Dietary salt intake modulates progression of antithymocyte serum nephritis through alteration of glomerular angiotensin II receptor expression

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Submitted 11 February 2003; accepted in final form 7 October 2003

Suzuki, Hiroyuki, Tatsuo Yamamoto, Naoki Ikegaya, and Akira Hishida. Dietary salt intake modulates progression of antithymocyte serum nephritis through alteration of glomerular angiotensin II receptor expression. Am J Physiol Renal Physiol 286: F267–F277, 2004. First published October 14, 2003; 10.1152/ajprenal.00059.2003.—Dietary salt intake modulates the renin-angiotensin system (RAS); however, little is known about the effect of salt intake on the progression of glomerulonephritis. We investigated the glomerular expression of TGF-β1, type I (TβRI) and II (TβRII) TGF-β receptors and RAS components in rats with antithymocyte serum (ATS) nephritis on normal (NSI)-, low (LSI)-, and high-salt intake (HSI) compared with those on NSI. Intrarenal renin and glomerular ANG II levels were significantly higher in LSI and lower in HSI rats. In ATS nephritis, HSI increased glomerular TβRII, TβRII, and ANG II type II receptor (AT2R), and decreased glomerular ANG II type 2 receptor (AT2R), whereas LSI decreased glomerular TGF-β1 and TβRII and increased glomerular AT2R. PD-123319 aggravated glomerular lesions and increased glomerular TGF-β1 and TβRII. Our results suggest that dietary salt intake influences progression of ATS nephritis by modulating glomerular TGF-β1 and TβR expression resulting, at least in part, from altered glomerular AT2R and AT1R expression.

dietary salt intake; angiotensin; transforming growth factor-β

ANG II IS THE primary effector of the renin-angiotensin system (RAS) and is produced systemically and locally in various tissues, including the heart, vascular wall, and kidney (28). ANG II plays an important role in the control of systemic blood pressure, electrolyte balance, and extracellular fluid volume (23). In addition, it has recently been shown that ANG II stimulates cell proliferation, matrix synthesis, oxidative stress (15), and generation of reactive oxygen species (45). Most of these actions are mediated through the ANG II type 1 receptor (AT1R). On the other hand, signaling through the ANG II type 2 receptor (AT2R) results in vasodilation, bradycardia, increased levels of nitric oxide and prostaglandins, antiproliferative effects, as well as an increase in apoptosis (3, 7).

Previous studies indicated that ANG II expression is involved in the progression of kidney diseases (5, 10). Furthermore, angiotensin-converting enzyme inhibitors and ANG II receptor blockers exhibit renoprotective effects and delay the progression to end-stage renal disease in human and animal kidney diseases (6, 20, 31). In the kidney, ANG II causes contraction of efferent rather than afferent arterioles and increases intraglomerular pressure (8). Glomerular hypertension has been shown to lead to progressive renal injury through several mechanisms (33). For example, high glomerular pressure enlarges the radius of the pores in the glomerular basement membrane by a mechanism that is mediated, at least in part, by ANG II (19) and impairs the size-selective barrier of the membrane so that protein content in the glomerular filtrate increases, which in turn increases the endocytosis of proteins by tubular cells, resulting in a nephritogenic effect (32). Glomerular hypertension also induces stretch or shear stress in glomerular cells and increases the expression of transforming growth factor-β (TGF-β), a major growth factor that underlies development of renal scarring resulting from pathological accumulation of extracellular matrix in chronic progressive kidney diseases (2). TGF-β exerts its biological effects through binding to specific cell surface receptor proteins composed of two transmembrane serine/threonine kinases, designated type I (TβRI) and type II (TβRII) TGF-β receptors (TβR). TβRI binds the ligand first, after which TβRII is recruited to form a heteromeric complex. ANG II-mediated expression of TGF-β in mesangial cells (12) and proximal tubular cells (43), and that of TβRII in proximal tubular cells (41), has also been reported. These data strongly suggest the pathogenic role of the RAS in the progression of renal sclerosis mediated by upregulation of TGF-β and TβR expression. However, it is also known that deoxycorticosterone acetate-salt-hypertensive rats exhibit high TGF-β expression in the kidney despite decreased renin activity (14).

Hypertension associated with high-salt intake can accelerate renal injury in patients with chronic progressive glomerulonephritis. However, a low-salt diet is not always necessary in patients with glomerulonephritis without overhydration or hypertension, and inappropriate restriction of salt intake should be avoided in patients with chronic renal failure and impaired urinary concentration ability because of the risk of dehydration. On the other hand, salt restriction is well known to enhance the circulatory RAS (13). At this stage, little is known about the effect of low- or high-salt intake per se, without changes in blood pressure, on the progression of glomerulonephritis. In the present study, we examined the effects of low- and high-salt intake on circulatory and intrarenal RAS, progression of glomerular lesions, and glomerular expression of TGF-β1, TβRI, and TβRII in antithymocyte serum (ATS) nephritis. Our results showed that dietary salt intake modulated...
the progression of ATS nephritis by altering glomerular TGF-β, TβRI, and TβRII expression, which was due, at least in part, to alteration of glomerular AT1R and AT2R expression.

