Resistance to oxidative stress by chronic infusion of angiotensin II in mouse kidney is not mediated by the AT₂ receptor

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ANGIOTENSIN II (ANG II) is a pivotal physiological regulator of blood pressure and cardiovascular and renal cell growth (46). An excess of ANG II or unopposed actions of ANG II (48), however, can induce hypertension, cardiac hypertrophy and remodeling, heart failure, vascular hypertrophy, atherosclerosis, and profound renal damage (24). Most of these actions are mediated via the AT₁ receptor (15), whereas the AT₂ receptor seems to oppose many of these processes. There is now strong evidence that actions of ANG II are exerted via generation of superoxide (O₂⁻) and are opposed by nitric oxide (NO) (10, 48). Several of the actions of the AT₂ receptor are mediated by stimulating NO synthesis (49).

Prooxidative actions of ANG II have been extensively studied in endothelial cells and vascular smooth muscle cells. Both cell culture studies and animal experiments indicate that ANG II induces O₂⁻ by enhancement of NADPH-oxidase (29, 34, 45). The kidney clearly is a target organ for the physiological actions of ANG II (i.e., blood pressure regulation) and for ANG II-mediated inflammation and cell damage. Nevertheless, pro- and antioxidant responses to ANG II in the renal cortex are less clear. Induction of components of NADPH-oxidase was found after 1 wk of ANG II infusion in rats (11). Renal tubular cells exposed to ANG II can modulate the cell cycle via NADPH-oxidase generated O₂⁻ (20). Information about antioxidant forces opposing ANG II-induced oxidative stress, however, is limited.

Chronic NO synthase (NOS) inhibition leads to profound renal damage in rats (3, 7, 54) but not in mice (13). The effects of NOS inhibition in rats can be completely prevented by AT₁ receptor blockade, indicating that damage is caused by unopposed actions of ANG II (48). We therefore reasoned that mice might well be relatively resistant to ANG II. The hypothesis of the present study is that elevated ANG II levels induce expression of antioxidative genes via the AT₂ receptor in the mouse renal cortex and thereby counteract prooxidative forces. To address this, we first studied renal cortical and aortic expression of antioxidative genes, the AT₂ receptor, heme oxygenase-1 (HO-1) and the AT₂ receptor were both induced in renal cortex and aorta. In contrast, ANG II suggestively increased AT₂ receptor expression in kidney but not in aorta. AT₂ receptor blockade enhanced hypertension in ANG II-infused mice, reversed ANG II effects on NOx excretion, but did not affect TBARS. Despite its prohypertensive effect, expression of NADPH oxidase components was decreased in renal cortex but induced in aorta. Heme oxygenase-1 (HO-1) was induced in both renal cortex and aorta. In contrast, ANG II suggestively increased AT₂ receptor expression in kidney but not in aorta. AT₂ receptor blockade enhanced hypertension in ANG II-infused mice, reversed ANG II effects on NOx excretion, but did not affect TBARS. Despite its prohypertensive effect, expression of prooxidative genes in the renal cortex decreased rather than increased after short-term AT₂ receptor blockade and renal HO-1 induction after ANG II was normalized. Thus chronic ANG II infusion in mice induces hypertension but not oxidative stress. In contrast to the response in aorta, gene expression of components of NADPH-oxidase was not enhanced in renal cortex. Although ANG II administration induced renal cortical AT₂ receptor expression, blockade of that receptor did not unveil the AT₂ receptor as intrarenal dampening factor of prooxidative forces.

mice; renin-angiotensin system; heme oxygenase-1; free radicals; NADPH-oxidase

METHODS

Animals. Female C57BL/6J mice (12 wk of age, 18 to 22 g; Charles River Laboratories, Maastrict, The Netherlands) received a standard diet containing 0.6% NaCl (wt/wt; RMH-TM; Hope Farms, Woerden, The Netherlands) and had free access to acidified tap water. Mice were housed in groups at 22°C, 60% humidity with a 12:12-h light-dark cycle. During the experiment, sentinel mice were regularly monitored for infection by nematodes and pathogenic bacteria, as well as antibodies to rodent viral pathogens. The Animal Ethical Committee of the University of Utrecht approved the protocols.

