Tubular Expression of Angiotensin II Receptors and Their Regulation in IgA Nephropathy


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Enhanced renal expression for the renin-angiotensin system (RAS) is detected in IgA nephropathy (IgAN). Previous data showed an altered glomerular expression of angiotensin II type 1 receptor (AT1R), suggesting a regulatory response to high intrarenal angiotensin II (Ang II) concentration in IgAN. In this study, the expression and regulation of Ang II receptors were examined in human proximal tubular epithelial cells (PTEC) in IgAN. Tubular expression of AT1R and Ang II type 2 receptor (AT2R) was increased in IgAN. In vitro culture experiment showed that the upregulation of Ang II receptors was not due to the direct effect of IgA but the indirect effect after IgA deposition on human mesangial cell. When PTEC were cultured with conditioned culture medium from human mesangial cells activated with IgA, Ang II production was upregulated, leading to inflammation and apoptosis via the AT1R and AT2R, respectively. Sequential expression of Ang II receptors determined the injury of PTEC induced by mediators in the conditioned medium. The initial interaction between Ang II and AT1R activated both protein kinase C and mitogen-activated protein kinase pathways, leading to inflammatory responses. This early AT1R-dependent event was followed by upregulation of AT2R expression and continued Ang II release. The interaction between Ang II and AT2R subsequently led to expression of cleaved poly[ADP-ribose] polymerase through downregulation of the mitogen-activated protein kinase pathway. The data suggest that appropriate control of Ang II receptor activities in PTEC may ameliorate tubulointerstitial injury in IgAN.


The intrarenal renin-angiotensin system (RAS) is pivotal in the pathogenesis of chronic renal injury, and angiotensin II (Ang II) plays an important role in regulating cell proliferation, apoptosis, and fibrosis (1–3). The Ang II type 1 receptor (AT1R) distributes widely in kidney cells, including mesangial, glomerular epithelial, endothelial, and vascular smooth muscle cells. Ang II can directly induce both cell growth and matrix accumulation in glomerular cells via the AT1R (4). In contrast, the AT2R is expressed at low levels in the endothelium in normal adult kidney and in cultured glomerular visceral epithelium. This receptor is often upregulated in response to injury and counterbalances the AT1R by increasing the production of bradykinin, nitric oxide, and cyclic guanosine monophosphate, thus mediating vasodilation, cell differentiation, and apoptosis (5–7). Pharmacologic blockade of the AT2R in wild-type mice and AT2R knockout mice shows decreased apoptosis and increased fibrosis in the kidney after injuries (8,9).

In rat proximal tubular epithelial cells (PTEC), Ang II induces cellular hypertrophy and activates relevant downstream signal transduction pathways (10,11). This Ang II–induced tubular cell hypertrophy is inhibited by losartan but not PD123319 (10–12), suggesting that the AT1R rather than the AT2R is contributory to the tubular cell hypertrophy. Rats that received Ang II infusion have an increased number of proliferating cell nuclear antigen– and transferase-dUTP-nick-end labeling–positive cells in proximal tubules (13). Similar findings have also been reported in cultured rat proximal tubular cells (14), suggesting that Ang II triggers both proliferation and apoptosis in tubular epithelial cells.

IgA nephropathy (IgAN) runs a variable clinical course with a slowly relentless and progressive renal failure in 30 to 50% of patients within 30 yr after the first clinical presentation (15). A subgroup with severe tubulointerstitial atrophy is often associated with rapid progression to end-stage renal failure (16). We recently reported an altered AT1R expression in human mesangial cells (HMC) in response to raised intrarenal Ang II concentration in IgAN (17). We proposed that an imbalance between AT1R and AT2R activities in HMC after binding to IgA could play a significant role in the pathogenesis of IgAN. In this study, we further examined the expression and regulation of Ang II receptors in PTEC and explored their role in the development of tubulointerstitial injury in IgAN.

Materials and Methods

Experimental Design

The study was conducted in accordance with the Declaration of Helsinki and was approved by the institutional ethics committee. All participants gave written informed consent for serum and tissue collection. Twenty milliliters of blood was collected from each control...
subject and from patient at clinical quiescence. The serum was frozen at $-20^\circ$C until isolation of IgA. Every IgA preparation was obtained separately from each individual patient or healthy control subject.

