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Upregulation of Renal Renin-Angiotensin System in Rats with Adriamycin-Induced Nephrosis

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The present study was aimed to investigate whether the adriamycin-induced nephrosis is associated with an altered regulation of local renin-angiotensin system (RAS) in the kidney. Rats were subjected to a single injection of adriamycin (2 mg/kg body weight, IV) and kept for 6 weeks to allow the development of nephrosis. They were then divided into two groups, and supplied with and without cilazapril, an angiotensin converting enzyme (ACE) inhibitor, in drinking water (100 mg/l) for additional 6 weeks. Another group without adriamycin-treatment served as control. The mRNA expression of renin, ACE, type 1 and type 2 angiotensin II receptors (AT1R, AT2R), and transforming growth factor (TGF) -β1 was determined in the cortex of the kidney by reverse transcription-polymerase chain reaction. Adriamycin treatment resulted in heavy proteinuria. Accordingly, the mRNA expression of renin, ACE, and AT1R was increased in the renal cortex, while that of AT2R was decreased. Co-treatment with cilazapril attenuated the degree of proteinuria. While not affecting the altered expression of renin, cilazapril decreased the expression of ACE to the control level. Cilazapril further increased the expression of AT1R, while it restored the decreased expression of AT2R. The expression of TGF-β1 was increased by the treatment with adriamycin, which was abolished by cilazapril. An altered expression of local RAS components may be causally related with the development of adriamycin-induced nephrosis, in which AT1R is for and AT2R is against the development of nephrosis.

Key Words: Adriamycin, Renin, Angiotensin converting enzyme, Type 1 and type 2 angiotensin II receptors, Transforming growth factor-β1

INTRODUCTION

The renin-angiotensin system (RAS) plays a major role in the pathogenesis of renal sclerosis, such as renal mass ablation (Lafayette et al, 1992), diabetic nephropathy (Anderson, 1998), and mesangioproliferative glomerulonephritis (Nakamura et al, 1999). It has been known that the kidney is not only a target organ of the circulating RAS, but also has its own local RAS (Kastner et al, 1984; Nakamura et al, 1989). Therefore, the circulating RAS activity does not necessarily reflect the local RAS activity in the kidney. The adriamycin-induced nephrosis may be attributed to an altered regulation of local RAS in the kidney (Hall et al, 1986; Li et al, 1999). However, the expression of RAS components in the kidney has not been completely examined in adriamycin-induced nephrosis. The present study was aimed to determine the expression of RAS components in the kidney in adriamycin-induced nephrosis.

METHODS

Animals

Male Sprague-Dawley rats (weighing 180~200 g) were used. Experimental protocols were approved by Institutional Committee of Laboratory Animal Care and Use. Rats were given a single injection of adriamycin (2 mg/kg body weight) via the tail vein. The control group received the vehicle only. The animals were kept for 6 weeks to allow the development of experimental nephrosis which was confirmed by moderate to severe proteinuria using sticks. They were then divided into two groups: cilazapril, an angiotensin converting enzyme inhibitor (Jeil Pharmaceutical; Seoul, Korea), was supplemented in drinking water (100 mg/l) in one group and not in the other. They were then kept for another 6 weeks. Twenty-four hour urine sample was collected in a metabolic cage on the last day of 12th weeks. Urine protein was measured by sulfosalicylic acid method.

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ABBREVIATIONS: RAS, renin-angiotensin system; RT-PCR, reverse transcription-polymerase chain reaction; ACE, angiotensin converting enzyme; ATIR, type 1 angiotensin II receptor; TGF, transforming growth factor.

