Kidney International, Vol. 65 (2004), pp. 2268–2278

Nitric oxide synthase expression in AT_2 receptor–deficient mice after DOCA-salt

MICHAEL OBST, VOLKMAR GROSS, ANTON BONARTSEV, JÜRGEN JANKE, DOMINIK N. MÜLLER, JOON-KEUN PARK, EVA KÄRGEL, and FRIEDRICH C. LUFT

Max Delbrück Center (MDC) for Molecular Medicine, Berlin, Germany; Department of Human and Animal Physiology, Faculty of Biology, Lomonosov Moscow State University, Moscow, Russia; Medical Faculty of the Charité, HELIOS-Klinikum-Berlin, Franz Volhard Clinic, Humboldt University, Berlin, Germany; Nephrology Division, Department of Medicine, Hannover Medical School, Hannover, Germany

Nitric oxide synthase expression in AT_2 receptor–deficient mice after DOCA-salt.

Background. Angiotensin II type 2 receptor–deficient mice (AT_2-/y) provide an opportunity to study the relationship between the angiotensin II type 1 receptor (AT_1) and nitric oxide synthase (NOS) isoforms without concomitant AT_2 receptor–related effects. To test this relationship, the expression of renal NOS isoforms (neural, inducible, and endothelial) in AT_2-/y and AT_2+/y mice was examined. The mice were challenged with deoxycorticosterone acetate (DOCA)-salt to stimulate NO generation.

Methods. Gene expression analyses by real-time polymerase chain reaction (PCR) (TaqMan) were performed in kidneys to characterize neuronal nitric oxide synthase (nNOS), epithelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and the AT_1 receptor. Pressure-natriuresis experiments were done to determine the physiologic background.

Results. AT_2-/y mice showed nNOS and iNOS up-regulation. DOCA-salt increased iNOS expression more in AT_2-/y mice than in AT_2+/y mice. Immunohistochemistry localized the iNOS expression with DOCA-salt mainly in the glomeruli. eNOS was not different between the groups, and was not affected by DOCA-salt. DOCA-salt increased mean arterial pressure more in AT_2-/y mice than in AT_2+/y mice. Concomitantly, the pressure-natriuresis relationship was shifted to the right in AT_2-/y and AT_2+/y mice after DOCA-salt. DOCA-salt decreased renal blood flow (RBF) and glomerular filtration rate (GFR) in both groups. iNOS blockade did not lower blood pressure.

Conclusion. We conclude that AT_2 receptor deletion and concomitant up-regulation of the AT_1 receptor is associated with up-regulation of nNOS and iNOS. Under DOCA-salt, renal iNOS expression was further increased. Because iNOS inhibition did not change blood pressure, iNOS may not be involved in the hemodynamics, but may contribute to organ damage.

Key words: AT_2 receptor knockout mouse, DOCA-salt, NO system, pressure natriuresis.

Received for publication May 15, 2003
and in revised form October 27, 2003, and December 15, 2003
Accepted for publication January 14, 2004

The renin-angiotensin system has close interrelationships with nitric oxide (NO) generation. Both are highly important in regulating renal function and maintaining sodium and water balance; both are important for regulation of blood pressure through extracellular fluid volume control. The effects of angiotensin (Ang) II are mediated by at least two receptor isoforms (AT_1 and AT_2). Through stimulation of aldosterone, Ang II enhances sodium reabsorption indirectly. On the other hand, the stimulation of the AT_2 receptor may serve a counter-regulatory protective role in response to the AT_1 receptor–dependent effects [1, 2]. In mice lacking the AT_2 receptor, renal blood flow (RBF) and glomerular filtration rate (GFR) are reduced, causing a shift to the right in pressure natriuresis and diuresis [3].

NO is released within the kidney and participates in the control of renal and glomerular hemodynamics, tubuloglomerular feedback, and renin release to influence sodium and water excretion [4]. We showed earlier that in mice lacking the AT_2 receptor, L-NAME–induced renal and blood pressure effects are aggravated, and that AT_1 receptor blockade prevents blood pressure increase [5]. This study indicated that the AT_1 receptor plays a major role in L-NAME-dependent hypertension. In a recent in vitro study [6], evidence was presented that the AT_2 receptor attenuates Ang II–induced vessel contraction via the AT_1 receptor by an NO-related mechanism. The authors suggested that NO counteracts AT_1 receptor–dependent Ang II effects. The AT_1 receptor is up-regulated in AT_2-/y mice [3, 7, 8]. Thus, the AT_2-/y mouse should be suitable to allow studying the unopposed AT_1 receptor effects on NOS isoform expression in the kidney, the organ which is responsible for long-term blood pressure regulation. We suspected that NOS isoforms might actually be down-regulated in mice missing the AT_2 receptor. To test this notion, we characterized the expression of eNOS, iNOS, and nNOS in the kidneys of AT_2-/y and AT_2+/y mice. We challenged the

mice with DOCA-salt to stimulate NO generation. To provide physiologic background information, we studied blood pressure changes and renal functions in these mice. Finally, we studied blood pressures in DOCA-salt mice under iNOS blockade with guanidinoethyldisulfide 2HCl (GED) to test the relevance of DOCA-salt dependent iNOS up-regulation on blood pressure.

METHODS

All animals were obtained from breeder pairs supplied by the Vanderbilt University Medical Center (Nashville, TN, USA) courtesy of Dr. T. Inagami. The mice were allowed free access to standard chow (0.25% sodium, SNIFF Spezialitäten; GmbH, Soest, Germany) and drinking water ad libitum. The experimental protocol was approved by the local council on animal care, whose standards correspond to those of the American Physiological Society. Genotypes were verified by polymerase chain reaction (PCR). For the experiments, we used only male mice. The body weights of the mice averaged 30.7 ± 0.4 g (AT_2+/y), 26.3 ± 0.5 g (AT_2−/y), 31.3 ± 0.6 g (AT_2+/y and DOCA-salt), 26.8 ± 0.5 g (AT_2−/y and DOCA-salt), 29.2 ± 1.2 g (AT_2+/y and DOCA-salt/GED), and 24.9 ± 0.8 g (AT_2−/y and DOCA-salt/GED).

Expression analyses

Gene expression analyses of the isoforms (nNOS, eNOS, iNOS) of the NO system ($N = 7$) and the renal AT_1 receptor ($N = 10$) were performed in kidneys of each group (AT_2+/y, AT_2−/y, AT_2+/y and DOCA-salt, AT_2−/y and DOCA-salt). RNA isolation from homogenized kidneys, reverse transcription (RT), PCR conditions, and quantification of gene expression analysis with the ABI 5700 sequence detection system (ABI, Darmstadt, Germany) for RT-PCR (TaqMan), and especially AT_1 receptor gene expression were performed as described [5]. Gene expression analysis for the NOS genes were performed with 2 ng/μL cDNA equal to reverse transcribed RNA. The sequence of the mouse NOS genes were derived from GenBank accession numbers NM008712 (*nNOS*), NM008713 (*eNOS*), and AY090567 (*iNOS*). The sequence of primers and probes and the final concentrations were: n*NOS* FP 5′-GAC TGA TGG CAA GCA TGA CTT C-3′ (900 nmol/L), RP 5′-GCC CAA GGT AGA GCC ATC TG-3′ (900 nmol/L), probe 5′ FAM-TGG AAC TCG CAG CTC ATC CGC TAT G-TAMRA 3′ (100 nmol/L). *eNOS* FP 5′-TCT GCG GCG ATG TCA CTA TG-3′ (900 nmol/L), RP 5′-CCA TGC CGC CCT CTG TT-3′ (900 nmol/L), probe 5′ FAM-AAC CAG CGT CCT GCA AAC CGT GC-TAMRA 3′ (150 nmol/L). *iNOS* FP 5′-TGA CGG CAA ACA TGA CTT CAG-3′ (900 nmol/L), RP 5′-GCC ATC GGG CAT CTG GTA-3′ (900 nmol/L), probe 5′ FAM-TGG AAT TCA CAG CTC ATC CGG TAC GCT-TAMRA 3′ (200 nmol/L). Rodent glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was chosen as the endogenous control (housekeeping gene). Results were evaluated per TaqMan plate. All data are given as mean ± SEM relative to total mean.