**MATERIALS AND METHODS**

**Experimental Design**

ATS was raised in a nonpregnant adult female England Suffolk sheep by subcutaneous immunization with 1 × 10⁴ Wistar rat thymocytes in complete Freund’s adjuvant, followed by four injections of 1 × 10⁶ thymocytes in complete Freund’s adjuvant at 4-wk intervals. Sera obtained 1 wk after the last injection were heat inactivated (56°C for 30 min) and absorbed three times with Wistar rat erythrocytes.

**Protocol 1.** Male Wistar rats weighing 80 g were purchased from SLC (Hamamatsu, Japan). They were randomly assigned into the following four groups: group A, in which 30 rats were fed normal rat chow containing 0.39% sodium chloride, given tap water as drinking water, and injected with ATS through the tail vein; group B, in which 35 rats were fed a low-salt diet containing 0.03% sodium chloride, given tap water as drinking water, injected with furosemide (2 mg/kg ip) 7 days before ATS injection, and then injected with ATS; group C, in which 35 rats were fed normal chow but given saltwater containing 1% sodium chloride as drinking water, injected with deoxycorticosterone acetate (10 mg/kg sc) 14 and 7 days before ATS injection, and then injected with ATS; and group D, in which 28 rats treated similarly to group C received candesartan cilexetil (1 mg/kg) orally every day after ATS injection. Before and 3, 7, 14, and 28 days after ATS injection, the rats were placed in metabolic cages for a 24-h urine collection under a 12:12-h light-dark cycle, and urinary protein excretion was measured using the Bradford method. Blood pressure was measured by the tail-cuff method in animals that were conscious and restrained. Under ether anesthesia, blood samples were collected from the abdominal aorta, and the kidneys were perfused with ice-cold sterile 0.1 M PBS at pH 7.4, dissected out, and subjected to histopathological and immunohistochemical analysis and glomerular isolation. Glomeruli were isolated using a graded sieving technique under sterile conditions (18).

**Protocol 2.** To investigate the role of glomerular AT1R on the progression of ATS nephritis, we compared the levels of proteinuria, renal lesions, cellular fibronectin deposition, and glomerular expression of TGF-β1 and its receptors in the following two groups. In group E, seven ATS nephritic rats on a low-salt intake were treated similarly to group B. In group F, seven ATS nephritic rats on a low-salt intake treated similarly to group B were implanted with microosmotic pumps (Alzet, Cupertino, CA) containing PD-123319, an AT1-R blocker, at a dose of 100 mg/mL, in the subcutaneous tissue of the back region after ATS injection. Seven days after ATS injection, the levels of urinary protein excretion were measured, and the kidneys were subjected to histopathological and immunohistochemical analysis and glomerular isolation as described above.

**Glomerular TGF-β1 Production**

Glomeruli (1 × 10⁴) were kept in RPMI-1640 supplemented with penicillin and streptomycin and incubated under a 95% air-5% CO₂ humidified atmosphere at 37°C. After a 24-h incubation, the medium was collected, centrifuged at 12,000 rpm for 10 min, and the supernatant was treated with 1 N HCl to activate latent TGF-β1 to active TGF-β1 detectable by the kit and allocated for the measurement of total TGF-β1 by ELISA using Quantikine (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Plasma Renin Activity**

Plasma renin activity was measured by determining the generation rate of angiotensin I per milliliter plasma per hour using an RIA kit (SRL, Tokyo, Japan).

**Histopathological and Immunohistochemical Analyses**

Kidney tissues were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Tissue sections (3-μm thick) were stained with periodic acid-Schiff for histopathological evaluation. Immunoreactivities for ED-1 and renin proteins were determined using the standard biotin-streptavidin-peroxidase method as described previously (9). Mouse anti-rat ED-1 antibody was purchased from Serotec (Raleigh, NC). Rabbit anti-mouse renin antibody was kindly provided by Dr. A. Fukamizu (Univ. of Tsukuba, Ibaraki, Japan). The secondary antibodies were affinity-purified biotinylated donkey anti-rabbit (Cortex Biochemical, San Leandro, CA) and donkey anti-mouse IgG (Cortex Biochemical). Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 30 min. Sections incubated with nonimmune rabbit sera instead of primary antibodies were used as controls. Nuclei were counterstained lightly with hematoxylin. All sections were stained under identical conditions together with a control incubation.

To evaluate the levels of extracellular matrix deposition before and after induction of nephritis, immunoreactivity for cellular fibronectin was determined by the immunofluorescence method in frozen sections as described previously (50). Mouse monoclonal antibody to ED-A of cellular fibronectin, ISTM-9, was kindly provided by Dr. L. Zardi (Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy), and FITC-conjugated donkey anti-mouse IgG was purchased from Chemicon (Temecula, CA).

To evaluate the difference in induction of ATS nephritis among the groups on normal-, low-, and high-salt intake, an additional five rats in each group were killed 24 h after ATS injection, and intraglomerular deposition of ATS (sheep IgG) and endogenous complement (rat C₃) was investigated by the immunofluorescence method. FITC-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rat C₃ were purchased from Cappel (Aurora, OH).

