

**EFFECTS OF ANG II TYPE I AND TYPE II RECEPTORS ON OXIDATIVE STRESS  
AND RENAL NADPH OXIDASE AND SOD EXPRESSION**

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## Summary

Oxidative stress accompanies Ang II infusion, but the role of AT<sub>1</sub> vs. AT<sub>2</sub> receptors (R) is unknown. *Methods:* We infused Ang II subcutaneously in rats for one week. Excretion of 8-IsoPGF<sub>2α</sub> (8-Iso) and malonyldialdehyde (MDA) were related to renal cortical mRNA abundance for subunits of NADPH oxidase and superoxide dismutases (SODs) using real time PCR. Subsets of Ang II-infused rats were given the AT<sub>1</sub>-R antagonist, candesartan (Cand) or the AT<sub>2</sub>-R antagonist, PD-123,319 (PD). *Results:* Compared to vehicle (Veh), Ang II increased 8-Iso excretion by 41% (Veh:  $5.4 \pm 0.8$  vs. Ang II:  $7.6 \pm 0.5$  pg·24h<sup>-1</sup>; p<0.05). This was prevented by Cand ( $5.6 \pm 0.5$ ; p < 0.05) and increased by PD ( $15.8 \pm 2.0$ ; p < 0.005). There were similar changes in MDA excretion. Compared to Veh, Ang II significantly (p<0.005) increased the renal cortical mRNA expression of p22<sup>phox</sup> (2-fold), Nox-1 (2.6-fold) and Mn-SOD (1.5-fold) and decreased Nox-4 (2.1-fold) and EC-SOD (2.1-fold). Cand prevented all these changes except for the increase in Mn-SOD. PD accentuated changes in p22<sup>phox</sup> and Nox-1 and increased p67<sup>phox</sup>. *Conclusion:* Ang II infusion stimulates oxidative stress via AT<sub>1</sub>-R, which increases the renal cortical mRNA expression of p22<sup>phox</sup> and Nox-1 and reduces abundance of Nox-4 and EC-SOD. This is offset by strong protective effects of AT<sub>2</sub>-R, which are accompanied by decreased expression of p22<sup>phox</sup>, Nox-1, and p67<sup>phox</sup>.

## Introduction

Oxidative stress accompanies angiotensin II (Ang II) infusion, but the role of AT<sub>1</sub> vs. AT<sub>2</sub> receptors on reactive oxygen species (ROS) generation is not clear. Rajagopalan, et al., demonstrated that administration of Ang II increases blood pressure and the formation of superoxide (O<sub>2</sub><sup>-</sup>) in aortic rings (1;2). These changes did not accompany an equivalent elevation in blood pressure with norepinephrine.

Oxidative stress implies an imbalance between the generation and the scavenging of ROS, such as O<sub>2</sub><sup>-</sup> or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). O<sub>2</sub><sup>-</sup> is metabolized by superoxide dismutases (SODs). ROS are implicated in cell signaling (3) and bioinactivation of nitric oxide (4). They contribute to aging, hypertension (5;6), and cardiovascular and kidney diseases (5;7). NADPH oxidase has been identified as a major source of O<sub>2</sub><sup>-</sup> in the vessel wall (8). This electron transport system was first described in phagocytes where it is composed of a membrane-associated glycoprotein, gp91<sup>phox</sup> with p22<sup>phox</sup>, and three cytosolic components, p40<sup>phox</sup>, p47<sup>phox</sup> and p67<sup>phox</sup> (8). Several isoforms of the gp91<sup>phox</sup> (now termed Nox-2) component have been described, including Nox-1 in vascular smooth muscle cells (formerly Mox-1) (9), gp91<sup>phox</sup> in endothelial cells (10), and Nox-4 in the kidney and colon (formerly RENOX) (11). The kidney cortex contains a complete complement of phagocyte-type NADPH oxidase and all three Nox isoforms (12).

O<sub>2</sub><sup>-</sup> interacts with esterified or free arachidonate to yield a family of isoprostanes including 8-isoprostane prostaglandin F<sub>2α</sub> (8-Iso). The steady state excretion of 8-Iso reflects oxidative stress (13). There is evidence of enhanced oxidative stress during prolonged infusion of Ang II (14) and in models of Ang II-dependent hypertension (15-19). However, the roles of AT<sub>1</sub> vs. AT<sub>2</sub> receptors in the generation of oxidative stress and the expression of NADPH oxidase and SOD in the kidneys

has not been established.

The first aim of this study was to determine the effects of AT<sub>1</sub>- and AT<sub>2</sub>-receptor blockade during a “slow-pressor” dose of Ang II on the excretion of 8-Iso. Since isoprostanes also can be generated by metabolism of arachidonate via cyclooxygenase (20;21), additional studies assessed the excretion of an arachidonate-independent marker of lipid peroxidation, malonyldialdehyde (MDA). The second aim was to investigate the effects of these perturbations on the renal expression of the mRNAs for the components of NADPH oxidase and SOD isoforms.

