A new transgenic rat model overexpressing the angiotensin II type 2 receptor provides evidence for inhibition of cell proliferation in the outer adrenal cortex

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Submitted 22 February 2011; accepted in final form 2 February 2012

Peters B, Podlich D, Ritter M, Müller A, Wanka H, Maser-Gluth C, Seitz C, de Boni L, Maier E, Gretz N, Peters J, Hoffmann SC. A new transgenic rat model overexpressing the angiotensin II type 2 receptor provides evidence for inhibition of cell proliferation in the outer adrenal cortex. Am J Physiol Endocrinol Metab 302: E1044–E1054, 2012. First published February 7, 2012; doi:10.1152/ajpendo.00080.2011.—This study aimed to elucidate the role of the AT2 receptor (AT2R), which is expressed and upregulated in the adrenal zona glomerulosa (ZG) under conditions of increased aldosterone production. We developed a novel transgenic rat (TGR; TGRCXmAT2R) that overexpresses the AT2R in the adrenal gland, heart, kidney, brain, skeletal muscle, testes, lung, spleen, aorta, and vein. As a consequence the total angiotensin II (Ang II) binding sites increased 7.8-fold in the kidney, 25-fold in the heart, and twofold in the adrenals. The AT2R number amounted to 82–98% of total Ang II binding sites. In the ZG of TGRCXmAT2R, the AT2R density was elevated threefold relative to wild-type (WT) littermates, whereas AT1R density remained unchanged. TGRCXmAT2R rats were viable and exhibited normal reproduction, blood pressure, and kidney function. Notably, a slightly but significantly reduced body weight and a moderate increase in plasma urea were observed. With respect to adrenal function, 24-h urinary and plasma aldosterone concentrations were unaffected in TGRCXmAT2R at baseline. Three and 14 days of Ang II infusion (300 ng·min−1·kg−1) increased plasma aldosterone levels in WT and in TGR. These changes were completely abolished by the AT1R blocker losartan. Of note, glomerulosa cell proliferation, as indicated by the number of Ki-67-positive glomerulosa cells, was stimulated by Ang II in TGR and WT rats; however, this increase was significantly attenuated in TGR overexpressing the AT2R. In conclusion, AT2R in the adrenal ZG inhibits Ang II-induced cell proliferation but has no obvious lasting effect on the regulation of the aldosterone production at the investigated stages.

zona glomerulosa; aldosterone production

ANGIOTENSIN II (Ang II) plays a key role in cardiovascular homeostasis and stimulates aldosterone production. Ang II acts through different Ang II receptors such as the AT1 (AT1R) and AT2 receptors (AT2R). AT1R is the predominant Ang II receptor in adults and mediates the classic Ang II effects; AT2R may be activated in response to Ang II but is expressed at lower levels and signal transduction pathways.

The elucidation of the AT2R actions is complicated due to the low degree of AT2R expression in the adult (18, 45). Thus, in contrast to AT1R, the functions and signaling mechanisms of AT2R remain elusive, and existing data are conflicting. AT2R has been shown to counterbalance AT1R functions (17, 52); however, under certain conditions AT1R and AT2R appear to transmit similar effects (47, 48). AT2R may be activated particularly under pharmacological AT1R inhibition, because under this condition Ang II levels increase markedly (56). The recent development of genetically engineered mouse models that either lack (6, 11) or specifically overexpress AT2R in the heart (21, 57, 58) or vascular smooth muscle (27, 55) provides a major step toward understanding AT2R function in these organs. However, models overexpressing AT2R in the adrenal gland, kidney, or brain are not yet available. Therefore, one aim of this study was to generate transgenic rats that overexpress AT2R in the adrenal gland, kidney, or brain during adulthood, allowing us to study tissue-specific AT2R effects and signal transduction pathways.

The adrenal gland is one of the few tissues in which AT2R expression persists into adulthood (for review, see Ref. 51). So far it is still quite unclear whether or not the AT2R is involved in the regulation of adrenal function. Ang II is one major regulator of aldosterone synthesis in the zona glomerulosa (ZG) acting via the AT1R (1, 4, 5). It is supposed that the AT2R is prevalent in adrenal cortex, whereas the AT1R dominates in adrenal medulla and contributes to the regulation of catecholamine synthesis (3, 5). However, we recently found AT2R expression in the ZG and demonstrated its upregulation following stimulation of aldosterone synthesis by bilateral nephrectomy (37) or a high-potassium diet (Peters B, personal communication). Furthermore, recent reports suggest an AngII effect on cortisol production via the AT2R in the zona fasciculata (ZF) in pigs (7). The functional importance of AT2R expression in the ZG and in ZF remains as yet elusive. Thus, another aim was to test the hypothesis that AT2R overexpressed in the adrenal cortex regulates aldosterone and/or corticosterone synthesis.

