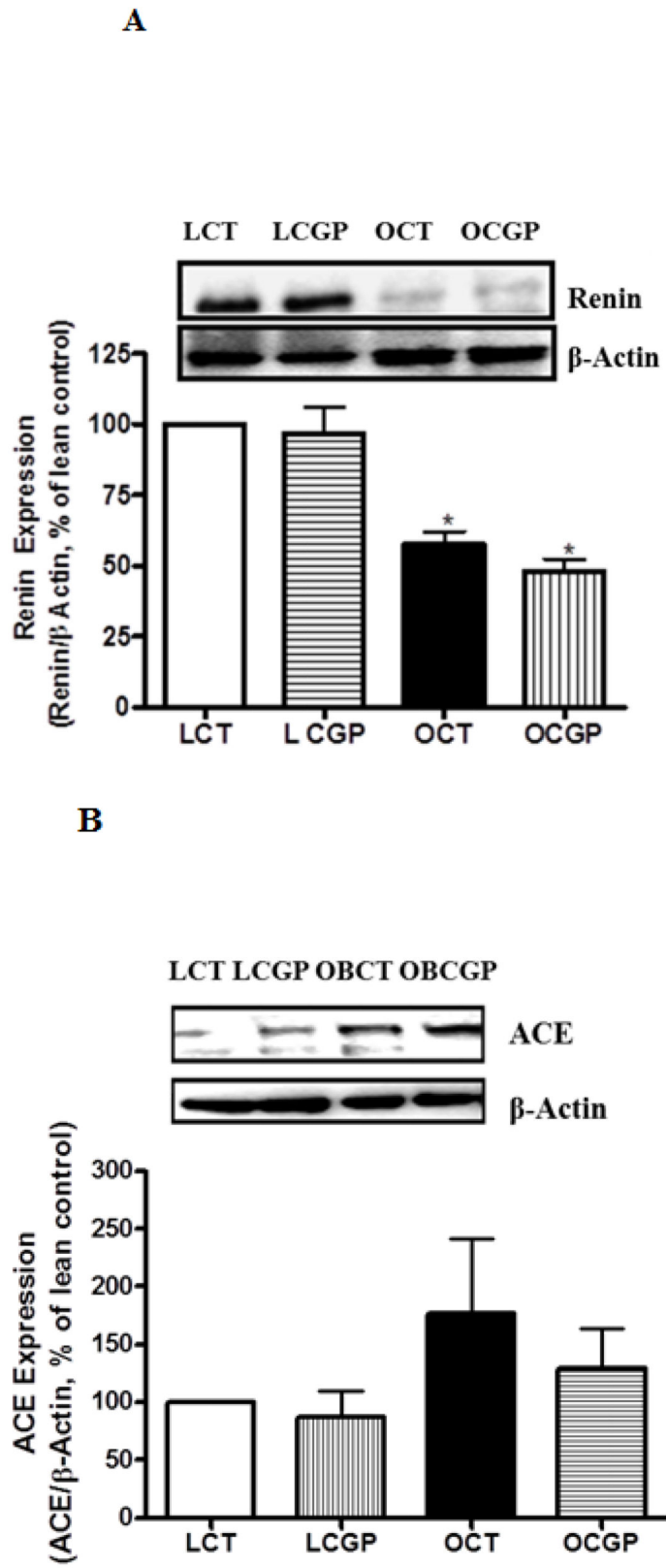


Fig. 4.

Expression of (A) renin and (B) ACE in the kidney cortex of control and CGP-treated lean and obese Zucker rats. Upper panels: 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. renin/ β -Actin, ACE/ β -Actin *significantly different compared with lean control rats. Values are represented as mean \pm SEM; One-way ANOVA followed by Neuman-Keuls test, $p < 0.05$; N=5-7 in each group). (LCT- lean control, LCGP-lean treated with CGP42112A, OCT-obese control, OCGP-obese treated with CGP42112A).