We first examined the tubular expression of AT1R and AT2R in patients with IgAN. Renal tissues were obtained from 13 normotensive patients who had mild IgAN (grade 1) and were admitted consecutively for renal biopsy with the presentation of microhematuria. The severity of renal pathology was classified into grade 1, 2, or 3 as described previously (18). These patients had normal creatinine clearance (80 ml/min per 1.73 m²) with proteinuria ranging from 0.8 to 1.7 g/d. They had not previously received angiotensin-converting enzyme inhibitor (ACEI) or AT1R antagonist. Control renal tissues were obtained from the intact pole of kidneys that were removed for circumscribed tumor in eight normotensive individuals (comparable in age, gender, and race). For studying the dose- and time-response profile of IgA-HMC–conditioned medium from patients with IgAN on the expression of Ang II receptors in cultured PTEC, total IgA1 were isolated from sera of five of these patients selected randomly. The same IgA preparations were used in subsequent studies of the expression of Ang II receptor modified by different antagonists and agonists after prolonged exposure to conditioned medium from HMC that were incubated with IgA.

For comparison of the immediate regulatory effect of IgA on the expression of Ang II receptors and Ang II release in cultured PTEC, IgA1 was prepared from sera of another 22 patients who had IgAN and were randomly recruited from the clinic. Their diagnosis was made at least 18 mo before the study, and their serum creatinine remained stable over the previous 12 mo. Their proteinuria ranged from 0.4 to 2.9 g/d. Their mean endogenous creatinine clearance was $72.8 \pm 23.2$ ml/min per 1.73 m². These patients varied in the histologic severity with grade 1 in 12, grade 2 in eight, and grade 3 in two. None of these patients with IgAN received ACEI or AT1R antagonist for BP control during the study period. Control subjects were age- and gender-matched healthy volunteers.

Morphometric Studies

Renal tissues were processed by standard methods, and the tubular expression of AT1R or AT2R was detected by immunohistochemistry using specific polyclonal antibodies for individual Ang II receptor (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (17). Two renal pathologists without previous knowledge of clinical or laboratory data evaluated the expression of AT receptor staining using an arbitrary 0 to 5+ scale. Ten consecutive cortical cross-sections were examined at high-power field. The preset grading criteria for tubular AT receptor staining were as follows: 0 if no tubular staining, 1 when <10% cortical tubular cells were positive; 2 when 10 to <20% cortical tubular cells were positive; 3 when 20 to <40% cells were positive; 4 when 40 to <60% cells were positive, and 5 when 60% or more cells were positive.

Cell Culture and Isolation of IgA1

Culture medium and FBS were obtained from Life Technologies (Rockville, MD). All other chemicals were obtained from Sigma (St. Louis, MO).

Isolation, characterization, and culture of human umbilical vein endothelial cells (HUVEC), HMC, and PTEC were performed as described previously (19,20). Human podocyte cell line was provided by Professor P.W. Mathieson (University of Bristol). In all experiments, cells that were grown to confluence were growth arrested in a six-well culture plate (0.5 $\times$ 10⁶ cells/well) with culture medium that contained 0.5%...
FBS for 48 h before commencement of experiments. Total IgA was isolated by affinity chromatography with jacalin agarose (Pierce, Rockford, IL) (17). The endotoxin levels for all IgA preparations were <1 pg/mg protein determined by a Limulus amebocyte lysate assay (Bio-Whittaker, Walkersville, MD).

Preparation of Conditioned Medium from HMC Activated by IgA

HMC, podocytes, and HUVEC were cultured with medium that contained IgA (final concentration 50 μg/ml) isolated from patients with IgAN or healthy control subjects for 48 h. The concentration of IgA preparation used was selected on the basis of our previous data that 50 μg/ml IgA was able to increase significantly the macrophage migration inhibitory factor (MIF) by cultured HMC (21). The conditioned media (IgA-HMC–conditioned medium, IgA-podocyte–conditioned medium, and IgA-HUVEC–conditioned medium) were collected and stored at −70°C until used. Conditioned medium from HMC that were cultured without the addition of IgA (control medium) was used as control. For all subsequent experiments that examined the effects of IgA–HMC or other conditioned medium on PTEC, conditioned medium first was diluted 10-fold with DMEM/F12 that contained 0.5% FBS before use for culturing PTEC. TNF-α in the IgA-HMC medium was determined using ELISA (R&D Systems, Minneapolis, MN).

Effect of IgA, Cytokines, or Conditioned Medium on Ang II Release

PTEC, podocytes, HUVEC, or HMC were cultured with an IgA preparation (50 μg/ml) for 96 h. In parallel experiments, PTEC were cultured with IgA–HMC–, IgA-podocyte–, and IgA-HUVEC–conditioned media or various cytokines (100 pg/ml TNF-α, 1 ng/ml TGF-β, or 10 ng/ml IL-6) for 96 h. After culture, the supernatants were stored at −70°C before assay for Ang II. Ang II was measured by an enzyme immunoassay using an anti–Ang II mAb labeled with acetylcholinesterase (SPI bio, Massy Cedex, France). The detection limit was 1 pg/ml, and the intra-assay coefficient of variation was 7% (17).