Infusion protocols. At 12 wk of age, the mice were trained for the tail cuff procedure. At 14 wk, all mice had reached a body weight of 20 g, the minimum requirement for subcutaneous implantation of...
osmotic minipumps (Alzet 2004; Charles River Laboratories). Mice were anesthetized with isoflurane and the minipumps were implanted subcutaneously in the nape of the neck and housed individually for 3 days after the operation.

In the first experiment, mice were continually infused with saline (control; n = 6) or a low (Low-ANG II; n = 5), medium (Med-ANG II; n = 5), or high (High-ANG II; n = 5) dose of ANG II (0.5, 1.0, and 1.5 mg·kg body wt−1·day−1, respectively; Sigma, Zwijndrecht, The Netherlands) for 28 days. Separate groups were also infused with Med-ANG II for 1 and 9 days (both n = 5). Osmotic minipump Alzet 1003D and Alzet 1002 were used for 1- and 9-day infusions, respectively.

In the second experiment, ANG II was infused at the medium dose (1.0 mg·kg body wt−1·day−1) for 28 days. Control mice were infused with saline. Half of the control and ANG II-infused mice were infused with saline. Half of the control and ANG II-infused mice were treated with PD-123,319 (PD; an AT2 receptor antagonist; Parke-Davis, Ann Arbor, MI) twice a day (50 mg·kg body wt−1·12 h−1), beginning at day 25. Each treatment group had five mice. ANG II and PD-123,139 were both dissolved in a sterile solution of 0.9% (wt/vol) NaCl with 0.01 N AcOH. The final concentration of PD-123,319 was 10 mg/ml.

Systolic blood pressure. Systolic blood pressure (SBP) was measured using the tail cuff method (ITTC, San Diego, CA) before the implantation procedure and at regular intervals during the rest of the experiment. Urine collections were obtained at regular intervals by placing the mice in metabolic cages for 14 h. During urine collection, mice had access to water containing 2%D-glucose. To prevent degradation of NO metabolites, urine was collected in tubes containing 4% formaldehyde for histological evaluation, and the middle 175 to 30 s. Total RNA was extracted from the TRIzol solution using 1-mm glass beads in a Mini beads. Total RNA was dissolved in 15 μl of dH2O.

Semiquantitative and real-time quantitative PCR. Total RNA isolated from the samples was pooled per group in equal amounts per subject. RT was done in batches of 5 μg total RNA for renal cortex and 1 μg for aorta; 1 μg of random hexanucleotide was added to total RNA in an end-volume of 11 μl. The solution was heated to 70°C for 10 min. Then, the RT was performed in 30 μl of reaction volume containing 500 μM dNTP (Ambion Europe, Huytington, UK), 20 U RNaseOUT recombinant ribonuclease inhibitor, 1× 1-strand buffer, 10 mM DTT, and 200 U Superscript II reverse transcriptase (Invitrogen) at 42°C for 2 h. The volume was heated to 95°C for 2 min and then immediately cooled on ice. The cDNA samples were stored at −20°C.

PCR was performed in a 50-μl reaction volume containing cDNA, 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 200 μM dNTP, 1.5 mM MgCl2, 1.25 U Taq DNA Polymerase (Invitrogen), and 125 ng sense and antisense primers (Sigma-Genosys, Haverhill, UK). Negative controls contained a PCR mix with cDNA but without polymerase or a PCR mix with polymerase but without cDNA. The primers used for each gene are listed in Table 1. The PCR reaction was performed with a hot start at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s using 35 or 40 PCR cycles. PCR samples were run on a 2% agarose gel containing ethidium bromide (17 μl/μl agarose; MP Biomedicals, Irvine, CA). Bio-Rad Chemidoc XRS photographed the gel, and the

RNA isolation from renal cortex. Frozen renal cortex (20–40 mg) was homogenized in 1 ml of TRIzol reagent (Invitrogen Life Technologies, Breda, The Netherlands) using 1-mm glass beads in a Mini Bead Beater (Merlin Diagnostic Systems, Breda, The Netherlands) for 20 to 30 s. Total RNA was extracted from the TRIzol solution using the procedure recommended by the supplier. Total RNA was finally dissolved in 50 μl of distilled H2O and stored at −80°C. Total RNA yields were determined on a UV Mini-1240 spectrophotometer (Shimadzu Deutschland, Duisburg Germany). The quality of the RNA samples was determined with the BioAnalyzer (Agilent, Amstelveen, The Netherlands) using the Eukaryote Total RNA Nano Assay.

