Renal expression of angiotensin type 2 (AT2) receptors during kidney damage

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Background. Activation of the renin angiotensin system has been described in pathologic conditions, including kidney damage. Angiotensin II (Ang II) acts through two receptors, AT1 and AT2. Most of the known actions of Ang II, including vasoconstriction and fibrosis, are due to AT1 activation. Recent data suggest that AT2 participates in the regulation of cell growth and renal inflammatory infiltration. Therefore, we investigated the renal expression of AT2 receptors in several models of renal injury.

Methods. Investigations were done in the following experimental models of kidney damage: systemic infusion of Ang II (inflammation), folic acid nephropathy (tubular cell death), and protein overload proteinuria. AT2 expression was determined by immunohistochemistry (protein) and reverse transcription-polymerase chain reaction (RT-PCR) (gene).

Results. In control animals, low levels of renal expression of AT2 were found. Ang II infusion resulted in an up-regulation of AT2 in tubular cells and de novo AT2 expression in glomeruli and vessels, associated with the presence of inflammatory cells. Acute tubular injury induced by folic acid was characterized by AT2 overexpression and apoptosis in tubular cells. Protein overload caused heavy proteinuria and tubular AT2 up-regulation.

Conclusion. AT2 is re-expressed in pathologic conditions of kidney damage, such as inflammation, apoptosis, and proteinuria, suggesting a potential role of this receptor during renal injury.

Activation of the local renin angiotensin system (RAS) has been described in pathologic conditions, including kidney damage. Angiotensin II (Ang II), the main effector peptide of the RAS, is a pleiotropic cytokine involved in the regulation of cell growth, fibrosis, and inflammatory response [1, 2]. Ang II binds to two main receptors: AT1 and AT2. Both receptor subtypes have been cloned and pharmacologically characterized. There are other potential Ang receptors, the AT3 and AT4, not yet included in a definitive classification [3, 4]. Most of the physiologic actions of Ang II, such as the regulation of blood pressure and water-electrolyte balance, are mediated by AT1. This receptor is widely distributed throughout the cardiovascular, renal, endocrine, and nervous system in humans. AT1 receptor is also involved in growth-promoting effects, fibrotic, thrombotic, and inflammatory processes through activation of various signal-transduction pathways, including calcium mobilization, activation of protein kinases, and production of reactive oxidant species [3, 4].

The biological functions and the signaling pathway of AT2 are not completely known. AT2 induces apoptosis and cell growth inhibition, vasodilatation, and regulates diuresis/natriuresis, renal nitric oxide (NO) production, and glomerular monocyte infiltration [3–6]. AT2 signaling activates phosphatases and production of ceramides, arachidonate, and kinin/NO/cGMP [3, 4]. The AT2 receptor is ubiquitously expressed in human fetal mesenchymal tissues, but its expression declines after birth [7]. In adults, AT2 receptor expression is detectable in the pancreas, heart, kidney, adrenals, brain, and vasculature [3, 4, 8]. AT2 is re-expressed in pathologic situations involving tissue remodeling or inflammation, such as neo-intima formation, heart failure, and wound healing [3, 9–11]. However, the role of AT2 receptors in renal disease remains to be defined.

Many investigators have demonstrated the presence of AT1 in the adult kidney and in cultured renal cells. However, there are still some controversial data about renal AT2 [1, 3, 4, 8]. Our aim was to investigate the renal expression of AT2 in experimental models of renal injury of different etiology, such as infusion of Ang II [6, 12], acute renal injury by folic acid, which is associated to apoptosis of tubular cells [13], and a model of heavy proteinuria caused by protein overload [14, 15].

METHODS

Experimental design

Angiotensin infusion was done during 3 days with osmotic subcutaneous minipumps (Alza Corp., Cupertino,
CA, USA). Studies were done in Wistar rats (Ang II at dose of 50 ng/kg/min), as previously described [6, 12], and in mice (1000 ng/kg/min). In these studies we used saline-infused animals as control (N = 8 each). Studies in mice were done in the following experimental groups: (I) AT1 receptor knockout male mice that were generated with a germ-line chimera derived from TT2 embryonic stem (ES) cells with a targeted mutation of the AT1A gene (Tanabe Seiyaku Corp, Osaka, Japan) [16]; and (2) wild-type (WT; C57BL/6). AT1 knockout mice were backcrossed for more than 6 generations with C57BL/6 mice. C57BL/6 and Balb/c mice were purchased from Harlan Interfauna Ibérica, S.A. (Barcelona, Spain). Eight animals from each group were studied.