Figure 3. Expression of AT1R and AT2R by proximal tubular epithelial cells (PTEC) that were cultured directly with IgA or with IgA–human mesangial cell (HMC)–conditioned medium. There was no difference in gene (A) and protein expression (B) of AT1R or AT2R in PTEC that were cultured with IgA that was purified from patients with IgAN (n = 22) when compared with that from healthy control subjects (n = 15). The gene (C) and protein expressions (D) of AT1R and AT2R were significantly upregulated in PTEC that were cultured with IgA–HMC–conditioned medium from patients with IgAN when compared with that from healthy control subjects. *P < 0.05.
II (10^-7 M) was completely abolished. After PTEC were incubated for 96 h, cell lysates were collected for detection of cleaved PARP by immunoblotting. The time-response effects were studied in growth-arrested PTEC (0.5 x 10^6 cells/well of six-well culture plate) that were exposed to IgA-HMC–conditioned medium that was prepared from patients with IgAN for different time intervals (from 12 h to 6 d) in the presence or absence of AT1R antagonist (losartan, 100 nM) or AT2R antagonist (PD123319, 10 μM). Each antagonist was added 30 min before the culture. At each time point, culture supernatants were collected for assay of Ang II, and cell lysates were collected for analysis of cleaved PARP, AT1R, and AT2R.

Effects of Recombinant TNF-α on IL-6 Production and Recombinant IL-6 on Ang II Receptor Expression by PTEC

PTEC were cultured with recombinant 5 to 160 pg/ml TNF-α (R&D Systems) for 12 h or at a concentration of 40 pg/ml for 3 h to 2 d. The supernatants were collected for assay of IL-6. PTEC were cultured with 0.25 to 2 ng/ml recombinant IL-6 (R&D Systems) for 12 h or at a concentration of 5 ng/ml for 3 h to 2 d. The cell lysate preparation was collected for determination of AT1R and AT2R expression.

Effect of Ang II on the Ang II Receptor Expression and Cleaved PARP Expression in PTEC

PTEC were cultured with Ang II (10^-12 to 10^-6 M) in the presence or absence of antagonists to AT1R (losartan, 100 nM) or AT2R (PD123319, 10 μM). The cells were cultured for 48 h, and cell lysates were prepared for determination of AT1R and AT2R protein. To study the effect of AT2R on cleaved PARP expression in PTEC, we performed similar experiments in cells that were incubated with an AT2R agonist CGP42112A (10^-12 to 10^-6 M), and the expression of cleaved PARP was analyzed.

Ang II Binding Studies

PTEC (1 x 10^5) were cultured with medium or IgA-HMC–conditioned medium for 2 d. After culture, cells were incubated with increasing concentrations (0.25 to 10 nM) of [3H]Ang II (50 Ci/mmol; American Radiolabeled Chemical, St. Louis, MO) in medium that was supplemented with 1% BSA at 20°C for 90 min. After incubation, unbound [3H]Ang II then were removed by washing with cold PBS. The cells then were lysed with 1 M NaOH plus 0.1% Triton X-100, and the radioactivity of the lysate was counted. Competitive binding assay was performed on cells that were incubated with 10 nM [H]Ang II with or without Ang II receptor antagonists (losartan 10 μM or PD123319 10 μM). For determining nonspecific binding of [3H]Ang II, cells were incubated in the presence of unlabeled Ang II (10^-5 M). The specific [3H]Ang II binding, expressed as fmol/mg protein, was calculated as the difference between the total and nonspecific binding. Scatchard plot analyses were performed to calculate the dissociation constant (Kd) and the maximum binding capacity (Bmax). The protein content was determined by a protein assay kit from Bio-Rad Laboratories (Hercules, CA).

Role of AT1R and AT2R on Soluble Intercellular Adhesion Molecule-1 Production and Cleaved PARP Expression in PTEC Cultured with IgA-HMC Medium

PTEC were cultured with IgA-HMC–conditioned medium from patients with IgAN at different time intervals (from 12 h to 6 d) in the presence of (1) losartan (100 nM), (2) PD123319 (10 μM), or (3) CGP42112A (10^-7 M) added 30 min before culture or (4) losartan (100 nM) added 30 min before culture and followed by addition of PD123319 (10 μM) 2 d after culture. At each time point, supernatants and cell lysates were collected and stored at -70°C for assay of soluble intercellular adhesion molecule-1 (sICAM-1) and cleaved PARP. The sICAM-1 concentration was determined by a commercial ELISA (Bender MedSystems, Vienna, Austria).