The number of ED-1-positive cells per glomerulus was counted under light microscopy at ×400 magnification. At least 30 glomeruli/sample were evaluated, and the mean values were determined. The levels of mesangial matrix deposition and the staining intensity of renin, cellular fibronectin, ATS (sheep IgG), and rat C₃ were graded using the following 0–4 scale in coded sections observed at ×400 magnification: 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining. Thirty randomly selected glomeruli were evaluated per sample, and the mean values of the staining scores were calculated. Evaluation of tissue sections was performed by a single investigator blinded to the experimental background.

Table 1. Body weight and mean arterial pressure of different groups of rats before and after injection of antithymocyte serum

<table>
<thead>
<tr>
<th>Group</th>
<th>Body wt, g</th>
<th>MAP, mmHg</th>
</tr>
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<tbody>
<tr>
<td>Day 0</td>
<td>150.8 ± 11.0</td>
<td>143.1 ± 3.1</td>
</tr>
<tr>
<td>Day 3</td>
<td>174.6 ± 11.0</td>
<td>141.1 ± 1.3†</td>
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<tr>
<td>Day 7</td>
<td>185.1 ± 8.5</td>
<td>159.0 ± 20.0†</td>
</tr>
<tr>
<td>Day 14</td>
<td>200.0 ± 7.2</td>
<td>158.6 ± 2.4†</td>
</tr>
<tr>
<td>Day 28</td>
<td>243.5 ± 10.3</td>
<td>210.6 ± 13.4†</td>
</tr>
</tbody>
</table>

MAP = mean arterial pressure; †P < 0.05 vs. group A.

Values are means ± SD. MAP, mean arterial pressure; NP, not performed. See MATERIALS AND METHODS for definitions of groups. *P < 0.05 vs. group A. †P < 0.05 vs. group B.
**Western Blot Analysis**

Isolated glomeruli were dissolved in RIPA buffer (0.5% deoxycholate, 0.5% Triton X-100, 25 mM Tris-HCl, pH 7.4, 0.1% SDS, and 150 mM NaCl) at 4°C containing 1 mM PMSF, 1 μM leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin. Samples were denatured by boiling for 10 min in LDS sample buffer (Novex, San Diego, CA) and separated on 4–12% polyacrylamide gradient gels. To analyze differences in the levels of glomerular TβRI, TβRII, AT1R, and AT2R expression among groups A, B, C, and D, samples obtained before and 7 days after ATS injection were applied on the same gel and examined in the same run. After electrophoresis, the proteins were electroblotted onto nitrocellulose membranes. The membranes were subsequently washed three times for 10 min each in TBS-T (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20), incubated in TBS containing 5% skim milk for 1 h at room temperature to block nonspecific binding, and subsequently incubated with the primary antibody (10 μg/ml overnight at 4°C). The primary antibodies used were rabbit anti-human TβRI and TβRII (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human AT1R and AT2R (Santa Cruz Biotechnology), and mouse anti-β-actin (Sigma, St. Louis, MO) antibodies. After the membranes were washed three times for 10 min each to remove excess antibodies, they were incubated with horse-radish peroxidase-conjugated rabbit anti-mouse IgG antibody (OEM Concepts, Toms River, NJ) or donkey anti-rabbit IgG (Amersham, Arlington Heights, IL) antibody diluted appropriately in TBS containing 5% skim milk for 1 h at room temperature. Antibodies bound to membrane-immobilized proteins were visualized by enhanced chemiluminescence (Amersham). Quantification of the band intensity was performed using National Institutes of Health Image software. The results were expressed as the densitometric ratios of TβRI, TβRII, AT1R, or AT2R relative to β-actin.

**Measurement of Glomerular ANG II**

The glomerular levels of ANG II were measured using RIA. Isolated glomeruli (1 × 10⁶) were homogenized with 1 ml of ice-cold saline containing 0.1 N HCl and 5% aprotinin and centrifuged at 12,000 g at 4°C for 30 min. The supernatant was collected, and the pellet was washed three times with distilled water, followed by acetone containing 0.5 N HCl and petroleum ether. The extract was dried under vacuum, reconstituted in Tris buffer (pH 8.5), and used for RIA. The incubation mixture consisted of samples or standard and rabbit anti-human ANG II antibody (SRL). The anti-ANG II antibody used in the present study has been shown to have very low cross-reactivity (0.037%) with ANG I (11). The incubation was carried out at 4°C for 24 h, followed by a normal-salt diet and then injected with ATS on day 0 (group A; hatched bars, n = 5); rats fed a low-salt diet (group B; open bars, n = 5), and high-salt diet (group C; filled bars, n = 5) 24 h after ATS injection. Values are means ± SD. No significant differences in the glomerular deposition of ATS and endogenous rat C3 were noted among the groups.