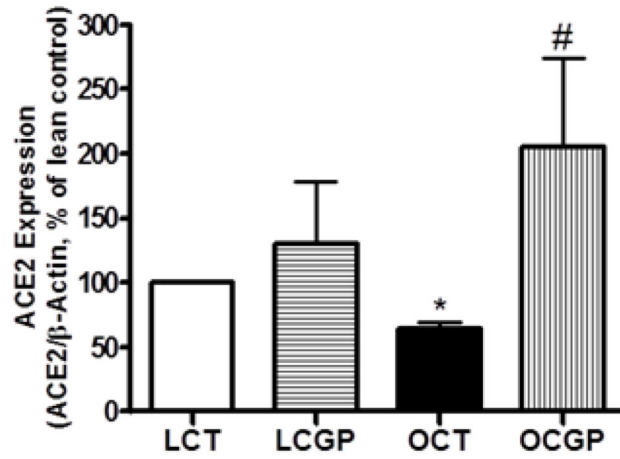
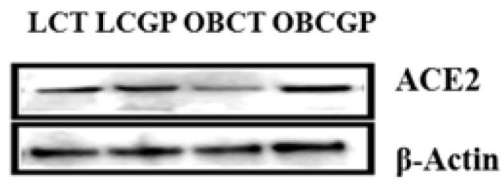
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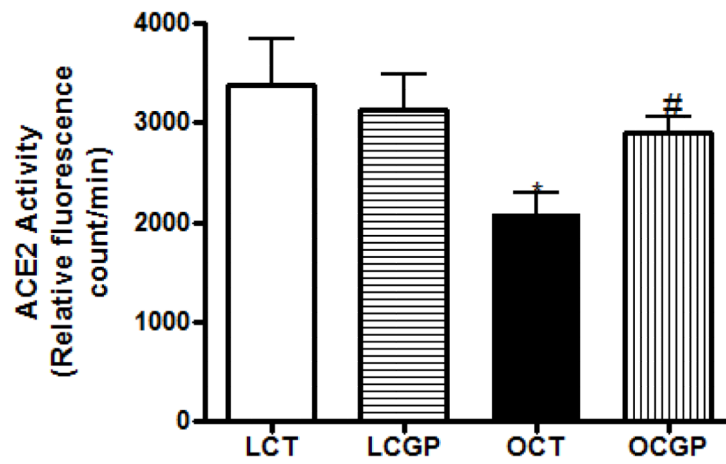
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A



B



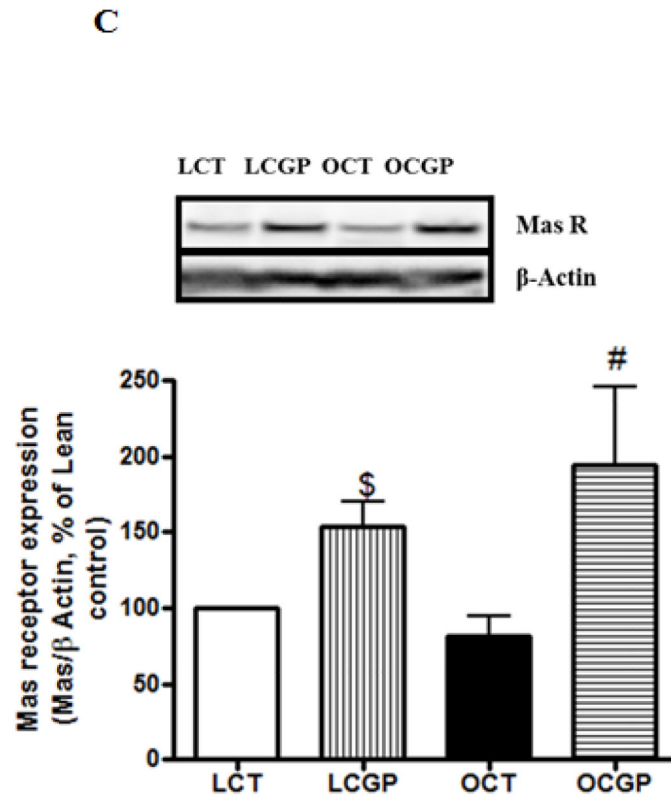


Fig. 5. (A) ACE2 expression (B) ACE2 activity and (C) Mas receptor expression in the kidney cortex of control and CGP-treated lean and obese Zucker rats. Upper panels: Representative Western blots for respective proteins with loading control β -actin. For Western blot only, bar graphs represent the ratios of densities of respective protein bands and β -Actin i.e. ACE2/ β -Actin, MasR/ β -Actin. ^{\$}significantly different compared with lean control rats, [#]significantly different compared with obese control rats. Values are represented as mean \pm SEM; One-way ANOVA followed by Neuman-Keuls test, $p < 0.05$; $N = 5-7$ in each group). (LCT- lean control, LCGP-lean treated with CGP42112A, OCT-obese control, OCGP-obese treated with CGP42112A).