## **Methods**

### ***Animal preparation***

Studies were approved by the Georgetown University Animal Care and Use Committee. They were performed according to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 93-23, revised 1985) and the Guidelines of the Animal Welfare Act.

Experiments were performed on male Sprague-Dawley rats weighing 210 - 300g. Rats (n= 8 each group) were maintained on a standard rat chow (Na<sup>+</sup> content: 0.3g·100g<sup>-1</sup>) for 8 to 10 days before being randomly assigned to different study protocols. Osmotic minipumps (Alzet, Model 2002) containing Ang II, vehicle (0.154M NaCl) (Veh) or PD-123,319 (PD) were placed for subcutaneous infusion on day 1 in the nape of the neck under sterile conditions during halothane anesthesia. The PD group had one minipump with Ang II and a second with PD. Ang II or vehicle were infused for 7 days, after which the animals were studied.

Rats were housed in individual cages under conditions of constant temperature and humidity. They were exposed to 12-hour cycles of light and dark. They had unrestricted water

intake. On the last day of study, rats were placed in clean, individual metabolism cages. A 24-hour urine was collected into containers with streptomycin (2000 IU), penicillin G (2000 IU) and amphotericin B (5 $\mu$ g) to prevent microbial overgrowth. The urine was centrifuged, separated from the sediments and stored at -70°C until analyzed. Urine was analyzed for volume, 8-Iso and MDA. At completion of the urine collections, rats were anesthetized and the kidneys flushed with ice-cold phosphate-buffered saline (PBS). They were removed immediately and the cortex separated by dissection for extraction of mRNA and stored at -70°C.

### ***Study protocols***

We tested the hypothesis that a prolonged subcutaneous infusion of Ang II increases the excretion of 8-Iso and MDA via type 1 or 2 receptors. Four groups of rats had osmotic minipumps inserted to infuse subcutaneously a Veh or Ang II at 200ng·kg<sup>-1</sup>·min<sup>-1</sup> for one week. The third group received the Ang II infusion and candesartan cilexetil (Cand). Cand was added to the drinking water in a dose calculated to deliver 10mg·kg<sup>-1</sup>·24h<sup>-1</sup>. This dose of Cand given to SHR over 10-14 days normalizes the elevated MAP and renal vascular resistance, and normalizes the depressed glomerular filtration rate (GFR) (17). The fourth group received separate subcutaneous infusions of Ang II and PD. PD was added to osmotic minipumps to deliver 60mg·kg<sup>-1</sup>·24h<sup>-1</sup>. This dose of PD was selected because, when given intravenously to normal rats, it has been shown to increase renal interstitial generation of nitric oxide metabolites and cyclic guanosine monophosphate (22). A lower dose of PD given by subcutaneous infusion to rats after a myocardial infarction reduces aortic compliance (23).

### ***mRNA Isolation and Real-time quantitative RT-PCR:***

RNA isolation and reverse transcription (RT) were performed as described previously (12). Briefly, total RNA was isolated from the kidney cortex with guanidinium isothiocyanate (QIAGEN, Valencia, CA) and treated with DNase. RT reactions were performed using the SuperScript Preamplification System for the first strand cDNA synthesis (Gibco BRL, Rockville, MD). Real time quantitative PCR was done using an ABI Prism 7700 Sequence detection system. Primers and probes for the NADPH oxidase subunits: p22<sup>phox</sup>, gp91<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup>, Nox-4, Nox-1, IC-SOD, Mn-SOD, EC-SOD were designed using Primer Express software 101 (Table 1). The probes were labeled with 6-carboxy-fluorecein (FAM) phosphoramidite as a reporter at the 5' end and with 6-carboxy-tetramethyl-rhodamine (TAMRA) as a quencher at the 3' end. ROX was used as a passive reference in each sample to normalize for non-PCR related fluctuations in fluorescence signal. As an active reference, endogenous 18S ribosomal RNA (r18S) or glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were amplified using specific primers and probes labeled with VIC<sup>™</sup> (ABI) at the 5' end as a reporter and TAMRA at the 3' end as a quencher. The sensitivity and specificity of the assays were assessed from serial dilutions of reference and target templates in separate PCR reactions. Optimal primer concentrations were determined as the minimum primer concentration giving maximum change in emission intensity (R<sub>n</sub>) and minimum cycle number of fluorescence signal of the product that crosses an arbitrary threshold set within the exponential phase of the PCR (C<sub>T</sub>). Optimal probe concentrations were chosen as those which gave minimum C<sub>T</sub>. The comparative C<sub>T</sub> method was used for relative quantification and statistical analysis (24).

Experiments were conducted in two series. Series 1 compared rats infused with vehicle or Ang II. Series 2 compared Ang II infused rats with Ang II vs. Cand and Ang II vs. PD.

