


Angiotensin type 2 receptor null mice express reduced levels of renal angiotensin converting enzyme-2/angiotensin (1-7)/Mas receptor and exhibit greater high-fat diet-induced kidney injury

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Abstract

Introduction: Renin–angiotensin system (RAS) components exert diverse physiological functions and have been sub-grouped into deleterious angiotensin-converting enzyme (ACE)/angiotensin II (Ang II)/angiotensin type 1 receptor (AT₁R) and protective ACE2/angiotensin (1-7) (Ang-(1-7))/Mas receptor (MasR) axes. We have reported that chronic activation of angiotensin type 2 receptor (AT₂R) alters RAS components and provides protection against obesity-related kidney injury.

Materials and methods: We utilized AT₂R knockout (AT₂KO) mice in this study and evaluated the renal expression of various RAS components and examined the renal injury after placing these mice on high fat diet (HFD) for 16 weeks.

Results: The cortical ACE2 activity and MasR expression were significantly decreased in AT₂KO mice compared to wild type (WT) mice. LC/MS analysis revealed an increase in renal Ang II levels and a decrease in Ang-(1-7) levels in AT₂KO mice. Cortical expression of ACE and AT₁R was increased but renin activity remained unchanged in AT₂KO compared with WT mice. WT mice fed HFD exhibited increased systolic blood pressure, higher indices of kidney injury, mesangial matrix expansion score, and microalbuminuria, which were further increased in AT₂KO mice.

Conclusion: This study suggests that deletion of AT₂R decreases the expression of the beneficial ACE2/Ang-(1-7)/MasR and increases the deleterious ACE/Ang II/AT₁R axis of the renal RAS in mice. Further, AT₂KO mice are more susceptible to HFD-induced renal injury.

Keywords

Renin–angiotensin system, angiotensin II type 2 receptor knockout, angiotensin (1-7), Mas receptor

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Introduction

The renin–angiotensin system (RAS) is comprised of peptide hormones, enzymes, and receptors, which have a diverse involvement in the maintenance of sodium homeostasis and regulation of blood pressure (BP).^{1,2} Recently, RAS components have been sub-grouped as angiotensin-converting enzyme (ACE)/angiotensin II (Ang II)/angiotensin type 1 receptor (AT₁R) and ACE2/angiotensin (1-7) (Ang-(1-7))/Mas receptor (MasR) axes with counter regulatory functions. While ACE/Ang II/AT₁R is known to play a critical role in the pathogenesis of various cardiovascular diseases, kidney injury, anti-natriuresis, and hypertension,^{3–5} emerging evidence supports a

protective role for ACE2/Ang-(1-7)/MasR axis in heart and kidney function, including natriuresis and hypertension.^{6–10} Inter-regulatory pathways have been suggested by which RAS components alter each other's expression and

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function. For example, AT₁R activation decreases ACE2 expression and Ang-(1-7) production^{11,12} leads to reduced MasR activation and function.^{13,14} Such changes in the RAS components cause a shift potentially impacting on the functional balance between deleterious versus protective arms of the RAS.

The angiotensin type 2 receptor (AT₂R) is another component of the RAS, and has not been “particularly” assigned to either of the RAS axes. However, numerous studies, including some from our laboratory, have shown that AT₂R is natriuretic, anti-hypertensive, and cardio- and reno-protective in various animal models.^{8,15–18} Therefore, AT₂R may be grouped with the protective ACE2/Ang-(1-7)/MasR axis. AT₂R is termed as ‘functional antagonist’ of AT₁R.¹⁹ This notion is based on the findings that AT₂Rs, via dimerization with AT₁R¹⁹ or cell signaling cross-talk, attenuates AT₁-mediated cellular responses in various cell types including the kidney epithelial cells.^{20,21} Additionally, chronic treatment with AT₂R agonist has been shown to cause a reduction in AT₁R expression, another mechanism by which AT₂R can reduce AT₁R function.²² Recently, we have reported that long-term pharmacological stimulation of AT₂R with preferential agonists caused a decrease in AT₁R-mediated renal function, but increased the expression/activity of ACE2/Ang-(1-7)/MasR axis in the kidney cortex of obese Zucker rats.⁸ These changes were associated with enhanced natriuresis, lowering of BP, and decreased renal injury in obese animals.^{8,16} In light of the beneficial effects mediated by AT₂R, the present study is designed to test the hypothesis that deletion of AT₂R (a) decreases the renal expression of ACE2/Ang-(1-7)/MasR axis and increases that of the ACE/Ang II/AT₁R axis and (b) makes the animals more susceptible to kidney injury in response to a high fat diet (HFD). To test this hypothesis, RAS components in the kidney cortices from AT₂KO mice and wild type (WT) controls from a similar background were measured. To demonstrate the reno-protective effect of AT₂R, WT and AT₂KO mice were fed either a normal fat diet or a HFD to induce obesity-related glomerulopathy.

