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Upregulation of Renin-angiotensin, Endothelin and C-type Natriuretic Peptide in Rat Glomerulus with Bilateral Ureteral Obstruction

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The present study was designed to investigate the effects renin-angiotensin-aldosterone system (RAAS), endothelin (ET) and local natriuretic peptide (NP) system for glomerulopathy induced in the experimental bilateral ureteral obstructive rats. Sprague-Dawley male rats (200~220 g body weight) were bilaterally obstructed by ligation of the proximal ureters for 24 hours. Control rats were treated in the same ways, except that no ligature was made. The glomeruli were isolated from cortex by graded sieve methods, and the mRNA expressions of local renin-angiotensin system (RAS), aldosterone synthase (CYP11B2), endothelin-1 (ET-1) and NP system were determined by real- time polymerase chain reaction. Following the bilateral ureteral obstruction, the mRNA expressions of renin, angiotensin converting enzyme 1 as well as ET-1 were increased, while that of angiotensin converting enzyme 2 was not changed. The expressions of CYP11B2 and angiotensin II receptors were not changed. C-type natriuretic peptide (CNP) expression was increased, while its receptors (natriuretic peptide receptor-B) were not changed. We suggest that the upregulation of local RAS and ET play a role in the progressive glomerular injury, and that the enhanced CNP activity also plays a compensatory role in obstructive uropathy in the glomerulus.

Key Words: Bilateral ureteral obstruction, Renin-angiotensin system, Aldosterone synthase, C-type natriuretic peptide, Endothelin, Glomerulus

INTRODUCTION

Deterioration of glomerular hemodynamics and decreased glomerular filtration rate are common manifestations in the obstructive uropathy, and glomerulosclerosis is one of the most common histologic findings in the ailment. These alterations may be the consequence of increased activation of vasoconstrictors such as angiotensin II (Ang II) and endothelin-1 (ET-1), (Yanagisawa et al, 1988; Yanagisawa et al, 1990; Reyes & Klahr, 1992) which are implicated in the fall of glomerular capillary plasma flow and ultrafiltration coefficient (Klahr, 1991). Circulating Ang II is the main effector of the renin-angiotensin system (RAS) and is involved in the global regulation of sympathetic activity, blood pressure as well as pathogenesis of progressive renal disease. In addition, Ang II is a renal growth factor that modulates cell growth and extracellular matrix synthesis and degradation (Ruiz-Ortega & Egido, 1997; Ruiz-Ortega et al, 2001). Thus, the role of Ang II as a vasoactive agent that participates in local and systemic hemodynamic regulation has recently been extended to include as a true cytokine to play an active role in renal pathology (Wolf & Neilson, 1993; Ruiz-Ortega et al, 2001). However, the classical view of the renin-angiotensin-aldosterone system (RAAS) has been challenged by the discovery of angiotensin converting enzyme 2 (ACE2) and aldosterone synthase (CYP11B2). Recently, ACE2 expression has been demonstrated in the glomeruli by the reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry (Li et al, 2005). ACE2 enzyme activitiy includes the degradation of both angiotensin I (Ang I) and Ang II, and the formation of Ang-(1-7), which is known to have biological effects opposite to those of Ang II (Ferrario et al, 1997; Iyer et al, 1998). In addition, CYP11B2 expression was, which is regulated by Ang II and low salt diet, demonstrated in the glomeruli by immunocytochemistry (Xue & Siragy, 2005). These findings provide evidence that a local renal autocrine or paracrine aldosterone system in close proximity influences renal structure and function. However, the role of renally produced aldosterone in the glomeruli is not clear. The changes of RAS components as well

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ABBREVIATIONS: ET-1, endothelin-1; RAAS, renin-angiotensin-aldosterone system; ACE2, converting enzyme 2; RT- PCR, reverse transcription-polymerase chain reaction; CYP11B2, aldosterone synthase; BUO, bilateral ureteral obstruction; CNP, C-type natriuretic peptide; TGF-β1, transforming growth factor-β1; AT1R, Ang-II type 1 receptor; AT2R, Ang-II type 2 receptor; MR, mineralocorticoid receptor; ECE, endothelin converting enzyme; ET_AR, endothelin $_A$ receptor; ET_BR, endothelin $_B$ receptor; NPR-B, natriuretic peptide receptor-B.

as ACE2 and CYP11B2 in the glomeruli isolated from bilateral ureteral obstruction (BUO) have not clearly been elucidated, and its causative role in the progressive glomerulopathy has not yet been completely examined.

Evidence indicates that renal ET plays a role in the process of progressive renal injury. However, the expression of ET components and their interaction with RAS have not been examined in the glomeruli of the obstructive uropathy, and more studies are needed to define the role of ET in the pathophysiology of human glomerulopathy.