**Fig. 1.** Staining intensity of antithymocyte serum (ATS; sheep IgG) and rat C3 in rats fed a normal-salt diet (group A; hatched bars, n = 5), low-salt diet (group B; open bars, n = 5), and high-salt diet (group C; filled bars, n = 5) 24 h after ATS injection. Values are means ± SD. No significant differences in the glomerular deposition of ATS and endogenous rat C3 were noted among the groups.

**Fig. 2.** Urinary protein excretion over 24 h in rats that were fed a normal-salt diet and then injected with ATS on day 0 (group A; hatched bars, n = 5); rats that were fed a low-salt diet and received furosemide (2 mg/kg ip) 7 days before ATS injection on day 0 (group B; open bars, n = 5); rats fed a high-salt diet, injected with deoxycorticosterone acetate (10 mg/kg sc) 14 and 7 days before ATS injection on day 0 (group C; filled bars, n = 5); and rats treated similarly as those in group C but treated with candesartan cilexetil (1 mg/kg po every day) after ATS injection (group D; stippled bars, n = 5). Values are means ± SD. *P < 0.05 group A vs. group C; §P < 0.05 group A vs. group B; #P < 0.05 group D vs. group C.
RESULTS

Body Weight and Mean Blood Pressure

The mean body weight of rats with ATS nephritis on a low-salt intake (group B) was less than that of other groups at all time points (Table 1). There were no significant differences in mean arterial blood pressure levels among the groups (Table 1).

Induction of ATS Nephritis

No significant differences in the levels of glomerular deposition of ATS (sheep IgG) and rat C3 24 h after ATS induction were noted among rats on a normal (group A)–, low (group B)–, and high (group C)–salt intake (Fig. 1). It is suggested that the induction of ATS nephritis did not significantly differ among the groups.

Urinary Protein Excretion

In rats with ATS nephritis on a normal-salt intake (group A) and those on a low-salt intake (group B), urinary protein excretion increased after ATS injection, reached a peak level on day 3, and decreased thereafter to normal levels by day 28 (Fig. 2). However, urinary protein excretion levels were lower in group B than group A throughout the study period. In rats with ATS nephritis on a high-salt intake (group C), urinary protein excretion levels were significantly higher than those of group A on days 14 and 28. Administration of candesartan cilexetil (group D) tended to reduce the level of proteinuria in rats with ATS nephritis on a high-salt intake on days 14 and 28, albeit insignificantly, compared with group C.

Histopathological Findings

There were no differences in mesangial cellularity and mesangial matrix deposition among all groups before ATS injection. In rats with ATS nephritis on a normal-salt intake (group A), mesangiolysis was noted 3 days after ATS injection, which was followed by mesangial cell proliferation and mesangial matrix accumulation, reaching a peak level at day 7 and returning to almost normal levels by day 28. The severity of these glomerular lesions was less in rats on a low-salt intake (group B; Fig. 3, A and B). In rats with ATS nephritis on a

Fig. 3. A: photomicrographs of glomeruli in kidney sections obtained 7 (a–d) and 14 days (e–h) after injection of ATS from rats on a normal-salt intake (group A; a and e), low-salt intake (group B; b and f), high-salt intake (group C; c and g), and high-salt intake and treated with candesartan cilexetil (group D; d and h). The severity of glomerular lesions consisting of mesangial cell proliferation and mesangial matrix accumulation was less in group B than that in group A. In contrast, glomeruli were enlarged and the glomerular lesions were markedly aggravated in group C. Candesartan cilexetil in rats with ATS nephritis on a high-salt intake (group D) resulted in amelioration of glomerular lesions. Original magnification: ×400. B: mesangial matrix scores in rats fed a normal-salt diet (group A; hatched bars, n = 6), low-salt diet (group B; open bars, n = 6), high-salt diet (group C; filled bars, n = 6), and high-salt diet and treated with candesartan cilexetil (group D; stippled bars, n = 6) 7 and 14 days after ATS injection. C: staining intensity of cellular fibronectin (cFN) in rats fed a normal-salt diet (group A; hatched bar, n = 6), low-salt diet (group B; open bar, n = 6), high-salt diet (group C; filled bar, n = 6), and high-salt diet and treated with candesartan cilexetil (group D) 7 days after ATS injection. Values are means ± SD. *P < 0.05 group A vs. group B. **P < 0.05 group A vs. group C. §P < 0.05 group B vs. group C. ¶P < 0.05 group C vs. group D.
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Fig. 4. Number of glomerular ED-1-positive infiltrating macrophages in rats with ATS nephritis on a normal-salt intake (group A; hatched bars, n = 4), low-salt intake (group B; open bars, n = 4), high-salt intake (group C; filled bars, n = 4), and high-salt intake and treated with candesartan cilexetil (group D; stippled bars, n = 4). Values are means ± SD. *P < 0.05 group A vs. group C. #P < 0.05 group A vs. group D. §P < 0.05 group C vs. group D.

