Th1 inflammatory response with altered expression of profibrotic and vasoactive mediators in AT1A and AT1B double-knockout mice

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1Division of Nephrology, Department of Medicine, University of Florida, Gainesville, Florida; 2Department of Medicine, Duke University, and Durham Veterans Affairs Medical Centers, Durham, North Carolina; 3Department of Nephrology, Ignacio Chavez, Mexico City, Mexico; and 4Nephrology Service, Hospital Universitario, Maracaibo, Venezuela

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Ouyang, Xiaosen, Thu H. Le, Carlos Roncal, Christine Gersch, Jaime Herrera-Acosta, Bernardo Rodriguez-Iturbe, Thomas M. Coffman, Richard J. Johnson, and Wei Mu. Th1 inflammatory response with altered expression of profibrotic and vasoactive mediators in AT1A and AT1B double-knockout mice. Am J Physiol Renal Physiol 289: F902–F910, 2005. First published May 31, 2005; doi:10.1152/ajprenal.00141.2005.—AT1 double receptor (AT1A and AT1B) knockout mice have lower blood pressure, impaired growth, and develop early renal microvascular disease and tubulointerstitial injury. We hypothesized that there would be an increased expression of vasoactive, profibrotic, and inflammatory mediators expressed in the kidneys of AT1 double-knockout mice. We examined the renal expression of various mediator systems in control (n = 6) vs. double-knockout mice (n = 6) at 3–5 mo of age by real-time PCR, immunohistochemistry, and Western blot analysis. AT1 double-knockout mice show activation of Th1-dependent pathways (with increased expression of IFN-γ, IL-2 mRNA) with increased expression of both monocyte (MCP-1 mRNA and T cell (RANTES mRNA) chemokines, infiltration of CD4+ and CD11b+ cells, increased fibrosis-associated mediators (CTGF, TGF-β and TNF-α mRNA) and extracellular matrix (collagens I and III mRNA and protein) deposition compared with controls (P < 0.05 for all markers). These changes were associated with increased mRNA expression of endothelin (ET)-1 and ET-A receptor (P < 0.05), cyclooxygenase (COX)-2/TA2 synthase (P < 0.05), NADPH oxidase (p40-phox, p67-phox, P < 0.05) and iNOS and nNOS (P < 0.05). COX-2 and nNOS protein were also increased in the kidneys of AT1 double-knockout mice by Western blot analysis (P < 0.05). Although renin and angiotensinogen mRNA expression were increased in the knockout mice, AT2 receptor mRNA expression was not significantly different from wild-type mice. In conclusion, the absence of the AT2 receptor is associated with marked renal alterations in vasoactive, profibrotic, and immune mediators with an inflammatory pattern favoring a Th1 phenotype.

endothelin; cyclooxygenase-2; NADPH oxidase

THE RENIN-ANGIOTENSIN SYSTEM (RAS) modulates a diverse set of physiological processes including development, blood pressure, renal function, and inflammation. In the kidney, all of the components of the RAS [renin, angiotensinogen, angiotensin-converting enzyme (ACE), ANG II, and ANG II type 1 and 2 receptors (AT1 and AT2)] are synthesized locally (2).

In humans, there is only a single AT1 receptor type, whereas in rodents, two subtypes of the AT1 receptor have been identified (AT1A and AT1B). To date, AT1 receptors have been shown to mediate most of the physiological actions of ANG II and this subtype is predominant in the control of ANG II-induced vascular functions (15). The AT1 receptor mediates most of the deleterious effects of ANG II, such as vasoconstriction, endothelial damage, and cell growth. AT1 receptor blockade appears to offer both active and passive therapeutic benefits (30). However, the absence of a functional RAS has been associated with development of microvascular disease and tubulointerstitial inflammation. This is observed in mice genetically lacking renin (33), angiotensinogen (11), ACE (12), and AT1 receptors (19). Renal microvascular disease has also been reported in marmosets or Wistar-Kyoto rats immunized against renin (16, 17) and in neonatal spontaneously hypertensive or Wistar rats treated with AT1 receptor inhibitors or ACEI (3, 5, 20, 29).

Characterization of the inflammatory changes in AT1A and AT1B double-knockout mice has not been previously described. We hypothesized that the inflammatory changes would be associated with marked alterations in vasoactive and profibrotic mediators. We further hypothesized that the inflammatory response might involve both T cells and macrophages.