C-type natriuretic peptide (CNP) was initially isolated from brain and, hence, thought to be a neurotransmitter (Sudoh et al, 1990). However, later studies detected CNP in human plasma and demonstrated its production in glomeruli among others, parts of renal vasculature as well as in the wall of large extrarenal vessels. It has been known that CNP inhibits mesangial cell proliferation and extracellular matrix expansion (Canaan-Kuhl et al, 1998). These findings suggest the existence of the glomerular natriuretic peptide (NP) system that may regulate tissue homeostasis and contribute to resolution of progressive glomerulopathy. However, the expression of CNP and its receptors has not been examined in the glomeruli isolated from BUO.

The purpose of this study was, therefore, to investigate the changes of the components in the local RAAS, ET and NP systems and its causative role for glomerulopathy in the glomerulus of experimental bilateral ureteral obstructive rats.

METHODS

Animals

Studies were performed with Sprague-Dawley male rats, weighing 200 to 220 g. Animals were kept in accordance with the Chonnam National University Guidelines of Experimental Animal Care and Use. The abdominal cavity was opened, and 2-0 silk ligature was placed at both proximal ureters under anesthesia with ketamine (50 mg/kg body weight, intraperitoneally). After closure of the abdomen, the animal were kept for 24-hours while they were given food and water ad libitum. Control rats were treated in the same ways, except that no ligature was made. Twenty-four hours later, the rats were considered to have successful ureteral obstruction, when the ureteral diameter was >2 mm and evidence of hydronephrosis was present (Kim et al, 2001a). The rats were then killed by decapitation in a conscious state. Then, the kidneys were quickly removed, and renal cortices were carefully dissected.

Isolation of RNA

The glomerulus was isolated from cortex by grade sieve methods (Torres et al, 1978; Kim et al, 2001b). In brief, the kidney was decapsulated, and the cortex was consecutively filtered through standard sieves (250, 150, 125, and 75 μm). The glomeruli on the 75 μm sieve were collected by centrifugation (1,000 $\times$ g for 15 min at 4°C). The glomerulus was homogenized in Trizol reagent (Invitrogen, Carlsbad, CA). RNA was extracted with chloroform, precipitated with isopropanol, washed with 75% ethanol, and then redissolved in distilled water. The isolated RNA was quantified spectrophotometrically.

Real-time polymerase chain reaction (PCR)

cDNA was made by reverse transcribing 5 ug of total RNA, using oligo (dT) priming and superscript reverse transcriptase II (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. cDNA was quantified using Smart Cycler II System (Cepheid, Sunnyvale, CA, USA), and SYBR Green was used for detection. Each PCR reaction contained the following final concentrations: 10 μM forward primer, 10 μM reverse primer, 2X SYBR Green Premix Ex Taq (TAKARA BIO INC, Seta 3-4-1, Japan), 0.5 μl of cDNA and H_2O to bring the final volume to 20 μl. Relative levels of mRNA were examined by real-time PCR, using a Rotor-Gene™ 3000 Detector System (Corbette research, Mortlake, New South Wales, Australia) according to the manufacturer's directions. Table 1 shows the nucleotide sequence

Table 1. Primers used in the polymerase chain reaction

Primers	Sequences
GAPDH	Sense: ATCAAATGGGGTGATGCTGGTGCTG
	Antisense: CAGGTTTCTCCAGGCGGCATGTCAG
Renin	Sense: AGGCAGTGACCCTCAACACCAG
	Antisense: CCAGTATGCAGGTCGTTCCT
ACE	Sense: GCCTCCCCAACAAGACTGCCA
	Antisense: CCACATGTCTCCCCAGCAGATG
ACE2	Sense: GGAGAATGCCCAAAAGATGA
	Antisense: CGTCCAATCCTGGTTCAAGT
AT1R	Sense: GCCAAAGTCACCTGCATCAT
	Antisense: AATTTTTTCCCCAGAAAGCC
AT2R	Sense: GGAGCGAGCACAGAATTGAAAGC
	Antisense: TGCCCAGAGAGGAAGGGTTGCC
CYP11B2	Sense: TGAGACGTGGTGTGTTCTTGC
	Antisense: GGCCTCCAAGAAGTCCCTTGC
MR	Sense: TGGATGTGTCTATCATCGTT
	Antisense: GGTCCTTCGTAGGCATAGA
TGF-β1	Sense: GGACTACTACGCCAAAGAAG
	Antisense: TCAAAAGACAGCCACTCAGG
ET-1	Sense: ATGGATTATTTTCCCGTGAT
	Antisense; GGGAGTGTTGACCCAGATGA
ECE	Sense: ATTCAAGCAGCAGACCGAGT
	Antisense: GGCTTAGACAAGATGGCTGC
ET_AR	Sense: GACCACAATGATTTTGGAGTG
	Antisense: GAACCAGCACCGCAACTCGTA
ET_BR	Sense: ACTGGCCATTTGGAGCTGAGAT
	Antisense: GACGTATGGTGAAAAGAAAGAC
CNP	Sense: CAAGAAGGGCTTGTCCAAAGG
	Antisense: AACATCCCAGACCGCTCATG
NPR-B	Sense: AACGGGCGCATTGTGTATATCTGCGGC
	Antisense: TTATCACAGGATGGGTCGTCCAAGT