RNA isolation from aorta. Frozen aortas (3–9 mg) were embedded in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and quickly frozen in liquid nitrogen. Cryo-static 5-μm slices were dissolved in 1 ml of TRizol. Further procedures were as mentioned above. Total RNA was dissolved in 15 μl of dH2O.

Table 1. Primer sequences for semiquantitative PCR

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<th>Synonym</th>
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<th>Antisense Primer</th>
<th>Expected Mass</th>
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<td>Kidney-specific gp91 isoform</td>
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<td>AGGATGATAGCTATGCTTGG</td>
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intensities of the bands were measured and quantified using the program Bio-Rad Quantity One (Bio-Rad Laboratories, Veenendaal, The Netherlands). Results were described as 18S-corrected mean intensities. None of the negative controls showed bands.

Real-time quantitative PCR (qPCR) was performed on aortic and renal cortical samples. Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA) were used. The genes studied were the AT₂ receptor (Agtr2, Mm00431727), p47phox (Ncf1, Mm00447921), heme oxygenase-1 (HO-1, Mm00516604), and 18S (18S, Hs99999901). qPCR was performed as recommended by the supplier. In short, duplicate samples of cDNA per individual per gene (50 ng of starting material total for AT₂ receptor, HO-1 and p47 or 80 pg for 18S) were mixed with Taqman Universal PCR Mastermix with AmpErase UNG and Taqman Gene Expression Assays in an end-volume of 25 μL. qPCR was performed using an ABI 7700 Single Reporter system. The gene threshold was determined by SDS 1.91 (Applied Biosystems).

Lipid peroxidation. Measurement of lipid peroxidation in mouse kidney was performed as described previously in rat kidney (7). In short, slices of kidney were placed in Krebs-HEPES buffer and the probe BoC11 was added to the buffer in final concentration of 10 μM. After 30 min of incubation, the buffer was refreshed and the BoC11 incorporation and oxidation in the kidney were observed using confocal laser-scanning microscopy (CLSM; Bio-Rad Laboratories, Hercules, CA) equipped with a krypton/argon laser. Slices were imaged in the red and green spectrum (nonoxidized and oxidized probe, respectively). Green and red fluorescence excitations were 488 and 568 nm, respectively, and green and red fluorescence emissions were 530 and 590 nm, respectively. Images were analyzed using Scion imaging software (Scion, Frederick, MD). Lipid peroxidation in each mouse was measured in duplicate.

Determination of NOₓ, thiobarbituric acid-reactive substances, proteins, and creatinine in urine. Urine protein was determined with a Bio-Rad Protein Assay Kit based on the Bradford method (Bio-Rad Laboratories). Creatinine was enzymatically determined with Creatinine F L-Type R1 and R2 (Wako Chemicals, Neuss, Germany). Total NOₓ was determined using the Nitrate/Nitrite Colorimetric Assay Kit of Cayman Chemical (ITK Diagnostics, Uithoorn, The Netherlands). Real-time quantitative PCR (qPCR) was performed on aortic and renal cortical samples. Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA) were used. The genes studied were the AT₂ receptor (Agtr2, Mm00431727), p47phox (Ncf1, Mm00447921), heme oxygenase-1 (HO-1, Mm00516604), and 18S (18S, Hs99999901). qPCR was performed as recommended by the supplier. In short, duplicate samples of cDNA per individual per gene (50 ng of starting material total for AT₂ receptor, HO-1 and p47 or 80 pg for 18S) were mixed with Taqman Universal PCR Mastermix with AmpErase UNG and Taqman Gene Expression Assays in an end-volume of 25 μL. qPCR was performed using an ABI 7700 Single Reporter system. The gene threshold was determined by SDS 1.91 (Applied Biosystems).