MATERIALS AND METHODS

Animals. Kidneys harvested from double homozygous Agtr1a−/− Agtr1b−/− mice were provided by the Thomas M. Coffman Laboratories (Duke University and Durham VA Medical Centers). These mice were produced as previously described (19). Because of poor survival of animals with combined AT1A/AT1B receptor deficiencies, animals for experiments were generated by selective breeding on mixed backgrounds of 129/SvEv and C57BL/6. Wild-type mice of similar mixed backgrounds were used as controls. All experiments were performed using male mice between 3–5 mo of age. All mice were bred and maintained in the American Association for Accreditation of Laboratory Animal Care-accredited animal facility of the Durham Veterans Affairs Medical Center under National Institutes of Health guidelines. Animals were anesthetized with isoflurane, blood was collected by a heart stick under the xiphoid using a 1-ml syringe and a 25-gauge needle, and kidney tissue samples were collected for RNA, protein, and immunohistochemical analysis.

Renal pathological and histological studies. Renal tissues were fixed in Methyl Carnoy’s fixative, and 3-μm paraffin sections were stained with periodic acid-Schiff (PAS) and hematoxylin. Immunohistochemistry was performed using the following affinity-purified primary antibodies: rabbit polyclonal antibodies against nNOS (Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal antibodies against cyclooxygenase (COX)-2 (Santa Cruz Biotechnology), rat polyclonal antibodies against CD11b (BD Pharmingen, San Diego, CA), rat polyclonal antibodies against CD4 (BD Pharmingen), goat

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polyclonal antibodies against collagen (Col) I and Col III (Southern Biotech, Birmingham, AL), monoclonal antibody against α-SMA (1A4, Sigma, St. Louis, MO). The following secondary antibodies (Rockland) were used in this study: horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated rabbit anti-goat IgG. For immunohistochemistry, renal tissues were fixed in 4% (wt/vol) buffered paraformaldehyde. Cryosections (4 μm) were stained as previously described (13). Sections were preincubated with

Table 1. Real-time PCR primers used in the study

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<th>Gene Symbol</th>
<th>Gene Description</th>
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<th>Reverse Primer Sequence (5′-3′)</th>
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Fig. 1. T and macrophage cell infiltration in AT1 receptor knockout mice. CD4-positive lymphocytes and CD11b-positive cells are increased in AT1 receptor knockout mice compared with wild-type (WT) mice ($A$: $P < 0.001$). Shown is a representative photomicrograph of kidney cortex from WT (B) and AT1 double receptor knockout (C) mice stained for CD4$^+$ cells (brown color, ×630).
10% fetal calf serum and 10% normal sheep serum for 20 min and then incubated with first antibody as described above overnight at 4°C. Sections were then washed in PBS, inactivated with endogenous peroxidase in 0.3% H2O2 in methanol, labeled with second antibody as described above followed by mouse peroxidase anti-peroxidase, and developed with DAB substrate kit (Vector Laboratories, Burlingame, CA) to produce a brown color, or developed with DCIP/NBT substrate kit (Vector Laboratories) to produce a blue color.

For quantification of immunohistochemistry staining, PAS-stained sections were imaged using a Axioplan 2 imaging microscope (Zeiss), CR5 digitized color camera, image analyzed using Zeiss Auto image software (Axiovision 4.1), and a 133-MHz Pentium computer (96 M RAM). For studies of vascular and glomerular morphology, cross sections of all the arteriole were traced and examined morphometrically. Both inner and outer areas (μm²) were measured for each arteriole, and outer area-inner area (designated as arterial wall thickness) was calculated and compared among wild-type and AT1 receptor knockout mice.

For studies of glomerular morphology, both glomerular tuft and whole glomerular areas (μm²) were measured, and whole glomerular area/glomerular tuft (designated as glomerular tuft/whole glomerular ratio) was calculated and compared among wild-type and AT1 receptor knockout mice. Single image frames (700 × 550 μm) were captured at ×200 magnification, and 20 frames/sample were used to count the number of CD4 and CD11b-positive cells [expressed as cell number/(700 × 550 μm)], and for quantification of Col I, Col III and α-SMA [positive area l/(700 × 550 μm)].