MATERIALS AND METHODS

Generation of transgenic rats. A 1.54-kb XbaI fragment of mouse AT2R genomic DNA containing 170 bp of the second intron and 1.37 kb of the third exon comprising the entire AT2R coding region (kind gift of Prof. Tadashi Inagami) was placed between the 1.60-kb hybrid CX promoter [composed of the cytomegalovirus enhancer and the −276–to +969-bp region of the chicken β-actin promoter (2, 16)] and the rabbit β-globin polyadenylation signal (Fig. 1A). The 3.80-kb

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E1044
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transgene was excised from the vector using SalI and HindIII, gel purified, and microinjected into the pronuclei of fertilized oocytes of Sprague-Dawley rats, as described previously (12), to generate transgenic rats (TGR; TGRCXmAT2R). Genotyping was performed by Southern blotting of EcoRI-digested tail DNA using a 32P-labeled 1.2-kb Apol fragment of the transgenic construct as a probe and by PCR using sense and antisense PCR primers designed from the third exon of the mAT2R gene (5′-GAGGCCACACAAATGATCT-3′) and the rabbit β-globin gene sequences (5′-GGGCATATGTTGCCAAACTC-3′).

RNA analysis by Northern blotting and real-time RT-PCR. Total RNA was extracted from snap-frozen heart, kidney, brain, adrenal gland, muscle, spleen, liver, lung, testes, aorta, vein, and thymus by means of guanidium isothiocyanate-cesium chloride centrifugation (TRIzol reagent; Life Technologies), fractionated on a 1% formaldehyde-agarose gel, and transferred to nylon membranes. Blots were hybridized with a specific, random-primer, 32P-labeled DNA probe consisting of a 1.2-kb Apol fragment of the transgenic construct, as described previously (14). Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used as loading standard. For Real-Time RT-PCR of promyelocytic leukemia zinc finger (PLZF) and GAPDH as internal control, RNA was treated with RNase-free DNase I (Qiagen), cleaned up with RNeasy spin columns (Qiagen), and reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen). PCR was performed on a Light Cycler (Roche Diagnostics) using the QuantiTect SYBR Green PCR Kit (Qiagen), following the manufacturers’ instructions in triplicate with the following primers: PLZF forward 5′-TTCATCCAGGAGCTGTT-3′, reverse 5′-ACCGTTTTCCACAGAGTTG-3′; and GAPDH forward 5′-TGG ACC AAC TGC TTA-3′, reverse 5′-GGA TGC AGG GAT GAT GTT C-3′. The amplification was performed with the following time course: 15 min, 95°C, and 40 cycles of 95°C (denaturation), 60 s; 55°C (annealing), 25 s, and 72°C (extension), 25 s. This was then followed by melting curve determination, 65°C for 15 s, and cooling, 40°C for 30 s.

Nonradioactive in situ hybridization. Nonradioactive in situ hybridization was performed to detect transgenic mAT2R mRNA, as described previously (36). A transgenic mAT2R transcript-specific probe was generated by PCR using the same primers as utilized for genotyping (as described above). The amplified 573-bp transcript corresponds to 195 bp of the mAT2R gene and to 387 bp of the rabbit β-globin gene sequences. DIGI-11-UTP-labeled sense and antisense riboprobes were synthesized from the linearized plasmids using T3 and T7 RNA polymerases, respectively. In situ hybridization was carried out on 7-μm-thick cryostat sections. The specificity of the in situ hybridization signal was verified by parallel incubation of antisense riboprobes on tissues of transgenic and nontransgenic animals. In situ hybridization for endogenous rat AT2R transcripts was performed as described previously (37).

Membrane preparation and binding assays. Membrane fractions were prepared from snap-frozen heart, kidney, adrenal gland, and adrenal capsules containing the ZG, as described previously (12). Subcapsular tissue with ZG was manually separated from the inner tissue, as described previously (36), resulting in contamination with ZF <5% (10). Adrenal capsules of two rats were pooled. Binding assay was performed as described elsewhere (13, 53). Briefly, mem-
branched fronds (5 adrenal gland), 10 (kidney), or 50 μg (heart) of protein were incubated with the nonspecific Ang II receptor antagonist [125I][Sar1-Ile8]-Ang II (5–6 points in 0.1–1.8 nmol/l for saturation experiment and 0.2 nmol/l for competition experiment, 2.200 Ci/mmol; DuPont-NEN) in a total assay volume of 200 μl for 90 min at 22°C. Specific binding was determined from the differences between counts in the absence and presence of 10 μmol/l unlabeled Ang II, 10 μmol/l of a nonpeptide antagonist of the AT-R PD 123319 (Sigma, München, Germany), and 10 μmol/l of the AT-R-specific nonpeptide antagonist CV 11974 (Alsedo Pharmaceutical Industries, Osaka, Japan). All assays were run in duplicate.

