AT1 receptor activation regulates the mRNA expression of CAT1, CAT2, arginase-1, and DDAH2 in preglomerular vessels from angiotensin II hypertensive rats

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Hultström M, Helle F, Iversen BM. AT1 receptor activation regulates the mRNA expression of CAT1, CAT2, arginase-1, and DDAH2 in preglomerular vessels from angiotensin II hypertensive rats. Am J Physiol Renal Physiol 297: F163–F168, 2009. First published April 22, 2009; doi:10.1152/ajprenal.00087.2009.—Previously, we found increased expression of l-arginine metabolizing enzymes in both kidneys from two-kidney, one-clip (2K1C) hypertensive rats (Helle F, Hultström M, Skogstrand T, Palm F, Iversen BM. Am J Physiol Renal Physiol 296: F78–F86, 2009). In the present study, we investigate whether AT1 receptor activation can induce the changes observed in 2K1C. Four groups of rats were infused with 80 ng/min ANG II or saline for 14 days and/or given 60 mg·kg⁻¹·day⁻¹ losartan. Gene expression was studied in isolated preglomerular vessels by RT-PCR. Dose-responses to ANG II were studied in isolated preglomerular vessels with and without acute NO inhibition [10⁴ mol/l N²-nitro-l-arginine methyl ester (l-NAME)]. Expressions of endothelial nitric oxide synthase (eNOS), caveolin-1, and arginase-2 were not changed by ANG II infusion. CAT1 (0.3 ± 0.07 to 0.73 ± 0.12, P < 0.05), CAT2 (1.14 ± 0.29 to 2.74 ± 0.48), DDAH2 (1.09 ± 0.27 to 2.3 ± 0.46), and arginase-1 (1.08 ± 0.17 to 1.82 ± 0.22) were increased in ANG II-infused rats. This was prevented by losartan treatment, which reduced the expression of eNOS (0.97 ± 0.26 to 0.37 ± 0.11 in controls; 0.8 ± 0.16 to 0.36 ± 0.1 in ANG II-infused rats) and caveolin-1 (2.49 ± 0.59 to 0.82 ± 0.24 in controls and 2.59 ± 0.61 to 1.1 ± 0.25 in ANG II-infused rats). ANG II (10⁻¹⁰ mol/l) caused vessels from ANG II-infused animals to contract to 53 ± 15% of baseline diameter and 90 ± 5% of baseline diameter in controls (P < 0.05) and was further enhanced by l-NAME to 4 ± 4% of baseline diameter (P < 0.05). In vivo losartan treatment reduced the reactivity of isolated vessels to 91 ± 2% of baseline in response to 10⁻⁷ mol/l ANG II compared with 82 ± 3% in controls (P < 0.05) and prevented the increased responsiveness caused by ANG II infusion. In conclusion, CAT1, CAT2, DDAH2, and arginase-1 expression in renal resistance vessels is regulated through the AT1 receptor. This finding may be of direct importance for NO and the regulation of preglomerular vascular function.

caveolin-1; afferent arteriole; contraction; losartan

THEafferent arterioles (AAs) are the main site for regulation of renal blood flow (RBF) and glomerular filtration rate (GFR). This control is mediated by three main mechanisms: first, hormonal factors (endocrine, autocrine or paracrine); second, the sympathetic nervous system; and third, the myogenic and tubuloglomerular feedback systems (11). ANG II is a key hormone in the regulation of RBF and GFR. However, its effect is in many situations balanced by vasodilators such as vasodilatory prostanoids and nitric oxide (NO) (6). Together these signaling substances are of major importance in the regulation of RBF in the normal kidney and probably more so in hypertensive models where the components of the renin-angiotensin system are activated or increased.

We have recently shown that ANG II-induced NO release controls renal vascular resistance by its ability to adjust the diameter of preglomerular vessels in the nonclipped kidney of two-kidney, one-clip hypertensive (2K1C) animals (6). The nonclipped kidney is subjected to high perfusion pressure and high vascular NO release, while vascular NO production is low in the clipped kidney.