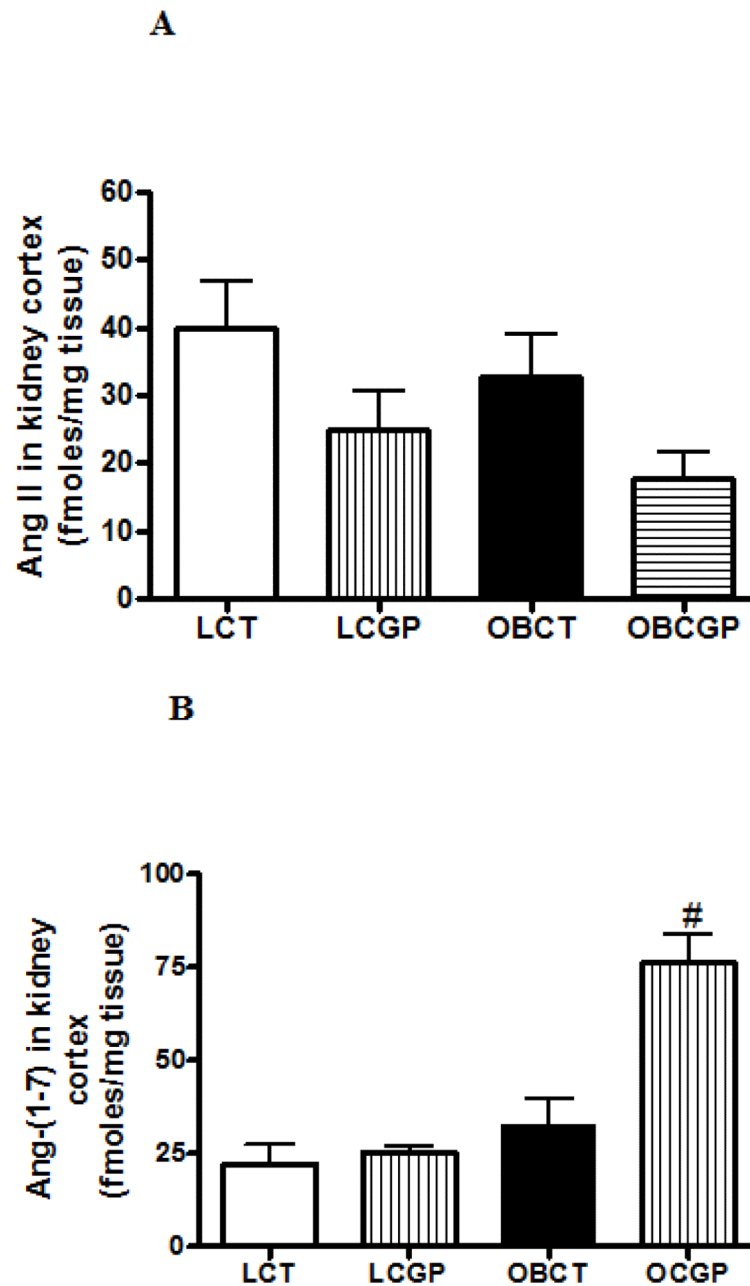
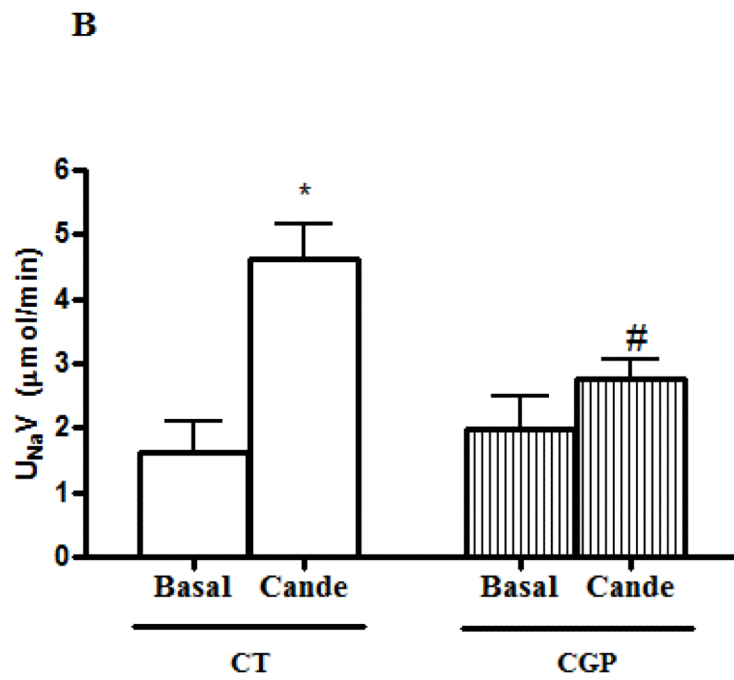