Materials and methods

Animals

Male AT₂R^{-/-} (AT₂KO) mice, which were kindly provided by Tadashi Inagami (Vanderbilt University, Nashville, Tennessee, USA) and wild type with similar background (C57BL/6) of 20 weeks of age (purchased from Harlan, Inc.) were used in the study. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Houston. These animals were euthanized and the kidneys were removed, washed in cold saline and kept frozen at –80°C for measuring RAS components.

Western blotting for AT₁R and MasR

The expression of AT₁R, and MasR in the kidney cortex was determined by western blotting. For this purpose, the kidney cortices were homogenized in a buffer containing (in mM) Tris 50, ethylene diamine tetra-acetic acid (EDTA) 10, Phenyl methyl sulphonyl fluoride (PMSF) 1 and protease inhibitors. Equal amounts of protein (20 µg for AT₁R and 40 µg for MasR) were subjected to SDS-PAGE and were transferred onto Immobilon P (blot). The blots were incubated with primary polyclonal antibodies for the AT₁R, and MasR. Following incubation with the primary antibodies, the blots were incubated with Horse radish peroxidase (HRP)-conjugated anti-rabbit Immunoglobulin type G (IgGs). The signal was detected by Enhanced chemiluminescence (ECL) system, recorded and analyzed by Fluorchem 8800 (Alpha Innotech Imaging System, San Leandro, California, USA) for the densitometric analysis. The blots were stripped, and re-probed with β-actin mouse monoclonal antibody.

Renin, ACE2, and ACE activity

Renin activity in the kidney cortex was measured by Sensolyte 520 Mouse renin and ACE2 activity was measured by Sensolyte 390 ACE2 assay kit (Anaspec Inc., Fremont, California, USA). The renin and ACE2 activity was determined using Mca/Dnp fluorescence resonance energy transfer (FRET) peptide. In the FRET peptide the fluorescence of Mca is quenched by Dnp, upon cleavage into two separate fragments by the enzyme, the fluorescence of Mca is recovered, and is monitored at excitation/emission at 330/390 nm for ACE2 and at 490/520 nm for renin. ACE concentration was measured by Enzyme linked Immunosorbent Assay (ELISA) kit (USCN Life Science Inc, Texas, USA) as per manufacturer’s instructions.

LC/MS quantification 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 protease inhibitors. The angiotensin peptides were then analyzed by LC/MS method as described in our earlier publication.^{23,24}

Diet-induced obesity protocol

Male WT and AT₂KO mice (four weeks old) were placed either on normal diet or HFD for 16 weeks to induce obesity. The HFD feed Teklad custom research diet (TD.06414) with adjusted calorie diet (60/Fat) containing 18.4% protein, 21.3% carbohydrate and 60.3% fat, and normal diet 7022 (code of normal diet) with isocaloric 29% protein, 56% carbohydrate and 15% fat were purchased from Harlan, Indianapolis, Indiana, USA. Both diets contained similar mineral mix AIN-93G-MX (94046) and vitamin mix

AIN93-VX (94047). The animals were individually housed in metabolic cages during the last week of diet administration to collect urine. At the end of the 16-week treatment, the mice were euthanized by cervical dislocation under isoflurane anesthesia following six hours of fasting. Each left kidney was fixed in formalin for histological analyses.