RNA extraction and RT-PCR

Rats were killed by decapitation in a conscious state, and the kidneys were quickly removed and stored at −70°C until used. Total RNA was isolated from the renal cortex according to the protocols of Ultraspec™ RNA isolation system (Biotecx Laboratories; Houston, TX, USA). The RNA concentration was determined by absorbance at 260 nm. The expression of renin, ACE, AT1R, AT2R and TGF-β mRNA was determined by reverse transcription (RT) polymerase chain reaction (PCR). For RT, 1 μg total RNA was incubated with reverse transcriptase (Gibco BRL; Grand Island, NY, USA; 200 U), RNasin (10 U), dNTP mix (10 mmol/l), DTT (0.1 mol/l), $MgCl_2$ (25 mmol/l), oligo (dT) (0.5 μg/l), and reaction buffer [200 mmol/l Tris-HCl (pH 8.4), 500 mmol/l KCl] in a final volume of 30 μl at 42°C for 50 min. After inactivation of reverse transcriptase at 72°C for 15 min, 2 μl cDNA was subjected to PCR amplification.

PCR was conducted in a final volume of 20 μl containing 10 pmole of each primer, dNTP mix (250 μmol/l), $MgCl_2$ (1.5 mmol/l), and Taq polymerase (0.3 U) using a thermal cycler (M.J. Research; Watertown, MA, USA). The amplification profile of renin consisted of 30 sec denaturation at 94°C, 60 sec annealing at 60°C, and 75 sec elongation at 72°C. For ACE, the profile consisted of 95°C for 2 min, 65°C for 1 min, and 72°C for 90 sec. For AT1R, it consisted of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. For AT2R, it consisted of 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min. For TGF-β, it consisted of 95°C for 45 sec, 50°C for 45 sec, and 72°C for 1 min. For β-actin, 94°C for 45 sec, 56°C for 45 sec, and 72°C for 90 sec. The final extension ended with 5 min of elongation at 72°C. Under the above conditions employed, RT-PCR allowed semiquantitative evaluation of renin, ACE, AT1R, TGF-β and β-actin mRNA with 0.1 μg of total RNA each. The amplification cycle was 35 in all the cases.

The primers were adopted as described by previous investigators (Table 1). PCR products were size fractionated by 1.2% agarose gel electrophoresis, and visualized under ultraviolet light with ethidium bromide staining. The quantification of cDNA was done using IMAGER™ & 1D MAIN (Bioneer; Cheongwon, Korea).

Statistical analysis

Results were expressed as means±SEM of 6 experiments. The statistical significance of differences between the groups was determined by ANOVA.

RESULTS

Development of proteinuria

Adriamycin treatment resulted in heavy proteinuria (317.0±49.1 vs. 44.9±8.8 mg/24 hr, n=6 each; $P<0.01$), the degree of which was attenuated by cilazapril treatment (89.5±22.7 mg/24 hr, n=6; $P<0.01$).

Alterations of RAS genes

Following the treatment with adriamycin, the mRNA

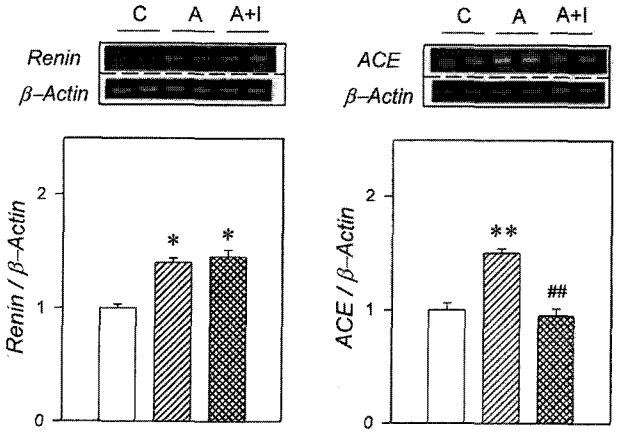

Fig. 1. Expression of renin and ACE mRNA in the renal cortex in control (C), adriamycin (A) and adriamycin+cilazapril (A+I)-treated rats. Fluorographs show ethidium bromide-stained RT-PCR products on agarose gels. Symbols are: (□) control, (▨) adriamycin, and (▩) adriamycin+cilazapril-treated. *$P<0.05$, **$P<0.01$, vs. control; ##$P<0.01$, vs. adriamycin.