The NO precursor, L-arginine, is transported into the cell by cationic amino acid transporters, CAT1 and CAT2, and we have recently shown that these transporters are upregulated in both the clipped and nonclipped kidney of 2K1C hypertensive rats, indicating a systemic control of these transporter systems. Furthermore, arginase-2, which is able to break down L-arginine to proline and ornithine, was also enhanced in both kidneys, a finding that also point to a systemic control system. Asymmetric dimethyl arginine (ADMA) is an inhibitor of NO synthase (NOS) and is broken down by dimethyl arginine dimethylaminohydrolase (DDAH). This enzyme is reduced in vessels from the nonclipped kidney, but not in the clipped kidney of 2K1C, suggesting that local regulation and hemodynamic mechanisms may be involved in the regulation of DDAHs.

The present study was designed to elucidate the role of ANG II in the regulation of transport and breakdown of L-arginine in ANG II-infused rats. We studied the effect of 2 wk of ANG II infusion (80 ng/min) on the enzymes involved in L-arginine metabolism in the renal resistance vessels and explored whether the effects of ANG II are mediated through the AT1 receptor by using the receptor blocker losartan. We hypothesized that genes upregulated in both the clipped and nonclipped kidney in 2K1C in our previous investigation would be primarily controlled by ANG II, while those that differed between the kidneys would be markers for the local hemodynamic situations in the kidneys. In addition, isolated resistance vessels were studied to explore whether these gene expression changes were connected to functional alterations of vascular function and NO release.

MATERIALS AND METHODS

Animals. Wistar rats from the Möllegaard breeding farm in Denmark (Taconic), weighing ~180 g at the start of the experiment, were used. The rats had free access to standard pelleted rat food and tap water. The experiments were evaluated and approved by the regional
ethics committee. At the start of the study, osmotic minipumps (no. 2002, Alzet) were subcutaneously implanted during isoflurane anesthesia (2% in 1:1 O₂ plus N₂O).

**Experimental groups.** The animals were divided into four groups, two groups that received 80 ng/min ANG II and two control groups that received isotonic saline solution for 14 days. One of the ANG II-infused groups and one of the control groups were given 60 mg·kg⁻¹·day⁻¹ losartan (Cozaar, Merck) in the drinking water. The animals were treated for 2 wk before death.

**Isolation and preparation of renal preglomerular vessels for diameter measurements.** Renal resistance vessels were isolated using an agarose infusion, enzyme digestion technique as described earlier (7). In short, rats were anesthetized with pentobarbital sodium (50–70 mg/kg). The renal resistance vessels were isolated using an enzyme digestion technique as described earlier (7). The abdominal aorta was freed, cannulated, and the kidneys were infused with 3–4 ml SeaPrep agarose solution (2%) in Ca²⁺-free Roswell Park Memorial Institute solution (RPMI; 37°C) to create an elastic core of agarose inside the renal microvessels. About 100-μm-thick slices were cut from the cortex of the kidney with a Thomas slicer (Thomas Scientific) and incubated at 37°C for 1 h in Ca²⁺-free RPMI containing 0.5 mg/ml protease (Sigma P3417, 0.5 U/ml), 0.3 mg/ml collagénase (Sigma CS5138, 700 U/ml), and 0.05 mg/ml trypsin inhibitor. Afferent fragments were picked with a 150-μm-diameter glass pipette and fastened by self-adhesion to a glass coverslip in a petri dish (MatTek P35G-0-14-C) containing 3 ml RPMI without Ca²⁺. Before the experiments, Ca²⁺ concentration in the medium was increased to 2 mM in three steps (2 μmol/l, 200 μmol/l, and 2 mM/l) with a 5-min incubation between each step. A typical preparation of agar-infused preglomerular vessels is seen in Fig. 1.

**Arteriolar diameter measurements.** Each experiment consisted of five periods, starting with a 120-s baseline followed by four 120-s treatment periods with successive doses of ANG II (10⁻⁸, 10⁻⁹, 10⁻⁸, and 10⁻⁷ mol/l). Images for diameter analysis were acquired using an Ultraview IIIu CCD camera with a resolution of 2,576 x 1,932 pixels at the start and end of the baseline period and at the end of each treatment period. Vessel diameter was measured at one responsive point using Olympus DP-Soft 5.0. A low number of arterioles with attached glomeruli made positive identification difficult, and all responding vessels with a diameter of ~20 μm were included in the study. The preparation therefore includes both afferent arterioles and terminal interlobular arteries, but not the larger parts of the interlobular artery or arcuate arteries that are significantly larger.