**RNA isolation, reverse transcription, and real-time PCR.** Total RNA was isolated from total kidney tissue using the SV Total RNA Isolation kit (Promega, Madison, WI) according to the manufacturer’s protocol. The RNA was eluted with 50 μl of RNase-free water. All RNA was quantified by spectrophotometer and the optical density (OD) 260/280 nm ratios were determined. Reverse transcription was performed in a one-step protocol using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s protocols. Reactions were incubated at 25°C for 5 min, 42°C for 30 min, 85°C for 5 min, and cooled at 4°C in a Thermocycler (Eppendorf, Hamburg, Germany). Primers (Table 1) were designed by Genetool software (BioTools, Alberta, Canada), and oligonucleotides were synthesized by Sigma Genosys. Real-time PCR analyses were performed using the Opticon PCR machine (MJ Research, Waltham, MA). The SYBR Green master mix kit (Bio-Rad) was used for all reactions with real-time PCR. Briefly, PCR was performed as follows: 94°C for 2 min followed by 40 cycles of denaturation, annealing, and extension.

![Fig. 2.](http://ajprenal.physiology.org/) Interstitial collagen (Col I and III) deposition in AT1 receptor double-knockout mice. Col I and Col III are markedly increased by both immunostaining (A; P < 0.01) and corresponded with an increase in mRNA by real-time PCR (D; fold increase in mRNA expression, P < 0.05) in AT1 receptor knockout mice compared with WT mice. Shown is a representative photomicrograph of kidney cortex from WT (B) and AT1 double receptor knockout (C) mice stained for Col III (brown, ×630).
at 94°C for 15 s, 64°C for 30 s, 72°C for 45 s, respectively, and final
extension at 72°C for 10 min. PCR reaction for each sample was done
in duplicate for all the product and for the GAPDH control. Ratios for
each product/GAPDH mRNA were calculated for each sample and
expressed as means ± SD. The data presented are expressed as the
fold-increase or fold-decrease in mRNA.

Western blot analysis. Whole kidney samples were mixed with
SDS-polyacrylamide gel electrophoresis sample buffer (Cell Signal-
ing), boiled for 5 min, and electrophoresed on a 7.5% SDS polyacryl-
amide gel (Bio-Rad). Proteins were transferred onto Immobilon-NC
Transfer Membrane (Millipore) with a Bio-Rad Transblot cell at 0.5
A overnight. The membrane was blocked in TBS (Bio-Rad) contain-
ing 0.05% Tween 20 for 2 h and then incubated overnight with rabbit
anti-mouse nNOS antibody or goat anti-mouse COX-2 antibody
(Santa Cruz Biotechnology). After being washed, the membrane was
incubated with a 1/5,000 dilution of fluorescence-conjugated goat
anti-rabbit IgG or rabbit anti-goat IgG in TBS containing 5% skim
milk powder and 0.05% Tween 20 for 1 h. Blots were developed using
Odyssey Infrared Imager (Odyssey) and analyzed by densitometry
using Odyssey Image Software (Version 1.2).

Serum uric acid measurement. Serum uric acid concentration was
determined by a carbonate phosphotungstate method and uric acid
standard (Sigma) (6).

Statistical analysis. All data are presented as means ± SD. Differ-
ences in the various parameters between groups were evaluated by
single-factor ANOVA. Significance was defined as $P < 0.05$.

RESULTS

Renal vascular disease and fibrosis in AT$_1$ receptor knock-
out mice. The routine light microscopy study of the kidney was
normal in the wild-type group. In contrast, the kidneys in the
AT$_1$ receptor knockout mice demonstrated focal areas of in-
terstitial fibrosis with mononuclear cell infiltration (Fig. 1).
Immunohistochemistry demonstrated expression of Col I and
Col III in the fibrotic areas (Fig. 2). An increase in Col I and

III mRNA was also observed in whole kidney RNA extracts
(Fig. 2). Preglomerular arteriolar vessels were thickened, re-
sulting in a threefold increased medial area compared with
wild-type controls (Fig. 3). The increase in medial wall thickness
was associated with dramatic increases in α-SMA expression
(Fig. 4). In addition, AT$_1$ receptor knockout mice displayed an
increase in α-SMA-positive cells in glomeruli (reflecting acti-
vated mesangial cells) and in areas of periglomerular and
interstitial fibrosis (reflecting myofibroblasts; Fig. 4). Glomer-
uuli displayed evidence of ischemia, with wrinkling of the
basement membrane and collapse of glomerular tufts (Fig. 3).

The ratio of glomerular tuft to total glomerular area (demar-
cated by Bowman’s space) was significantly lower in the AT$_1$
receptor knockout mice, reflecting the higher frequency of
glomerular collapse in these mice (Fig. 3).