For immunohistochemistry, the kidneys were washed with ice-cold saline, blotted dry, weighed, frozen in isopentane (−35°C), and stored at −80°C. Ice-cold acetone-fixed cryosections (6 μm) were air dried and immersed in 0.05 mol/L Tris buffer, 0.15 mol/L NaCl, pH 7.6 (TBS). All incubations were performed in a humid chamber at room temperature. At first, the sections were incubated in 10% normal donkey serum (Dianova, Germany) for 30 minutes to block any nonspecific binding. The sections were incubated for 60 minutes with polyclonal iNOS antibody (Santa Cruz, CA, USA). After washing with TBS, the sections were incubated with Cy3-conjugated secondary antibodies (donkey anti-rabbit IgG-Cy3; Dianova, Germany) for 60 minutes. After a final washing with TBS, slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Preparations were analyzed under a Zeiss Axioplan-2 microscope (Carl Zeiss, Jena, Germany), and digitally photographed using AxioVision 2 multi channel image processing system (Carl Zeiss).

Blood pressure

Blood pressure was measured by telemetry in four AT_2−/y mice and in five AT_2 +/y mice. The mice were anesthetized with isoflurane, and the left common carotid artery was isolated. The catheter of the telemetric device was inserted into the carotid artery below the bifurcation, and advanced to the point where the small notch on the tubing resided at the vessel opening so that the tip of the catheter was placed inside of the thoracic aorta. After securing the catheter at this position, the transmitter body was placed into a subcutaneous pouch along the animal's right flank.

After seven to eight recovery days when normal day/night rhythms of blood pressure and heart rate were restored and the mice gained weight again, baseline values were continuously recorded for three days. Thereafter, 50 mg DOCA tablets (Innovative Research of America, Saratosa, FL, USA) were implanted, and the mice were provided with 1% NaCl solution to drink [9, 10]. To characterize the effect of a selective inhibitor of iNOS [11] on DOCA-salt induced blood pressure changes, GED (Inotek Corp., Beverly, MA, USA) was given in the third week of DOCA-salt. We chose this time schedule because after two weeks of DOCA-salt, blood pressure and sodium excretion does not increase further [9]. GED 10 mg/kg intraperitoneally three times daily was given over five days, and blood pressure measurements

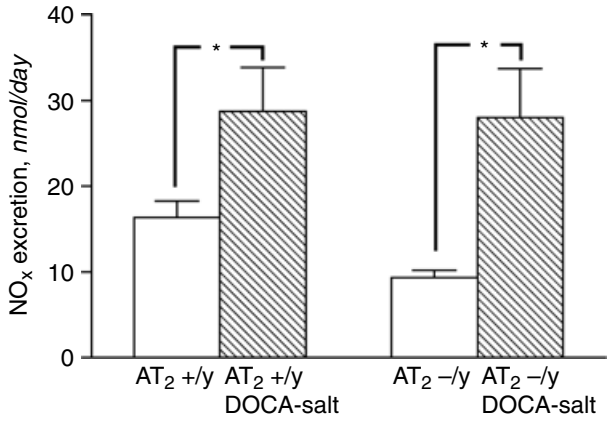

Fig. 1. Nitrite and nitrate (Nox) excretion before and after deoxycorticosterone acetate (DOCA)-salt in AT_2−/y and AT_2+/y mice. NOx excretory values represent the average of two 24-hour urine collections. DOCA salt increased NOx excretion in both strains. $^*P < 0.05$.

were continued (personal communication, Dr. G. Southan, Inotek Corp.). All values were sampled every five minutes for 10 seconds continuously day and night, with a sampling rate of 1000 Hz. Values are shown as 24-hour means.

NOx measurements

In a subgroup of seven AT_2−/y mice and nine AT_2+/y mice we verified that the urinary NOx excretion was elevated by DOCA-salt treatment, as has been described for rats [12]. The 24-hour urine collections were obtained over two days after adaptation to the metabolic cages (UNO Roestvaststaal; Zevenaar, The Netherlands). Urine was collected for NOx measurements under baseline conditions and after DOCA-salt. Nitrite was measured as a stable end product of NO using the Griess reaction (CellTrend, Luckenwalde, Germany). Briefly, 150 µL diluted urine sample was incubated with 75 µL 1% sulfanilamide and 75 µL 0,1% N-(1-naphthyl) ethylenediamine at room temperature. Nitrite content was monitored spectrophotometrically at 520 nm and compared with a sodium nitrite standard curve. Urinary NOx excretion was calculated as NOx concentration multiplied by urine volume.

Pressure-diuresis-natriuresis, renal blood flow, glomerular filtration rate

To gain insight into the DOCA-salt–related effects, we analyzed pressure-diuresis-natriuresis relationships, RBF (AT_2−/y: $N = 10$; AT_2−/y and DOCA-salt: $N = 8$; AT_2+/y: $N = 10$; AT_2+/y and DOCA-salt: $N = 6$) and GFR (AT_2−/y: $N = 8$; AT_2−/y and DOCA-salt: $N = 12$; AT_2+/y: $N = 7$; AT_2+/y and DOCA-salt: $N = 10$) in ketamine- (50 µg/g intraperitoneally, Parke-