Renal histology and morphometry

The formalin-fixed tissue was embedded in paraffin and 4 μ m sections were prepared. The slides were further stained with Periodic Acid Schiff (PAS) reagents or Masson's trichrome stain (Dako North America, Inc.) according to the manufacturer's instructions. All tissue samples were evaluated independently by two investigators in a blinded fashion by light microscopy ($\times 400$). For each animal, 30 glomeruli from each of the three consecutive sections were assessed. A semi-quantitative scoring method described by Raij et al.²⁵ was used to evaluate the degree of damage to the glomeruli. This was graded according to the severity of the glomerular damage: 0, normal; 1, slight glomerular damage of the mesangial matrix and/or hyalinosis with focal adhesion involving <25% of the glomerulus; 2, sclerosis of 25% to 50%; 3, sclerosis of 50% to 75%; and 4, sclerosis >75% of the glomerulus. A mesangial matrix expansion (MME) score was assigned in a similar manner on a scale of 0–4 depending upon the area of the glomerulus occupied by mesangial matrix when compared to lean control glomeruli. The glomerulosclerosis index or mesangial score was calculated by averaging the grades assigned to all glomeruli fields using the formula:

$$\text{sclerosis index / MME score} = \frac{(N1 \times 1 + N2 \times 2 + N3 \times 3 + N4 \times 4)}{n}$$

where N1–N4 represent the number of glomeruli with the respective score, n is the total number of glomeruli.

Measurement of microalbuminuria

An indirect competitive ELISA kit (Exocell, Philadelphia, Pennsylvania, USA) was used to measure urinary albumin excretion according to the manufacturer's instructions.

BP

BP was measured by tail-cuff method using CODA system which is clinically validated and provides 99% correlation with telemetry and direct BP measurements.^{26,27} The CODA tail-cuff BP system utilizes volume pressure recording (VPR) sensor technology to measure the mouse tail BP. BP was measured during the last two weeks (three days in each week with three readings each day on each animal) of the feeding regimen. For each animal, the data for each animal was averaged and treated as one value, which was used to calculate mean \pm standard error of the mean (SEM).

Chemicals and reagents

Antibodies for MasR and β Actin were purchased from Alomone Labs Ltd, Israel and Santa Cruz, California, USA respectively. AT₁R antibody was custom raised (Biomolecular Integration, Arizona, USA). Standards for Ang II and Ang-(1-7) were purchased from Sigma Aldrich, St Louis, Missouri, USA. All other chemicals were of standard grade.

Statistical analysis

Data are presented as mean \pm SEM and were analyzed using GraphPad Prism 4 and subjected to t -test. $n=5-6$ in each group, as detailed in figure legends. A p value of less than 0.05 was considered statistically significant.

Results

Effect of deletion of AT₂R on RAS components

ACE2, Ang-(1-7) and MasR. The cortical ACE2 activity (Figure 1(a)) was significantly reduced (WT: 113 \pm 8, AT₂KO: 41 \pm 11, RFU/min) in AT₂KO mice. The LC/MS quantification of angiotensin peptides revealed that the levels of Ang-(1-7) (Figure 1(b)) was also significantly reduced (WT: 33 \pm 5, AT₂KO: 14 \pm 0.8 fmoles/mg tissue) in the kidney cortex of AT₂KO compared to WT mice. Similarly, cortical MasR band at 47 kDa was significantly reduced in AT₂KO mice (Figure 1(c)).

ACE, Ang II and AT₁R. ACE in the kidney cortex of AT₂KO was significantly elevated compared to WT mice (WT 1.9 \pm 0.02, AT₂KO: 3 \pm 0.4 ng ACE/ μ g tissue). Ang II peptide levels (Figure 2(b)) were significantly higher in AT₂KO mice kidney cortex (WT: 31 \pm 2.7, AT₂KO: 47 \pm 2.7 fmoles/mg tissue). Western blot of AT₁R at 41 kDa revealed almost a two-fold increase in the kidney cortex of AT₂KO compared to WT mice (Figure 2(c)).

Renin. Renin activity in the kidney cortex remained unchanged between wild type and AT₂KO mice (Figure 3).