Folic acid-induced nephropathy. Acute tubular injury was induced by a single injection of folic acid (250 mg/kg) in Balb/c mice as described [13], and animals were studied after 24 hours. Saline injection was used as control in all models.

Overload proteinuria was induced in Sprague-Dawley rats by daily intraperitoneal injections of 2 g bovine serum albumin (BSA) for 7 and 14 days [14]. At the end of the study period, animals were sacrificed under pentobarbital anesthesia and kidneys were removed and processed in liquid nitrogen for RNA studies or treated to obtain paraffin sections for histology and immunohistochemistry.

\textbf{AT2 protein expression by immunohistochemistry}

Paraffin-embedded renal tissue sections (4 µm) were mounted on poly-l-lysine-coated slides. The slides were deparaffinized with xylene and graded concentrations of ethanol and then rehydrated. The endogenous peroxidase was blocked by incubating in 3% H2O2/methanol (1:1) at 25°C for 30 minutes. The slides were treated with 0.01% trypsin in HCl for 15 minutes at 37°C. To reduce non-specific background staining, slides were incubated in buffer A [phosphate-buffered saline (PBS) with 2% horse serum, 4% BSA, and 0.1% trypsin] for 1 hour at room temperature, and incubated overnight at 4°C with a specific goat anti-AT2 antibody (Santa Cruz, #sc-1173). After washing with PBS, the sections were incubated with secondary anti-immunoglobulin G (IgG) biotinylated-conjugated antibody diluted 1:200 without horse serum for 30 minutes. Detection was done by incubation with ABC complex (Dako Corp., Carpinteria, CA, USA) for 30 minutes, after 0.05% 3,3′-diaminobenzidine (Dako Corp.) in 0.3% H2O2 for 5 minutes. The sections were counterstained with Mayer’s hematoxylin and mounted in Pertex (Medite, Burgdorf, Germany). In each experiment, negative control without the primary antibody, or using an unrelated antibody, were included to check for nonspecific staining.

In overload nephropathy, the immunolabeled surface area was evaluated by quantitative image analysis using a KZ 300 Imaging System 3.0 (Zeiss, München-Hallbergmoos, Germany), and the degree of staining was calculated by the ratio of suitable binary threshold images and the total field area integrating the intensity of the staining in the specific areas. For each sample, the mean staining was obtained by analysis of 20 different fields (at 10×), excluding glomeruli and vessels. Quantification was done twice, independently. The staining score was expressed as density/mm².

\textbf{Determination of mRNA expression of AT2}

Total RNA was obtained from the renal cortex by homogenization and isolation with Trizol (Invitrogen, Carlsbad, CA, USA). AT2 mRNA expression was analyzed by reverse transcription-polymerase chain reaction (RT-PCR), with specific primers 5′-TGGAGTT GCTGCAGTTCAAT-3′ (sense) and 5′-TCCCCGAAA ATAAAGTGGG-3′ (antisense), yielding a 568 bp product. PCR amplification (1 minute at 94°C, 1 minute at 58°C, and 2 minutes at 68°C) was linear up to 35 cycles, and data corresponding to cycle 35 were used for calculations. Control experiments were done with RNA samples, but without avian myeloblastosis virus (AMV) reverse transcriptase.

\textbf{RESULTS}

Renal AT2 expression is up-regulated in different experimental models of renal injury

Protein levels of AT2 in the kidney were evaluated by immunohistochemistry. In renal sections of control rats and mice there was positive immunostaining for AT2 located in tubuloepithelial cells, while no staining was observed in glomeruli (Figs. 1 and 2).

In Ang II–infused rats (Fig. 1), increased staining for AT2 was observed in tubuloepithelial cells. In addition, induction of AT2 was found in glomeruli, mainly in the mesangial area and vascular smooth muscle cells (VSMC) of renal vessels. Infusion of Ang II for 3 days also caused infiltration of inflammatory cells in glomerular and tubulointerstitial areas [6].

In Ang II–infused mice (Fig. 2), AT2 over-expression was also observed, mainly in tubular cells. In contrast, there was no induction in glomeruli. In this model we have described focal interstitial infiltration, mainly of macrophages, but inflammatory cells were absent in the glomeruli [12]. In AT1 knockout mice, AT2 expression was also elevated in some tubuli by Ang II infusion (Fig. 2). By RT-PCR we analyzed AT2 gene levels in the mouse model. In control kidneys a band corresponding to the AT2 mRNA was observed. In Ang II–infused mice AT2 mRNA was up-regulated, both in WT and AT1 knockout mice (Fig. 3). These data suggest an association between the presence of infiltrating cells and renal AT2 over-expression.
Fig. 1. Systemic infusion of angiotensin II (Ang II) into rats up-regulates renal AT2 expression. Immunohistochemistry was done as described in Methods. In control rats, AT2 is present in low levels in some tubular cells. In Ang II–infused rats for 3 days AT2 is over-expressed in tubular cells and appeared in glomeruli. Negative control samples were incubated in the absence of primary antibody.