Table 1. Oligonucleotide sequences used in PCR amplification

Gene	Primer sequence	References
Renin (374 bp)		
Sense	5'- TGCCACCTTGTTGTGTGAGG-3'	Tank et al, 1998
Antisense	5'- ACCCGATGCGATTGTTATGCCG-3'	
ACE (389 bp)		
Sense	5'-GCCTCCCCAACAAGACTGCCA-3'	Passier et al, 1995
Antisense	5'-CCACATGTCTCCCCAGCAGATG-3'	
AT1R (497 bp)		
Sense	5'-GCCAAAGTCACCTGCATCAT-3'	Kitami et al, 1992
Antisense	5'-AATTTTTTCCCCAGAAAGCC-3'	
AT2R (445 bp)		
Sense	5'-GGAGCGAGCACAGAATTGAAAGC-3'	Miyata et al, 1999
Antisense	5'-TGCCCAGAGAGGAAGGGTTGCC-3'	
TGF-β1 (294 bp)		
Sense	5'-GGACTACTACGCCAAAGAAG-3'	Fukuda et al, 2001
Antisense	5'-TCAAAAGACAGCCACTCAGG-3'	
β-Actin (423 bp)		
Sense	5'-GACTACCTCATGAAGATCCTGACC-3'	Mistry et al, 1998
Antisense	5'-TGATCTTCATGGTGCTAGGAGCC-3'	

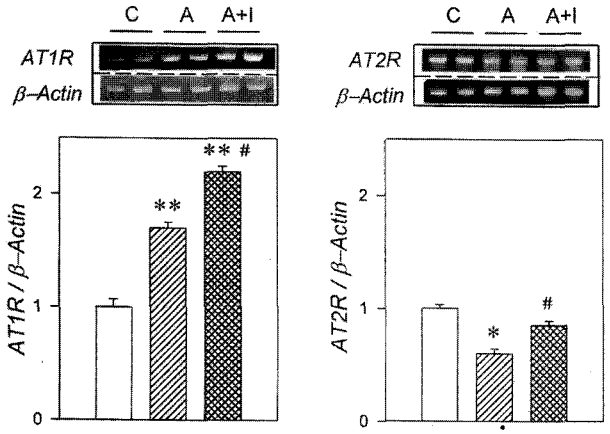

Fig. 2. Expression of AT1 and AT2 receptor mRNA in the renal cortex in control, adriamycin and adriamycin+cilazapril-treated rats. Legends are the same as in Fig. 1. $^{*}P<0.05$, $^{**}P<0.01$, vs. control; $^{\#}P<0.05$, vs. adriamycin.

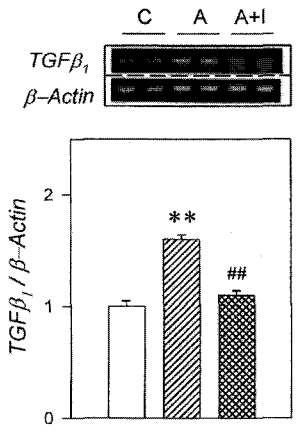

Fig. 3. Expression of TGF-β1 mRNA in the renal cortex in control, adriamycin and adriamycin+cilazapril-treated rats. Legends as in Fig. 1. $^{**}P<0.01$, vs. control; $^{\#\#}P<0.01$, vs. adriamycin.

expression of renin, ACE, and AT1R was increased in the renal cortex, while that of AT2R was decreased (Figs. 1 and 2). Co-treatment with cilazapril did not affect the altered expression of renin, but decreased that of ACE to the control level. Cilazapril further increased the expression of AT1R, while it restored the decreased expression of AT2R. The expression of TGF-β1 was increased by the treatment with adriamycin, which was abolished by cilazapril (Fig. 3).

DISCUSSION

In the present study, adriamycin treatment induced a heavy proteinuria, which was ameliorated by cilazapril. These results are in good agreement with those in many previous studies which examined the renoprotective effects of ACE inhibitors in progressive renal disease rat models (Anderson et al, 1986; Kakinuma et al, 1992). Our study further demonstrated that the mRNA expression of RAS components including renin and ACE was increased by adriamycin.