Statistics. Results are expressed as means ± SE. Data were compared using one-way ANOVA or two-way repeated-measures ANOVA where appropriate followed by a Student-Newman-Keuls post hoc test. P < 0.05 was considered significant. In semiquantitative PCR, the bands of the PCR products were photographed and a program calculated the intensities of the bands. This semiquantitative approach does not allow statistical analysis. Because of this reason, variation in replicates is not included in graphs.

RESULTS

ANG II increased blood pressure and hematocrit and induced hypertrophy of aorta. ANG II infusion dose dependently increased blood pressure (Fig. 1A). On day 10 the SBP of High-ANG II was increased compared with control and Low-ANG II (P < 0.01). For control, Low-ANG II, Med-ANG II, and High-ANG II the SBP at day 26 was 99 ± 3, 103 ± 4, 120 ± 2 (P < 0.01 Med-ANG II vs. both control and Low-ANG II), and 140 ± 4 mmHg (P < 0.01 vs. all other groups), respectively. No significant elevation of tail cuff pressure was found before day 10 in any of the AGF II-infused groups. At 1 and 9 days, there was no effect of Med-ANG II on aorta weight. However, at 28 days aorta weight was dose dependently increased (P < 0.005 Med-ANG II vs. control and Low-ANG II and P < 0.005 High-ANG II vs. control and Low-ANG II and P < 0.05 Med-ANG II; Fig. 1B). There were no effects on body or kidney weight. Hematocrit was increased in all ANG II-infused mice, even at day 1. Hematocrit for Med-ANG II vs. control at days 1, 9, and 28 was 53.1 ± 0.3 vs. 48.0 ± 0.3% (P < 0.001), 50.8 ± 0.4 vs. 48.5 ± 0.2% (P < 0.001), and 53.1 ± 1.3 vs. 48.6 ± 0.2% (P < 0.001), respectively.

Chronic ANG II infusion dose independently decreased urinary NOₓ without renal damage. Urine NOₓ, TBARS, and protein were determined (Table 2). There were no differences in plasma creatinine and there were no changes in creatinine clearance (data not shown). Urinary excretions were corrected for urinary creatinine excretion. No significant differences were found after 1 and 9 days of Med-ANG II infusion (data not shown). Urinary NOₓ were decreased in all doses of ANG II-infused mice at end of infusion period (P < 0.05 vs. control); there were no difference between doses of ANG II. There were no increases in protein or TBARS excretion, and none of the ANG II-infused mice showed histological evidence of renal damage (Fig. 2). There was no change in renal cortical lipid peroxidation in 28-day Med-ANG II mice (n = 4). However, 9-day Med-ANG II mice (n = 4) showed ANG...
the AT2 receptor in renal cortex was markedly increased by infusion. In Fig. 4, gene expression is shown for pro- and antioxidative genes and for components of the renin-angiotensin system after 28 days of ANG II infusion. The semiquantitative PCR (RT) was performed in triplicate on pooled samples and was corrected using 18S gene expression. Because there was no dose dependency for any of the tested genes, data were pooled for all ANG II doses (n = 11). Prooxidative genes were clearly not induced, the NADPH oxidase components p22 and p47 were decreased, and Rac2 tended to decrease. Expression of HO-1 was increased in this first experiment. Expression of the AT2 receptor in renal cortex was markedly increased by 50% (Fig. 4A) but this was independent of ANG II dose (Fig. 5A). AT2 receptor expression was not induced by the medium ANG II dose up to 9 days, but again was induced at day 28 (Fig. 5B). Quantitative real-time PCR was performed on renal cortex for the AT2 receptor, HO-1, p47, and 18S (Fig. 5C). No significant changes were observed due to great variation between individuals and small sample size per treatment group. When all ANG II data were pooled and compared with control data, the AT2 receptor or HO-1 was significantly changed (P < 0.05 and P < 0.005, respectively). In the aorta of Med-ANG II mice several prooxidative genes were induced, in particular p47 (Fig. 6, A and B). HO-1 was also induced during ANG II infusion. In contrast to the kidney cortex, the AT2 receptor was not induced in the aorta following 28 days of ANG II infusion. The induction of aortic p47 and HO-1 in ANG II-infused mice was confirmed by qPCR (P < 0.05; Fig. 6C).