Total RNA Extraction and Reverse Transcription–PCR and Immunoblotting

Total cellular RNA was extracted using NucleoSpin RNA II total RNA extraction kit (Macherey-Nagel, Duren, Germany), and reverse transcription–PCR (RT-PCR) was performed using designed primers for AT1R, AT2R, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as described previously (17). The gene expression was semiquantified as the ratio of AT1R or AT2R amplicon to GAPDH amplicon. Immunoblotting was performed as described previously (17). Rabbit polyclonal anti-AT1R and AT2R receptors were obtained from Santa Cruz Biotechnology. Monoclonal anticleaved PARP and polyclonal rabbit anti-phospho p42/p44 MAPK and anti-phospho pan-protein kinase C (PKC) were obtained from Cell Signaling Technology (Beverly, MA). Monoclonal anti-actin was obtained from Neomarkers (Fremont, CA). Briefly, 10 μg of total protein from the

Figure 4. (A) The synthesis of angiotensin II (Ang II) was not upregulated in PTEC, podocytes, or human umbilical vein endothelial cell (HUVEC) that were cultured directly with IgA alone but was significantly upregulated in HMC. (B) The synthesis of Ang II was significantly upregulated in PTEC that were cultured with IgA-HMC–conditioned medium that was prepared from patients with IgAN (n = 22) for 96 h when compared with that from healthy control subjects (n = 15). The Ang II concentration in the IgA-HMC–conditioned medium (diluted 10-fold with DMEM/F12 that contained 0.5% FBS before use for culturing PTEC) was 0.45 pg/ml. There was no upregulation of Ang II release by PTEC that were cultured with IgA-podocyte–conditioned medium, IgA-HUVEC–conditioned medium, or various cytokines (TNF-α, TGF-β, or IL-6).
extract was electrophoresed and transferred to a polyvinylidene difluoride membrane before probing with anticleaved PARP (1:500), anti-AT1R (1:1000), anti-AT2R (1:1000), anti-actin (1:1000), anti–phospho p42/44 MAPK (1:5000), or anti–phospho pan-PKC (1:4000) antibody in PBS-Tween. The images were scanned, and the densitometric results were reported as average arbitrary integrated values (units) after normalization with the average arbitrary integrated values of the actin signal.

**Activation of p42/p44 MAPK and PKC Signal Transduction Pathways in PTEC**

PTEC were exposed to IgA-HMC–conditioned medium or Ang II (10^{-10} M) for 24 h. In parallel experiments, individual or a combination of antagonists to AT1R (losartan, 100 nM) and AT2R (PD123319, 10 μM) were added 30 min before the addition of stimulants. For studying the effect of AT2R agonist CGP42112A on the activation of p42/p44 MAPK and PKC pathways in PTEC by IgA-HMC–conditioned medium, PTEC were exposed to IgA-HMC–conditioned medium in the presence of CGP42112A (10^{-12} to 10^{-5} M) for 24 h. At the end of experiment, the expression of phospho-p42/p44 and phospho-PKC by PTEC was determined by immunoblotting.

**Statistical Analyses**

All data were expressed as means ± SD unless otherwise specified. The significance of differences between groups was determined using one-way ANOVA. P < 0.05 was considered statistically significant.

**Results**

**Tubular Expression of Ang II Receptors**

Immunoreactive ATIR or AT2R protein was found in tubular epithelium cells of normal renal tissue (Figure 1). An increase in staining for both Ang II receptors in PTEC was observed in biopsies from patients with IgAN. With the use of a six-point scale, tubular AT1R immunostaining was marginally higher in IgAN (mean score 1.75 in IgAN versus 1.37 in control subjects; P = 0.0217; Figure 2A). In contrast, tubular immunostaining for AT2R was significantly increased in patients with IgAN (mean score 3.5 in IgAN versus 2.2 in control subjects; P = 0.0004; Figure 2B).

**Expression of Ang II Receptors in Cultured PTEC**

There was no increase in gene and protein expression of AT1R or AT2R in PTEC that were cultured with IgA that was...
isolated from patients with IgAN when compared with control subjects (Figure 3, A and B). However, the gene and protein expression of AT1R or AT2R were upregulated when PTEC were cultured with IgA-HMC–conditioned medium from patients with IgAN ($P < 0.05$; Figure 3, C and D). There was increased release of Ang II in HMC that were cultured with IgA that was isolated from patients with IgAN ($P < 0.05$ versus controls; Figure 4A). Similar increased production of Ang II was not observed in PTEC, podocytes, or HUVEC. There was increased release of Ang II in PTEC that were cultured with IgA-HMC–conditioned medium from patients with IgAN ($P < 0.05$; Figure 4B). Nonetheless, similar increased production of Ang II was not observed in PTEC that were cultured with IgA-podocyte– or IgA-HUVEC–conditioned medium or recombinant TGF-$\beta$, TNF-$\alpha$, or IL-6 (Figure 4B).