Fig. 2. Systemic infusion of angiotensin II (Ang II) into mice up-regulates tubular AT2 expression. In Ang II–infused mice AT2 expression was elevated in tubular cells compared to control mice. Similar results were found in AT1 knockout mice.

In acute renal injury induced by folic acid in mice, renal AT2 mRNA over-expression was observed (Fig. 3). AT2 staining was also increased, mainly in tubular cells (Fig. 4), and correlated with elevated levels of apoptotic proteins [13].

In rats with overload proteinuria, AT1 was distributed in the cortex and medulla, mainly in distal tubules and to a lesser extent in proximal tubuli and blood vessels. AT2 staining was observed more intensely in the cortex than in the medulla, and its distribution was stronger in distal tubules and blood vessels, reaching significant differences both after the 7th and 14th injection (Fig. 5).

Fig. 3. Analysis of AT2 mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR) in murine models of renal damage. Total RNA from renal cortex of control mice expressed AT2 mRNA. In both models—infusion of (A) Ang II and injection of (B) folic acid—AT2 mRNA expression was increased compared to control animals.

Fig. 4. Angiotensin II receptor type 2 (AT2) is up-regulated in folic acid–induced renal injury. In control Balb-c mice, a weak staining for AT2 in tubular cells was observed. Injection of folic acid up-regulated tubular AT2 expression.
These animals presented heavy proteinuria, elevated blood pressure, severe tubulointerstitial inflammation, and activation of the fibrogenesis process [14].

**DISCUSSION**

Our data show that AT$_2$ is up-regulated during renal injury independently of the initial cause of damage, and is associated with different features, such as inflammation, proteinuria, and apoptosis. These data, together with the information provided by AT$_2$ blockers and AT$_2$ knockout mice [3, 4, 18, 19], support an important role for AT$_2$ in the pathophysiology of the kidney.

Systemic infusion of Ang II for 3 days into rats increases renal AT$_2$ expression in tubular cells. The AT$_2$ up-regulation occurs simultaneously with the presence of inflammatory cells in glomeruli and in tubulointerstitial areas in a focal manner [5, 6], and the elevation of pro-inflammatory parameters in the kidney, including nuclear factor kappa B (NF-$\kappa$B) activation and production of chemokines and cytokines [5, 6, 20]. Interestingly, AT$_2$ blockade diminished renal inflammatory cell infiltration, NF-$\kappa$B activation, and over-expression of related genes caused by Ang II infusion [5, 6, 12]. All these data show that a potential mechanism involved in Ang II–induced renal inflammation is the AT$_2$/NF-$\kappa$B pathway, extensively discussed in another work in this supplement [12]. The potential clinical implications of these findings need to be addressed in human diseases.

Persistent proteinuria contributes to tubulointerstitial damage in human and experimental renal diseases [21, 22]. However, the mechanisms by which proteinuria could cause interstitial inflammation and fibrosis are still not fully understood. The experimental model of protein overload in rats is characterized by heavy and sustained proteinuria. In this model, we have observed up-regulation of AT$_2$ in tubular cells. We have previously shown...
that persistent proteinuria elicited renal RAS activation, shown by elevated angiotensin-converting enzyme (ACE) in tubular epithelial cells [15]. One potential mechanism by which persistent proteinuria may participate in the progression of renal disease is through the Ang II/NF-κB pathway. In this sense, rats and mice with protein overload nephropathy present elevated renal NF-κB activity and over-expression of chemokines, which were prevented by ACE inhibition treatment [14, 23]. In AT1 knockout mice with overload nephropathy, proteinuria, tumor necrosis factor-α (TNF-α) mRNA expression, and NF-κB activity was similar to those found in WT. Moreover, interstitial infiltration was higher in AT1 knockout than in WT mice [14]. These data suggest that heavy proteinuria may cause NF-κB activation and related proinflammatory responses independently of AT2, further supporting the idea that the AT2/NF-κB pathway participates in renal inflammatory processes, at least in that condition. In contrast, AT2 knockout mice with overload nephropathy had significantly less activator protein-1 (AP-1) activation and mRNA expression of transforming growth factor-β (TGF-β) and extracellular matrix proteins [14], showing that AT2 regulates fibrosis, as observed in response to AT1 blockade in different models of renal injury [1].