**Real-time RT-PCR.** mRNA levels for CAT1, CAT2, arginase-1, arginase-2, DDAH1, and DDAH2 was measured in preglomerular vessels isolated with an iron oxide method (3, 16). Vessels were collected from 10 rats for each group. The same three-step dilution of the cDNA standard was used as a reference in all reactions. The results were expressed as the quantity of mRNA relative to the 18S ribosomal RNA in the same sample.

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The isolated tissue was immediately transferred to RNA-later (Qiagen) and frozen at −80°C. Total RNA was extracted with an RNeasy minikit (Qiagen) and stored at −80°C until used. Reverse transcription was performed with an RT-core kit (Eurogentec) using random nonamers as primers. Quantitative PCR (qPCR) was performed on an ABI prism (Applied Biosystems) using a qPCR Mastermix for SYBR Green I (Eurogentec). Primers for PCR of 18S were constructed using Primer Express software (ABI). The primers for arginase-1 (ABI product number: Rn00567522_m1), arginase-2 (Rn00569814_m1), the dimethylarginine dimethylaminohydrases [DDAH1 (Rn00574200_m1) and DDAH2 (Rn01525775_g1)], cat-ionic aminoacid transferases [CAT1 (Rn00565399_m1) and CAT2 (Rn00432039_g1)], ANG II receptors [AT₁a (Rn01435427_m1), AT₁b (Rn02132799_s1), and AT₂ (Rn00560677_s1)], and endothelial NOS (Hamil) (Rn00561646_m1) were ordered as ready-made gene expression assays (ABI). The primers used for 18S ribosomal RNA (18S) were sense 5'-agtcgagctgtgtcactac-3' and antisense 5'-gagctgagcctcactacaa-3'.

**Statistics.** The data is presented as means ± SE. The curves were analyzed with two-way ANOVA to test for contraction over time and difference between curves. Tukey-HSD was used as a post hoc test between points on the curves. Gene expressions were analyzed using ANOVA, and Student’s t-test was used to compare controls and ANG II-treated animals, as well as the losartan-treated groups with their respective controls. P < 0.05 was considered significant. Statistics were calculated using R 2.8.1 (13).

**RESULTS**

**Animals.** At the time of death, the rats weighed ~250 g; there was no difference between groups. Blood pressure was significantly increased in the ANG II-infused animals, and losartan prevented this increase, but it had no effect in controls (Fig. 2).

**Real-time RT-PCR.** As shown in Fig. 2, CAT1, CAT2, and DDAH2 doubled in expression after 2 wk of ANG II infusion (P < 0.05). Similarly, arginase-1 increased by ~70% after ANG II infusion. Treatment with losartan prevented the increase in CAT1 and -2, DDAH2, and arginase-1 (Fig. 3). The expression of arginase-2 and DDAH1 was not changed by ANG II infusion (Table 1).

Although eNOS did not change during ANG II infusion, losartan reduced the expression of eNOS by more than half in both controls and ANG II-infused rats (P < 0.05). Similarly, caveolin-1 was unaffected by ANG II infusion but was reduced to ~50% by losartan treatment (Fig. 4). There was no change in the expression of the AT₁a receptor for ANG II in preglomerular vessels after ANG II infusion. The expression of the ANG II receptor AT₁b was not changed nor was the expression of the AT₂ receptor (Table 1). Expression of iNOS mRNA could not be detected in isolated preglomerular vessels from any of the groups.

**Preglomerular vascular diameter.** Stimulation with ANG II concentrations from 10⁻¹⁰ to 10⁻⁷ mol/l induced progressively stronger constriction, up to 82 ± 3% of baseline diameter at 10⁻⁷ mol/l in control vessels (n = 7, Fig. 5). N²-nitro-L-arginine methyl ester (L-NAME) had no effect on ANG II-induced constriction in vessels from control animals, giving a maximal contraction to 70 ± 7% of baseline diameter (n = 7). In ANG II-infused rats, ANG II elicited a significantly stronger constriction. At 10⁻¹⁰ mol/l ANG II, vessels contracted to 54 ± 15% (n = 8, P < 0.05 compared with controls) of baseline diameter in untreated vessels and to 4 ± 4% of baseline diameter in l-NAME-treated vessels.
animals without L-NAME). The constriction of vessels from ANG II-treated animals to 10\(^{-7}\) mol/l ANG II, and vessels from animals with both ANG II and losartan (\(P < 0.05\) compared with untreated ANG II-infused animals (\(P < 0.05\)). L-NAME, was stronger than in vessels from control animals but not different between groups (7 ± 4 vs. 3 ± 3% of baseline diameter).