Fig. 5. Levels of transforming growth factor (TGF)-β1 production in glomeruli isolated from rats with ATS nephritis on a normal-salt intake (group A; hatched bars, n = 5), low-salt intake (group B; open bars, n = 5), high-salt intake (group C; filled bars, n = 5), and high-salt intake treated with candesartan cilexetil (group D; stippled bars, n = 5). Values are means ± SD. *P < 0.05 group A vs. group B. #P < 0.05 group B vs. group C. §P < 0.05 group B vs. group D. ¶P < 0.05 group C vs. group D.

high-salt intake (group C), enlargement of glomeruli and worsening of glomerular lesions were evident. Compared with group C, administration of candesartan cilexetil (group D) resulted in amelioration of the glomerular lesions.

Cellular fibronectin was not detected in the glomeruli of all groups before induction of nephritis (data not shown). In rats with ATS nephritis on a normal-salt intake (group A), glomerular deposition of cellular fibronectin was increased significantly 7 days after induction of nephritis. The levels of glomerular deposition of cellular fibronectin were less severe in rats on a low-salt intake (group B) and more in rats on a high-salt intake (group D) compared with group A (Fig. 3C). Compared with group C, administration of candesartan cilexetil (group D) significantly reduced the deposition of cellular fibronectin.

Glomerular Infiltration of ED-1-Positive Macrophages

ATS injection was associated with a subsequent increase in glomerular infiltration of ED-1-positive macrophages, which reached peak levels at day 7 in all groups (Fig. 4). Rats with ATS nephritis on a high-salt intake (group C) showed persistent infiltration of macrophages in glomeruli and a significantly higher number of infiltrating macrophages 14 days after ATS injection compared with group A. Administration of candesartan cilexetil significantly suppressed glomerular macrophage infiltration in rats with ATS nephritis on a high-salt intake (group D) at days 3, 7, and 14 compared with those in group C.

Glomerular TGF-β1 Production

Glomerular production of total TGF-β1 increased after ATS injection, reaching peak levels at day 7, but returned to normal levels by day 28 in all groups (Fig. 5). The glomerular TGF-β1 level was significantly lower in rats with ATS nephritis on a low-salt intake (group B) than in those on a normal-salt intake (group A) on days 3, 7, and 14. No significant differences in the levels of glomerular TGF-β1 were noted between rats in group A and those on a high-salt intake (group C). Administration of candesartan cilexetil significantly decreased TGF-β1 production in rats with ATS nephritis on a high-salt intake (group D) at days 3 and 14 compared with those of group C.

Glomerular Expression of TβRI and TβRII

Compared with control rats, the levels of glomerular TβRI expression increased in rats with ATS nephritis on a normal-salt intake (group A) at day 7, whereas no remarkable changes were noted in the levels of glomerular TβRII expression (Fig. 6). These results were identical to our previous report (25).

Before ATS injection, slight decreases in glomerular TβRI and TβRII expression were noted in rats on a low-salt intake.
and a slight increase in glomerular TβRI was observed in rats on a high-salt intake (Fig. 7). Seven days after ATS injection, when the glomerular lesions were at maximum levels, the expression of glomerular TβRI was less in rats with ATS nephritis on a low-salt intake (group B) than that in ATS nephritic rats on a normal-salt intake (group A) (Fig. 7). In contrast, glomerular expression of both TβRI and TβRII increased markedly in rats with ATS nephritis on a high-salt intake (group C) at day 7. Administration of candesartan cilexetil reduced glomerular expression of TβRII in rats with ATS nephritis on a high-salt intake at day 7 (group D).

Glomerular Expression of AT1R and AT2R

Compared with control rats, the levels of glomerular AT1R expression increased markedly in rats with ATS nephritis on a normal-salt intake (group A) at day 7 (Fig. 6). An increase in glomerular AT2R expression also was noted in group A at day 7.

Before ATS injection, no remarkable changes in glomerular AT1R and AT2R expression were observed in rats on a high- or low-salt intake compared with that in rats on a normal-salt intake (Fig. 7). However, 7 days after ATS injection, overexpression of glomerular AT1R was noted in rats with ATS nephritis on a high-salt intake (group C) compared with that in normal-salt-intake ATS nephritic rats (group A), whereas no marked change in glomerular AT1R expression was observed in rats with ATS nephritis on a low-salt intake (group B) (Fig. 7). The increase in glomerular AT1R expression in ATS nephritic rats on a high-salt intake at day 7 was not significant with administration of candesartan cilexetil (group D). In contrast, the levels of glomerular AT2R expression increased markedly in rats with ATS nephritis on a low-salt intake (group B) and decreased in those on a high-salt intake (group C) at day 7 (Fig. 7). Administration of candesartan cilexetil increased the levels of glomerular AT2R in high-salt-intake ATS nephritic rats (group D).

Plasma Renin Activity, Renal Renin Expression, and Glomerular ANG II Levels

Plasma renin activity was significantly higher in group B and lower in group C than that in group A before and after induction of ATS nephritis (Table 2). No significant differences in the levels of plasma renin activity were noted between high-salt-intake ATS nephritic rats treated with candesartan cilexetil (group D) and those untreated (group C). Immunoreactive renin protein was found in glomerular arterioles constituting the juxtaglomerular apparatus. The staining intensity of intrarenal renin roughly paralleled the levels of plasma renin activity in all groups (Table 2). Glomerular ANG II levels also roughly paralleled the levels of plasma renin activity and intrarenal renin staining intensity; it was significantly higher in rats with ATS nephritis on a low-salt intake (group B) and lower in those on a high-salt intake (group C) than those on a normal-salt intake (group A) (Table 2). In all samples obtained from high-salt-intake ATS nephritic rats treated with candesartan cilexetil at day 7 (group D; n = 6), the glomerular ANG II levels were lower than the lower limit for detection by the method used in the present study.