Folic acid nephropathy is a classic model of acute tubular injury. In mice, injection of folic acid increased renal AT2 gene and protein, mainly in tubular cells. Folic acid caused apoptosis and changes in the expression of apoptosis regulatory genes (Bcl2) in tubular epithelium [13]. This resulted in a decreased Bel2/Bax ratio that favored cell death. In cultured cells, AT2 stimulation caused apoptosis via caspase activation and ceramide production [4], and induced dephosphorylation of Bel-2 [24]. In cultured tubular cells, serum deprivation, TNFα, and Ang II stimulation caused apoptosis [13, 25]. Although no studies are found in cultured tubular cells, serum deprivation up-regulated AT2 in mesangial cells and VSMC [26, 27], showing that factors involved in apoptosis could also be responsible for the tubular AT2 over-expression found in folic acid nephropathy. AT2 may also be involved in apoptosis through the NF-κB pathway. In most cell types, NF-κB mediates cell survival signals, protecting cells from apoptosis, but in other conditions it may also induce apoptosis [28]. Our data clearly show that during acute renal injury, there was an AT2 up-regulation associated with apoptosis of tubular cells, suggesting that a potential relationship merits further investigation.

In other models of renal damage, AT2 up-regulation has previously been described. In rats with immune complex glomerulonephritis, which presented an activation of the renal RAS [29], a strong glomerular staining with the anti-AT2 antibody was noted [6]. In the adult rat, sodium depletion was associated with increased renal AT2 [8], suggesting that homeostatic adaptations to changes in salt and water balance, which may trigger activation of the RAS, may result in increased expression of the AT2. Up-regulation of AT2 has also been found in other pathologic conditions, including vascular damage and wound healing [3, 9–11]. Future studies are needed to determine the expression levels of AT2 in human kidney diseases.

The mechanisms of the regulation of AT2 mRNA expression in renal cells are poorly understood. However, mouse fibroblast R3T3 cells have been extensively studied. In these cells, interleukin-1β (IL-1β), insulin, and Ang II up-regulate AT2; expression, while serum (10%), fibroblast growth factor, phorbol ester, and lysophosphatidic acid reduced it [30, 31]. The competitive binding of interferon regulatory factor (IRF)-1 and IRF-2 transcriptionally regulates AT2 expression. IRF-1 increases growth-dependent AT2 expression, whereas IRF-2 inhibits it [32]. In these cells, up-regulation of IRF-1 and AT2 exert proapoptotic effects [33]. In the promotor region of the mouse AT2 gene there are potential cis DNA elements that respond to IL-1β (CCAAT enhancer binding protein site), insulin (insulin response sequence of phospho(enol)pyruvate carboxykinase gene), and phorbol ester (AP-1 site). In cultured mesangial cells and VSMC, prolonged serum depletion induced AT2 mRNA expression. This effect was only found in cells from Wistar-Kyoto rats, but not from spontaneously hypertensive rats (SHR) [26, 27]. In VSMC, insulin-like growth factor up-regulates AT2 [34], and in rat neonatal myocytes, protein kinase C activators, such as Ang II down-regulated AT2 mRNA level [35]. These data show that AT2 expression is regulated by many stimuli, in positive and negative directions, which vary in the different cell types.

The role of AT2 in cell growth is not completely defined. In mesangial cells the stage of growth modulates AT2 expression, therefore, in confluent cells AT2 expression was greatly increased [21]. In vitro studies have demonstrated that AT2 stimulation inhibits the growth of various cell types, including mesangial, VSMC, endothelial cells, cardiomyocytes, and cardiac fibroblasts [3, 4]. However, the results of in vivo studies in the cardiovascular system are controversial [11]. Some experimental studies, using in vivo transfer of the AT2 gene and AT2 knockout mice showed that AT2 could inhibit VSMC growth in vivo. AT2 antagonist administrates to AT2 knockout mice decreases vascular lesion to a lesser extent than in WT, supporting the hypothesis of AT2 stimulation during AT1 blockade [36]. However, other works show opposite results, implicating the AT2 receptors in cardiac hypertrophy and fibrosis [37]. In AT2 knockout mice with Ang II–induced hypertension, left ventricular hypertrophy and interstitial collagen type I was absent [38]. These contrasting results emphasize that the role of AT2 in human diseases needs to be investigated.
CONCLUSION

Our data show that after diverse initiating insults, AT₂ over-expression is a common feature of kidney injury. Future research on the role of AT₂ in human renal diseases is necessary, particularly in view of the emerging importance of the RAS and its inhibition in clinical practice.

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