**T cell and monocyte infiltration in AT$_1$ receptor knockout mice:**
association with a Th1 phenotype and oxidative stress.
Infiltration of both monocytes (CD11b-positive cells) and
CD4-positive T cells were present in the cortex of AT$_1$ receptor
knockout mice, especially in areas of interstitial fibrosis, com-
pared with the wild-type mice (Fig. 1). Real-time PCR on the
whole kidneys demonstrated marked expression of proinflam-
atory cytokines and mediators of oxidative stress (Fig. 5). An
increase in chemotactic factors for both monocytes (monocyte
chemotactic factor 1, or MCP-1) and T cells (RANTES) was
observed. There was also a marked increase in the mRNA for
enzymes involved in oxidative stress, including p40-phox,
p67-phox, and gp91-phox (the phagocyte NADPH oxidase)
and the xanthine oxidase (XO)/xanthine dehydrogenase (XDH)
systems (Fig. 5). The increase in XO expression was associated
with higher serum uric acid levels, although this did not reach
statistical significance (1.1 ± 0.3 vs. 1.6 ± 0.5 mg/dl, $P = 0.06$).
Interestingly, there was a relative downregulation of
NOX4, which is the major catalytic component of an endothelial NAD(P)H oxidase. Consistent with the inflammatory and profibrotic response, we observed an increase in TNF-α mRNA and TGF-β mRNA, respectively, in the AT1 receptor knockout mice (Fig. 5).

Given the infiltration of T cells in the kidneys of AT1 receptor knockout mice, we performed real-time PCR to determine whether the phenotype was consistent with a Th1 or Th2 response (Fig. 5). AT1 receptor knockout mice demonstrated a Th1 phenotypic pattern in their kidneys, as manifested by increased mRNA expression of IFN-γ and IL-2 (Th1 cytokines), whereas IL-4 and IL-10 (Th2 cytokine) mRNA levels were not changed.

AT1 receptor knockout mice have evidence for activation of vasoactive pathways in their kidneys. Consistent with previous studies, AT1 receptor knockout mice had an increase in renin and angiotensinogen mRNA in their kidneys compared with wild-type mice (Fig. 6). Interestingly, neither ACE nor AT2 receptor mRNA expression was altered (Fig. 6). There was also evidence for activation of the endothelin system, with upregulation of ET-1 and ET-A receptor mRNA but not ET-B receptor mRNA, consistent with activation of the endothelin vasoconstriction. AT1 receptor knockout mice also demonstrated increased expression of nNOS protein (Fig. 7) and COX-2 (Fig. 8), consistent with previous studies suggesting activation of these pathways involved in inflammation and tissue damage.
tubuloglomerular feedback (10). Inducible NOS, which is present in tubules and inflammatory cells and has been reported to be increased in conditions associated with renal inflammation and injury (4), was also increased. Interestingly, endothelial NOS (eNOS), an important vasodilatory enzyme, was not altered (Fig. 6).

DISCUSSION

ANG II, the main effector of the RAS, plays a central role in the hemodynamic and nonhemodynamic mechanisms of chronic renal disease and is currently the main target of interventions aimed to prevent the onset of chronic nephropathies and their progression to end-stage renal disease (24). ANG II is known as a multifunctional hormone that influences the function of cardiovascular cells through a complex series of intracellular signaling events initiated by the interaction of ANG II with AT1 and AT2 receptors. AT1 receptor activation leads to cell growth, vascular contraction, inflammatory responses, and salt and water retention (27). AT1 receptor blockade appears to offer both active and passive therapeutic benefits in humans, but interestingly, the absence of AT1 receptor has been associated with development of microvascular disease and tubulointerstitial inflammation in kidney, the same phenomenon observed in experimental and human salt-sensitive hypertension (9, 23, 32). In these latter models, a key role for T cells (bearing a Th1 phenotype), oxidative stress, and renal vasoconstriction has been reported (22). We thus hypothesized that there would be an alteration in vasoactive and profibrotic

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Fig. 5. Cytokines, oxidative enzymes, and inflammatory mediators in AT1 receptor knockout mice. The graph shows the fold-increase or decrease in mRNA expression of various cytokines, oxidative enzymes, and inflammatory mediators in whole kidneys obtained from AT1 receptor double-knockout mice compared with WT control mice as quantified by real-time PCR (*P < 0.05; **P < 0.01; ***P < 0.001). Five to six animals per group were studied and the analyses were performed in duplicate.