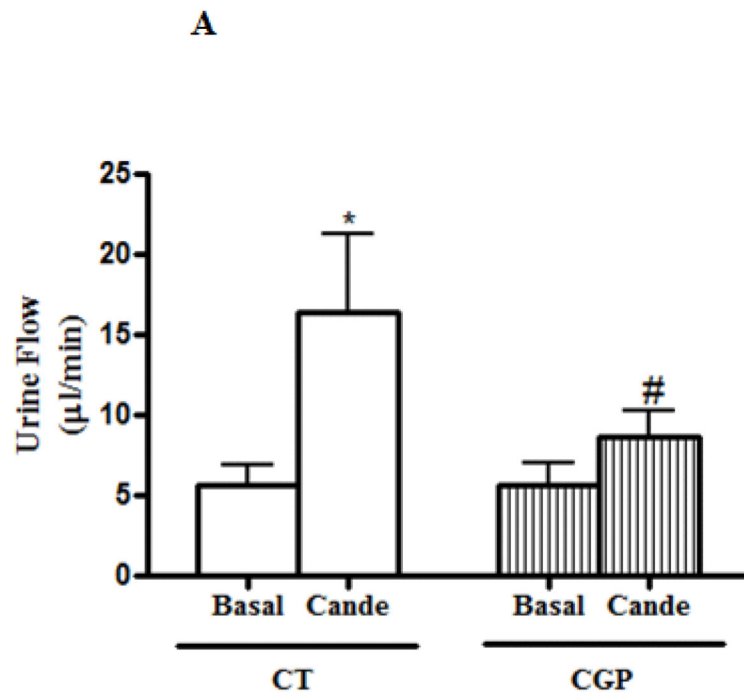