**Time-Course Study of PTEC Cultured with IgA-HMC–Conditioned Medium**

Figure 5A shows the time course of the response of PTEC that were cultured with IgA-HMC–conditioned medium. AT1R expression in PTEC was increased significantly on days 1 and 2 after incubation with IgA-HMC–conditioned medium ($P < 0.05$) but returned thereafter to basal level on day 6. AT2R expression in PTEC increased gradually after culture with IgA-HMC–conditioned medium, and the expression was significantly greater than the basal level on days 4 and 6. Rapid increase in IL-6 production by PTEC was evident after 12 h after incubation with IgA-HMC–conditioned medium ($P < 0.05$; Figure 5B). In parallel, the expression of cleaved PARP in PTEC was enhanced from day 4 after incubation with IgA-HMC–conditioned medium ($P < 0.05$). Blockade of AT1R with losartan further amplified this upregulation of cleaved PARP.

**TNF-$\alpha$ Increased IL-6 Production by PTEC**

Recombinant TNF-$\alpha$ significantly increased the IL-6 production by PTEC at a concentration of $\geq 40$ pg/ml ($P < 0.05$; Figure 6A). Incubation of PTEC with 40 pg/ml TNF-$\alpha$ significantly increased IL-6 production by 12 h and thereafter (Figure 6B). It should be noted that the concentration of TNF-$\alpha$ in the IgA-HMC–conditioned medium ($41 \pm 8.6$ pg/ml TNF-$\alpha$ used for culturing PTEC) could stimulate the production of IL-6 by PTEC after 12 h of culture.

**IL-6 Upregulated Ang II Receptor Expression by PTEC**

Recombinant IL-6 significantly increased the AT1R or AT2R expression by PTEC at concentrations $\leq 5$ and $\geq 40$ ng/ml, respectively ($P < 0.05$; Figure 6C). Recombinant IL-6 at 5 ng/ml significantly increased the expression of AT1R and AT2R receptors by PTEC after 12 and 48 h of culture, respectively (Figure 6D).

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![Figure 6.](image)

Figure 6. (A) Recombinant TNF-$\alpha$ significantly increased the IL-6 production by PTEC at a concentration of $\geq 40$ pg/ml. (B) Incubation of PTEC with 40 pg/ml TNF-$\alpha$ significantly increased IL-6 production by the 12 h and thereafter. (C) Recombinant IL-6 significantly increased the AT1R or AT2R expression by PTEC at concentrations of $\leq 5$ and $\geq 40$ ng/ml, respectively. (D) Recombinant IL-6 at 5 ng/ml significantly increased the expression of AT1R and AT2R receptors by PTEC after 12 and 48 h of culture, respectively. The results represent the mean $\pm$ SD of four separate experiments. *$P < 0.05$ versus data from day 0 or medium control.
Binding of Ang II to PTEC Cultured with IgA-HMC–Conditioned Medium

Binding of \[^{3}H\]Ang II to PTEC was specific and saturable (Figure 7A). The results from Scatchard plot analysis (Figure 7B) showed that incubation of PTEC with IgA-HMC–conditioned medium caused a significant increase in B\(_{max}\) of \[^{3}H\]Ang II (medium 452 ± 57 fmol/mg protein; IgA-HMC–conditioned medium 582 ± 18 fmol/mg protein; \(n = 4; P < 0.05\)). There was no change in K\(_d\) after PTEC was incubated with IgA-HMC–conditioned medium (medium 2.64 ± 0.12 nM; IgA-HMC–conditioned medium 2.58 ± 0.6 nM; \(n = 4; P = 0.88\)), suggesting that there was no alteration in Ang II receptor affinity of PTEC after incubation with IgA-HMC–conditioned medium.

The relative amount of AT receptors on PTEC was determined using competitive displacement assay with \[^{3}H\]Ang II in the presence of losartan or PD123319 (Table 1). Losartan displaced 43.72 ± 8.8% of \[^{3}H\]Ang II binding to PTEC and PD123319 displaced 53.29 ± 6.79% of \[^{3}H\]Ang II at day 0 of the culture. There was no significant difference in the relative amount of AT receptors after incubation of PTEC with IgA-

<table>
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<tr>
<th>Days after Incubation</th>
<th>PD123319</th>
<th>Losartan</th>
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<tr>
<td>0</td>
<td>53.29 ± 6.79(^b)</td>
<td>43.72 ± 8.80</td>
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<tr>
<td>2</td>
<td>54.45 ± 7.24</td>
<td>43.62 ± 6.52</td>
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\(^a\)Ang II, angiotensin II; PTEC, proximal tubular epithelial cells; HMC, human mesangial cells.