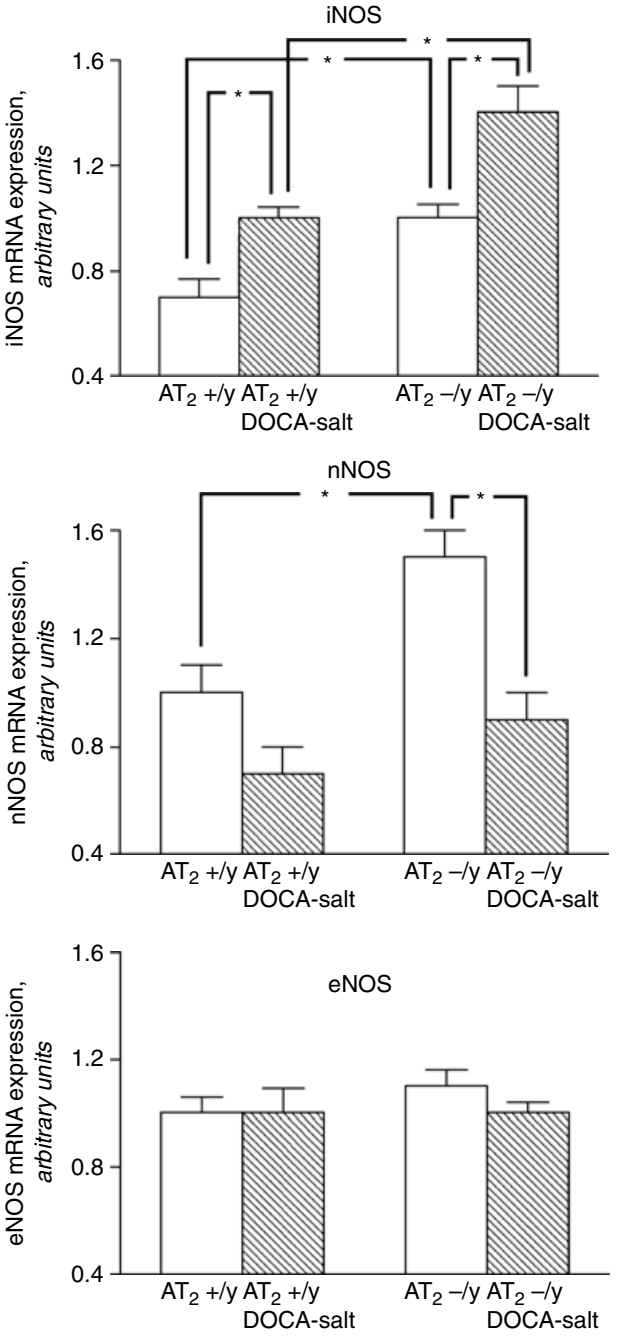

Fig. 2. Renal gene expression of inducible nitric oxide synthase (iNOS) (*top*), neuronal nitric oxide synthase (nNOS) (*middle*), and epithelial nitric oxide synthase (eNOS) (*bottom*) in AT_2−/y and AT_2+/y mice before and after deoxycorticosterone acetate (DOCA)-salt are shown. iNOS and nNOS were up-regulated in AT_2−/y mice. DOCA-salt increased iNOS expression in both AT_2−/y and AT_2+/y mice. Renal nNOS expression was only significantly decreased by DOCA-salt in AT_2−/y mice. Renal eNOS expression was not different between the groups and not affected by DOCA-salt. $^*P < 0.05$.

Davis GmbH, Berlin, Germany) and inaction- (100 µg/g intraperitoneally; Res Biochemical, Inc., Natick, MA, USA) anesthetized mice. We relied on techniques described earlier [3]. GFR was measured by inulin

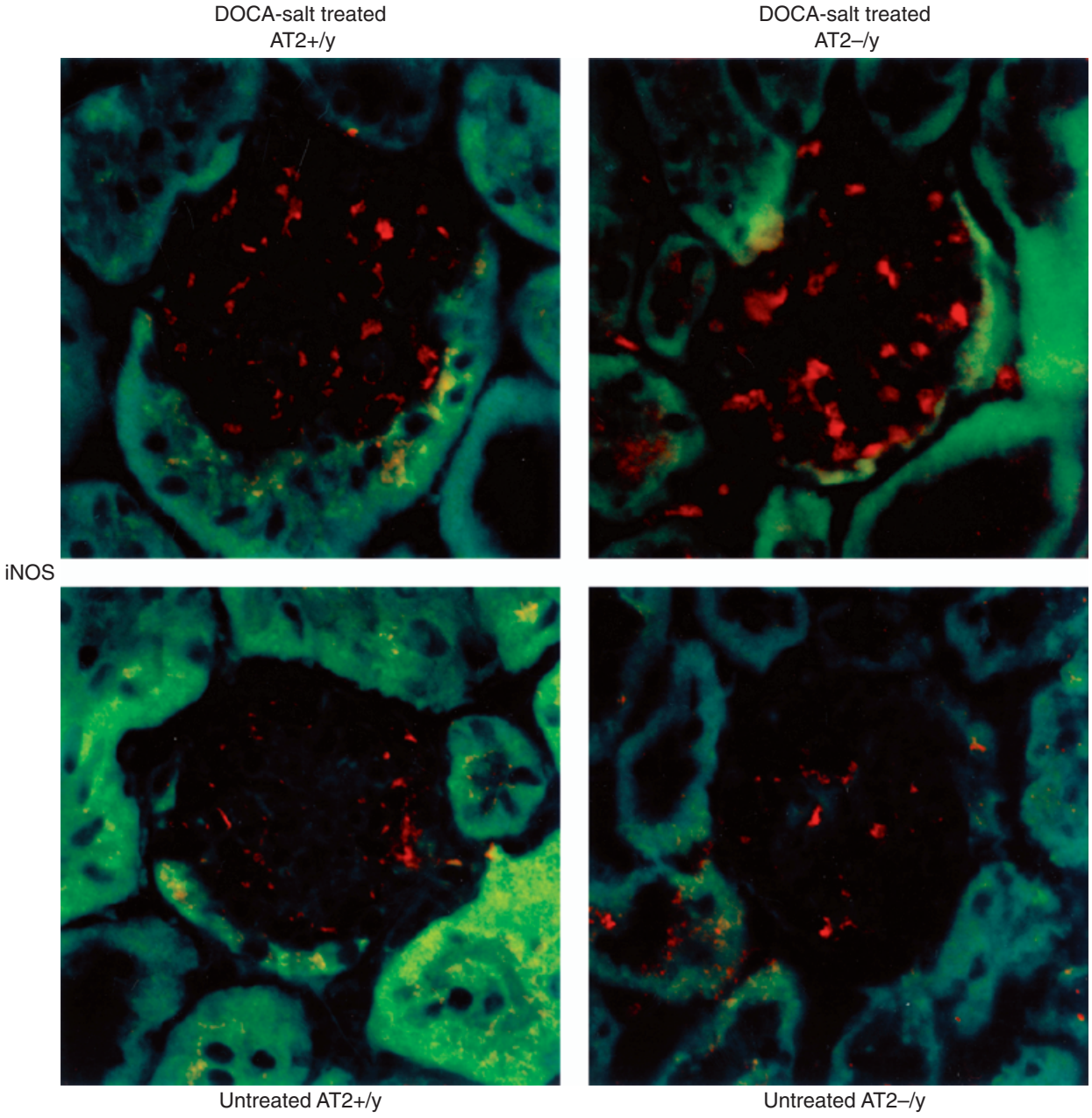

Fig. 3. Representative immunofluorescence staining of renal inducible nitric oxide synthase (iNOS) expression is presented. Deoxycorticosterone acetate (DOCA)-salt–treated AT_2–/y mice showed the highest iNOS expression within the glomerulus (*red staining*). DOCA-salt–treated AT_2+/y mice also showed more intense staining compared to the untreated control strains. Green fluorescence represents renal autofluorescence.

clearance, and RBF was determined using 0.5-mm V-series flow probe (Transonic Systems, Inc., Ithaca, NY, USA). After surgery and a 30- to 45-minute equilibration period, mean arterial pressure (MAP) and RBF were recorded continuously, and urine was sampled in two 10- to 30-minute collecting periods. Renal perfusion pressure (RPP) was then increased by tying off the mesenteric and celiac arteries, and thereafter by occluding the aorta below the kidney. Blood pressure and renal blood flow were calculated for each period by averaging all recorded values during that time period. Renal vascular resistance (RVR) was calculated as the MAP-to-RBF ratio. Urine was sampled and determined gravimetrically. Urinary sodium concentrations were determined by ion-selective electrode (Konelab Microlyte 3+2, Frankfurt, Germany). Urine flow, sodium excretion (UNaV), GFR, and RBF were normalized per gram kidney wet weight (kwt).