Fig. 6. A: representative photographs of Western blot analysis showing glomerular expression of type I (TβRI) and II (TβRII) TGF-β receptors, angiotensin II type 1 (AT1R) and type 2 receptors (AT2R), and β-actin before and 7 days after ATS injection in rats on a normal-salt intake. B: mean densitometric ratios of the band intensities of TβRI/β-actin (b), TβRII/β-actin (d), AT1R/β-actin (c), and AT2R/β-actin (e) before and 7 days after ATS injection. Mean values were obtained from 3 independent experiments.

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Effect of AT_2 R Blocker on Rats with ATS Nephritis on a Low-Salt Intake

Because the decrease in urinary protein excretion and amelioration of renal lesions were associated with increased glomerular AT_2 R expression in ATS nephritic rats on a low-salt intake, we investigated the effect of PD-123319, an AT_2 R blocker, on the progression of ATS nephritis under a low-salt intake. Administration of PD-123319 did not affect blood pressure levels and changes in body weight during the course of ATS nephritis in rats on a low-salt intake (Table 3). Significant increases in mesangial cell proliferation and matrix accumulation, urinary protein excretion (group E, 31.2 mg/day, vs. group F, 63.2 mg/day, \( P < 0.05 \)), deposition of cellular fibronectin, and production of glomerular TGF-\( \beta_1 \) were noted in low-salt-intake ATS nephritic rats treated with PD-123319 (group F) compared with those untreated (group E) 7 days after ATS injection (Fig. 8). No remarkable differences in glomerular TBJRI expression were noted between groups E and F. However, glomerular expression of TBJRII increased significantly in low-salt-intake ATS nephritic rats treated with PD-123319 (group F) at day 7 (Fig. 9). The data for immunohistochemical staining of TBJRI and TBJRII also showed similar results; no remarkable changes in TBJRI and marked increases in TBJRII in glomeruli of PD-123319-treated, low-salt-intake nephritic rats on day 7 (data not shown). These results suggest that increased glomerular AT_2 R expression in low-salt-intake nephritic rats played some protective role in the progression of ATS nephritis.

Fig. 7. Western blot analysis showing glomerular expression of TBJRI and AT_1 R, and AT_2 R before and 7 days after ATS injection in rats on a normal-salt intake (group A; hatched bars), low-salt intake (group B; open bars), high-salt intake (group C; filled bars), and high-salt intake treated with candesartan cilexetil (group D; stippled bars). A: representative photographs of Western blot analysis showing glomerular expression of TBJRI, TBJRII, AT_1 R, AT_2 R, and \( \beta \)-actin before (a) and 7 days after (b) ATS injection. B: mean densitometric ratios of the band intensity of TBJRI/\( \beta \)-actin (c and d), TBJRII/\( \beta \)-actin (e and f), AT_1 R/\( \beta \)-actin (g and h), and AT_2 R/\( \beta \)-actin (i and j) before (c, e, g, and i) and 7 days after (d, f, h, and j) ATS injection. Mean values were obtained from 3 independent experiments.

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Pressur also increased in these rats, although it was not more

nephritic rats on a high-salt intake. Glomerular TGF-

fection, progressing to the amelioration of glomerular RAS, resulting in exacerbation of ATS nephritis with increased glomerular TGF-β and TβR expression during a high-salt intake. Amelioration of nephritis with decreased glomerular TGF-β and TβRII expression in high-salt-intake nephritic rats treated with candesartan cilexetil, an AT(R) blocker, further supports this concept. The marked decrease of glomerular ANG II may be also involved in the amelioration of nephritis resulting from treatment with candesartan cilexetil. Previous studies showed that administration of an AT(R) blocker decreased renal ANG II levels in ANG II-infused rats (48). ANG II-mediated expression of angiotensinogen was also demonstrated in cardiomyocytes (39). The decrease of glomerular AT(R) expression noted in nephritic rats on a high-salt intake might also play some additive role in the aggravation of nephritis, because most signals mediated by AT(R) are antagonistic to those mediated by AT(R) (22).