**Immunohistological staining for Ki-67.** Proliferating cells were stained with an antibody against rat Ki-67 immunohistochemically, as described previously (29). Briefly, deparaffinized sections were treated with 0.3% hydrogen peroxide, microwaved for antigen retrieval (20 min, 10 mM citrate buffer, pH 6.0–6.2), and blocked with 2% BSA-1× PBS for 1 h. The slides were incubated with anti-Ki-67 antibody (rat Ki-67, 1:50; Dako) for 1 h and then with a biotinylated secondary antibody (anti-mouse IgG, 1:200; Sigma). Incubation with avidin-biotin complex (ABC) reagent and colorimetric detection was accomplished according to the manufacturer’s instructions (Vectastain Elite ABC-Peroxidase Kit, DAB Substrate Kit; Vector Laboratories). Proliferating cells were counted in 15 visual fields using a ×40 objective.

**Animal experiments.** All animal experiments were conducted in accordance with federal and local laws and institutional regulations and were approved by the Regierungspärisidium Karlsruhe, Land Baden-Württemberg. Sprague-Dawley rats were derived from a commercial source (Janvier). The generated transgenic lines and their wild-type (WT) littermates were bred and kept in our own animal facility at the Medical Faculty Mannheim. The rats were kept on a 12:12 h light-dark cycle with 55% humidity at an ambient temperature of 23 ± 2°C and had free access to a standard pellet diet (Sniff, Ferdinand-Gabriel-Weg 16, D-59494 Soest) and tap water. Ten-week-old male TGRXmAT2R line 199 (n = 6), line 235 (n = 12), and age-matched WT littermates (line 199, n = 14; line 235, n = 8) were used to determine body weight, heart, and kidney weights, and biochemical parameters from blood plasma. Creatinine and urea clearance were calculated using 24-h urine specimens collected from rats placed in metabolic cages. The same parameters, as well as blood pressure, were obtained from 9- to 11-mo-old male TGRXmAT2R (line 199, n = 12; line 235, n = 8) and age-matched WT rats (n = 7). Furthermore, rats were treated with either 300 ng Ang II·min·kg·day (experiment A) or 300 ng Ang II (experiment B) in a 100-μl volume via a second catheter (PE 0.58) that was implanted into the left vena femoralis. Blood pressure was taken for 5 min at baseline and for 5 min following a bolus injection of 5 ng of Ang II in a 100-μl volume via a second catheter (PE 0.58) that was implanted into the left aorta femoralis. Blood pressure was measured on 4 consecutive days in freely moving 3-mo-old male rats and was kept in a Rattun Sampling Cage.

**Statistical analysis.** Data are expressed as means ± SD or as median (25th/75th percentile). Differences between TGRXmAT2R and WT animals were analyzed by the unpaired sampled t-test or ANOVA, followed by Bonferroni correction for multiple comparisons; P < 0.05 was considered statistically significant. Saturation isotherms and the Scatchard plots were generated, and Kd and maximum binding capacity (Bmax) were estimated by nonlinear regression using the InPlot program (GraphPad Software for Science; GraphPad, San Diego, CA).

**RESULTS**

**Generation of transgenic rats that overexpress AT2R.** Four transgenic TGRXmAT2R lines (199, 215, 235, and 238) harboring the mAT2R under the control of the CX promoter (Fig. 1A) were generated. Northern blotting revealed transgene expression in numerous tissues, with high levels in adrenal gland, lung, kidney, brain, skeletal muscle, blood vessels, and heart and testes (Fig. 1B). Further studies were conducted using TGRXmAT2R lines 199 and 235 with the highest transgene expression.

Figure 2A shows the Ang II receptor densities in the heart, kidney, and adrenal glands of 8- to 10 wk-old male rats. In WT, the highest Ang II receptor density was found in the adrenal glands, followed by the kidney, whereas Ang II receptor density in the heart was very low. TGRXmAT2R (lines 199 and 235) demonstrated a marked increase in Bmax in all tissues tested: in the kidney, 7.9- and 6.5-fold; in the adrenal gland, 2.0- and 1.9-fold; and in the heart, 14- and 22-fold, respectively, vs. WT (Fig. 2A). Affinities (Kd) were not changed significantly and agreed with previously published data (42). Consequently, the proportions of AT2R relative to AT1R densities were increased markedly in TGRXmAT2R rats, as demonstrated by competitive binding studies (Fig. 2B). Transgene expression was detected in newborn rats and persisted at high levels ≤15 mo of age as assessed by Northern blotting and saturation binding studies (data not shown). Thus, the
AT2R is the predominant Ang II receptor in cardiovascular-relevant tissues in adult TGRCXmAT2R but not WT rats. Assuming that the observed Ang II action is the net effect of activity at AT1R and AT2R, in TGRCXmAT2R we would expect to see a blunting of those AT1R-mediated effects that are opposed by AT2R and an enhancement of those Ang II effects that is based on concerted actions of AT1R and AT2R.