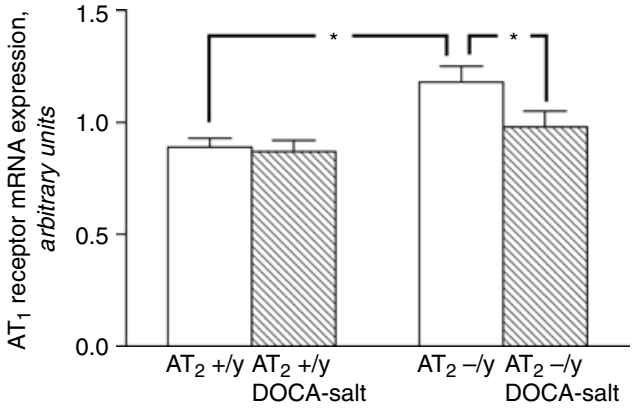

Fig. 4. Angiotensin II type 1 receptor antagonist (AT_1) receptor gene expression in the kidneys of AT_2+/y and AT_2−/y mice with and without deoxycorticosterone acetate (DOCA)-salt. The AT_1 receptor was up-regulated in AT_2−/y mice. DOCA-salt decreased renal AT_1 receptor gene expression in AT_2−/y mice. $^*P < 0.05$.

Statistics

For statistical analysis, we relied on two-way analysis of variance (ANOVA) and the Duncan test. Significance was accepted at $P < 0.05$. Data are mean ± SEM.

RESULTS

We tested the relationship between the AT_1 receptor and NOS isoforms without concomitant AT_2 receptor–related effects under baseline conditions, and after challenging the mice with DOCA-salt to stimulate NO generation. Figure 1 shows 24-hour urinary NOx excretion before and after DOCA-salt treatment. Urinary NOx levels in AT_2+/y mice leveled at 16.40 ± 1.87 compared with 9.4 ± 0.84 nmol/day in AT_2−/y mice. In both strains, urinary NOx excretion increased with DOCA-salt to about 28 nmol/day. Thus, DOCA-salt administration increased the NOx excretion.

Gene expression

The gene expression of renal NOS isoforms (nNOS, iNOS, eNOS) is shown in Figure 2. Renal iNOS mRNA was significantly higher in AT_2−/y than in AT_2 +/y mice at 1.00 ± 0.05 versus 0.7 ± 0.07 arbitrary units, respectively (upper panel). DOCA-salt increased the expression of renal iNOS (1.4 ± 0.10 vs. 1.0 ± 0.04 arbitrary units) in AT_2−/y and in AT_2+/y mice significantly. Renal nNOS expression was also increased in AT_2−/y mice compared with AT_2+/y mice (1.5 ± 0.1 vs. 1.0 ± 0.1 arbitrary units). DOCA-salt decreased the nNOS mRNA values (middle panel) in AT_2−/y mice; AT_2+/y mice showed a trend in the same direction. Renal eNOS expression was not different between AT_2−/y and AT_2+/y mice, and was not influenced by DOCA-salt (lower panel). Figure 3 shows that iNOS was found mainly in the glomeruli. The highest signals were found with DOCA-salt treatment in AT_2−/y mice. These data suggest that iNOS and nNOS genes were up-regulated in AT_2−/y mice compared with AT_2+/y mice. iNOS gene expressions increased, and nNOS expression decreased with DOCA-salt administration. iNOS expression was most pronounced in the glomeruli.

The renal AT_1 receptor was up-regulated in AT_2−/y mice compared with AT_2+/y mice (1.2 ± 0.07 vs. 0.9 ± 0.04 arbitrary units). DOCA-salt decreased the renal AT_1 receptor gene expression slightly to 1.0 ± 0.07 arbitrary units in AT_2−/y mice to levels not different from AT_2+/y mice, as shown in Figure 4. These data suggest that the AT_1 receptor was up-regulated in AT_2−/y mice, and that DOCA-salt administration decreased AT_1 receptor gene expression when the AT_2 receptor is absent.

Blood pressure

Figure 5 (upper panel) shows MAP in AT_2−/y and AT_2+/y mice before and after DOCA-salt and the iNOS inhibitor GED. MAP leveled at 123 ± 2 and at 114 ± 6 mm Hg in AT_2−/y and AT_2+/y mice before DOCA-salt, and increased significantly to 142 ± 3 and to 123 ± 7 mm Hg, respectively. GED did not influence the blood pressure levels in either strain. Figure 5 (lower panel) shows the respective heart rates. Heart rates ranged between 566 and 588 beats/min in AT_2−/y and AT_2+/y mice before DOCA-salt, and decreased to about 500 beats/min as blood pressure increased under DOCA-salt. As a reflection of the stronger increase in blood pressure, heart rate decreased more in AT_2−/y than in AT_2+/y mice under DOCA-salt. GED did not change these levels.

Pressure-diuresis-natriuresis, renal blood flow, glomerular filtration rate

In untreated AT_2+/y mice, urine flow and sodium excretion leveled at 23.94 ± 3.79 μL/min/g kwt and 4.48 ± 0.72 μmol/min/g kwt at a renal perfusion pressure (RPP) level of 103 ± 2 mm Hg, respectively. Increasing RPP to 141 ± 2 mm Hg increased urine flow and sodium excretion to 109.24 ± 6.88 μL/min/g kwt and 19.63 ± 1.73 μmol/min/g kwt. At RPP levels of 107 and 147 mm Hg, AT_2−/y mice had urine flows and sodium excretions of 19.56 ± 3.16 μL/min/g kwt and 3.40 ± 0.44 μmol/min/g kwt, or 85.93 ± 7.63 μL/min/g kwt and 18.12 ± 1.62 μmol/min/g kwt, respectively. These values were reduced compared with AT_2+/y mice. With DOCA-salt the pressure-diuresis-natriuresis curves were shifted to the right in AT_2+/y and AT_2−/y mice, as shown in Figure 6.

Figure 7 (upper panel) represents the effect of changing RPP on RBF in AT_2+/y mice and AT_2−/y mice (lower panel) with and without DOCA-salt. In all groups, RBF was auto-regulated over the entire range of pressures.