Effect of deletion of AT₂R on renal injury

Markers of early renal injury. Renal sections were stained with PAS to assess glomerular injury. Early pathological changes, including glomerular hypertrophy, mesangial expansion, and basement membrane thickening, were observed in WT and AT₂KO mice maintained on the HFD while the renal morphology of ND-fed WT and AT₂KO mice was normal (Figure 4(a) and (b)). MME scores were higher in HFD-fed AT₂KO mice compared to WT mice fed the HFD (Figure 4(a) and (b)). Masson's trichrome stain was used to evaluate tubulointerstitial collagen deposition. No tubulointerstitial fibrosis was detected in any of the treatment groups (data not shown). WT mice on HFD

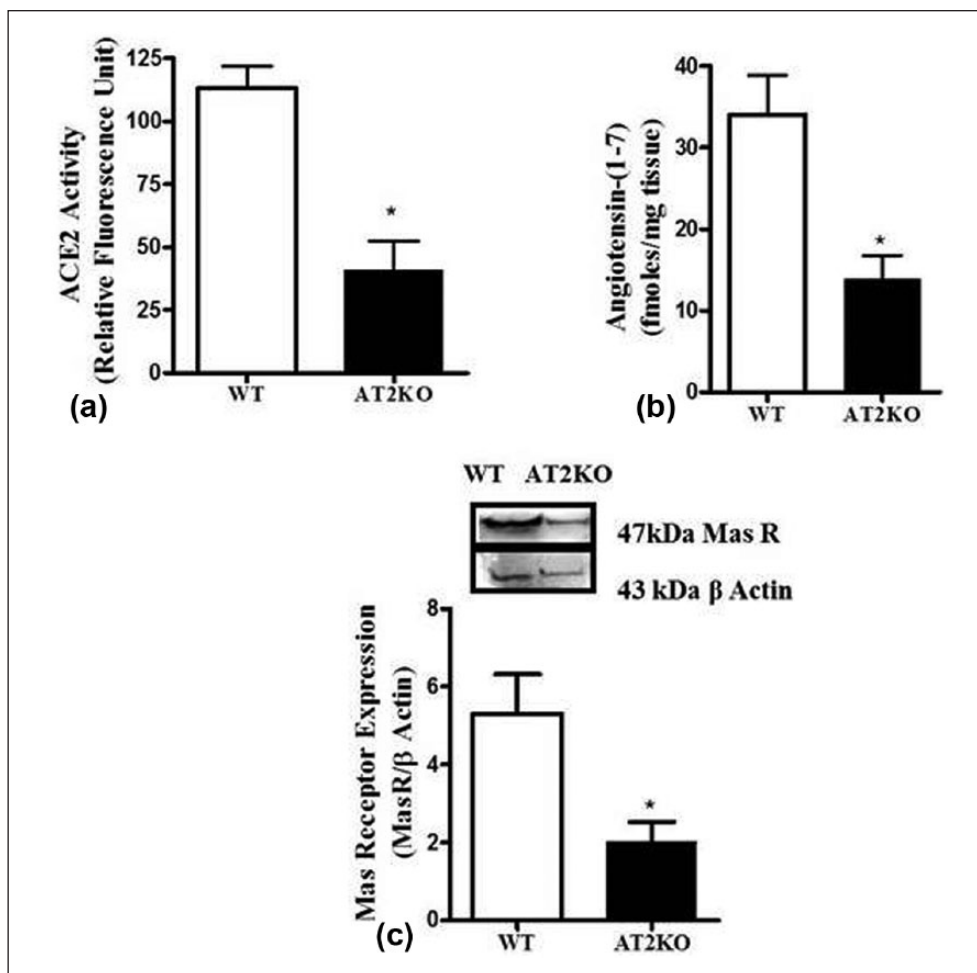


Figure 1. (a) Angiotensin-converting enzyme (ACE)2 activity (b) LC/MS quantification of angiotensin-(1-7) and (c) Mas receptor (MasR) expression in the kidney cortex of wild type and angiotensin II type 2 receptor knockout (AT₂KO) mice. Upper panels: representative western blot of MasR protein with loading control β-actin. Bar graph: represent the ratio of density of the protein band and β actin i.e. MasR/β actin *Significantly different compared with wild type (WT) mice. Values are represented as mean±standard error of the mean (SEM); *t*-test, *p*<0.05; *n*=5 in each group.

excreted increased amounts of albumin in the urine, and lack of AT₂R in HFD-fed mice modestly increased the urinary albumin excretion (Figure 4(c)). The urinary albumin excretion was within normal values in WT and AT₂KO mice on ND (Figure 4(c)).