Losartan decreased the contractility of the preglomerular vasculature (Fig. 6). Vessels from animals receiving losartan only contracted to 91 ± 2% (\(n = 6\), \(P < 0.05\) compared with vessels from control animals) of baseline diameter in response to \(10^{-7}\) mol/l ANG II, and vessels from animals with both ANG II and losartan treatment contracted to 92 ± 4% (\(n = 8\), \(P < 0.05\) compared with vessels from ANG II-treated animals). L-NAME had no effect on the contraction of vessels from losartan-treated rats. The contraction to \(10^{-7}\) mol/l ANG II reached 90 ± 3 and 89 ± 2% in vessels from losartan only (\(n = 9\)) and ANG II plus losartan (\(n = 6\))-treated rats, respectively.

**DISCUSSION**

The main finding of the present study is that ANG II induced an AT\(_1\) receptor-dependent upregulation of the gene expressions of CAT1, CAT2, and arginase-2. These genes are of major importance in the L-arginine metabolism. Their expression may influence the NO-release capacity in response to ANG II and may moderate the impact of this hormone on the function of afferent arterioles and renal hemodynamics. In addition, we show that the increased reactivity to ANG II found in isolated vessels from ANG II hypertensive rats is AT\(_1\) dependent and that the ANG II reactivity of isolated preglomerular vessels may be blunted by AT\(_1\) blockade in vivo.

The present study confirms the earlier information that ANG II does not induce an upregulation of eNOS and that CAT1 and CAT2 expression may be ANG II dependent as it is also increased in both the clipped and nonclipped kidney in 2K1C (6). This holds true for arginase-1 as well (Hultström M, unpublished observations). The ability of losartan to prevent the expression changes in these genes without affecting the level of gene expression in control rats is a clear indication that the effect is AT\(_1\) receptor dependent.

On the other hand, arginase-2 was upregulated in both kidneys from 2K1C (6) while we were unable to find an effect on this enzyme in the present investigation. Furthermore, in vessels from 2K1C rats the expression of DDAH1 was increased only in the nonclipped kidney (Hultström M, unpublished observations) while DDAH2 was unchanged (6). These findings at the mRNA level are in contrast to the data obtained in the present study where we found DDAH1 unchanged and DDAH2 upregulated by ANG II. This may indicate that both

<table>
<thead>
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<th>Gene</th>
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<th>ANG II</th>
<th>Losartan</th>
<th>ANG II + Losartan</th>
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<td>Arginase2/18S</td>
<td>0.90±0.27</td>
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<td>AT(_1)/18S</td>
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<td>0.93±0.28</td>
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<tr>
<td>AT(_2)/18S</td>
<td>1.55±0.39</td>
<td>1.43±0.31</td>
<td>0.68±0.16</td>
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<tr>
<td>AT(_4)/18S</td>
<td>1.68±0.47</td>
<td>1.45±0.33</td>
<td>0.74±0.19</td>
<td>1.05±0.27</td>
</tr>
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Values are means ± SE. Neither gene was affected by 14 days of 80 ng/min ANG II infusion or by losartan treatment.
DDAH1 and -2 are influenced by the hemodynamic situation in the respective kidneys during 2K1C hypertension.

The present study was not designed to show whether L-arginine entry through CAT1 and CAT2 is indeed necessary for NO synthesis or may be influenced by an increased transport capacity. However, it seems plausible that the increased transport capacity may play a role in the rate of NO synthesis, but further studies are needed to fully understand this mechanism.

It is well established that NO serves as a potent vasodilator and plays an important role in maintaining vascular tone and counteracting ANG II-induced vasoconstriction in renal vessels in the ANG II hypertensive rat (8), which helps to maintain RBF despite high circulating levels of ANG II (4). In the kidney, NO is synthesized primarily by eNOS and neuronal NOS (12), while iNOS seems to have a less important role. The ANG II-induced pressor response in isolated, perfused kidneys is enhanced by L-NAME treatment and in eNOS/H11002/H11002 mice, in which L-NAME has no effect (14), supporting the notion that ANG II-induced NO release is important in the regulation of...
RBF and that eNOS is the most important of the NOS isoforms in this response.