Fig 6. LC/MS quantification of (A) Angiotensin II and (B) Angiotensin-(1-7) in the kidney cortex of control and CGP-treated lean and obese rats. [#]significantly different compared with obese control rats, Values are represented as mean \pm SEM; One-way ANOVA with Newman-Keuls test, $p < 0.05$; $N = 5-7$ in each group. (LCT- lean control, LCGP-lean treated with CGP42112A, OCT-obese control, OCGP-obese treated with CGP42112A).



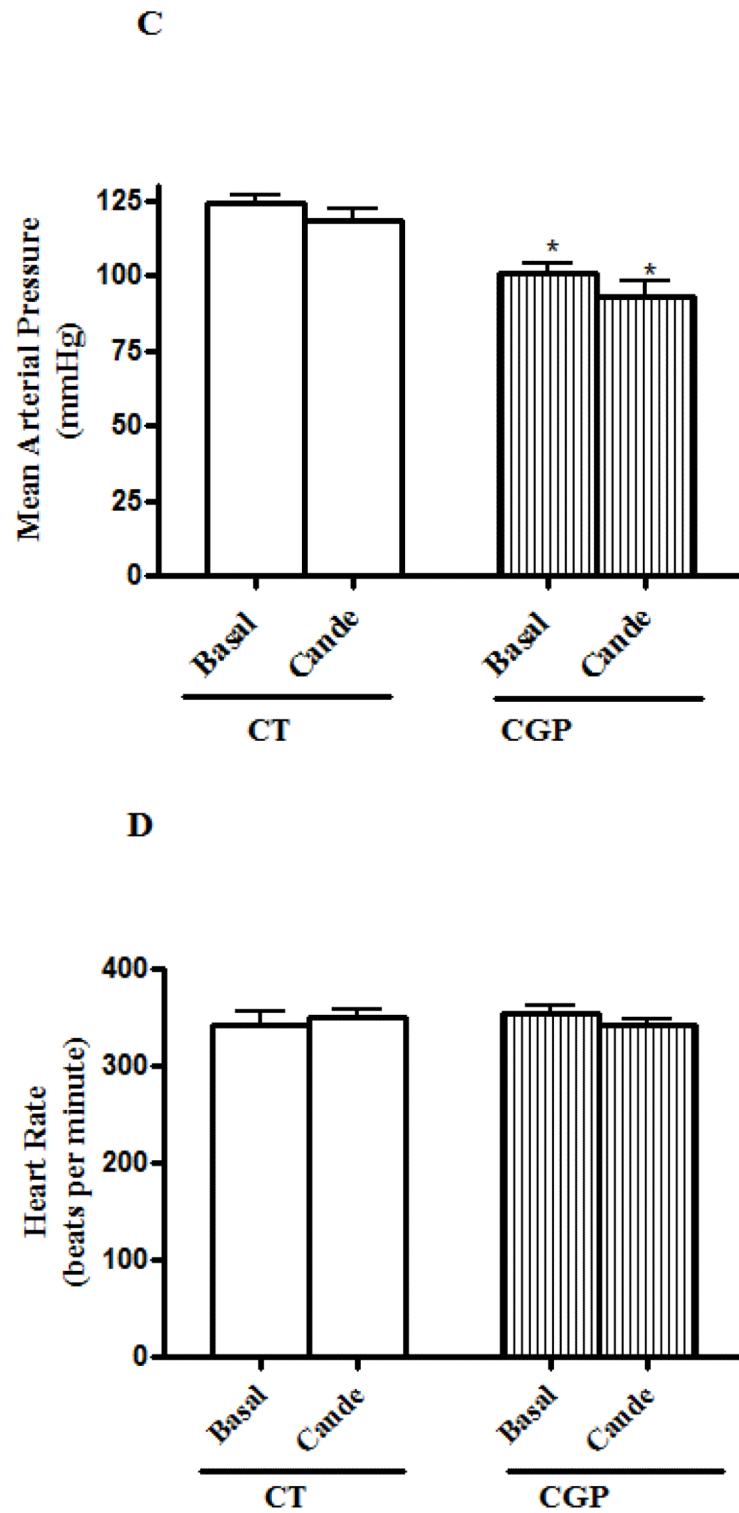


Fig 7. Effect of candesartan (100 $\mu\text{g}/\text{kg}$ bolus) on (A) diuresis (B) natriuresis (C) mean arterial pressure and (D) heart rate in control and CGP-treated obese Zucker rats. *significantly