### ***Chemical methods***

*8-IsoPGF<sub>2α</sub>*: Urine for 8-Iso was extracted, purified, and analyzed with an enzyme immunoassay kit (Cayman Chemical) using methods described in detail and validated in a prior study (25). Our assay has a limit of detection of 1pg·ml<sup>-1</sup>, an intra-assay coefficient of variation of 8% (n=10) and an inter-assay coefficient of variation of 10% (n=6). A blinded comparison of 12 rat urine samples analyzed both with our RIA method and by GCMS (kindly undertaken by Jack Roberts, M.D., University of Vanderbilt) showed a good correlation (r=0.85; p<0.001) without systematic bias. The variation includes that due to RIA and to GCMS. Briefly, samples were diluted to fall in the mid portion of the standard curve (10 to 100 pg·ml<sup>-1</sup>). They were extracted using a polyboronic acid column, eluted with ethyl acetate containing 1% methanol, and evaporated under nitrogen. 8-Iso was assayed using an enzyme linked competitive binding assay (ELISA) with mouse anti-rabbit IgG monoclonal antibody in a 96-well plate. Concentrations of the reaction product were determined from absorbency at 405nm using a standard curve. Samples were assayed in duplicate. Recovery of [<sup>3</sup>H]-8-Iso averaged 76 ± 3% (n=12).

*MDA*: Malonyldialdehyde (MDA) in the urine was measured by a commercial kit (Oxi-Tek TBARS assay kit, Zeptomatrix Corp., Buffalo, NY) that utilizes the measurement of thiobarbituric acid reactive substances (TBARS). Briefly, urine (100 μl) was mixed with 100 μl of 8.1% sodium dodecyl sulfate (SDS). The TBA/buffer reagent was prepared by mixing 0.5 g of thiobarbituric acid with 50 ml of acetic acid and 50 ml of NaOH. 2.5 ml of TBA/buffer reagent was added to 200 μl of sample/SDS mixture and incubated at 95°C in capped tubes for 60 minutes. Thereafter, the sample was cooled to room temperature on in an ice bath for 10 min and centrifuged at 3000 rpm for 15 min. The supernatant was removed and its absorbance measured

at 532 nm in semi-micro cuvettes in a spectrophotometer (Genesys ® 10 Vis). The concentration of TBARS was expressed in  $\text{nmol}\cdot\text{ml}^{-1}$  of MDA equivalents by interpolation from standard curve of MDA in concentration from 0-10  $\text{nmol}\cdot\text{ml}^{-1}$ .

### ***Drugs***

Ang II (Sigma Chemical Inc., St. Louis, MI) was dissolved in 0.9% saline. Candesartan cilexetil was a generous gift of Peter Morsing, Ph.D. of AstraZeneca (Moldal, Sweden). It was prepared daily and mixed in the drinking water according to the manufacturer's instructions as described previously (26). PD-123,319-121B (trifluoroacetate salt) was a generous gift from Joan Keiser, Ph.D., Parke Davis (Chicago, IL). It was prepared as described previously (22), and was added to the osmotic minipumps.

### ***Statistical methods***

Results are reported as mean  $\pm$  SE. Data were analyzed using SPSS. Comparison between multiple groups was by analysis of variance (ANOVA). When appropriate, *post hoc* comparisons between groups were made by Dunnett's t test. The mRNA expression for Ang II infused animals relative to vehicle, and for the co-administration of Cand and PD with Ang II, are reported as the ratio  $\pm$  the central-limit SE with the 95% confidence intervals based on the assumption of an approximate log-normal distribution of the ratios.

### **Results**

The body weight of rats averaged  $277 \pm 8$  g and did not differ between groups. The 24-hour urine volume of vehicle-infused rats was  $3.2 \pm 0.3$   $\text{ml}\cdot 24\text{h}^{-1}\cdot 100\text{g}^{-1}$ . It was increased in those

infused with Ang II ( $4.7 \pm 0.3 \text{ ml}\cdot 24\text{h}^{-1}\cdot 100\text{g}^{-1}$ ;  $p < 0.025$ ). Among rats infused with Ang II, the urine volume was not affected by Cand, but was increased by PD ( $11.8 \pm 0.9 \text{ ml}\cdot 24\text{h}^{-1}\cdot 100\text{g}^{-1}$ ;  $p < 0.001$ ).

Compared to vehicle-infused rats, subcutaneous Ang II infusion increased the excretion of 8-Iso by 41% (Veh:  $5.4 \pm 0.8$  vs. Ang II:  $7.6 \pm 0.5 \text{ pg}\cdot 24\text{h}^{-1}$ ;  $p < 0.05$ ) (fig 1A). The increase with subcutaneous Ang II was prevented by Cand ( $5.6 \pm 0.5$ ;  $p < 0.05$ ), but during PD the increase with Ang II was 2.1-fold greater than with Ang II alone ( $15.8 \pm 2.0$ ;  $p < 0.005$ ). Subcutaneous Ang II also increased the excretion of MDA by 47% (Veh:  $17.9 \pm 1.2$  vs. Ang II:  $26.4 \pm 1.5 \text{ nmol}\cdot 24\text{h}^{-1}$ ;  $p < 0.002$ ) (fig 1B). This too was prevented by Cand ( $20.2 \pm 1.9 \text{ nmol}\cdot 24\text{h}^{-1}$ ), but during PD the increase with Ang II was increased further ( $33.2 \pm 1.3 \text{ nmol}\cdot 24\text{h}^{-1}$ ;  $p < 0.002$ ). We conclude that Ang II increased oxidative stress, as reflected by changes in excretion of 8-Iso and MDA, and that these effects are dependent on the AT<sub>1</sub>-receptor and are offset by the AT<sub>2</sub>-receptor.