General characterization of TGRCXmAT2R. TGRCXmAT2R exhibited normal reproduction and development, and there was no evidence of compromised lifespan. Mean systolic baseline blood pressure did not differ between TGR and WT littermates, and it did not matter whether it was measured in 9- to 11-mo-old conscious rats by tail-cuff plethysmography (TGRCXmAT2R line 199, 150 ± 14 mmHg; line 235, 147 ± 10 mmHg; WT (line 199 ± 235), 144 ± 8 mmHg) or in awake, freely moving, 3-mo-old rats via a catheter cannulated into the left aorta femoralis. Bolus injection of Ang II challenged a similar blood pressure response in TGRCXmAT2R and WT rats (Fig. 3).

No gross morphological or histological alterations in the kidney, heart, or adrenal gland were observed (data not shown), and heart and kidney growth expressed as organ-to-body weight ratio was unaltered in TGRCXmAT2R (Table 1). Notably, transgenic animals displayed a moderately lower body weight and a significantly increased plasma urea concentration and plasma urea/creatinine ratio relative to their WT littermates at the ages of 10 wk (Table 1) and 9–11 mo (body weight in g: line 235, 603 ± 37; line 199, 510 ± 87; WT, 615 ± 70; plasma urea in mg/dl: line 235, 42.0 ± 10, P < 0.05; line 199, 43.4 ± 3.7, P < 0.05; WT, 34.6 ± 2.7; plasma urea/creatinine ratios: line 235, 150 ± 59, P < 0.001; line 199, 143 ± 23, P < 0.001; WT, 86.9 ± 16). The changes in plasma urea occurred in the absence of differences in urinary urea excretion between transgenic lines 235 and 199 and WT. There were no obvious differences between TGRCXmAT2R and WT in other biochemical parameters analyzed (Table 1).

Transgenic mAT2R is expressed in the adrenal ZG. Transgene expression in the adrenal glands was studied in more detail. In situ hybridization revealed that WT rats express endogenous AT2R in the ZG and medulla (Fig. 4A). Using the same probe, we found more intense staining in the adrenal cortex of TGRCXmAT2R because the rat AT2R probe cross-

Fig. 2. Angiotensin II (Ang II) receptor densities in different tissues of 8- to 10-wk-old TGR and WT littermates. A: Ang II receptor densities [maximum binding capacity (B max)] as determined by saturation binding assays on membranes from kidney, adrenal gland, and heart from TGR lines 199 and 235 and age-matched WT using 125I-[Sar1,Ile8]-Ang II as a ligand. B: %B max for AT2R on B max for total Ang II binding in different organs from TGR and WT animals, as determined by competitive binding studies using CV-11974 and PD-123319 as competitors. Results are expressed as means ± SD. Each sample was assayed in duplicate. **P < 0.001 vs. WT; 4 rats/group.

Fig. 3. Blood pressure in awake, freely moving, 3-mo-old catheterized male rats at baseline (measured for 5 min) and following a bolus injection of 5 ng of Ang II via a 2nd catheter in TGR (n = 4) and WT littermates (n = 3). Experiments were repeated on 4 consecutive days. *P < 0.05 vs. baseline.
reacted with the transgenic AT₂R, as shown in Fig. 4B. The transgene-specific AT₂R probe did not cross-react with endogenous AT₂R, as seen in WT samples (Fig. 4C). In TGRCXmAT₂R samples, intense signals for the transgenic AT₂R were found in the whole adrenal cortex, including the cells of the ZG and the ZF (Fig. 4D).

Next, we estimated the number of AT₁R and AT₂R binding sites in the total adrenal gland (Fig. 5, A and B) and in the capsules containing the ZG (Fig. 5, C and D) by saturation binding studies assessing the selective displacement of ¹²⁵I-[Sar¹-Ile⁸]-Ang II by ligands of the two receptors CV-11974 and PD-123319. In WT, the AT₂R density in whole adrenal glands was relatively high (B_max: 0.25±0.17 pmol/mg) compared with other organs and comprised 37.5±3.0% of total adrenal Ang II binding sites. In TGRCXmAT₂R line 199 and 235, the number of adrenal AT₂R binding sites was roughly six-fold (line 235) and 5.6-fold (line 199) higher, respectively, compared with WT. There were no significant differ-