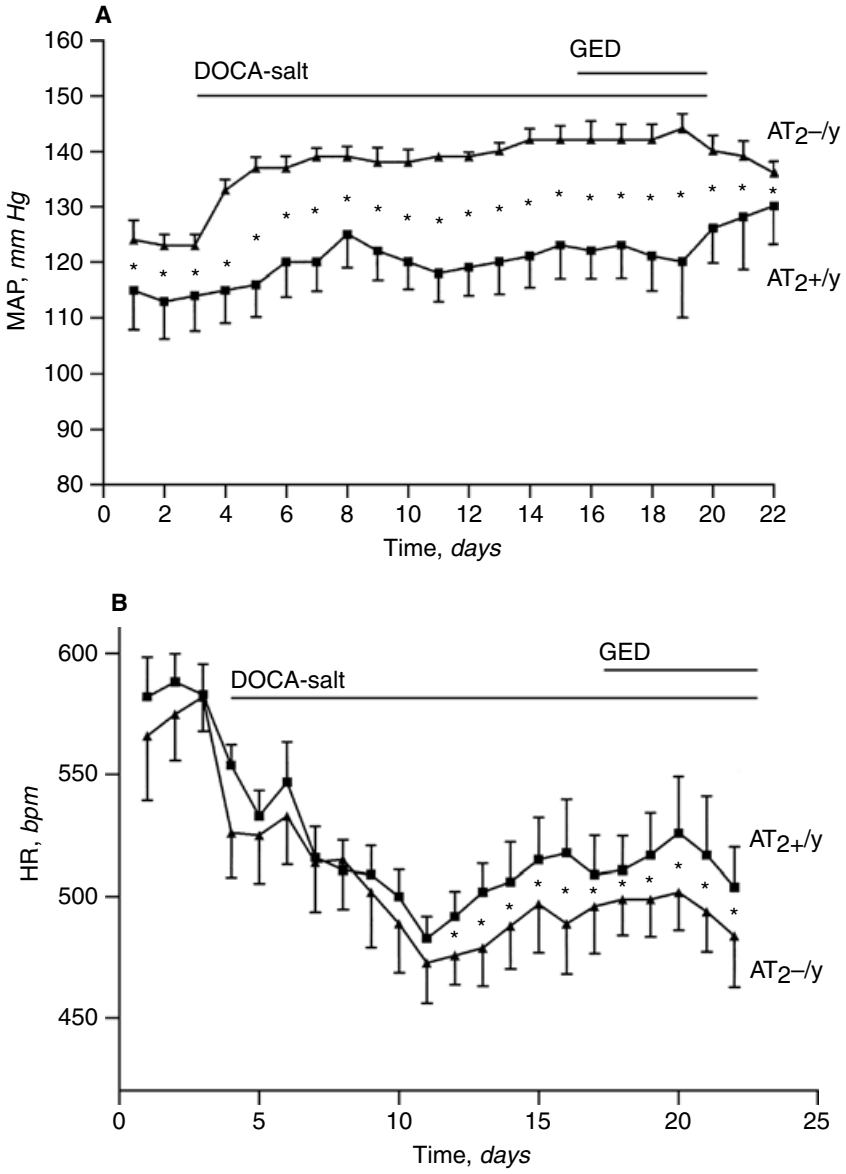

Fig. 5. Mean arterial pressure (MAP) (*A*) and heart rate (*B*) in AT_2−/y and AT_2+/y mice before and after deoxycorticosterone acetate (DOCA)-salt and inducible nitric oxide synthase (iNOS) inhibition by guanidinoethyldisulfide 2HCl (GED, 10 mg/kg, three times a day, intraperitoneally) are shown. MAP values were measured continuously (24-hour) by telemetry. DOCA-salt increased blood pressure stronger in AT_2−/y mice than in AT_2+/y mice. GED did not change blood pressure or heart rate levels. $^*P < 0.05$.

RBF averaged, over the pressure range studied, 5.82 ± 0.33 mL/min/g kwt in AT_2+/y mice, and 4.52 ± 0.31 mL/min/g kwt in AT_2−/y mice, respectively. DOCA-salt administration decreased RBF in AT_2+/y mice to 4.43 ± 0.31 mL/min/g kwt, and in AT_2−/y mice to a mean value of 3.86 ± 0.18 mL/min/g kwt. The autoregulation in RBF was caused by an increase in RVR, as RPP was increased (data not shown). These data indicate that RBF was reduced in AT_2−/y mice compared with AT_2+/y mice, and that DOCA-salt reduced RBF in AT_2+/y and AT_2−/y mice. RVR reacted adequately in both strains to maintain autoregulation of RBF in the pressure ranges studied.

Figure 8 (upper panel) shows the relationships between RPP and GFR of AT_2+/y and AT_2−/y (lower panel) mice with and without DOCA-salt. No significant GFR differences were observed when AT_2+/y and AT_2−/y mice were compared. GFR at all pressure levels averaged 1.10 ± 0.09 in AT_2+/y, and 1.03 ± 0.09 mL/min g kidney wt in AT_2−/y mice, respectively. DOCA-salt decreased GFR in both groups significantly. After DOCA-salt, GFR averaged 0.76 ± 0.04 in AT_2+/y mice and 0.72 ± 0.04 mL/min g kidney wt in AT_2−/y mice. These data show that GFR was reduced by DOCA-salt in AT_2−/y and AT_2+/y mice.

DISCUSSION

We studied nNOS, iNOS, and eNOS, the three major isoforms of NOS in AT_2−/y mice with up-regulated AT_1 receptor expression. We challenged the mice with DOCA-salt to increase NO production. We demonstrated that in AT_2−/y mice iNOS and nNOS were up-regulated compared with AT_2+/y mice. iNOS expression

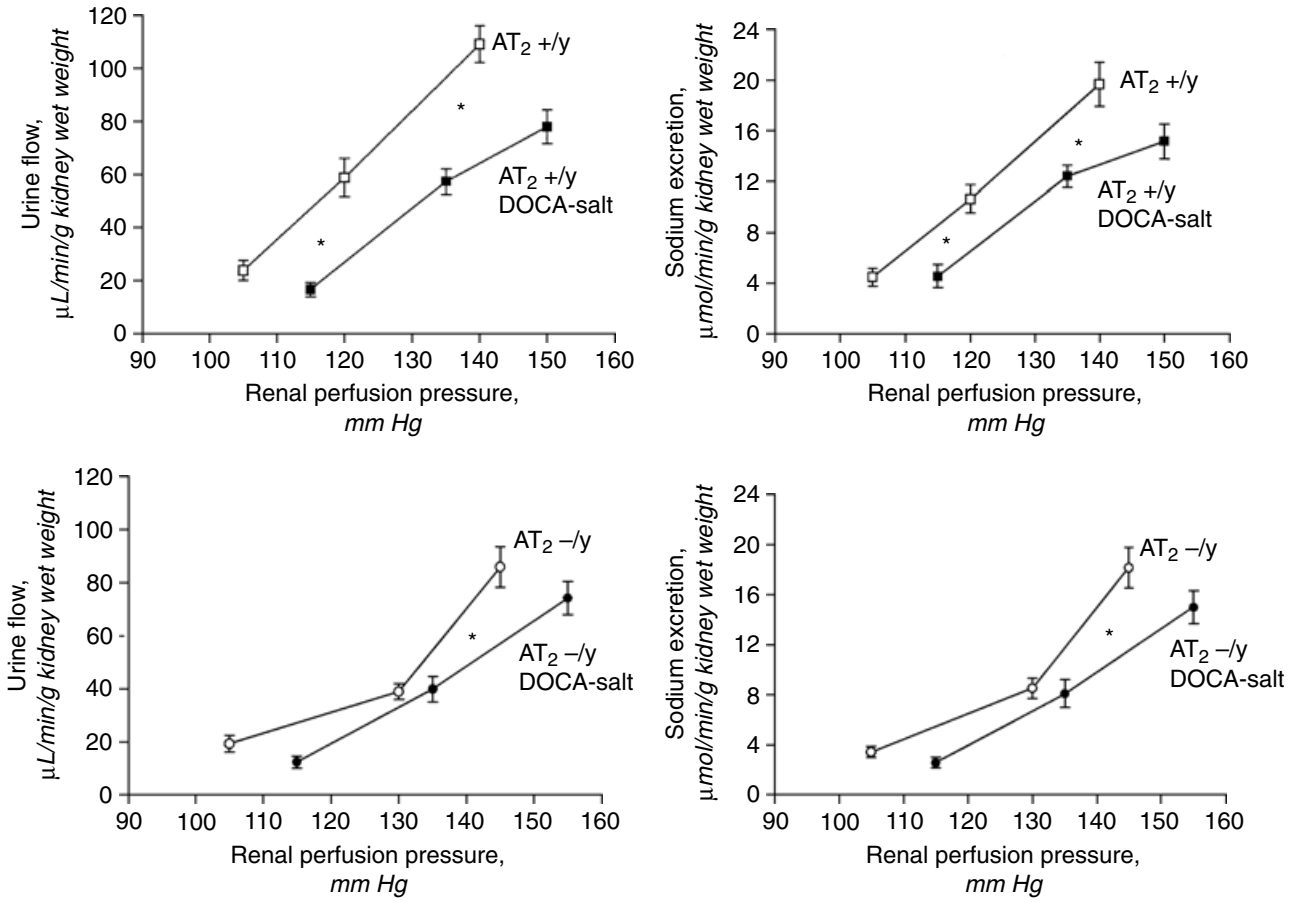

Fig. 6. Deoxycorticosterone acetate (DOCA)-salt treatment and the relationship between renal perfusion pressure and urine flow (*left panel*) and sodium excretion (*right panel*) in AT_2+/y mice (*upper panels*) and AT_2−/y mice (*lower panels*). $P < 0.05$, values compared at equivalent renal perfusion pressure levels. DOCA-salt shifted pressure natriuresis-diuresis curves toward right. *$P < 0.05$.