The data for mRNA expression in the kidney cortex, relative to GAPDH and r18S are presented in Table 2 and in figures 2 and 3. Since these figures represent changes compared to the mean values for the vehicle or Ang II alone groups, only mean changes can be calculated. A unit increase in cycle value represents a 2-fold increase in mRNA abundance. Reported probability values for mRNA expression in Table 2 should be interpreted cautiously in light of the increased chance of a type-I error because of multiple comparisons. A nine-fold Bonferroni adjustment for the examination of nine subunits requires  $p < (0.05/9)$  or  $p < 0.0056$  for a decisive conclusion of significance. Only those results that remain significant under the Bonferroni adjustment are shown in figures 2 and 3. Compared to Veh, subcutaneous infusion of Ang II significantly ( $p < 0.0056$ ) increased the expression of p22<sup>phox</sup> (2-fold), Nox-1 (2.6-fold), and Mn-

SOD (1.5-fold) and decreased the expression of Nox-4 (2.1-fold) and EC-SOD (2.1-fold). All of the changes caused by Ang II, except for Mn-SOD, were reversed in rats receiving Ang II plus Cand ( $p < 0.005$ ). Co-infusion of PD with Ang II significantly ( $p < 0.0056$ ) increased the expression of p22<sup>phox</sup> (2-fold), Nox-1 (2.3-fold) and p67<sup>phox</sup> (1.9-fold).

## Discussion

The main new findings of this study are that the increased excretion of 8-Iso during prolonged subcutaneous infusion of Ang II is prevented by blockade of AT<sub>1</sub>-R, but is enhanced by blockade of AT<sub>2</sub>-R. There are similar changes in excretion of MDA. Ang II increases the renal cortical expression of the mRNAs for p22<sup>phox</sup> and Nox-1 and decreases the expression of Nox-4. In Ang II infused rats, changes in NADPH oxidase are prevented by Cand, whereas increases in p22<sup>phox</sup> and Nox-1 are accentuated by PD which also enhances expression of p67<sup>phox</sup>. Ang II infusion enhances expression of Mn-SOD by an action that is not affected by Cand or PD, and decreases EC-SOD which is prevented by Cand.

One limitation of this study is the use of mRNA rather than protein. However, we found good general agreement between changes in mRNA and protein for NADPH oxidase subunits in the kidney of the SHR (12). Since our antibodies to gp91<sup>phox</sup> cross react with other isoforms, we elected for the specificity of the mRNA method. Another limitation is the absence of physiological data. We elected not to instrument the animals to prevent confounding effects on mRNA expression. Moreover some effects of Ang II infusion under similar conditions and the effects of AT<sub>1</sub>- or AT<sub>2</sub>-R blockade on renal function and BP have been reported. This dose of Ang II leads to a slow pressor response that raises the BP by approximately 22mmHg and reduces the renal blood flow by approximately 20% over 7-12 days (27). The BP during Ang II

infusion is reportedly reduced by AT<sub>1</sub>-R blockade, but is not altered by AT<sub>2</sub>-R blockade (28). Therefore, in this study, changes in oxidative stress with Ang II, and Ang II + Cand likely were complicated by parallel changes in blood pressure. However, we have shown recently that oxidative stress in the kidney cortex of the SHR is reversed in full by two weeks of administration of an AT<sub>1</sub>-receptor antagonist, but not by equally effective antihypertensive therapy that does not block Ang II (29). Moreover, both in the young SHR (12) and in the young SHR stroke-prone (30), the oxidative stress and/or enhanced expression of NADPH oxidase preceded the development of hypertension. Therefore, the effects of AT<sub>1</sub>-receptor blockade have been dissociated from blood pressure.

O<sub>2</sub><sup>-</sup> interacts with arachidonate that is esterified into membrane phospholipids and yields free isoprostanes after hydrolysis by phospholipases (13). Therefore, a short-term increase in 8-Iso excretion may relate to release of preformed isoprostanes. Ang II is a potent stimulus to phospholipase activity (31). However, the sustained increase in 8-Iso detected after a week of Ang II likely represents increased generation. Isoprostanes also can be generated enzymically by COX-1 in platelets (32) or COX-2 in monocytes (33) or isolated glomeruli (34). However, we found the same pattern of changes in excretion of 8-Iso and MDA which is not generated by cyclooxygenase (fig 1A and B). Moreover, the enhanced excretion of 8-Iso in the SHR (25), and the Ang II-infused mouse (35), is reversed by co-infusion of a membrane permeable SOD mimetic tempol to obviate associated oxidative stress. These markers of lipid peroxidation apparently reflect systemic, rather than renal-limited, oxidative stress (13;36).