### Table 1. Clinical and laboratory data of 10-wk-old male TGR and WT rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Line 199</th>
<th>Line 235</th>
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<tr>
<td>Body weight, g</td>
<td>342 ± 25</td>
<td>337 ± 43</td>
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<tr>
<td>Kidney/body weight ratio</td>
<td>3.7 ± 0.2</td>
<td>3.58 ± 0.2</td>
</tr>
<tr>
<td>Heart/body weight ratio</td>
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<td>3.25 ± 0.3</td>
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<td>45.0 ± 6.0**</td>
</tr>
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<td>Plasma creatinine, mg/dl</td>
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<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>Plasma cholesterol, mg/dl</td>
<td>62.0 ± 9.9</td>
<td>62.6 ± 10.0</td>
</tr>
<tr>
<td>Plasma triglyceride, mg/dl</td>
<td>175 ± 45</td>
<td>140 ± 25</td>
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<tr>
<td>Plasma phosphate, mmol/l</td>
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<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Plasma Na, mmol/l</td>
<td>141 ± 4.0</td>
<td>139 ± 6.3</td>
</tr>
<tr>
<td>Plasma K, mmol/l</td>
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<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>Plasma protein, mg/dl</td>
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<td>60 ± 3.7</td>
</tr>
<tr>
<td>Plasma urea/creatinine ratio</td>
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<td>196 ± 29**</td>
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<td>Urinary urea, mg/24 h</td>
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<td>Clearance creatinine, ml·min⁻¹·100 g⁻¹</td>
<td>0.98 ± 0.26</td>
<td>1.05 ± 0.23</td>
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</table>

Data are means ± SD. WT, wild type; TGR, TGRCXmAT₂R (transgenic). *P < 0.05 vs. WT; **P < 0.02 vs. WT.
ences in AT1R density between TGRCXmAT2R line 199, line 235, and WT (Fig. 5, A and B). The adrenal levels of endogenous AT2R, AT1R, ACE1, ACE2, and angiotensinogen mRNA, as assessed by real-time PCR, were not modified by overexpression of transgenic AT2R mRNA (data not shown).

Figure 5C shows the AT1R and AT2R densities in the adrenal capsule containing the ZG. In WT, Ang II binding in ZG was fourfold higher than in the whole adrenal glands, but the proportion of AT2R was somewhat lower (25 ± 3.7%). AT1R binding sites in the ZG did not differ between TGRCXmAT2R and WT, but the number of AT2R binding sites was increased significantly 3.1-fold in TGRCXmAT2R/235 and 2.6-fold in TGRCXmAT2R/199 relative to WT; consequently, the AT2R/AT1R ratio increased 2.7- and twofold, respectively (Fig. 5, C and D). The saturation-binding curves for AT1R and AT2R in the ZG from TGRCXmAT2R and WT rats are shown in Fig. 5, E and F, respectively.

Effect of AT2R overexpression in the outer adrenal cortex on aldosterone levels and Ang II-induced proliferation. Basal plasma aldosterone and renin levels (Table 2) as well the 24-h urinary excretion of aldosterone (line 199: WT 5.8 ± 1.8 ng/24 h, TGR 4.2 ± 1.3 ng/24 h; line 235: WT 4.2 ± 0.5 ng/24 h, TGR 3.4 ± 0.9 ng/24 h) were not altered in TGRCXmAT2R/235 and TGRCXmAT2R/199 relative to their WT littermates.

It is well accepted that Ang II stimulates aldosterone production and hyperplasia of ZG cells via the AT1R (26). To investigate a possible implication of the AT2R, we continuously infused Ang II (300 ng·kg body wt−1·day−1) for 3 days into TGRCXmAT2R/199 and TGRCXmAT2R/235 and their respective WT littermates and for 14 days into TGRCXmAT2R/235 and Sprague-Dawley rats as WT controls. As expected, plasma renin levels became markedly suppressed by Ang II (Table 2) so that the plasma Ang II was determined exclusively by the Ang II infusion. In all TGR and WT rats, the number of Ki-67-positive cells increased in the ZG and in the zone between ZG and ZF after 3 as well as after 14 days of Ang II stimulation, as expected (Fig. 6, A and D–H). Remarkably, this proliferative response was significantly attenuated in the TGR at both stages. Also, the plasma aldosterone levels in TGR and WT increased in response to Ang II. After 3 days of Ang II infusion, the aldosterone levels varied considerably between the rats within a group, reflecting an early stage of stimulation (Table 2 and Fig. 6B, left). Fourteen days of Ang II infusion evoked a striking raise in aldosterone levels up to >2,000 pg/ml in all TGR and WT rats (Table 2 and Fig. 6B, right). Interestingly, the increases in plasma aldosterone were blunted in TGR of both lines at 3 days of Ang II infusion, although these differences reached statistical significance in only one
line. After 14 days of Ang II infusion, the aldosterone response in terms of factor of stimulation did not differ between TGR and WT (18 ± 10-fold in TGR vs. 25 ± 15-fold in WT), although the absolute aldosterone levels were significantly higher in TGR. We would like to stress here that the factor of stimulation is more meaningful than the absolute aldosterone levels. Unlike all the other experiments performed in this study, in the 14-day Ang II stimulation experiment, Sprague-Dawley rats in place of nontransgenic littermates were used as WT controls. In the course of breeding, WT littermates of Sprague-Dawley rats in place of nontransgenic littermates were used as study, in the 14-day Ang II stimulation experiment, Sprague-Dawley rats obtained from exter-
nal sources (Table 2 and Fig. 6B, right). This difference reflects changes only in the genetic background.