Effect of deletion of AT₂R on systolic BP. As shown in Figure 5, systolic BP in AT₂KO on normal diet was modestly but significantly higher compared to WT. High fat diet significantly increased systolic BP in both WT and AT₂KO. However, the BP levels were similar in WT HFD and AT₂KO HFD mice.

Discussion

The most notable finding of the current study is that AT₂R deficient mice expressed a decreased level of ACE2/Ang-(1-7)/MasR while ACE/Ang II/AT₁R increased in the kidney

cortex. This shift in the balance of RAS components resulted in greater susceptibility to obesity-induced renal injury, which highlights the critical role of AT₂R in regulating renal RAS components and exerting reno-protective effects.

ACE2 is an important RAS enzyme which converts Ang II to Ang-(1-7). Similar to AT₂R, Ang-(1-7) via the MasR, can oppose the actions of Ang II mediated via AT₁R²⁸⁻³¹ and can exert vasodilatation, natriuresis, and reno-protection. It has been shown that stimulation of MasR inhibits the AT₁R-mediated regulation of ERK1/2 activity,³² a critical enzyme involved in cell growth and tissue remodeling under various pathological conditions. MasR is considered reno-protective, and blockade or deletion of this receptor has been shown to cause reduced natriuresis, increased proteinuria, and reduced renal blood flow.³³⁻³⁵ AT₁R expression in the kidney is markedly increased along with reduced glomerular tuft diameter, increased collagen and fibronectin in MasR knockout