was further increased in both strains by DOCA-salt. With immunohistochemistry, we found strong iNOS staining in the glomeruli, especially after DOCA-salt in AT_2−/y mice. We observed little expression in other renal structures, which may be related in part to the antibody we used. eNOS was not different between the strains and was not affected by DOCA-salt. Concomitant blood pressure values increased more in DOCA-salt–treated AT_2−/y mice compared with control mice. Pharmacologic iNOS inhibition did not influence DOCA-salt blood pressures. Pressure-natriuresis relationships shifted right in DOCA-salt–treated mice. DOCA-salt reduced RBF and GFR in AT_2+/y mice and AT_2−/y.

NO and NOS isoforms are important in the regulation of cardiovascular and renal homeostasis. In the kidney, NO regulates salt and fluid reabsorption, renal and glomerular hemodynamics, tubuloglomerular feedback responses, renin release, and renal sodium and water excretion [4]. NO and Ang II have many opposing effects [13]. Strong evidence has been presented that the AT_2 receptor mediates the renal production of NO in the rat [14]. A role for nNOS was proposed. These data raised the possibility that NOS expression might be decreased in kidneys of AT_2−/y mice. Such a decrease might explain their hypertension. However, this result did not appear to be the case. Instead, we found an up-regulation of iNOS and nNOS in AT_2−/y mice. These changes may be a counter-regulatory reaction to the up-regulated AT_1 receptor in these mice. Experiments with rat glomerular mesangial cells have shown that Ang II dose dependently inhibited iNOS mRNA and protein expression. The effect was abolished by AT_2 receptor blockade. On the other hand, shifting the AT receptor expression toward the type 1 receptor led to an increased iNOS expression. These experiments suggested that induced NO production was enhanced by the AT_1 receptor and diminished by the AT_2 receptor [15]. The changes in AT_1 receptor gene expression and/or altered NOS isoform expression may have been involved in hemodynamic and functional changes we described earlier for AT_2−/y mice [3].

DOCA-salt increased blood pressure more in AT_2−/y mice than in AT_2+/y mice. Our acute pressure-natriuresis/diuresis experiments in AT_2−/y and AT_2+/y mice confirmed earlier observations that DOCA-salt

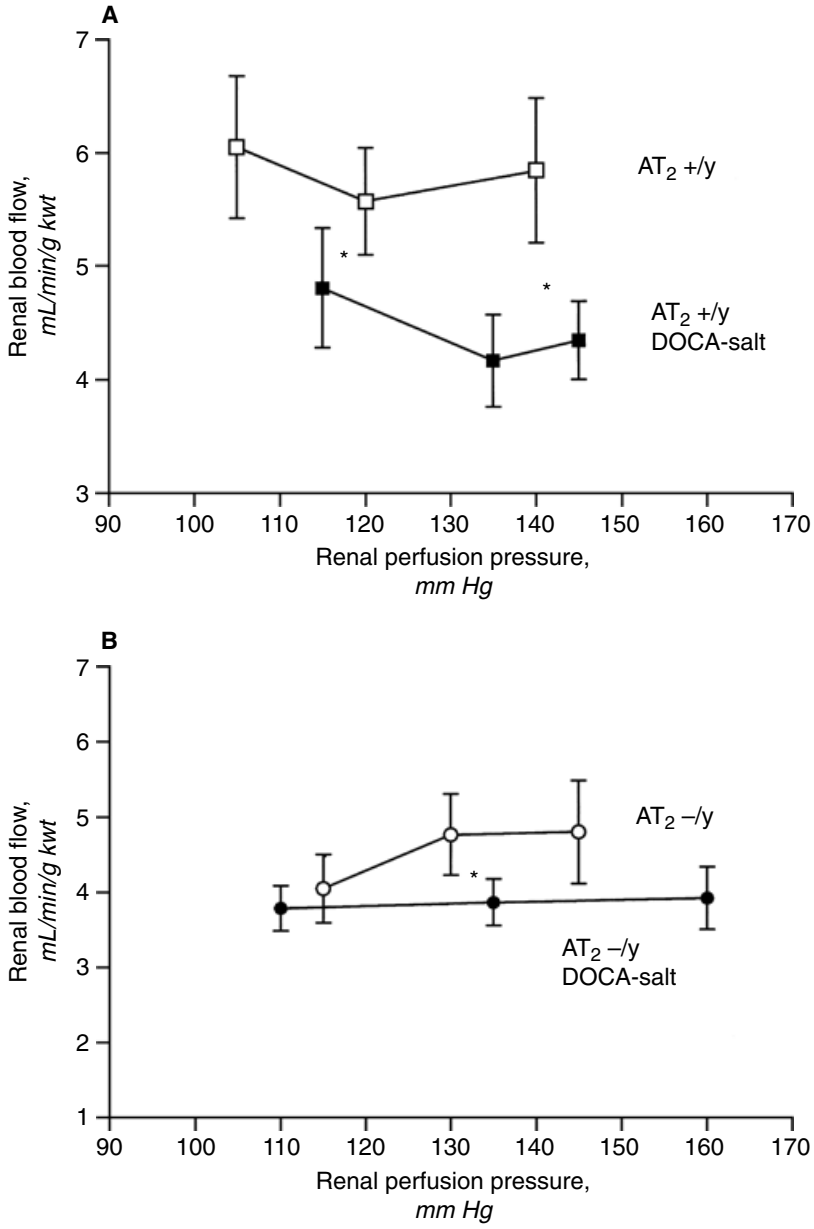

Fig. 7. Relationships between renal perfusion pressure and renal blood flow in AT_2+/y (*A*) and in AT_2−/y mice (*B*) with and without deoxycorticosterone acetate (DOCA)-salt. Renal blood flow was lower in AT_2−/y than in AT_2+/y mice. DOCA-salt decreased renal blood flow (RBF) in AT_2+/y and in AT_2−/y mice. $^*P < 0.05$. kwt is kidney wet weight.

shifts these curves toward higher renal perfusion pressures [16]. This shift to the right was accompanied by a decrease in RBF and GFR and an increase in RVR. This result underscores the crucial role of the kidney for long-term blood pressure regulation [17].

An increase in salt intake increases NO production and NO levels within the kidney [18, 19]. DOCA-salt treatment results in an experimental form of salt-dependent hypertension with increased urinary NOx excretion [12]. Correspondingly, DOCA-salt administration also increased urinary NOx excretion in AT_2−/y and AT_2+/y mice. Because the mice were not receiving a standardized NOx intake, and we did not measure plasma NOx, the NOx excretion values should be interpreted with caution. The method is relatively crude. We therefore did not concentrate on the relatively small differences in NOx excretion between AT_2−/y and AT_2+/y mice, and did not correlate these values with the NOS isoform expressions.