\(^b\)Results were expressed as percentage of displaceable specific \[^{3}H\]Ang II binding to PTEC compared with that of the binding in the absence of antagonists. Values represent mean ± SD from four experiments.

HMC–conditioned medium for 2 d (43.62 ± 6.52% for losartan and 54.45 ± 7.24% for PD123319).

Effect of Ang II on Ang II Receptor Expression and Cleaved PARP Expression in PTEC

The expression of AT1R, AT2R, and cleaved PARP was studied in PTEC that were incubated with increasing concentrations of Ang II with or without antagonists to AT1R or AT2R. Ang II at a concentration of \(\geq 10^{-9}\) M significantly increased the AT1R expression (\(P < 0.05\)), and the enhanced AT1R expression was blocked by losartan but not PD123319 (Figure 8A). Ang II at concentration of \(\geq 10^{-10}\) M significantly increased the AT2R expression (\(P < 0.05\); Figure 8B). The increased expression of AT2R by Ang II was blocked by PD123319 but not losartan. Furthermore, Ang II at a concentration of \(\geq 10^{-11}\) M increased the expression of cleaved PARP (\(P < 0.05\); Figure 8C). The increased apoptosis induced by Ang II as determined by the expression of cleaved PARP was readily suppressed by PD123319 but not losartan. The role of AT2R in inducing cleaved PARP expression of PTEC was further supported by finding that exposure to increasing concentrations of an AT2R agonist, CGP42112A, significantly increased the expression of cleaved PARP at concentrations of \(\geq 10^{-9}\) M (Figure 9).

Signal Transduction Pathways Involved in the Activation of PTEC by IgA-HMC–Conditioned Medium

Figure 10A depicts the detection of phospho-p42 and phospho-p44 subunits of MAPK or phospho-PKC in lysates from PTEC that were incubated previously with IgA-HMC–conditioned medium prepared from patients with IgAN or Ang II (\(10^{-10}\) M). Activation of either signal transduction pathway was reduced by AT1R blockade but was enhanced after AT2R blockade. Activation of the p42/p44 MAPK but not PKC pathway in PTEC that were preincubated with IgA-HMC–conditioned medium was blunted with addition of CGP42112A at a concentration of \(\geq 10^{-9}\) M (Figure 10B).

Role of AT1R and AT2R on sICAM-1 Synthesis and Cleaved PARP Expression in PTEC Cultured with IgA-HMC–Conditioned Medium

For studying the role played by individual Ang II receptor in the inflammatory response and apoptosis of PTEC induced by
IgA-HMC–conditioned medium, PTEC were cultured with IgA-HMC–conditioned medium in the presence of (1) losartan, (2) PD123319, (3) CGP42112A, or (4) first losartan then addition of PD123319 on day 2 after culture. IgA-HMC–conditioned medium induced an inflammatory response in PTEC shown by increased synthesis of sICAM-1 (Figure 11). Addition of PD123319 further amplified the synthesis of sICAM-1. CGP42112A decreased the sICAM-1 synthesis induced by IgA-HMC–conditioned medium after 2-d incubation. The sICAM-1 synthesis induced by IgA-HMC–conditioned medium was aborted by either losartan alone or losartan supplemented sequentially with PD123319. PTEC that were incubated with IgA-HMC–conditioned medium exhibited a gradual increase of cleaved PARP. The expression of cleaved PARP was amplified further by either losartan or CGP42112A. PD123319 suppressed the expression of cleaved PARP. The increased cleaved PARP expression after early addition of losartan was subsequently blunted with sequential addition of PD123319.

**Discussion**

The RAS is involved in the development of progressive renal fibrosis in IgAN (22,23). Local Ang II hyperactivity has been demonstrated in patients with IgAN (24). We demonstrated previously that IgA from patients with IgAN is capable of upregu-
lating the TGF-β production via increased Ang II release by HMC after binding to polymeric IgA (pIgA) (25). We have also demonstrated an altered AT1R expression in HMC in response to raised intrarenal Ang II in IgAN (17). Although there is an immediate downregulation of mesangial AT1R expression in IgAN after an acute exposure to pIgA, this adaptive mechanism is lost with chronic exposure to Ang II released by pIgA in IgAN. Furthermore, there is a defective counterbalance of AT1R by AT2R as the intrarenal Ang II concentration is insufficient to upregulate the mesangial AT2R expression in IgAN. Hence, the imbalance of AT1R and AT2R activities in HMC after exposure to pIgA may play a significant pathogenetic role in the glomerular inflammation in IgAN. The severity of tubulointerstitial damage in IgAN correlates closely with the declining renal function and the long-term clinical outcome (26). Most recently, we demonstrated no binding of IgA to and absence of IgA receptors in PTEC (19). Other than the tubulotoxic effect of proteinuria, the tubulointerstitial damage in IgAN is an indirect sequel as a result of a novel glomerulotubular cross-talk mediated through soluble factors released by HMC after IgA deposition (19).