In rats on a low-salt intake, progression of ATS nephritis was mitigated and glomerular expression levels of TGF-β and TβRI were decreased markedly, although no remarkable difference in glomerular TβRII expression was noted. A low-salt intake has been shown to increase the expression of AT(R) in proximal tubular cells (4) and adrenal cortical cells (37), resulting in increases of sodium reabsorption and aldosterone secretion, respectively, due to increased responsiveness to ANG II in these target cells. It was also reported that a low-salt intake decreases expression of AT(R) in afferent arterioles, mesenteric arteries, and aorta (4, 35), resulting in decreases of ANG II-mediated vascular contraction (35). On the other hand, the effect of a low-salt intake on glomerular AT(R) expression has been controversial. Amiri and Garcia (1) reported that glomerular AT(R) expression did not change after a low-salt intake. However, Cheng et al. (4) reported a decrease in the expression during a low-salt intake. In the present study, no significant change was noted in the expression of glomerular AT(R) in ATS nephritic rats on a low-salt intake compared with those on a normal-salt intake, when progression of ATS nephritis and glomerular expression of TGF-β and TβRII were mitigated in low-salt-intake nephritic rats. The glomerular ANG II levels paralleled renin expression and were high in rats

of TGF-β and TβR expression has been shown in mesangial cells (12) and proximal tubular cells (41, 43). Considering the fact that glomerular ANG II levels were not increased in rats with ATS nephritis on a high-salt intake, the increased glomerular AT(R) expression seems to explain the increased activity of glomerular RAS, resulting in exacerbation of ATS nephritis with increased glomerular TGF-β and TβR expression during a high-salt intake. Amelioration of nephritis with decreased glomerular TGF-β and TβRII expression in high-salt-intake nephritic rats treated with candesartan cilexetil, an AT(R) blocker, further supports this concept. The marked decrease of glomerular ANG II may be also involved in the amelioration of nephritis resulting from treatment with candesartan cilexetil. Previous studies showed that administration of an AT(R) blocker decreased renal ANG II levels in ANG II-infused rats (48). ANG II-mediated expression of angiotensinogen was also demonstrated in cardiomyocytes (39). The decrease of glomerular AT(R) expression noted in nephritic rats on a high-salt intake might also play some additive role in the aggravation of nephritis, because most signals mediated by AT(R) are antagonistic to those mediated by AT(R) (22).

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DISCUSSION

ANG II is a primary effector in RAS and has recently been shown to play an important role in the progression of kidney diseases (5, 10). Salt restriction enhances circulatory RAS and a high-salt intake suppresses it. In addition to circulatory RAS, recent studies by Navar and co-workers (24) highlighted the crucial role of intrarenal RAS in the paracrine regulation of renal function and the pathophysiology of hypertension as well as progressive kidney diseases. Discrepancy between circulatory and intrarenal RAS activity has been reported in high-salt intake rats receiving chronic ANG II injection (16). Dietary sodium manipulation was shown to induce organ-specific modulation of AT(R) and AT(R) expression in the kidney, dietary and renal gland (35, 46). Clinically, dehydation due to inappropriate sodium restriction often induces aggravation of underlying kidney diseases. However, it is unclear whether modulation of salt intake, which does not change arterial blood pressure, could influence the progression of nephritis. In the present study, we examined the effects of a low- and high-salt intake, which did not change significantly the mean blood pressure levels, on circulatory and intrarenal RAS, progression of renal lesions, and glomerular expression of TGF-β1, TβRI, and TβRII in ATS nephritis. The role of glomerular AT(R) under a low-salt intake was also investigated.

Induction of ATS nephritis was not significantly different among rats on a normal-, low-, and high-salt intake 24 h after ATS injection. However, exacerbation of nephritis with increased glomerular expression of AT(R), TβRI, and TβRII, and decreased glomerular expression of AT(R) were noted in ATS nephritic rats on a high-salt intake. Glomerular TGF-β1 expression also increased in these rats, although it was not more than the level seen in nephritic rats on a normal-salt intake. A high-salt intake has been shown to increase AT(R) expression in glomeruli, afferent arterioles, mesenteric arteries, and aorta (35, 46). In this context, Nickenig et al. (25) reported that sodium chloride upregulated AT(R) expression directly in vascular endothelial cells. In addition, ANG II-mediated induction

Table 2. Plasma renin activity, renin staining score, and glomerular ANG II levels in different groups of rats before and after injection of antithymocyte serum

<table>
<thead>
<tr>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRA, ng/ml·1/h</td>
<td>PRA, ng/ml·1/h</td>
<td>PRA, ng/ml·1/h</td>
<td>PRA, ng/ml·1/h</td>
</tr>
<tr>
<td>Day 0</td>
<td>33.5 ± 15.4</td>
<td>66.3 ± 24.5†</td>
<td>23.2 ± 19.8†</td>
</tr>
<tr>
<td>Day 3</td>
<td>16.0 ± 4.5</td>
<td>46.6 ± 19.1†</td>
<td>1.2 ± 1.1†</td>
</tr>
<tr>
<td>Day 7</td>
<td>13.0 ± 8.9</td>
<td>42.1 ± 14.9†</td>
<td>3.1 ± 1.1†</td>
</tr>
<tr>
<td>Day 14</td>
<td>15.5 ± 9.2</td>
<td>50.6 ± 19.4†</td>
<td>1.8 ± 1.7†</td>
</tr>
<tr>
<td>Day 28</td>
<td>17.2 ± 6.4</td>
<td>76.3 ± 14.2‡</td>
<td>2.2 ± 1.7†</td>
</tr>
</tbody>
</table>

Values are means ± SD. PRA, plasma renin activity; RSS, renin staining score; UD, undetectable. *P < 0.05 vs. group A. †P < 0.05 vs. group B. ‡P < 0.05 vs. groups A and B.