AT2 receptor activation antagonizes ANG II-induced hypertension. In the second experiment, we attempted to dissect the function of secondary AT2 receptor expression in the renal cortex. PD was administered during the final 3 days of ANG II infusion when blood pressure had reached a plateau. Once again, ANG II infusion significantly increased SBP (Fig. 7) at day 25 compared with control (105 ± 5 vs. 91 ± 3 mmHg, P < 0.01). At day 28 of continued ANG II infusion, SBP had not increased further (106 ± 4 mmHg). PD treatment significantly increased blood pressure in ANG II-infused mice, but not in control mice. ANG II mice treated with PD had SBP of 104 ± 3 mmHg at day 25, before initiation of PD treatment and 117 ± 4 mmHg (P < 0.01 vs. day 25 and P < 0.05 vs. ANG II alone day 28) after 3 days of PD treatment. Hematocrit was elevated in ANG II-infused mice (53.1 ± 3 vs. 48.6 ± 0.2; P < 0.001) and was not affected by AT2 receptor blockade (52.7 ± 0.6 vs. 45.8 ± 1.0; P < 0.001).

Urinary NOx was not significantly affected by AT2 receptor antagonist. NOx, TBARS, and protein measured in urine in the second experiment are listed in Table 2. Similar to the first experiment, NOx was decreased in ANG II-infused mice vs. control on day 28 (P < 0.05), and this was partially corrected by PD (not significant vs. control). PD did not enhance TBARS in either the ANG II or the control group.

Table 2. Urinary NOx, TBARS, and protein

<table>
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<th>Treatment</th>
<th>TBARS/Creatinine, μmol/mmol</th>
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<tr>
<td>Control (n = 6)</td>
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<td>Low-ANG II (n = 5)</td>
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<td>Med-ANG II (n = 5)</td>
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<td>NOx/creatinine, μmol/mmol</td>
<td>444±137</td>
<td>268±31</td>
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<td>TBARS/creatinine, μmol/mmol</td>
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Urinary data before and at the end of infusion period are shown. In the second experiment, urinary data at the end of infusion period are shown. NOx, nitric oxide metabolites; TBARS, thiobarbituric acid-reactive substances; PD, PD-123, 319, the AT2 receptor antagonist. *P < 0.05 vs. control.
Responses of renal cortical and aortic gene expression to AT2 receptor antagonist. In the second experiment, gene expression of components of the renin-angiotensin system, of NADPH oxidase, and of HO-1 was evaluated in control mice (n = 10) and in mice infused with Med-ANG II (n = 10), with and without concomitant treatment with PD from day 25 to day 28 (n = 5 for each group). Gene expression in renal cortex was performed in duplicate on pooled samples, corrected by 18S, and compared with control. Similar to the first experiment, chronic ANG II infusion induced expression of HO-1 and nNOS (Fig. 8A) and depressed expression of p22 and Rac2 (Fig. 8B). Remarkably, inhibition of the AT2 receptor led to decreased expression of all tested prooxidative genes except XO in controls and accentuated decreased expression of many prooxidative genes (gp91, NOX4, p47, and XO) in ANG II-infused mice (Fig. 8B). Thus antagonizing the AT2 receptor did not induce but decreased the expression of prooxidative genes. In control mice, AT2 receptor blockade led to depressed inducible NOS (Fig. 8A). PD cotreatment in ANG II-infused mice normalized HO-1 expression and decreased inducible NOS and endothelial NOS, but not neuronal NOS. Representative

**Fig. 3.** Lipid peroxidation in renal cortex measured using BoC11 in 1 and 9 days control together (n = 7), 1-day ANG II (1d-ANG II, n = 5), 9 days ANG II (n = 4), and 28 days ANG II (n = 4). Mice infused with ANG II (1.0 mg·kg\(^{-1}\)·day\(^{-1}\)) for 9 days showed increased renal cortical lipid peroxidation. #P < 0.05 vs. all other groups.