Of note, the AT$_1$R antagonist losartan completely abolished the aldosterone response in both TGRCXmAT2R/R and WT rats (Table 2). As anticipated, the losartan-induced increase in plasma renin levels was similar in TGR and WT rats even during Ang II infusion.

Previous studies identified the antiproliferative-acting and growth-promoting PLZF (43, 46, 59) as a novel direct inter-
action partner of the AT$_2$R that, for example, mediates the hypertrophic action of the AT$_2$R in the heart (44). To evaluate whether PLZF, which is downregulated after birth in many organs except for the heart, may have a potential role in AT$_2$R signal transduction are largely contextual and the AT$_1$R prev-

DISCUSSION

We report the generation of a new transgenic rat model that overexpresses the mAT$_2$R in kidney, heart, brain, aorta, vein, testes, and adrenal gland throughout life.

Currently, few data are available concerning the role of AT$_2$R in the adult (24, 34). The elucidation of the AT$_2$R-related actions is complicated, since the AT$_2$R effects and signal transduction are largely contextual and the AT$_1$R pre

We did not detect obvious functional and structural distur-
ances in the TGRCXmAT$_2$R, although the AT$_2$R/AT$_1$R ratio increased dramatically in many tissues throughout life. We focused particularly on the role of AT$_2$R in the adrenal ZG because the adult ZG exhibits a considerable number of AT$_1$R in the physiological function, which has so far been unappreciated. Furthermore, the regulation of aldosterone production has recently acquired importance due to pathological implication of inappropriate aldosterone secretion and its direct link to cardiac fibrosis and hypertrophy (8, 60). We provide evidence that AT$_2$R overexpression in adrenal ZG inhibits the rise in cell proliferation following Ang II stimulation for 3 and 14 days. Aldosterone response to Ang II seems to be delayed in TGR but has remained unchanged in the long run.

To drive transgene expression, we used the CX promoter that was developed originally to be a strong promoter for the pCAGGS expression vector and has utilized features of the cytoplasmic β-actin promoter, which is highly active in a wide range of cell types (25, 33). The transgenic AT$_2$R is highly expressed in important tissues and causes a shift from AT$_1$R to
AT2R predominance. This is particularly obvious in the kidney and heart, which express AT2R at negligible levels in WT animals. Importantly, the absolute numbers of AT1R are unaffected by the transgene expression. The Ang II effect is assumed to be determined by the ratio of AT1R/AT2R, although both receptors often mediate antagonistic actions (20). Interestingly, the prevalence of the AT2R did not cause obvious developmental abnormalities or morphological and histological alterations, indicating that the AT2R excess has no detrimental effects. Mouse models with AT2R deficiency (6, 15) or tissue-specific overexpression (21, 55) were also not affected by reduced survival or lifespan. The development of cardiac failure in a transgenic mouse line with ventricular overexpression of AT2R was related to the level of transgene expression. Whereas a line with a AT2R density of Bmax ≈ 884 fmol/mg protein developed cardiac disease, a second line with slightly lower transgene expression (Bmax ≈ 706 fmol/mg protein) was healthy (57). In our TGRCXmAT2R the cardiac density of AT2R was even lower (Bmax ≈ 302 fmol/mg protein), which might explain the lack of phenotype in the heart. Although we found AT2R to be expressed in blood vessels, the transgenic rats were normotensive and exhibited a largely similar pressure response to Ang II as WT rats. This seems to be in contrast to previous studies describing an enhanced vasoconstrictive effect of Ang II in AT2R-knockout mice (11, 15) or a vasodilatation due to AT2R overexpression in vascular smooth muscle cells (55). However, even in these models no or only minor changes in baseline blood pressure were found. Unlike these models, in our TGRCXmAT2R the transgenic receptor driven by the CX promoter is not expressed essentially in each cell, and the expression levels vary between tissues. Consequently, we did not inevitably expect a modification in systemic blood pressure. Thus, any differences between the studies may still be explained by degree and specific site of AT2R overexpression in the vessels. Ongoing studies will elucidate the role of the transgenic AT2R in the heart, kidney, and circulation under challenged and pathological conditions that are more detailed.