AT<sub>1</sub>-R and AT<sub>2</sub>-R are widely distributed in the cortex and medulla of the kidneys of adult rats, except for the glomerulus, which lacks the mRNA or immunocytochemical staining for AT<sub>2</sub>-R (37). The increase in O<sub>2</sub><sup>-</sup> by Ang II in cultured endothelial cells is prevented by blockade of

AT<sub>1</sub>-R and enhanced by blockade of AT<sub>2</sub>-R (38). AT<sub>2</sub>-R in the kidney are upregulated by salt depletion or Ang II infusion (39). An enhanced expression of AT<sub>2</sub>-R may explain why blockade of the AT<sub>2</sub>-R had such potent effects to enhance oxidative stress during Ang II infusion. Our results assign the oxidative stress during Ang II infusion to activation of AT<sub>1</sub>-R. They demonstrate that the relatively modest effects of prolonged Ang II are due to offsetting activation of AT<sub>2</sub>-R.

Three isoforms of a gp91<sup>phox</sup> family (Nox-1, gp91<sup>phox</sup> and Nox-4) have been reported in blood vessels or kidney. They form the catalytic center of the enzyme (8). The normal rat kidney expresses the mRNA for each of these (12). There is a prominent expression of gp91<sup>phox</sup> in vascular smooth muscle cells (VSMCs) of resistance arterioles from humans (40), in fibroblasts (41) and endothelial cells (10). gp91<sup>phox</sup> is upregulated by Ang II in microvessels (40). Our failure to detect this change in kidney cortex may relate to the small contribution of microvessels to whole kidney mRNA. Nox-1 is expressed in VSMCs and the colon of rodents (42). The Nox-1 is a major isoform in larger blood vessels where it can replace gp91<sup>phox</sup> and mediate Ang II-induced O<sub>2</sub><sup>-</sup> formation and redox signaling (42). We confirmed that Ang II upregulates Nox-1 in the kidney and showed that this can be ascribed to effects of AT<sub>1</sub> receptors that are offset by opposite effects of AT<sub>2</sub> receptors. Nox-4 has been located in the proximal tubules and medullary collecting ducts (11). Nox-4 in VSMCs is upregulated by Ang II in VSMCs of transgenic rats with extra renin genes (43) whereas we detected a reduction in Nox-4 in the kidney with Ang II. This may relate to differences in the kidney compared to VSMCs or to the fact that Ang II stimulates Nox-4 in VSMCs only for the first 24 hours (44). Moreover, renin transgenic rats have a severe, malignant form of hypertension in contrast to the relatively moderate increase in BP associated with Ang II infusion in our study. Indeed, our observation is in agreement with

Lassegue et al (42) who reported down-regulation of Nox-4 by Ang II in VSMCs. The increase in renal cortical Nox-1 mRNA expression in Ang II infused rats was accompanied by increased p22<sup>phox</sup>. This change is apparently mediated by AT<sub>1</sub>-R, since it is blocked by Cand, similar to results reported in the vasculature (6). Blockade of AT<sub>2</sub>-R with PD accentuates the effects of Ang II to increase expression of p22<sup>phox</sup> and Nox-1. The parallel upregulation of p22<sup>phox</sup> and Nox-1 by Ang II, that is prevented by Cand and accentuated by PD, are accompanied by parallel changes in oxidative stress. This suggests that p22<sup>phox</sup> and Nox-1 may be critical in transducing the effects of AT<sub>1</sub>-R to increase, and AT<sub>2</sub>-R to decrease, the activity of NADPH oxidase to generate O<sub>2</sub><sup>-</sup> in the kidney.

We found that Ang II infused with PD stimulates the expression of p67<sup>phox</sup> in the kidney. Ang II upregulates p67<sup>phox</sup> in adventitial fibroblasts (45) and aorta (46). While the function of the subunits of NADPH oxidase in non-phagocytic cells remains controversial, p67<sup>phox</sup> facilitates the transfer of electrons to the flavine center of the cytochrome *b* and contains an NADPH binding site (8;47). Absence or dysfunction of this subunit results in impaired phagocyte production of superoxide and the clinical condition of chronic granulomatous disease (48). Although p47<sup>phox</sup> appears to be crucial for the enzyme overall activity, it can be substituted for high abundance of p67<sup>phox</sup> (8). Upregulation of p67<sup>phox</sup> might contribute to oxidative in Ang II infused rats given PD.