**Fig. 4.** Gene expression changes in renal cortex at 28 days. Semiquantitative PCR on pooled samples (A; n = 11 for ANG II and n = 6 for control), related to 18S gene expression, and compared with control. B: representative PCR. Gene synonyms are shown in Table 1.

**Fig. 5.** Dose- (A) and time-dependent (B) effects of ANG II on expression of the AT2 receptor gene in the renal cortex. Time-dependent effects were measured in response to Med-ANG II. C: quantitative PCR was performed for renal cortical genes of AT2 receptor, HO-1, p47, and 18S.
PCR of some of these genes is shown in Fig. 8C. Quantitative real-time PCR showed gene induction of HO-1 by ANG II (P < 0.001 vs. control), which was normalized by AT2 receptor blockade (P < 0.05; Fig. 8D).

As mentioned, in the aorta prooxidative genes were induced in ANG II-infused mice (Fig. 9, A and B). Interestingly, expression of these genes decreased during AT2 receptor blockade (ANG II+PD), being that expression remained higher than in mice treated with AT2 receptor antagonist only (control+PD). Aortic HO-1 induction by ANG II infusion was not affected by AT2 receptor blockade.

DISCUSSION

Knowledge about the protection of the kidney from hypertensive damage is limited. While glomerular hypertension has been recognized to be pivotal for the development of damage, prooxidative forces are also becoming recognized as relevant players. Interestingly, mice seem to be relatively protected from damage caused by hypertension (13) or renal ablation (26, 36). In the present study, mice chronically infused with different doses of ANG II clearly show dose-dependent changes in SBP and hypertrophy of the aorta. Yet, creatinine clearance was normal, proteinuria was absent, and there was no detectable histological damage in the kidney, even at the highest dose of ANG II. Gene expression of NADPH-oxidase components and xanthine oxidase (XO) was not induced in the kidney during ANG II infusion, but upregulation of the AT2 receptor, HO-1 and nNOS was observed. In contrast, expression of NADPH-oxidase components p22 and p47 was induced in the aorta. Thus the mouse kidney seems to be strongly protected against elevated ANG II, and hypertension and damage are dissociated. This is quite different from the rat where ANG II infusion induces pressure-dependent injury (23, 38). To dissect the direct effects of ANG II and systemic hypertension, an angiotensin-independent hypertension model (e.g., norepinephrine) would be required, but that was not necessary in the present study as low dose of ANG II did not result in hypertension or any damage but resulted in the same gene responses and urinary NOx excretion as higher doses of ANG II.

Initiation of oxidative stress under conditions where ANG II is increased appears to involve activation of NAPDH oxidase (19). Components of this O2−-generating complex are transcriptionally induced, in particular p22 in vascular smooth muscle cells (18), and p47 in vascular smooth muscle cells (47) and endothelial cells (28, 32). Evidence for this mechanism is particularly solid in (components of) the vascular wall, and data are now available that ANG II may induce NADPH-oxidase in the kidney as well (20). The first step in the present study was to evaluate the transcriptional induction of components of the NADPH-oxidase in the kidney and vasculature. In line with previous observations, expression of the p22 and p47...
components of NADPH-oxidase was increased in the mouse aorta after ANG II infusion. In contrast, ANG II did not regulate p22 and p47 in the renal cortex. Oxidative stress, assessed by TBARS excretion in the urine, was not altered after 28 days of ANG II infusion, despite persistent increases in blood pressure. Closer evaluation of oxidative stress by use of the probe BoC11, which is sensitive for lipid peroxidation (7), revealed only slight increased oxidative stress at day 9 that was not sustained. Thus blood pressure on the one hand, and damage and oxidative stress on the other, was dissociated. This seems to corroborate the view that oxidative stress is an essential key pathogenic factor for the establishment of hypertensive renal injury. This should not be overinterpreted in the sense that renal injury cannot occur in the absence of oxidative stress during ANG II-induced hypertension. Testing this would require, for instance, a p47phox knockout mouse.