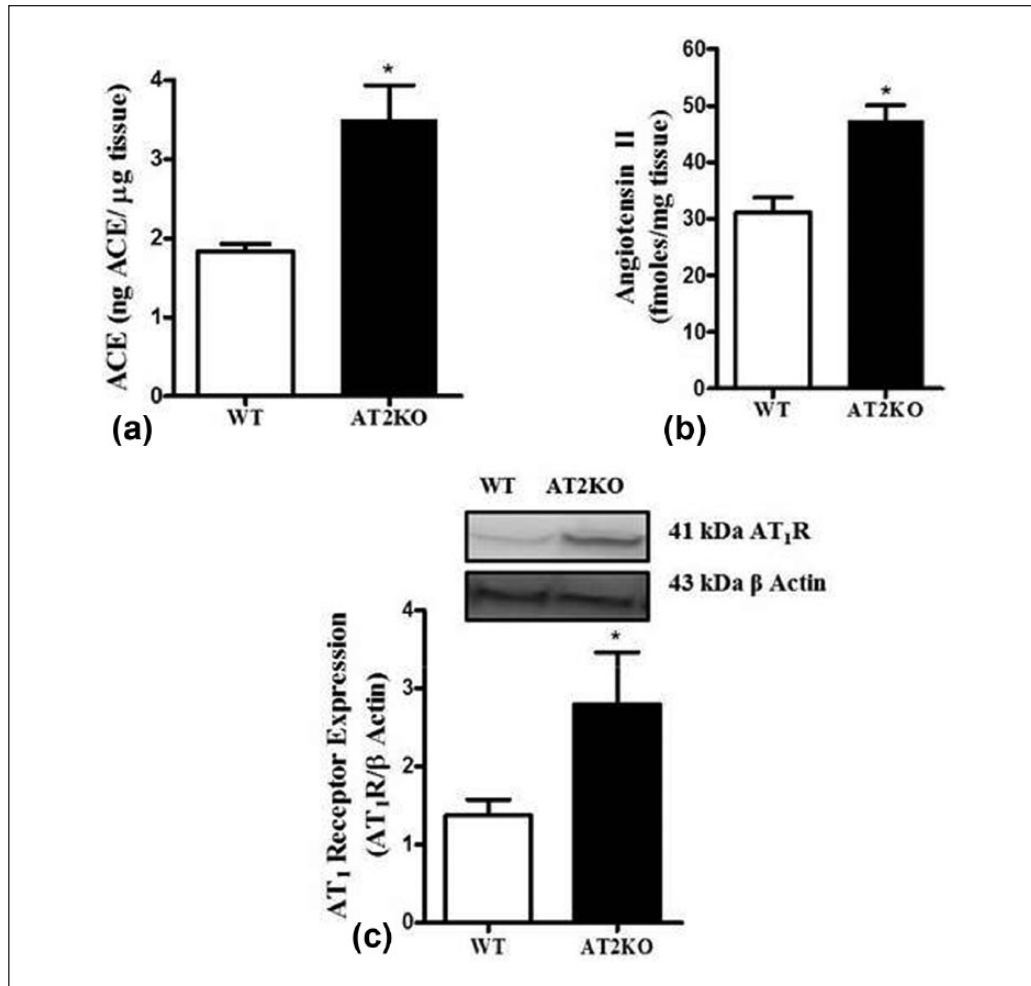


Figure 2. (a) ACE activity (b) LC/MS quantification of angiotensin II (Ang II) and (c) angiotensin type I receptor (AT₁R) expression in the kidney cortex of wild type (WT) and angiotensin II type 2 receptor knock-out (AT₂KO) mice. Upper panels: representative western blot of AT₁R protein with loading control β-actin. Bar graph: represent the ratio of density of the protein band and β actin i.e. AT₁/β actin *Significantly different compared with WT mice. Values are represented as mean±standard error of the mean (SEM); t-test, $p < 0.05$; $n = 5$ in each group.

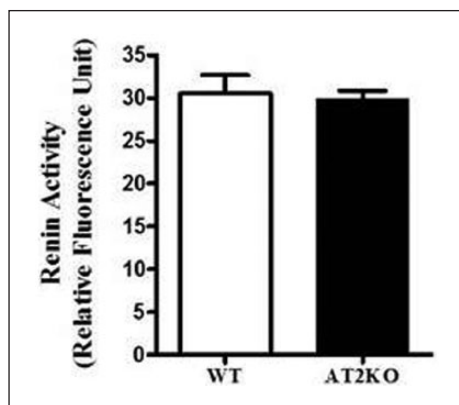


Figure 3. Renin activity in the kidney cortex of wild type (WT) and angiotensin II type 2 receptor knock-out (AT₂KO) mice. *Significantly different compared with WT mice. Values are represented as mean±standard error of the mean (SEM); t-test, $p < 0.05$; $n = 5$ in each group.

mice.³³ These findings suggest that MasR activation not only opposes the actions mediated by AT₁R, but the levels of MasR may inversely influence the expression of AT₁R. Adding to this RAS inter-regulatory complexity, we demonstrate for the first time that deletion of AT₂R decreases ACE2 activity, MasR expression, and decreases Ang-(1-7) levels in the kidney cortex of mice. Moreover, consistent with other reports,^{36,37} we also observed that deletion of AT₂R significantly increased AT₁R expression in the kidney cortex. Additionally, Ang II levels were also elevated in the kidney cortex of AT₂KO mice.

The inter-regulatory role of AT₂R in the two arms of RAS seems to be complex but can be plausibly explained. The enhanced AT₁R expression in AT₂KO mice kidney could be a result of the reduced expression on MasR in these animals, as earlier study has shown that deletion of MasR causes an increase in the AT₁R expression.³³ Subsequently, it is likely that the increase in AT₁R