In addition, the expression of AT1R was increased while that of AT2R was decreased. AT1R is involved in the known biological functions of angiotensin II. On the other hand, physiological role of AT2R remains rather undefined. In isolated and perfused afferent arterioles, AT2R stimulation mediates an endothelium-dependent dilation (Arima et al, 1997). The vasodilator effect of AT2R may antagonize AT1R-dependent contraction in these arterioles (Endo et al, 1998). In this context, the increased expression of AT1R in association with a decreased AT2R may lead to an increased sensitivity to angiotensin II, contributing to the glomerular hypertension and sclerosis in adriamycin-induced nephrosis.

Adriamycin-induced nephrosis has been a model of chronic progressive focal glomerulosclerosis (Okuda et al, 1986). There has been a substantial evidence to support a pathogenetic role of TGF-β1 in the accumulation of extracellular matrix, leading to fibrosis and renal dysfunction in several experimental models of glomerular injury and in human kidney diseases (Yamamoto et al, 1993; Neimir et al, 1995; Wu et al, 1997). In the present study, the expression of TGF-β1 was also found to increase following the treatment with adriamycin. Angiotensin II induces TGF-β expression in a variety of cell types that may contribute to the pathogenesis of progressive renal injury (Wolf et al, 1993; Kagami et al, 1994; Ruitz-Ortega & Egido, 1997). Furthermore, the stimulation of matrix protein synthesis which accompanies angiotensin II-induced induction of TGF-β transcription can be blocked by either neutralizing TGF-β antibody or by angiotensin II receptor antagonism (Kagami et al, 1994). Similiarly, infusion of angiotensin II leads to increased TGF-β and matrix protein expression (Noble & Border, 1997), further suggesting a link between activation of RAS, TGF-β expression and extracellular matrix synthesis. Therefore, the enhanced expression of TGF-β1 can be related with an enhanced local activity of RAS in adriamycin-induced nephrosis.

The inhibition of RAS by AT1R blockers and ACE inhibitors elevates plasma renin activity (Gasc et al, 1993). Renin mRNA levels in the enalapril-treated rats are higher than in the control (Gomez et al, 1988). They may thus be an indicator of the extent of RAS inhibition. However, in the present study, the increased expression of renin mRNA was not further affected by cilazapril treatment. On the other hand, ACE mRNA expression was decreased by cilazapril. It has been known that ACE inhibitor diminishes renal ACE activity (Hirsch et al, 1992). Therefore, the decreased mRNA expression of ACE by cilazapril may account for the diminished ACE activity. Its underlying mechanisms remain to be further elucidated.

The treatment with ACE inhibitors further increased the expression of AT1R. This finding is in line with that in the previous study which showed an increased expression of AT1R mRNA in vascular smooth muscle of spontaneously hypertensive rats by the treatment with enalapril (Negoro et al, 1994). A decreased formation of angiotensin II by ACE inhibitors may have resulted in an upregulation of AT1R. On the contrary, the adriamycin-induced decrease of AT2R expression was ameliorated by cilazapril. It has been known that AT2R stimulates the formation of bradykinin, nitric oxide and cyclic GMP in the vascular smooth muscle cells (Tsutsumi et al, 1999), and been suggested that AT2R plays an inhibitory role in regulating ACE activity in the kidney in mice (Hunley et al, 2000). In this context, ACE and AT2R may be reciprocally regulated in adriamycin-induced nephrosis. The recovery of AT2R expression may

then play a role in ameliorating the adriamycin-induced renal injury. Indeed, the expression of TGF-β1 mRNA was also decreased to the control level by ACE inhibitors.

In summary, the mRNA expression of renin, ACE, and AT1R was increased in the kidney following the treatment with adriamycin. The expression of AT2R was decreased, which was restored by cilazapril. The mRNA expression of TGF-β1 was increased, which was abolished by cilazapril. The increased local RAS activity may be causally related with the adriamycin-induced nephrosis, in which AT1R is for and AT2R is against the development of nephrosis.

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