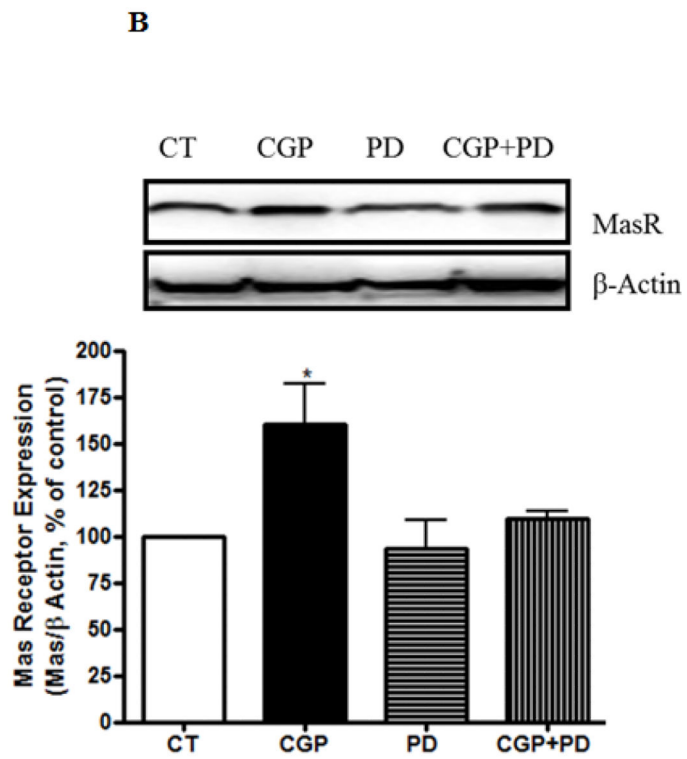
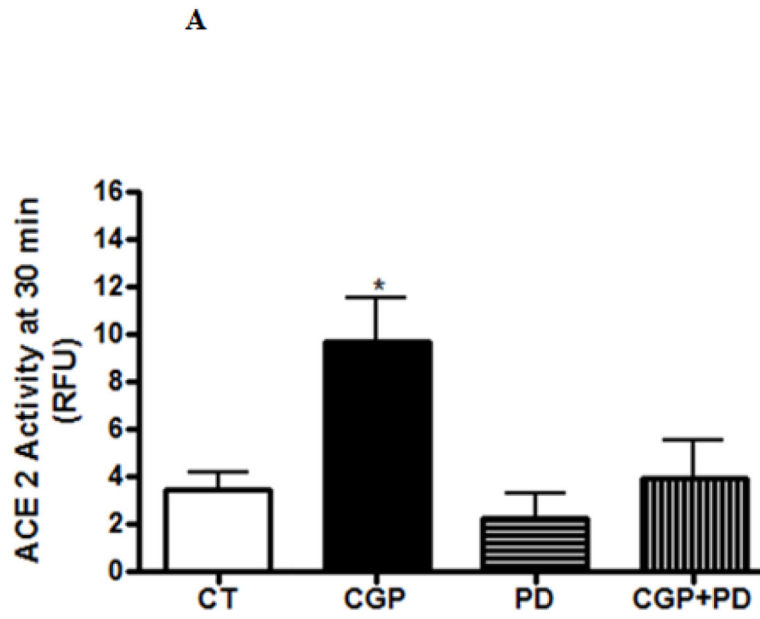
different compared with control basal, #significantly different compared with control candesartan. Values are represented as mean \pm SEM; One-way ANOVA followed by Neuman-Keuls test, $p < 0.05$, $N = 5$ rats in each group). (OBCT-obese control, OCGP-obese treated with CGP42112A).