**Fig. 6.** Ang II’s effect on no. of Ki-67-positive cells/visual field (×40 objective) in the ZG and on plasma aldosterone. A and B: Ki-67-positive cells/visual field in the ZG (A) and plasma aldosterone levels (B) at baseline and following 3- (left) and 14-day periods (right) of continuous Ang II infusion (300 ng·kg body wt⁻¹·day⁻¹) in male (TGR) and WT rats. *P < 0.05 and **P < 0.001, Ang II vs. control; #P < 0.05, TGR vs. WT. C: quantitative evaluation of promyelocytic leukemia zinc finger (PLZF) mRNA expression related to GAPDH mRNA as internal standard in different tissues of TGR and WT rats, using real-time RT-PCR. *P < 0.05 and **P < 0.001, n = 5 rats/group (3 repeats/sample). D–H: representative immunohistochemistry for Ki-67 in TGR (D and F) and WT (E, G, and H) at baseline (D and E) and following a 3-day period of Ang II infusion (F–H). H: negative control without Ki-67 antibody. Data are expressed as means ± SD (A and C) or as median (25th/75th percentile; B). ND, not determined.
involvement of the AT$_2$R in Ang II-induced muscle wasting. Corticosterone involvement is unlikely because we did not find alterations in corticosterone levels in TGRCXmAT$_2$R.

We demonstrated further that the adrenal glands, particularly the ZG, exhibited the highest AT$_2$R density compared with other tissues in WT rats. Aldosterone production in ZG, tightly controlled by Ang II via the AT$_1$R, was found to correlate with the AT$_2$R transcript levels (37), which suggests an involvement of AT$_2$R in regulating aldosterone production. This assumption is also supported by Naruse et al. (28), who found that aldosterone levels transiently decreased following AT$_1$R antagonist treatment but returned to baseline over time (aldosterone escape phenomenon). Under these circumstances the AT$_2$R expression was elevated and the aldosterone escape phenomenon prevented by combined treatment with AT$_1$R and AT$_2$R antagonists. However, studies by others concerning the role of AT$_2$R in aldosterone production provided conflicting data (4, 23), and an indirect AT$_2$R action on aldosterone via catecholamine stimulation was speculated (25).

Despite a roughly threefold increase in AT$_2$R binding sites and in the AT$_2$R/AT$_1$R ratio in the ZG of TGRCXmAT$_2$R relative to WT, the plasma aldosterone levels and aldosterone/renin ratios at baseline were not altered. When challenged by Ang II for 3 and 14 days, plasma aldosterone levels as well as the number of Ki-67-positive ZG cells increased in both TGRCXmAT$_2$R and WT. It has been well established that, in addition to regulating the steroidogenic function of the ZG, Ang II exerts mitogenic effects (24, 31, 54). How this mitotic activity relates to the aldosterone production is not fully understood. Ang II stimulates aldosterone production of ZG cells via the AT$_1$R by two main mechanisms: 1) by the depolarization of the cell membrane through inhibition of the Na$_+-$K$^+$-ATPase and of K$^+$ channels such as the TWIK-related acid-sensitive K$^+$ channel and 2) by the induction of a potent Ca$^{2+}$ signal (50), which might also be related to the cell cycle control. In this manner, Ang II evokes a rapid aldosterone response. In contrast, the AT$_2$R causes hyperpolarization and repolarization of cell membranes by opening potassium channels in neuronal cells and vascular smooth muscle cells (9). Thus, the AT$_2$R might potentially antagonize the AT$_1$R-mediated aldosterone increase. However, the glomerulosa cells are functionally an inhomogeneous cell population. Only part of them is enabled to produce aldosterone. Thus, in addition to increasing the aldosterone production per cell as a rapid response, Ang II stimulates the aldosterone production by increasing the absolute number of aldosterone-producing cells. This is accomplished by enhancing cell proliferation and/or by a recruitment of steroidogenic inactive cells to aldosterone production (31, 37, 50). Remarkably, the AT$_2$R overexpression in TGRCXmAT$_2$R significantly attenuated the Ang II stimulation of glomerulosa cell proliferation, but the aldosterone production was, if ever, only transiently blunted. The antimitotic effects of the AT$_2$R have been demonstrated previously in distinct tissues like vascular neointima and coronary endothelial cells (44, 52). Furthermore, studies indicate that Ang II exerts a mitotic action through the AT$_1$R only if the AT$_2$R is absent (52). Otis et al. (34) demonstrated in vitro that the Ang II-promoting effect on protein synthesis occurs with a concomitant arrest in basal cell proliferation, suggesting a shift of pathways involved in proliferation to those involved in protein synthesis (34, 35). This implies that hampered proliferation of ZG cells could go along with a rise in cells that differentiate and grow. We demonstrated in an earlier study that the elimination of circulating active renin by bilateral nephrectomy leads to increased AT$_2$R expression in the ZG, but the number of proliferating ZG cells was lowered to even undetectable levels. Nonetheless, the number of aldosterone-producing cells as well as aldosterone production was increased markedly, which was explained by a recruitment/differentiation of steroidogenic inactive cells (37). The assumption that AT$_2$R in the ZG could play a role for differentiation can be strengthened by our data, but visualizing the precise mechanism needs further elucidation.