Which factors in the kidney could have dampened the prooxidative forces of ANG II, which were clearly observed in the aorta? Several candidates were considered: NOS, HO-1, or the angiotensin AT2 receptor. There is ample evidence in rats that the renal vasoconstrictive and sodium-retaining actions of ANG II are opposed by NO (2, 10, 31, 48). Furthermore, L-arginine supplementation has been shown to completely inhibit the salt loading-induced induction of p47 and gp91 in the kidney of Dahl-sensitive rats (17). In contrast to these indications that NO could oppose the actions of ANG II, NOx excretion decreased rather than increased in chronically ANG II-infused mice in the present study. Furthermore, only renal cortical nNOS gene expression seemed increased, which is unlikely a sufficient explanation for a global dampening of NADPH-oxidase, as cortical expression of nNOS is limited to the juxtaglomerular apparatus (5, 6, 25, 39) and NADPH-oxidase is expressed widely in the tubular system (12). Together, these findings suggest that NO is not primarily responsible for renal protection in ANG II-infused mice.

HO-1 and the AT2 receptor were also considered as suitable candidates for renal protection against ANG II-induced damage. HO-1, which can be induced by hypertension (22), can attenuate ANG II-mediated increase in oxidative injury in the thick ascending loop of Henle (40) and is able to reduce the pressor responsiveness to ANG II (52). HO-1 is important for protection of renal (8, 37) and vascular systems against hypertensive injury (14, 53). In line with this, HO-1 was upregulated in the rat by ANG II infusion, inhibition of HO-1-exacerbated...

Fig. 8. Renal cortical gene expression of components of the renin-angiotensin system, NADPH oxidase, and HO-1 in control mice (Con; n = 10) and in mice infused with ANG II (1.0 mg·kg⁻¹·day⁻¹; n = 10), with and without treatment with the AT2 receptor antagonist PD (100 mg·kg⁻¹·day⁻¹; n = 5 for each group) corrected by expression of 18S and compared with 28 days control (n = 5, A). Of the 6 prooxidative genes, 3 (p22, Rac2, and XO) were decreased in ANG II-infused mice. However, after concomitant AT2 receptor blockade in ANG II-infused mice, all 6 prooxidative genes decreased (B). The impact of AT2 receptor blockade on the reduction of prooxidative genes was much greater in control than in ANG II mice. C: representative PCR. Quantitative PCR showed gene induction of HO-1 in ANG II-infused mice and was normalized by AT2 receptor blockade. #P < 0.001 vs. control. *P < 0.05 vs. ANG II (D).
injury, while induction of HO-1 was protective (1). In the present study, ANG II administration seemingly induced HO-1 gene expression in the renal cortex and was also induced in the aorta. Systemic ANG II administration indicated an induction of gene expression of the AT2 receptor in the renal cortex; however, it did not affect AT2 receptor gene expression in the aorta. Although to the best of our knowledge aortic AT2 receptor expression has not been studied during ANG II-induced hypertension, ANG II infusion in rats has been shown to increase AT2 receptor expression in (mesenteric) resistance arteries (9). Nevertheless, in the present study, the AT2 receptor was induced only in renal cortex after 28 days, while HO-1 was upregulated in both aorta and kidney. This differential response suggests that a secondary increase in the AT2 receptor could be an important component of endogenous renal defense in the mouse, as only the aorta showed injury, namely, hypertrophy, suggesting that the AT2 receptor perhaps specifically in the mouse, as only the aorta showed injury, namely, hypertrophy, suggesting that the AT2 receptor might be partly responsible. Administration of the AT2 receptor antagonist PD-123,319 after 25 days of ANG II infusion exacerbated hypertension, while in control mice blood pressure did not change. This supports the concept that AT2 receptor activation is a secondary effect because for up to 21 days of administration, effects of ANG II on blood pressure were reported to be unaffected by concomitant PD administration (27, 33, 42). The blood pressure response is a clear indication that, at least for systems controlling blood pressure, induction of gene expression of the AT2 receptor is accompanied by a functional response.