We found no effect of ANG II on eNOS mRNA expression. However, earlier studies have shown an upregulation of eNOS protein as well as immunohistochemical staining for this enzyme in the endothelium of cortical vessels (15) after a 6-day infusion of ANG II in Sprague-Dawley rats. This was followed by upregulation of DDAH expression, and the effects were inhibited by losartan, indicating a receptor-operated mechanism. This may indicate that there is an early upregulation of eNOS after 1 wk of ANG II treatment that normalizes with time, i.e., 2 wk, but it may also be caused by significant differences in the experimental protocols used. Tojo et al. (15) used another strain of rats, a lower ANG II dose (200 ng·kg⁻¹·min⁻¹ compared with 400 ng·kg⁻¹·min⁻¹ in the present investigation) and a shorter treatment time (6 vs. 14 days). Interestingly, in rats given a high-salt (6% NaCl) diet, which suppresses ANG II levels, the same authors found no significant effect of losartan (15).

Losartan was able to reduce both eNOS and caveolin-1 expressions in rats that received no ANG II infusions. This indicates a receptor-independent mechanism that is not an inhibition of ANG II stimulation, which is also inconsistent with the findings in Sprague-Dawley rats given a high-salt diet (15). While AT₁-independent effects of losartan are not unknown, this direct effect on gene expression is a novel finding. It is known that losartan is metabolized into two compounds, EXP3174 and EXP3179, and that the latter has effects not mediated through the AT₁ receptor (17). Studies using these metabolites were not performed in the present investigation but would be of great interest for future studies. An alternative explanation is that the baseline AT₁ receptor activation by normal circulating levels of ANG II is needed for normal expression of eNOS, as has been indicated by Tojo et al. (15). We found no expression of iNOS in isolated vessels, which confirms our findings in vessels from 2K1C rats (Hultström M, unpublished observations). We suggest that a possible upregulation of iNOS in these kidneys may be localized to infiltrating cells in the interstitium and not to the vessels.

Vessels from ANG II-infused rats showed a higher sensitivity to ANG II, with significant constriction already at 10⁻¹⁰ M ANG II, as well as a significantly stronger maximal constriction. Blockage of the AT₁ receptor with losartan prevented not only this effect but reduced the reactivity of vessels from control animals. We further show that this is not caused by changes in ANG II receptor mRNA expression. The increased contractility is consistent with previous studies in renal vessels (6) as well as in other vascular beds (1). Thus long-time ANG II infusion appears to produce positive feedback to make vascular responses to ANG II even stronger than normal in isolated tissues. This is mediated through the AT₁ receptor, and baseline activation of this receptor appears necessary for renal vascular sensitivity to ANG II. The increased responsiveness found in isolated vessels is, however, not borne out in vivo: 2K1C rats have reduced RBF responses to ANG II (2), and a 12-day ANG II infusion (270 ng·kg⁻¹·min⁻¹) does not decrease RBF (4, 10), or only decreases it moderately (5).

1-L-NAME-treated vessels from ANG II-infused animals had an enhanced contractile response to 10⁻¹⁰ M ANG II, indicating that vascular effects of ANG II were blunted by NO release in isolated vessels. This supports the conclusion that there is an ANG II-induced NO release in vessels from ANG II hypertensive animals that is not present in control animals. At higher doses of ANG II, however, there was no difference between untreated and 1-NAME-treated vessels from ANG II-infused rats. Whether this is because of a saturated NO-release system while the contractile activation can be further activated, or because of a fall in NO synthesis is not readily deducible from the present investigation. In our earlier paper, we show that eNOS, DDAH₂, and arginase-2 exhibit significant posttranscriptional regulation of protein expression (6). Therefore, it is not certain that the AT₁-mediated regulation of gene expression we describe is the only mode of regulation that influences ANG II-induced NO release after 14 days of ANG II infusion.

In conclusion, 14-day ANG II infusion causes an upregulation of several enzymes involved in l-arginine metabolism and an increased NO release from renal resistance vessels, as well as an increased reactivity to ANG II in isolated vessels. These effects are substantially AT₁ receptor mediated. The changed gene expressions lead to an increased ANG II-induced NO release that partially blunts the ANG II-induced contraction in renal resistance vessels isolated from ANG II-infused rats.

REFERENCES