An up-regulation of eNOS and iNOS mRNA levels has been reported earlier with DOCA-salt administration [12, 20]. We found no change in eNOS expression after DOCA-salt. DOCA-salt induced an up-regulation of iNOS. Immunohistochemistry confirmed strong iNOS staining in the glomeruli, especially after DOCA-salt administration in AT_2−/y mice. We found little expression in other renal structures, although we have not performed confirmatory studies with Western blotting. In rats, iNOS

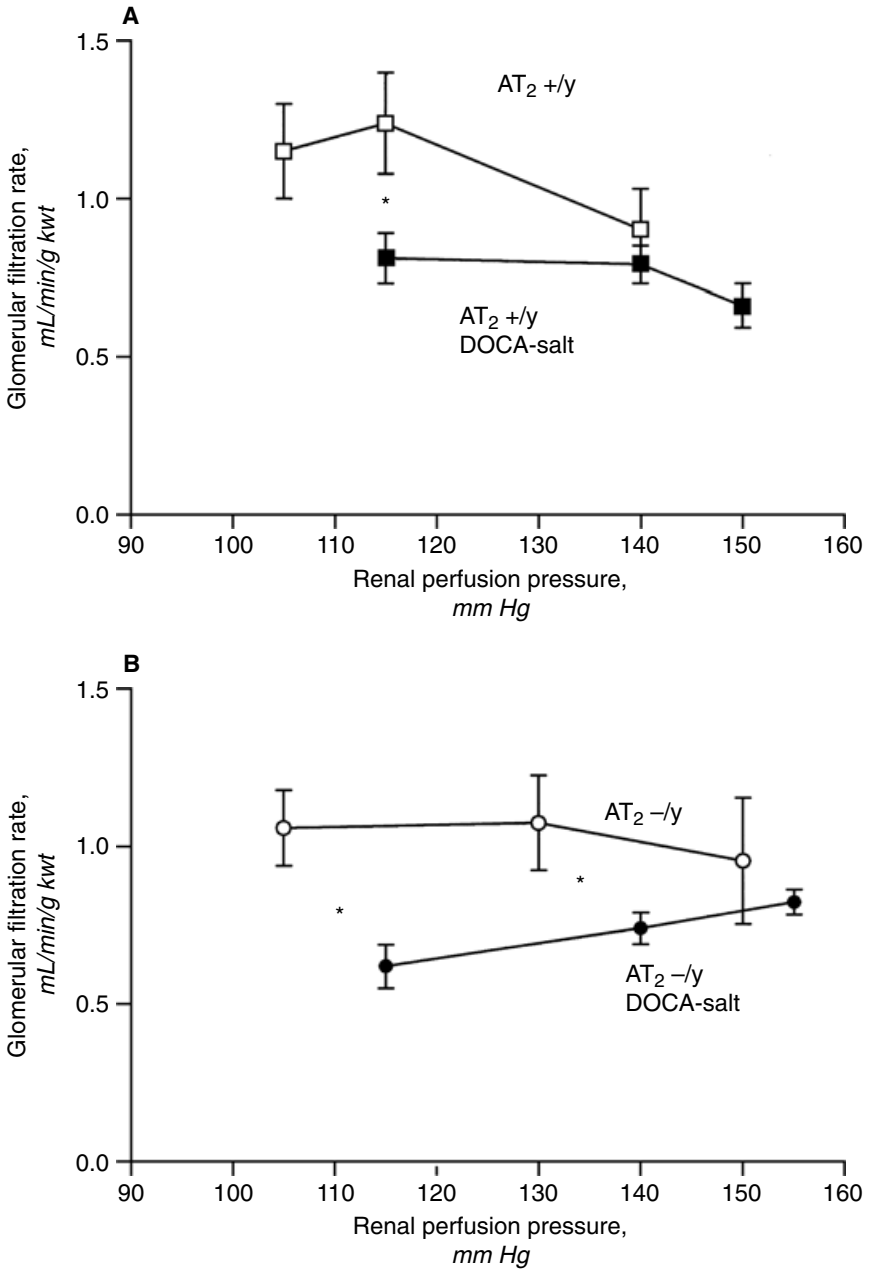

Fig. 8. Deoxycorticosterone acetate (DOCA)-salt and the relationship between renal perfusion pressure and glomerular filtration rate (GFR) in AT_2+/y (*A*) and AT_2−/y mice (*B*). DOCA-salt decreased GFR inAT_2+/y and AT_2−/y mice. $^*P < 0.05$. kwt is kidney wet weight.

was identified in renal vascular and tubular segments. iNOS was implicated in sodium homeostasis as well as in salt-induced hypertension [21, 22]. On the other hand, an "interstitial" fraction of iNOS was described, which may be proinflammatory [23]. Because pharmacologic iNOS inhibition did not change blood pressure in AT_2−/y and AT_2+/y DOCA-salt–treated mice, we believe that the proinflammatory interstitial fraction of iNOS, and not the tubular iNOS fraction that is involved in sodium excretion, was up-regulated by DOCA-salt. GED iNOS blockade in our AT_2−/y and AT_2+/y mice did not lower blood pressure. This view agrees with experiments in rats, in which application of aminoguanidine also did not affect blood pressure [23, 24]. We suggest that for hemodynamic and renal changes in our mice, AT_1 receptor–dependent effects were more important than effects related to changes in iNOS.

The focus of our study was not directed at end-organ damage. Nevertheless, iNOS up-regulation may be relevant in this setting [25]. The role of mineralocorticoid receptor signaling in that regard is a topic of major interest. DOCA-salt is known to stimulate nuclear factor kappa B (NF-κB) signaling and the treatment stimulates NAD(P)H oxidase [26]. Conceivably, we observed

mechanisms by which DOCA-salt induces end-organ damage. These mechanisms appear to have little or no effect on blood pressure.

The AT_1 receptor mediates Ang II–induced sodium and water reabsorption in the renal tubules, and Ang II is also involved in RBF regulation and tubuloglomerular feedback responses, as well as in changes in renal interstitial hydrostatic pressure [27–31]. The AT_1 receptor is up-regulated in AT_2−/y mice. The up-regulation of the AT_1 receptor in AT_2−/y mice was found in various tissues and seems to be a general phenomenon in these mice [3, 7, 8, 32]. The AT_1 receptor response after DOCA-salt appears to be quite variable. For hyper-mineralocorticoid states, an up-regulation, no change, or a down-regulation of the AT_1 receptor were observed [9, 32–36]. The reasons for these differences are not clear. They may depend on strain differences or on the tissues examined. In our experiments, DOCA-salt reduced renal AT_1 receptor mRNA in AT_2−/y mice. Despite similar AT_1 receptor expression levels in AT_2−/y and AT_2+/y mice under DOCA-salt, the Ang II–related effects may not be the same. AT_2–/y mice exhibit absence of AT_2 receptor–related effects that counter-regulate AT_1 receptor–dependent Ang II effects.

CONCLUSION

We showed that the deletion of the AT_2 receptor alone, or with up-regulation of the AT_1 receptor and the administration of DOCA-salt, are strong stimuli for iNOS induction. We suggest that up-regulation of iNOS is not involved in hemodynamic changes caused by DOCA-salt. iNOS inhibition did not affect blood pressure and heart rate changes under DOCA-salt. Nevertheless, the iNOS up-regulation may be related to subsequent organ damage, a hypothesis worthy of testing.

ACKNOWLEDGMENT

This study was supported by a grant-in-aid from the Deutsche Forschungsgemeinschaft to Volkmar Gross. Anton Bonartsev was supported by a grant from the Deutscher Akademischer Austauschdienst. We are grateful to Sabine Grüger and Ilona Kamer for technical assistance.