AT2 receptor inhibition did not affect the expression of p22 in the renal cortex and the aorta; however, it led to an unexpected decreased expression (by RT-PCR) of p47, gp91, Nox4, and Rac2 in the kidney, in both control and ANG II-infused mice. TBARS excretion displayed a tendency to decrease during concomitant administration of ANG II and AT2 receptor antagonist. These findings suggest that the AT2 receptor is not the specific renal protection factor in this model. On the contrary, the observation that AT2 receptor blockade was followed by a decreased expression of prooxidative genes suggests that in the renal cortex the net effect of the AT2 receptor may even be prooxidative. This is in discordance with a study in rats showing that administration of a AT2 receptor antagonist during 1 wk of low-dose ANG II infusion increased urinary excretion of markers of oxidative stress, malonyldialdehyde and 8-iso-prostanoids, and increased expression of the NADPH-oxidase components p22, p67, and the gp91 isoform NOX1 (11). Besides methodological differences (species, different dosage, and duration of ANG II administration), blood pressure was strongly increased in the current experiments and the AT2 receptor was suggestively induced. Another option is that the AT2 receptor transmitted a prooxidative signal through NF-kB. High-dose ANG II infusion in mice has been shown to induce NF-kB in the renal cortex, a process that could be completely inhibited by concomitant AT2 receptor blockade (16, 43). Similarly, NF-kB activation could be inhibited by an AT2 receptor antagonist in the kidney-derived cell line Cos7 (51). The other view that can be derived from these experiments is that the AT2 receptor induced an oxidative force that activated NF-kB, which in turn activated the transcription of HO-1 (21, 30). It has been shown previously that ANG II induces HO-1 in the aorta (22) and kidney (1). The present data support that under particular circumstances, this is mediated, at least in part, via the AT2 receptor, as AT2 receptor blockade inhibited the induction of HO-1 in renal cortex. Many questions around AT1/AT2 and redox balance remain unanswered. The present data suggest that the scheme wherein the AT2 receptor simply opposes actions of the AT1 receptor is too simple (35).

Interesting in the present study is also that hypertension and oxidative stress do not seem to correlate. The report that chronic ANG II infusion in rats induced oxidative stress and that concomitant infusion of recombinant heparin-binding superoxide dismutase ameliorates the increase in blood pressure (18) has introduced the idea that (part of) the actions of ANG II are mediated via the generation of O2•-. Accordingly, inhi-
bition of assembly of p47 with gp91 (41) and deletion of p47 expression (28) both alleviate ANG II-induced hypertension in mice. Moreover, blood pressure was normalized in rats with two-kidney, one-clip Goldblatt hypertension by administration of the SOD mimetic tempol (50). Our data clearly show that conventional signaling of ANG II may induce hypertension without increased oxidative stress caused by NADPH-oxidase. Others have found hypertension during ANG II infusion much earlier than we did in the present study (44). These studies used male mice or other strains (e.g., 129/Sv), which were more sensitive to ANG II. Note that in the present study hematocrit was already elevated after 1 day of ANG II infusion, indicating that the osmotic minipumps were effective within 1 day.

Taken together, the present study shows that infusion of ANG II in wild-type female mice induces hypertension, but not oxidative stress. In contrast to the aorta, gene expression of components of NADPH-oxidase was not enhanced in the renal cortex. The angiotensin AT2 receptor tended to be induced in the kidney; however, blockade of that receptor did not reveal this to be the factor that dampened prooxidative forces. Thus the factor responsible for renal protection during chronic ANG II administration in female mice remains unknown. A possible candidate is HO-1 but this remains to be tested.

Perspectives

Without doubt unopposed actions of ANG II will lead to target organ damage. The mouse model, rather than frustrating us because of lack of renal injury, could form a valuable source of information about mechanisms that can be activated in response to a primary noxious agent. In this light, experiments in mice can lead to insights into protective systems that potentially can be manipulated in a beneficial direction in other species including humans. Besides, the experiments indicate that the downstream actions of the AT2 receptor may be highly species dependent; in mice the renal cortial AT2 receptor is potentially prooxidative, which raises the question whether conditions exist in humans where use of AT1 receptor antagonists may be disadvantageous.

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