The finding that Ang II infusion decreases the expression of EC-SOD and that this is reversed by Cand suggests that EC-SOD may contribute to the protective effects of AT<sub>1</sub> receptor blockade on oxidative stress in the kidneys (17). Losartan has also been reported to increase EC-SOD activity in humans (49). In contrast, Fukai et al (50), reported that losartan prevents the increased expression of EC-SOD in blood vessels of mice infused with Ang II (50). The cause

for these differences is not clear. Moreover, it is uncertain whether EC-SOD is fully effective in the rat (23). Interestingly, nitric oxide increases the activity of vascular EC-SOD (51). Cand increases bioactive nitric oxide in the hypertensive rat kidney (17). Therefore, the enhanced EC-SOD expression induced by Cand during Ang II may be a response to increased nitric oxide within the kidney.

Ang II increased Mn-SOD expression in the kidney cortex. An increase in Mn-SOD occurs in response to oxidative stress which could underlie this finding. However, the increased expression was not affected by Cand which suggests that the effects of Ang II may be mediated via an independent mechanism, such as by Ang (1-7).

In conclusion, these studies confirm that Ang II causes oxidative stress. They ascribe the increased oxidative stress to activation of AT<sub>1</sub>-R, which is offset by AT<sub>2</sub>-R. Ang II infusion enhances the renal cortical mRNA expression of p22<sup>phox</sup>, Nox-1, and Mn-SOD, and reduces the expression of Nox-4 and EC-SOD. Activation of AT<sub>1</sub> receptors contributes to increased expression of p22<sup>phox</sup> and Nox-1 and decreased expression of Nox-4 and EC-SOD whereas activation of AT<sub>2</sub> receptors blunt the increase in p22<sup>phox</sup> and Nox-1 and reduce the expression of p67<sup>phox</sup>. Thus, there are powerful, counterveiling effects of type 1 and 2 receptors on oxidative stress in the kidney.

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## Figure Legends

Figure 1: Mean  $\pm$  SEM values in groups (n=8) of rats showing 24 hour excretion of 8-Isoprostane PGF<sub>2 $\alpha$</sub>  (8-Iso) (panel A) or malonyldialdehyde (MDA) (panel B) in rats were infused with a vehicle (closed boxes) or angiotensin II (200ng·kg<sup>-1</sup>·min<sup>-1</sup>) given alone (open boxes) or with Candesartan (10mg·kg<sup>-1</sup>·24h<sup>-1</sup>) (gray boxes) or PD-123,319 (60mg·kg<sup>-1</sup>·24h<sup>-1</sup>) (vertical line boxes). Significance of change from vehicle: \*, p < 0.05; \*\*\*, p < 0.005.

Figure 2: Expression of NADPH oxidase subunits (panel A) and SODs (panel B) that remained significant under the Bonferroni adjustment, presented as fold differences in  $\Delta C_T$  values in groups of rats (n=6-7) infused with angiotensin II compared to vehicle. Significance of change from vehicle: \*\*\*, p < 0.0056.

Figure 3: Expression of NADPH oxidase subunits and SODs expression that remained significant under the Bonferroni adjustment, presented as fold differences in  $\Delta C_T$  values in groups of rats (n=6-7). Data compare Ang II plus Candesartan (gray boxes) or Ang II plus PD-123,319 (vertical line boxes) with Ang II alone. Compared to Ang II alone; \*\*\*, p < 0.0056.

**Table 1: Oligonucleotides and related products**

<i>Gene</i>	Oligonucleotide sequences for forward (F) and reverse (R) primers (5'-3')	Location of product (bp)	GenBank Accession No.
Nox-1	F 5'- GGAGTTGCAGGAGTCCTCATTTT - 3'	888-910 bp	AF152963
	R 5'- TTCTGCCGGGAGCGATAA - 3'	1005-988 bp	
	Probe: 5'-6FAM-CAACCCCTGAGTCTTGGAAGTGGATC-3'	917-943 bp	
Nox-4	F 5' - AGAATGAGGATCCCAGAAAGCTT-3'	489-511 bp	AY027527
	R 5' - ATGAGGAACAATACCACCACCAT-3'	570-548 bp	
	Probe: 5' -6FAM - CTTCACAAC TGTTCGGGCCTGACA –TAMRA-3'	514-538 bp	
gp91 <sup>phox</sup>	F 5' -AAAGGAGTGCCAGTACCAAAGT-3'	762-784 bp	AF298656
	R 3' -TACAGGAACATGGGACCCACTAT-3'	839-817 bp	
	Probe 5'-6FAM - CCGGAAACCCTCCTATGACTTGGAAAT G – TAMRA-3'	788-815 bp	
p22 <sup>phox</sup>	F 5'-ACCTGACCGCTGTGGTGAA-3'	259-277 bp	U18729
	R 5'-GTG GAG GAC AGC CCG GA-3'	326-310 bp	
	Probe: 5'-6FAM-CTGTTCCGGGCCCTCACCAGAAATTAC T – TAMRA-3'	279-306 bp	
p47 <sup>phox</sup>	F 5'-ACGCTCACCGAGTACTTCAACA-3'	291-312 bp	AY029167
	R 5'-TCATCGGGCCGCACTTT-3'	385-369 bp	
	Probe 5'-6FAM-CCCCTGCCCACACCTCTTGAAC T – TAMRA-3'	340-363 bp	
p67 <sup>phox</sup>	F 5'-GCTTCGGAACATGGTGTCTAAGA-3'	1215-1237bp	AB002664
	R 5'-AGAGTCAGGCAGTAGTTTTTCACTTG-3'	1364-1339 bp	
	Probe: 5'-FAM6-AACACACTAAACTGAGCTACCGGCGTCG-TAMRA-3'	1256-1283bp	
IC-SOD	F 5'-TGTGTCCATTGAAGATCGTGTGA-3'	384-406 bp	NM-017050