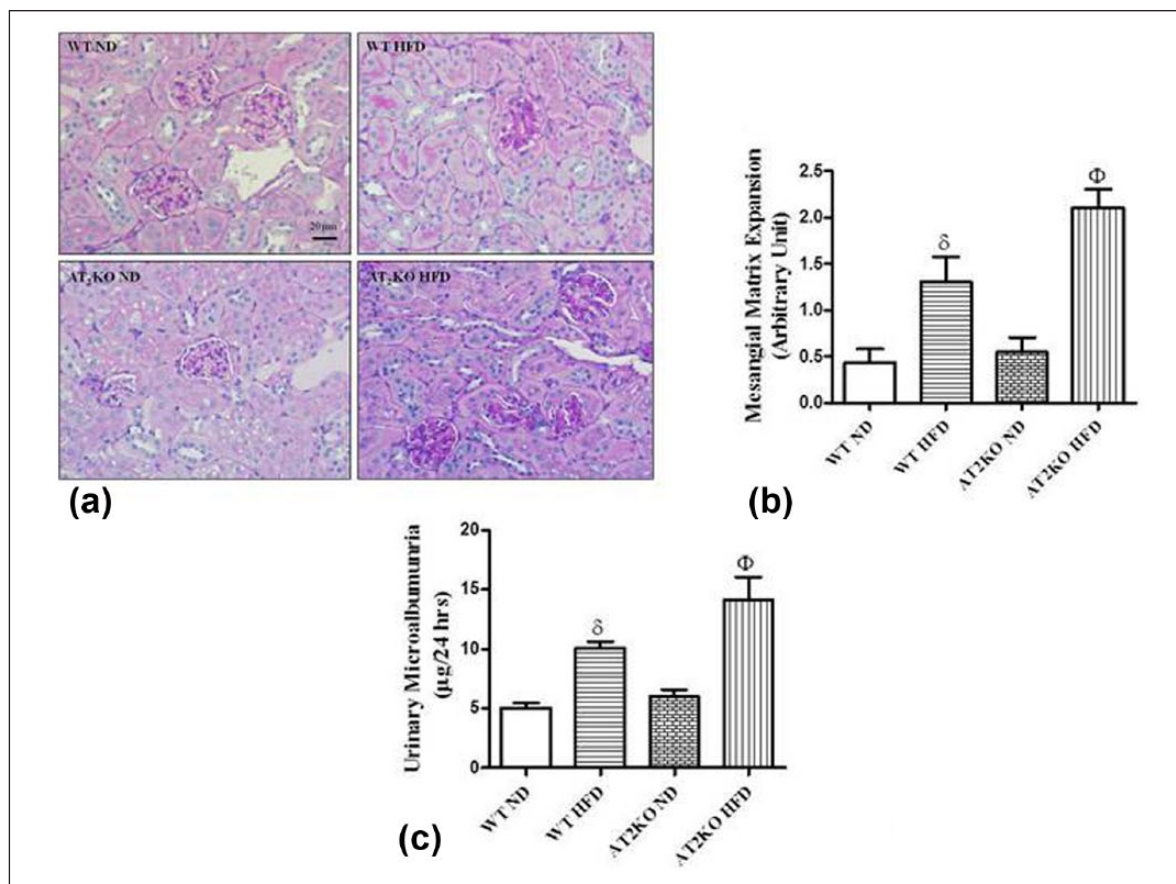


Figure 4. Markers of obesity-linked early renal injury in wild type (WT) and angiotensin II type 2 receptor knock-out (AT₂KO) mice. Renal morphology (a) and mesangial matrix expansion (MME) score (b) and 24-hour urinary albumin excretion (c) from WT and AT₂KO mice on a normal diet (ND) or high-fat diet (HFD). Sections measuring 4 µm were stained with periodic acid Schiff (PAS) stain (magnification×400). MME score was assigned based on a semiquantitative scale described in the methods section. Data are represented as mean±standard error of the mean (SEM) (n=6). The symbol δ indicates p<0.05 vs WT ND and Φ indicates p<0.05 vs KO ND mice. Scale bar - 50 µm.

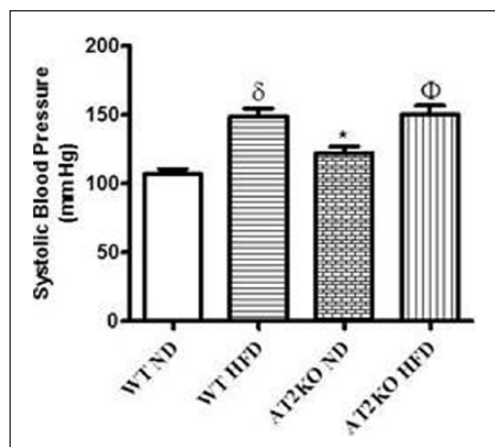


Figure 5. Systolic blood pressure in wild type (WT) and angiotensin type 2 receptor knock-out (AT₂KO) mice on a normal diet (ND) or high-fat diet (HFD). Data are represented as mean±standard error of the mean (SEM) (n=3–5). Both δ and * indicate p<0.05 vs WT ND, and Φ indicates p<0.05 vs KO ND mice.