Figure 9. Immunoblot analysis of cleaved PARP expression in PTEC that were incubated with culture medium that contained increasing concentrations of CGP42112A. CGP42112A at concentrations of \( \geq 10^{-9} \) M significantly increased the expression of cleaved PARP. The results represent the mean ± SD of five separate experiments. *\( P < 0.05 \) versus medium control.

Figure 10. Detection of phospho-p42 and phospho-p44 subunits of mitogen-activated protein kinase (MAPK) or phospho-PKC in PTEC lysate after incubation with plain medium alone, IgA-HMC–conditioned medium prepared from patient with IgAN, or Ang II (10^{-10} M). (A) Activation of the p42/p44 MAPK and protein kinase C (PKC) pathways was detected in PTEC that were incubated with IgA-HMC–conditioned medium or Ang II. The activation was reduced by losartan but was enhanced by PD123319. (B) After incubation with IgA-HMC–conditioned medium that was prepared from patients with IgAN for 24 h in the presence of increasing concentrations of CGP42112A, activation of the p42/p44 MAPK but not the PKC pathway was blunted in PTEC that were incubated with IgA-HMC–conditioned medium in the presence of CGP42112A at concentrations of \( \geq 10^{-9} \) M.
In this study, we demonstrated that there is constitutive expression of AT1R and AT2R in renal tubules with increased expression in IgAN. The glomerular AT1R was reduced in IgAN, whereas there was no change in the expression of glomerular AT2R (data not shown), confirming our previous results (17).

In vitro studies showed no change in Ang II receptor expression when PTEC were cultured with IgA, yet these receptors were upregulated after incubation with IgA-HMC–conditioned medium prepared from the same patient with IgAN. These findings further support the notion that disturbance of tubular RAS in IgAN is an indirect event through signals released by HMC after IgA deposition. Dissociation in tubular expression of Ang II receptors is evident by the time course of in vitro experiments of PTEC that were cultured with IgA-HMC–conditioned medium that was prepared from patients with IgAN. AT1R expression was upregulated in the first 2 d and decreased gradually to the basal level toward day 6. In contrast, significant increase in the AT2R expression was detected only after day 2 and thereafter. Results from the [3H]Ang II binding assay demonstrate that IgA-HMC–conditioned medium increased the Ang II receptor density without affecting the affinity or the relative proportion of AT1R and AT2R on PTEC after being cultured with IgA-HMC–conditioned medium for 2 d. Simultaneously, there was also increase in IL-6 production as early as 12 h after culture. The Ang II release and the expression of cleaved PARP were increased from day 2, associated with increased AT2R expression induced by IgA-HMC–conditioned medium. Our data suggest that although the initial Ang II level in PTEC that were cultured with IgA-HMC–conditioned medium was not high enough to upregulate AT receptor expression immediately, the concentration of TNF-α in the IgA-HMC–conditioned medium could induce a rapid and early increase of IL-6 level that in turn upregulates AT1R and AT2R expression in “primed PTEC.” Increased Ang II production through increased Ang II and its receptor binding could further upregulate the production of Ang II. We also demonstrated that IgA-HMC–conditioned medium induced cleaved PARP expression in PTEC as measured by an apoptotic marker, the cleaved PARP. We found that cleaved PARP was amplified by AT1R antagonist but abolished by AT2R antagonist. Normally, apoptosis mediated by AT2R counterbalances growth-stimulatory effects of Ang II through AT1R in renal proximal tubular cells (27,28). Pharmacologic blockade of the RAS, either by ACEI or AT1R antagonist, may retard the progression to glomerulosclerosis (29). AT2R modulates the actions of chemokine RANTES (30), matrix protein osteopontin (13), and nitric oxide (31), and blockade of the AT2R may reduce kidney injury. Our data revealed that AT2R antagonist could block the cleaved PARP expression in PTEC after incubation with IgA-HMC–conditioned medium. The role of AT2R in inducing apoptosis in PTEC was strengthened further by our observation that expression of cleaved PARP was increased with an AT2R agonist (CGP42112A).