Fig. 6. Vasoactive mediator mRNA expression in AT1 receptor double-knockout mice. The graph shows the fold-increase or decrease in mRNA expression of various vasoactive mediators in whole kidneys from AT1 receptor double-knockout mice compared with WT control mice by quantitative real-time PCR (*P < 0.05; **P < 0.01; ***P < 0.001). Five to six animals per group were studied and the analyses were performed in duplicate.
mediators and that the inflammatory response would include both T cells and macrophages, with the former expressing a Th1 phenotype.

We first confirmed previous studies that demonstrated that AT1 receptor knockout mice develop premature vascular disease and interstitial fibrosis (19, 28). The vascular disease was marked and was associated with an increase in \( \alpha \)-SMA-positive cells and by the marked increase in arteriolar medial area and medial:lumen ratios (Figs. 1 and 3). The vascular disease was also associated with glomerular collapse with shrinkage of the glomerular tuft, suggesting glomerular ischemia. Focal area of fibrosis was also present, as demonstrated by the increase in Col I and III staining, and an increase in TGF-\( \beta \) mRNA by quantitative real-time PCR.

The first new finding was the characterization of the inflammatory cells and cytokines in the AT1 receptor knockout mice. Specifically, we found that the renal tissue had elevated levels of both monocyte (MCP-1) and T cell (RANTES) cytokine mRNA expression in association with an increase in monocytes and CD4-positive T cells. The T cells were likely of a Th1 phenotype, as the kidneys also have a marked induction of mRNA expression of Th1 cytokines (IFN-\( \gamma \) and IL-2) but not Th2 (IL-4 and IL-10) cytokines. There was also evidence for the increased expression of monocyte/macrophage inflammatory cytokines (TNF-\( \alpha \) mRNA). The local inflammatory response was associated with upregulation of oxidative enzymes, including the NADPH oxidase system and the XO enzymes. Thus the renal injury in AT1 receptor knockout is associated with a T cell and monocyte response with local activation of inflammatory mediators and oxidative enzyme systems.

The second new finding was the observation that there was an induction of cytokines and vasoactive mediators. An upregulation of proximal pathways involved in the RAS, such as renin and angiotensinogen, was expected. However, we also found an increased expression of the renal endothelin system.
(including ET-1 and the ET-A receptor) consistent with a vasoconstrictive response. Although eNOS expression was not modulated in AT1 receptor-deficient state, nNOS and COX-2 mRNA and protein were increased in our AT1 receptor knockout mice consistent with a vasodilatory response. An increase in the nNOS and COX-2 pathways has been previously reported in rodents in which the RAS is blocked, and this is thought to represent a consequence of interrupting the negative feedback of ANG II on the nNOS-COX-2 pathways involved in tubuloglomerular feedback (1, 10, 31). Moreover, the afferent arteriolar diameter might be directly regulated by NO derived from nNOS in the macula densa (26), and COX-2 participates in tubular flow-dependent afferent arteriolar tone via interaction with nNOS (7).

An interesting observation in the current study was the finding that both the NADPH oxidase isoform P40-phox and P67-phox mRNA were increased in the AT1 receptor knockout mice, whereas a crucial flavin-containing catalytic subunit of NADPH oxidase, Nox4, was reduced. Unlike other subunits, neither Nox1 nor Nox4 is present in leukocytes but is highly expressed in vascular cells and upregulated in vascular remodeling, such as that found in hypertension and atherosclerosis (8). This suggests that the upregulation of NADPH oxidase in the kidney of AT1 receptor knockout mice may largely represent activation by infiltrating monocytes and macrophages, which have been shown to produce oxidants in other models of salt-sensitive hypertension (14, 24, 25, 34). One might posit that NADPH oxidases should also be induced in the preglomerular vessels, as activation of these oxidases has been shown to play a critical role in vascular remodeling (18). However, most studies suggest that the induction of NADPH oxidases in vascular cells is dependent on ANG II (21), raising the possibility that other mechanisms may be required when the RAS is completely blocked.

In conclusion, while ANG II has been definitively shown to have a role in mediating inflammation and fibrosis in the kidney, in this study we demonstrate that a complete absence of AT1 receptor is also associated with the development of preglomerular vascular disease and interstitial inflammation. Characterization suggests the induction of chemokines driving a Th1 and monocyte/macrophage response coupled with oxidative stress, an alteration in vasoactive mediators, and fibrosis.

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