expression and activation by higher Ang II concentration, as observed in this study, causes a reduction in ACE2 expression and activity leading to the reduced Ang-(1-7) production. Earlier studies have shown such relationship in that Ang II via activation of AT₁R causes a reduction in ACE2 expression and activity, thus reduces Ang-(1-7) production in cardiac myocytes.¹¹ However, we have recently reported that the AT₂R activation may have direct positive regulation of ACE2 expression and activity, without the involvement of AT₁Rs.⁸ Thus it's likely that AT₂R may have a direct as well as indirect role in regulating the protective and deleterious arms of the RAS. However, systemic studies are needed to delineate the role of AT₂R in regulating various RAS components.

The functional relevance of AT₂R deletion and of the shift in the two arms of the RAS was studied in animals subjected to high fat diet, which is known to cause renal injury. As expected HFD fed WT mice developed renal injury evidenced by MME and microalbuminuria in these animals. However, AT₂KO mice on HFD developed

greater renal injury, suggesting a protective role of AT₂R. Since AT₂KO per se had no changes in MME and microalbuminuria, it could be argued that AT₂R-mediated protection occurs only under pathogenic conditions, such as obesity. This notion is further supported by other studies,³⁸ including ours,¹⁶ which show that pharmacological activation of AT₂R protects against nephropathy in obese rats and diabetic animal models and had no injurious effects in normal animals. Hypertension is a known risk factor for renal injury.³⁹ It is likely that the injury in HFD fed WT mice was related to elevated BP in these animals. However, AT₂KO mice on HFD had similar elevated BP as WT HFD, yet exhibited greater renal injury, suggesting BP-independent protection by AT₂R. This AT₂R-mediated protection could be related to its anti-oxidative stress and anti-inflammatory activity, as reported recently.^{16,40} Consistent with a previous study,⁴¹ AT₂KO mice had modest elevation in BP, which had no significant impact on kidney structure and albuminuria. In the present study we found an imbalance of RAS components with AT₂KO without alteration in mesangial area and albuminuria. Although the levels of renal Ang II and AT₁R were increased, this may not be enough to induce kidney injury and MME. In a recent study, Kim et al.,⁴² demonstrated that even onset of early chronic kidney disease in an experimental model is not sufficient to cause glomerular injury or collagen deposition. This suggests that a simple increase in a deleterious RAS component may not lead to kidney damage. Indeed, we have recently shown that obese rats treated with high salt leads to increase in superoxide via Nicotinamide adenine dinucleotide phosphate-oxidase (NOX) and increased oxidative stress in kidney.⁴³ Superoxide can directly affect the glomerular barrier and increases Na reabsorption leading to hypertension and renal injury in obese rats and Dahl salt sensitive rats. Further, activation of AT₂R by the non-peptide agonist C21 reduces the Ang II level, expression of p47phox-NOX subunit, superoxide formation and salt-sensitive rise in BP,^{22,43} but had no effects on Ang II levels or oxidative stress in obese rats treated with normal diet.⁸ This suggests that the adverse effect of AT₂R deletion can be seen only under pathogenic conditions. The limitation of this study is the fact that we did not design this study to assess renal or plasma components of RAS in AT₂KO HFD mice, therefore it is unclear whether RAS components have changed in response to HFD per se and whether those changes might have further contributed to the renal injury in AT₂KO mice. However, collectively our data highlights the importance of AT₂R under pathogenic conditions, such as obesity, where it plays a protective role. The contribution of various components of the RAS in the face of opposing changes in obesity warrants a detailed systematic study.

In summary, we observed that deletion of AT₂R caused an elevation in renal ACE/Ang-II/AT₁R and reduction in ACE2/Ang-(1-7)/MasR expression leading to a shift from beneficial to deleterious axis of RAS. Thus, owing to the

beneficial role of renal AT₂R, it could be considered as a potentially important therapeutic target in preventing obesity related renal injury.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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