	R 5'-TCTTGTTTCTCGTGGACCACC-3'	467-447 bp	
	Probe: 5'-FAM6-CTCAGGAGAGCATTCCATCATTGGCC-TAMRA-3'	414-439 bp	
Mn-SOD	F 5'-TTAACGCGCAGATCATGCA-3'	193-211 bp	NM-017051
	R 5'-CCTCGGTGACGTTTCAGATTGT-3'	267-247 bp	
	Probe: 5'-FAM6-CACAGCAAGCACCACGCGACCTAC-TAMRA-3'	219-242 bp	
EC-SOD	F-5'-GGCCCAGCTCCAGACTTGA-3'	385-403 bp	X94371
	R 5'-CTCAGGTCCCCGAACTCATG-3'	487-468 bp	
	Probe: 5'-FAM6-CAGCCGAGCAGAACACCTCCAACC-TAMRA-3'	430-453 bp	

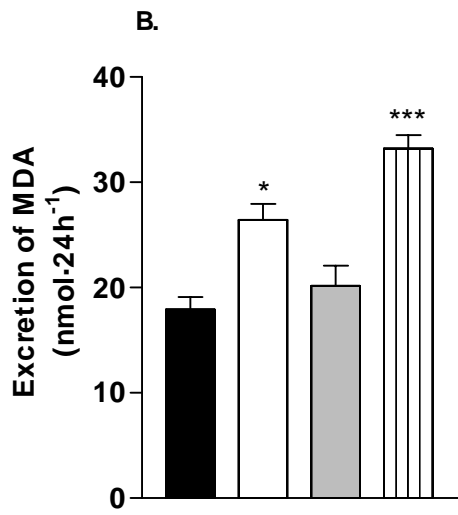
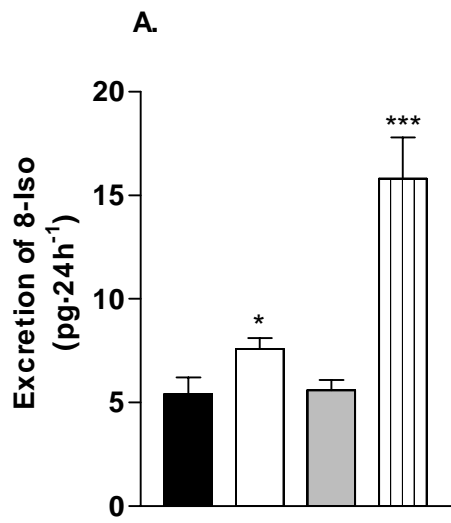
**Table 2: The mRNA (expressed as  $\Delta C_T$ ) for NADPH oxidase subunits and SODs in kidney cortex: effects of Ang II, and of AT<sub>1</sub>- or AT<sub>2</sub>-receptor blockade during Ang II**