The MAPK pathway is of pivotal importance in cell mitogenesis and hypertrophy. Ang II increases the tyrosine kinase and phosphatase activities (32). In addition to MAPK, phospholipase C-γ
(PLC-γ) can be phosphorylated and results in the stimulation of PKC by Ang II (33). Our study showed that IgA-HMC–conditioned medium from patients with IgAN or exogenous Ang II alone activated p42/p44 MAPK and PKC pathways in PTEC. The expression of phospho-p42 and phospho-p44 subunits of MAPK as well as phospho-PKC was enhanced by the AT2R antagonist but was reduced by the AT1R antagonist under an identical experimental setting. However, adding an AT2R agonist to PTEC that were preincubated with IgA–HMC–conditioned medium blunted the activation of phospho-p42 and phospho-p44 subunits of MAPK but not phospho-PKC, indicating that AT2R only counteracted the AT1R-mediated signal transduction pathway of MAPK but not PKC.

It seems that the temporal sequence of Ang II receptor expression will determine the fate of PTEC after exposure to mediators released from IgA-activated HMC. On the basis of our recent data (19) and our study, we propose a hypothetical mechanism of IgA-induced tubulointerstitial injury in IgAN (Figure 12). HMC develop inflammatory injury when chronically exposed to pathogenic IgA from IgAN as a result of the loss of an adaptive downregulation of the AT1R. There is also increased release of mediators by HMC that include TNF-α and Ang II. In PTEC, HMC-derived TNF-α first upregulates IL-6 production by PTEC. The released IL-6 in turn increases the AT1R expression and also enhances Ang II production gradually through a positive feedback loop. The early interaction between Ang II and AT1R activates both PKC and MAPK pathways, leading to subsequent inflammatory responses. This is supported by our finding of increased release of sICAM-1 from PTEC that were incubated with IgA–HMC–conditioned medium that is readily abolished by losartan. The early phase of AT1R-dependent inflammation is followed by subsequent upregulation of AT2R expression with continued Ang II release. The interaction between Ang II and AT2R then will lead to cleaved PARP expression through downregulation of the MAPK pathway. One of the proposed mechanisms of downregulation of MAPK is the increased activation of MAPK phosphatase-1 (34), which will counterbalance the AT1R-induced MAPK activation. Initial inflammatory responses in tubular cells are followed by later stages of tubular lysis or apoptosis, depending on the balance between activation of the MAPK pathway by AT1R and the suppression of the MAPK by an AT2R-dependent mechanism. If our hypothesis is correct, then sequential suppression of AT1R and AT2R could be a better way to reduce the IgA-induced PTEC damage. Indeed, this novel approach is supported by our preliminary in vitro data that sequential administration of losartan followed by PD123319 is effective in reducing early inflammatory responses (determined by sICAM-1) and preventing late onset of apoptosis caused by AT2R overexpression (determined by cleaved PARP).

In conclusion, our in vitro model suggests that dysregulation of the RAS in PTEC is due to the effects of cytokine release from HMC after IgA incubation. This dysregulation of the RAS in PTEC is pivotal in the pathogenesis of tubulointerstitial injury in IgAN. The tubulointerstitial injury could be ameliorated by a novel therapeutic approach of sequential targeting the AT1R and AT2R expression. Further in vivo study is warranted to test the hypothesis.

In conclusion, our in vitro model suggests that dysregulation of the RAS in PTEC is due to the effects of cytokine release from HMC after IgA incubation. This dysregulation of the RAS in PTEC is pivotal in the pathogenesis of tubulointerstitial injury in IgAN. The tubulointerstitial injury could be ameliorated by a novel therapeutic approach of sequential targeting the AT1R and AT2R expression. Further in vivo study is warranted to test the hypothesis.

Figure 12. Hypothetical model of tubular regulation of Ang II receptors in IgAN. HMC that are exposed to pathogenic IgA from patients with IgAN stimulate the synthesis and release of TNF-α and Ang II. An immediate downregulation of mesangial AT1R expression will ameliorate the proliferative and inflammatory changes induced by Ang II released by IgA. However, this adaptive downregulation of AT1R gradually disappears after chronic exposure to pathogenic IgA (@) and is likely to permit the development of proliferative and inflammatory processes in the glomerulus subsequently. In the tubular lumen, TNF-α released by HMC first increases the IL-6 production. The IL-6 in turn increases the AT1R expression and then gradually enhances Ang II production. The interaction of Ang II and early expressed AT1R will activate the PKC and MAPK pathways, leading to inflammatory responses. The early phase of AT1R-dependent inflammation is followed by subsequent upregulation of AT2R expression with continued release of Ang II. The interaction between Ang II and AT2R leads to apoptosis through downregulation of the MAPK pathway, and this will counterbalance the AT1R-induced MAPK activation (double arrow denotes interaction).

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