Reprint requests to Friedrich C. Luft, M.D., Franz Volhard Clinic, Wiltberg Strasse 50, 13125 Berlin, Germany.
E-mail: luft@fvk-berlin.de

REFERENCES

1. Carey RM, Wang ZQ, Siragy HM: Role of the angiotensin type 2 receptor in the regulation of blood pressure and renal function. *Hypertension* 35:155–163, 2000
2. De Gasparo M, Catt KJ, Inagami T, *et al*: International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev* 52:415–472, 2000
3. Gross V, Schunck WH, Honeck H, *et al*: Inhibition of pressure natriuresis in mice lacking the AT2 receptor. *Kidney Int* 57:191–202, 2000
4. Zou AP, Cowley AW, Jr.: Role of nitric oxide in the control of renal function and salt sensitivity. *Curr Hypertens Rep* 1:178–186, 1999
5. Obst M, Gross V, Janke J, *et al*: Pressure natriuresis in AT(2) receptor-deficient mice with l-NAME hypertension. *J Am Soc Nephrol* 14:303–310, 2003
6. Hannan RE, Davis EA, Widdop RE: Functional role of angiotensin II AT2 receptor in modulation of AT1 receptor-mediated contraction in rat uterine artery: Involvement of bradykinin and nitric oxide. *Br J Pharmacol* 140:987–995, 2003
7. Saavedra JM, Hauser W, Ciuffo G, *et al*: Increased AT(1) receptor expression and mRNA in kidney glomeruli of AT(2) receptor gene-disrupted mice. *Am J Physiol Renal Physiol* 280:F71–F78, 2001
8. Tanaka M, Tsuchida S, Imai T, *et al*: Vascular response to angiotensin II is exaggerated through an upregulation of AT1 receptor in AT2 knockout mice. *Biochem Biophys Res Commun* 258:194–198, 1999
9. Gross V, Lippoldt A, Bohlender J, *et al*: Cortical and medullary hemodynamics in deoxycorticosterone acetate-salt hypertensive mice. *J Am Soc Nephrol* 9:346–354, 1998
10. Johns C, Gavras I, Handy DE, *et al*: Models of experimental hypertension in mice. *Hypertension* 28:1064–1069, 1996
11. Szabo C, Bryk R, Zingarelli B, *et al*: Pharmacological characterization of guanidinoethyldisulphide (GED), a novel inhibitor of nitric oxide synthase with selectivity towards the inducible isoform. *Br J Pharmacol* 118:1659–1668, 1996
12. Allcock GH, Hukkanen M, Polak JM, *et al*: Increased nitric oxide synthase-3 expression in kidneys of deoxycorticosterone acetate-salt hypertensive rats. *J Am Soc Nephrol* 10:2283–2289, 1999
13. Millatt LJ, Abdel-Rahman EM, Siragy HM: Angiotensin II and nitric oxide: A question of balance. *Regul Pept* 81:1–10, 1999
14. Siragy HM, Carey RM: The subtype 2 (AT2) angiotensin receptor mediates renal production of nitric oxide in conscious rats. *J Clin Invest* 100:264–269, 1997
15. Schwobel J, Fischer T, Lanz B, *et al*: Angiotensin II receptor subtypes determine induced NO production in rat glomerular mesangial cells. *Am J Physiol Renal Physiol* 279:F1092–F1100, 2000
16. Gross V, Lippoldt A, Luft FC: Pressure diuresis and natriuresis in DOCA-salt mice. *Kidney Int* 52:1364–1368, 1997
17. Cowley AW, Jr.: Long-term control of arterial blood pressure. *Physiol Rev* 72:231–300, 1992
18. Shultz PJ, Tolins JP: Adaptation to increased dietary salt intake in the rat. Role of endogenous nitric oxide. *J Clin Invest* 91:642–650, 1993
19. Deng X, Welch WJ, Wilcox CS: Renal vasoconstriction during inhibition of NO synthase: Effects of dietary salt. *Kidney Int* 46:639–646, 1994
20. Nadaud S, Mao C, Luvara G, *et al*: Isoform-specific regulation of nitric oxide synthase mRNA in the kidney by sodium and blood pressure. *J Hypertens* 16:1315–1323, 1998
21. Ahn KY, Mohaupt MG, Madsen KM, *et al*: In situ hybridization localization of mRNA encoding inducible nitric oxide synthase in rat kidney. *Am J Physiol* 267:F748–F757, 1994
22. Mohaupt MG, Elzie JL, Ahn KY, *et al*: Differential expression and induction of mRNAs encoding two inducible nitric oxide synthases in rat kidney. *Kidney Int* 46:653–665, 1994
23. Fujihara CK, Mattar AL, Vieira JM, Jr., *et al*: Evidence for the existence of two distinct functions for the inducible NO synthase in the rat kidney: Effect of aminoguanidine in rats with 5/6 ablation. *J Am Soc Nephrol* 13:2278–2287, 2002
24. Waz WR, Van Liew JB, Feld LG: Nitric oxide-inhibitory effect of aminoguanidine on renal function in rats. *Kidney Blood Press Res* 20:211–217, 1997
25. Lirk P, Hoffmann G, Rieder J: Inducible nitric oxide synthase—Time for reappraisal. *Curr Drug Targets Inflamm Allergy* 1:89–108, 2002
26. Beswick RA, Dorrance AM, Leite R, *et al*: NADH/NADPH oxidase and enhanced superoxide production in the mineralocorticoid hypertensive rat. *Hypertension* 38:1107–1111, 2001
27. Dukacz SA, Kline RL: Differing effects of enalapril and losartan on renal medullary blood flow and renal interstitial hydrostatic pressure in spontaneously hypertensive rats. *J Hypertens* 17:1345–1352, 1999

28. Harris PJ, Navar LG, Ploth DW: Evidence for angiotensin-stimulated proximal tubular fluid reabsorption in normotensive and hypertensive rats: Effect of acute administration of captopril. *Clin Sci (Lond)* 66:541–544, 1984
29. Navar LG, Inscho EW, Majid SA, *et al*: Paracrine regulation of the renal microcirculation. *Physiol Rev* 76:425–536, 1996
30. Peti-Peterdi J, Warnock DG, Bell PD: Angiotensin II directly stimulates ENaC activity in the cortical collecting duct via AT(1) receptors. *J Am Soc Nephrol* 13:1131–1135, 2002
31. Schnermann JB, Traynor T, Yang T, *et al*: Absence of tubuloglomerular feedback responses in AT1A receptor-deficient mice. *Am J Physiol* 273:F315–F320, 1997
32. Gross V, Walther T, Milia AF, *et al*: Left ventricular function in mice lacking the AT2 receptor. *J Hypertens* 19:967–976, 2001
33. Asano N, Ogura T, Mimura Y, *et al*: Renal AT1 receptor: Computerized quantification in spontaneously hypertensive rats and DOCA-salt rats. *Res Commun Mol Pathol Pharmacol* 100:171–180, 1998
34. Hara K, Kobayashi N, Watanabe S, *et al*: Effects of quinapril on expression of eNOS, ACE, and AT1 receptor in deoxycorticosterone acetate-salt hypertensive rats. *Am J Hypertens* 14:321–330, 2001
35. Lee J, Kim S, Jung M, *et al*: Altered expression of vascular natriuretic peptide receptors in experimental hypertensive rats. *Clin Exp Pharmacol Physiol* 29:299–303, 2002
36. Lippoldt A, Gross V, Schneider K, *et al*: Nitric oxide synthase and renin-angiotensin system gene expression in salt-sensitive and salt-resistant Sabra rats. *Hypertension* 30:409–415, 1997