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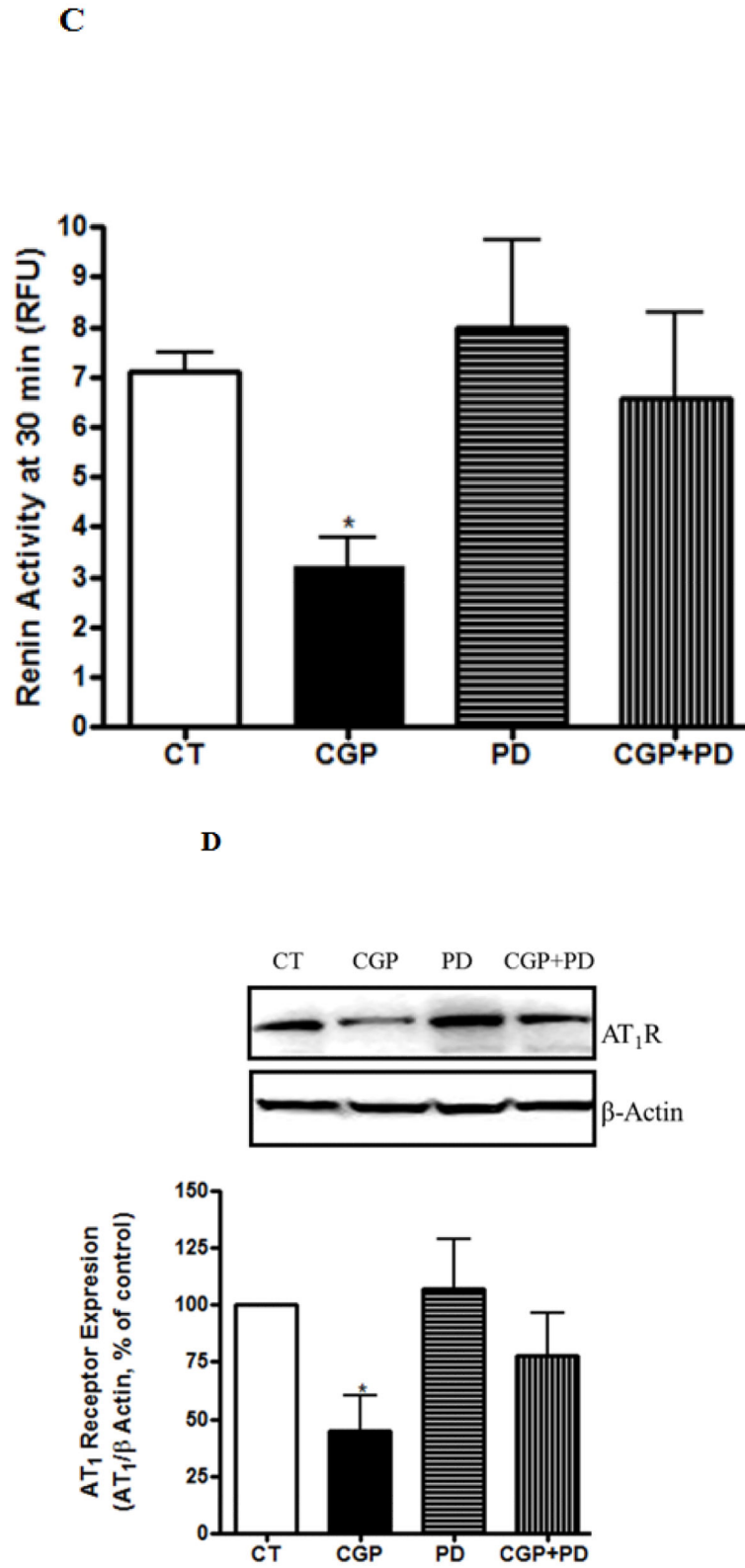


Fig 8.

Effect of AT₂R agonist and antagonist on (A) ACE2 activity (B) MasR expression (C) Renin activity and (D) AT₁R expression. Upper panels: 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 AT₁/ β -Actin, Mas/ β -Actin. *significantly different compared with control. Values are represented as mean \pm SEM; One-way ANOVA followed by Neuman-Keuls test, $p < 0.05$, N=6-7 in each group). (CT-control, CGP-CGP42112A, PD-PD123319).

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Table 1

Parameters/Rat groups	LCT	LCGP	OCT	OCGP
MAP, mm Hg	ND	ND	106 ± 3	93 ± 3 [#]
Heart rate, beats per min	ND	ND	392 ± 11	409 ± 9 ^{ns}
Renal blood flow, ml/hr	3±0.3	4±0.4	7.3 ± 0.8*	9 ± 2 ^{ns}
Kidney wt, gm	2.2±0.1	2.3±0.1	2.8±0.1*	2.7±0.1 ^{ns}
Body wt, g	295±8	291±8	473±12*	461±11 ^{ns}
Urinary Nitrates at day 2, nmol/min	0.1±0.06	0.1±0.04	0.4±0.11*	1±0.26 [#]
Urinary Nitrates at day 14, nmol/min	0.07±0.01	0.08±0.01	0.58±0.3*	0.47±.03 ^{ns}

ND: Not Determined

* compared to LCT

[#] compared to OCT (LCT-lean control, LCGP-lean CGP42112A-treated, OCT-obese control, OCGP-obese CGP42112A-treated), ns-not significant-compared to OCT.