Putative signaling pathways involved in AT$_2$R-mediated antiproliferative effects may imply a direct inhibition of AT$_1$R-mediated signaling by dephosphorylating ERK1/2 via activation of the protein phosphatases SH2 domain-containing protein tyrosine phosphatase-1, protein phosphatase 2A, and MAP kinase phosphatase-1 or by a direct physical interaction (i.e., heterodimerization) with the AT$_1$R (35, 40, 41). Another possible mechanism may involve the novel AT$_2$R-interacting transcription factor PLZF. Following Ang II stimulation, PLZF is activated, interacts physically with the AT$_2$R, and then translocates to the nucleus, where it stimulates the phosphatidylinositol 3-kinase-p85α expression, leading to increased protein synthesis and cell growth (44). Remarkably, in vitro and in vivo studies established an antiproliferative effect of PLZF in different cells and tissues, which seems to be mediated by the suppression of cyclin A and proproliferative genes (43, 46, 59). PLZF, highly expressed in embryonic life, is downregulated in most adult organs but expressed considerably in the heart. Here, we demonstrate that PLZF is expressed robustly in the adrenal gland and thus could mediate AT$_2$R effects on growth and differentiation. In such a case, despite limiting cell proliferation, AT$_2$R could still affect the percentage of aldosterone-producing cells by recruiting previously steroidogenic inactive cells to aldosterone production. The TGRCXmAT$_2$R may well represent a useful model to elucidate this complex regulatory network.

Notably, we furthermore did not find any effect of transgenic AT$_2$R expression in the ZF on corticosterone production. We just observed an increase of corticosterone production by Ang II, as expected, but no effect of AT$_2$R overexpression. AT$_2$R expression was not found in the ZF of WT animals, suggesting that necessary components of the AT$_2$R signaling network are absent in fasciculata cells. Furthermore, the absence of any effect of AT$_2$R overexpression in the ZF implies that the previously observed Ang II-dependent increase in glucocorticoid production (7) is mediated by the AT$_1$R.

In summary, we describe a novel transgenic rat model that overexpresses the AT$_2$R in important cardiovascular tissues. This model is a powerful tool for analyzing the relevance and mechanisms of AT$_2$R activity in the adult organism under both physiological and pathophysiological conditions. Moreover, this TGRCXmAT$_2$R model is an excellent source for distinct AT$_2$R-overexpressing cells and tissues for in vitro studies. Using this model, we demonstrate that AT$_2$R overexpression in the adrenal ZG counteracts the AT$_1$R-mediated proliferative response of ZG to Ang II and has minor effects on aldosterone production that may vary in the course of Ang II stimulation. We demonstrated further that AT$_2$R overexpression in the ZF is functionless with regard to corticosterone production.

AJP-Endocrinol Metab • doi:10.1152/ajpendo.00080.2011 • www.ajpendo.org
ACKNOWLEDGMENTS

We express gratitude to Nina Pluntke, Victoria Skude, Cathleen Fichtner, Heike Rauser, and Brigitte Sturm for their excellent technical assistance. We thank Tadashi Inagami for the mAT2R cDNA.

GRANTS

This study was supported by the German Research Foundation Deutsche Forschungsgemeinschaft Grant FOR 406 (to S. C. Hoffmann) and the German Federal Ministry for Education and Research (BMBF/NBL3; FKZ: 01 ZZ 0403).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

B.P., D.P., M.R., A.M., H.W., L.d.B., E.M., and S.C.H. performed the experiments; B.P., D.P., J.P., and S.C.H. analyzed the data; B.P., J.P., and S.C.H. interpreted the results of the experiments; B.P. and S.C.H. prepared the figures; N.G., J.P., and S.C.H. edited and revised the manuscript; J.P. and S.C.H. did the conception and design of the research; J.P. and S.C.H. approved the final version of the manuscript; S.C.H. drafted the manuscript.

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