Table 3. Body weight and MAP of different groups of rats before and after injection of antithymocyte serum in protocol 2

<table>
<thead>
<tr>
<th>Group E</th>
<th>Group F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>Body wt, g</td>
</tr>
<tr>
<td>Day 0</td>
<td>181.4 ± 4.9</td>
</tr>
<tr>
<td>Day 3</td>
<td>190.0 ± 3.2</td>
</tr>
<tr>
<td>Day 7</td>
<td>194.2 ± 4.3</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>MAP, mmHg</td>
</tr>
<tr>
<td>Day 0</td>
<td>95.8 ± 5.3</td>
</tr>
<tr>
<td>Day 3</td>
<td>98.5 ± 6.9</td>
</tr>
<tr>
<td>Day 7</td>
<td>95.4 ± 5.9</td>
</tr>
</tbody>
</table>

Values are means ± SD. No statistical difference was detected among the groups.
with ATS nephritis on a low-salt intake. Considered together, these results suggest that the ameliorative effect of a low-salt intake was not attributable to the reduced glomerular ANG II activity mediated by AT1R.

Several recent studies have demonstrated the important role of AT2R in the repair process of various organs with tissue injuries. Increased expression of AT2R has been reported in postsischemic kidney, heart, and brain lesions (17, 26, 47). Accelerated fibrosis and collagen deposition develop in the renal interstitium of AT2R null mutant mice during ureteral obstruction (21). Upregulation of glomerular AT2R was also demonstrated in rats on a low-salt intake (30). In the present study, the increase of glomerular AT2R expression was noted in ATS nephritic rats on a low-salt intake. AT2R blockade by PD-123319 resulted in aggravation of nephritis and increased glomerular expression of TGF-β1 and TβRII in these rats. These data suggest that the increased glomerular AT2R expression induced by a low-salt intake played a role in amelioration of ATS nephritis through, at least in part, downregulation of glomerular TGF-β1 and TβRII expression. Su et al. (38) reported that overexpression of AT2R altered TβRII expression in vascular smooth muscle cells of spontaneous hypertensive rats and these changes might be attributable to the imbalance between AT1R and AT2R abundance.

Our results also showed that candesartan cilexetil significantly reduced glomerular macrophage infiltration in ATS nephritis. ANG II has been shown to induce the expression of monocyte chemoattractant protein-1 in mesangial cells (36) and on activation-regulated normal T cell expressed and secreted (RANTES) in glomerular endothelial cells (42). TGF-β also has a chemoattractant activity for neutrophils, T cells, monocytes, and fibroblasts (34). Hilgers et al. (8) reported that valsartan reduced the expression of monocyte chemoattractant protein-1 and interstitial infiltration of macrophages in the two-kidney one-clip model. Because macrophages have AT1R (29), it is reasonable to conclude that candesartan cilexetil inhibited the infiltration of these cells by reducing the expression of chemoattractants and might also have suppressed the

![Figure 8](http://ajprenal.physiology.org/)

Fig. 8. A: photomicrographs of glomeruli in kidney sections obtained from a low-salt-intake ATS nephritic rat at day 7 (group E) and a low-salt-intake ATS nephritic rat treated with PD-123319 at day 7 (group F). Magnification: ×400. B: mesangial matrix scores in groups E (n = 7) and F (n = 7) 7 days after ATS injection. C: glomerular staining intensity of cellular fibronectin (cFN) at day 7 in groups E (n = 7) and F (n = 7). D: urinary protein excretion over 24 h 7 days after injection of ATS in groups E (n = 7) and F (n = 7). E: levels of TGF-β1 production in glomeruli isolated from groups E (n = 7) and F (n = 7) 7 days after injection of ATS. Values are means ± SD. *P < 0.05 group E vs. group F.
amplified progression of ATS nephritis and was associated with decreased glomerular expression of TGF-β and TβRI and increased glomerular expression of AT2R. Blocking of AT2R by PD-123319 aggravated the glomerular lesions and increased glomerular TGF-β1 and TβRII expression. Our results suggest that increased glomerular AT1R and decreased glomerular AT2R expression are involved in the aggravation of ATS nephritis under a high-salt intake and that upregulation of glomerular AT1R may participate in the amelioration of the nephritis under a low-salt intake.

ACKNOWLEDGMENTS

We thank Dr. A. Fukamizu, University of Tsukuba (Ibaraki, Japan), for kindly providing the rabbit anti-mouse renin antibody, Dr. L. Zardi (Genoa, Italy) for kindly providing IST-9, and Takeda Chemical Industries (Osaka, Japan) for generously providing candesartan cilexetil.

GRANTS

This study was supported in part by Salt Science Research Foundation Grant 0048.

DISCLOSURES

Parts of this work were presented in abstract form at the 33rd and 34th Annual Meetings of the American Society of Nephrology (Toronto, Ontario, Canada, 2000, and San Francisco, CA, 2001).

REFERENCES