Group	p22phox	gp91phox	NOX-1	NOX-4	p47phox	p67phox	IC-SOD	Mn-SOD	EC-SOD
<b>Series 1 – Vehicle</b>									
mRNA $\Delta C_T$	5.44 ± 0.18 (6)	6.20 ± 0.26 (7)	14.10 ± 0.21 (7)	1.25 ± 0.16 (6)	6.38 ± 0.18 (7)	11.89 ± 0.22 (7)	3.54 ± 0.18 (7)	1.30 ± 0.09 (6)	0.95 ± 0.13 (6)
<b>Series 1 – Angiotensin II</b>									
mRNA $\Delta C_T$	4.45 ± 0.09 (7)	6.70 ± 0.09 (7)	12.67 ± 0.07 (7)	2.35 ± 0.08 (7)	6.27 ± 0.10 (8)	11.22 ± 0.11 (8)	2.98 ± 0.10 (7)	0.70 ± 0.06 (8)	2.00 ± 0.13 (7)
ratio relative to vehicle	0.85 ± 0.03 (0.78--0.93) p = 0.005	1.08 ± 0.05 (0.98--1.19) p = 0.107	0.90 ± 0.01 (0.87--0.93) p < 0.001	1.88 ± 0.25 (1.40--2.53) p < 0.001	0.98 ± 0.03 (0.92--1.05) p = 0.597	0.95 ± 0.02 (0.91--1.00) p = 0.045	0.84 ± 0.05 (0.74--0.96) p = 0.022	0.54 ± 0.06 (0.42--0.68) p < 0.001	2.12 ± 0.32 (1.52--2.94) p < 0.001
<b>Series 2 – Angiotensin II</b>									
mRNA $\Delta C_T$	2.57 ± 0.03 (6)	6.54 ± 0.19 (6)	13.26 ± 0.13 (6)	2.68 ± 0.19 (6)	7.39 ± 0.16 (6)	11.97 ± 0.17 (6)	4.00 ± 0.16 (6)	2.33 ± 0.11 (7)	2.98 ± 0.11 (6)
<b>Series 2 – Angiotensin II+Candesartan</b>									
mRNA $\Delta C_T$	3.72 ± 0.13 (6)	6.57 ± 0.12 (7)	14.76 ± 0.08 (7)	1.34 ± 0.10 (7)	7.58 ± 0.09 (7)	11.95 ± 0.16 (7)	4.18 ± 0.19 (7)	2.29 ± 0.08 (7)	2.04 ± 0.20 (6)
ratio relative to Ang-II	1.45 ± 0.05 (1.34--1.57) p < 0.001	1.00 ± 0.03 (0.93--1.08) p = 0.992	1.11 ± 0.01 (1.08--1.14) p < 0.001	0.50 ± 0.05 (0.40--0.63) p = 0.001	1.03 ± 0.03 (0.97--1.08) p = 0.655	1.00 ± 0.02 (0.96--1.04) p = 0.994	1.05 ± 0.06 (0.92--1.20) p = 0.977	0.98 ± 0.06 (0.87--1.12) p = 0.949	0.69 ± 0.07 (0.55--0.86) p = 0.005
<b>Series 2 – Angiotensin II+PD-123,319</b>									
mRNA $\Delta C_T$	1.65 ± 0.04 (6)	6.26 ± 0.17 (6)	11.99 ± 0.31 (6)	1.74 ± 0.31 (7)	6.65 ± 0.25 (6)	11.05 ± 0.11 (6)	4.56 ± 0.26 (6)	2.15 ± 0.12 (6)	2.66 ± 0.22 (6)
ratio relative to Ang-II	0.64 ± 0.02 (0.61--0.68) p < 0.001	0.96 ± 0.04 (0.88--1.05) p = 0.387	0.90 ± 0.02 (0.84--0.95) p < 0.001	0.65 ± 0.12 (0.43--0.99) p = 0.016	0.90 ± 0.04 (0.82--0.99) p = 0.017	0.92 ± 0.02 (0.89--0.96) p = 0.001	1.18 ± 0.08 (1.01--1.37) p = 0.11	0.92 ± 0.07 (0.78--1.08) p = 0.371	0.90 ± 0.08 (0.73--1.10) p = 0.403

mRNA  $\Delta C_T$  expression summaries are shown as mean ± SEM (N)

Changes in the mRNA expression are shown as ratio ± SE / (95% confidence interval for ratio) / significance for ratio.

Figure 1.



**Figure 2.**

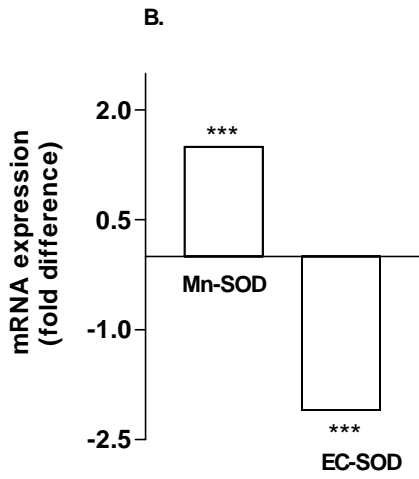
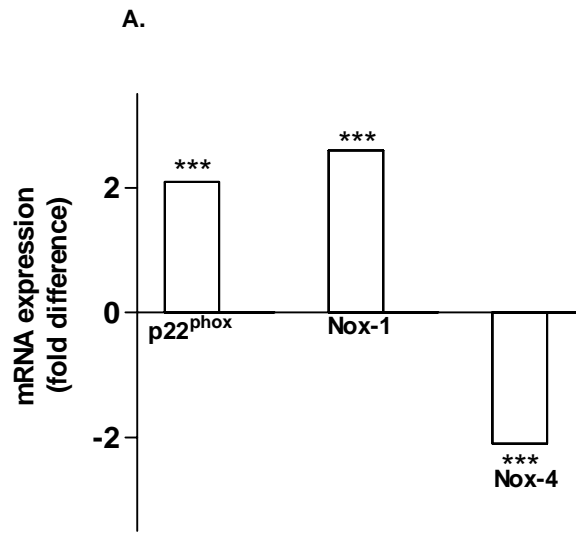


Figure 3.