GAPDH, glyceraldehydes-3-phosphate dehydrogenase; ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme2; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; CYP11B2, aldosterone synthase; MR, mineralocorticoid receptor; TGF-β1, transforming growth factor-β1; ET-1 endothelin-1; ECE, endothelin converting enzyme; ET_AR, endothelin $_A$ receptor; ET_BR, endothelin $_B$ receptor; CNP, C-type natriuretic peptide; NPR-B, natriuretic peptide receptor-B.

of primers. The real-time PCR was performed according to the following steps: 1) 95°C for 5 min; 2) 95°C for 20 s; 3) 58 to 60°C for 20 s (optimized for each primer pair); 4) 72°C for 30 s; and 5) 85°C for 6 s to detect SYBR Green (non-specific products melt at <85°C, therefore, are not detected). Steps 2~5 were repeated for an additional 64 cycles, while temperature was increased from 60 to 95°C to produce a melt curve at the end of the last cycle. The ratio of each gene and GAPDH level (relative gene expression number) was calculated by subtracting the threshold cycle number of the target gene from that of GAPDH and raising 2 to the power of this difference.

Statistical analysis

Values were presented as means±SEM. Comparisons between two groups were made by unpaired *t*-test. p values <0.05 were considered significant.

RESULTS

mRNA expression of RAAS

Fig. 1 shows the expressions of renin, ACE1 and ACE2 mRNA in the glomerulus. The expressions of renin and ACE1 mRNA were significantly increased in the glomerulus of BUO compared with that of controls, whereas ACE2 mRNA expression was not changed. The expressions of transforming growth factor-β1 (TGF-β1), CYP11B2, Ang-II type 1 receptor (AT1R), Ang-II type 2 receptor (AT2R) and mineralocorticoid receptor (MR) mRNA revealed no significant difference between BUO and control (Table 2).

mRNA expression of ET system

The abundance of ET-1 mRNA was significantly increased in the glomerulus of BUO compared with controls, but the expressions of endothelin converting enzyme (ECE) (not shown), endothelin $_A$ receptor (ET_AR) and endothelin $_B$ receptor (ET_BR) mRNA were not changed (Fig. 2).

Table 2. The expression of TGF-β1, CYP11B2, AT1R, AT2R, and MR mRNA

	Control group (n=3)	BUO group (n=4)	p values
TGF-β1	1.00±0.05	1.06±0.06	NS
CYP11B2	1.00±0.03	1.02±0.04	NS
AT1R	1.00±0.02	0.99±0.01	NS
AT2R	1.00±0.01	1.05±0.04	NS
MR	1.00±0.04	0.99±0.04	NS

Values are expressed as mean±SEM. NS, not significant. Abbreviation as in Table. 1.

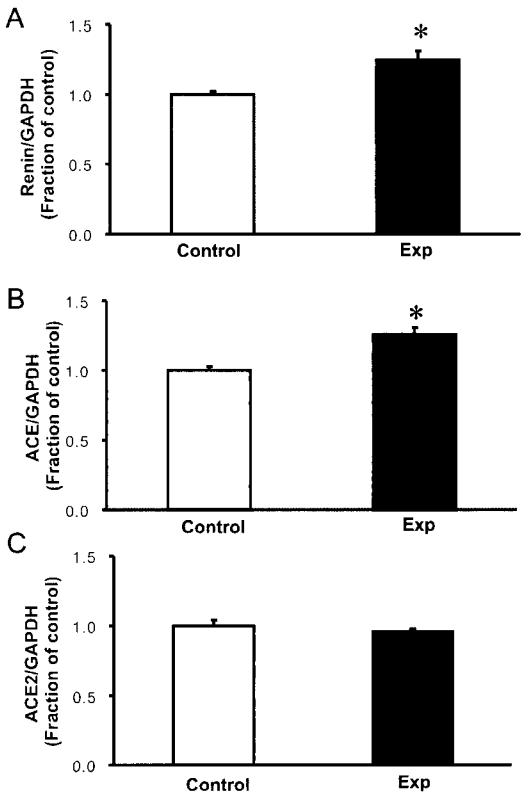

Fig. 1. Expressions of renin, ACE and ACE2 mRNA in the glomeruli of rat kidney. *p<0.05 vs. control.

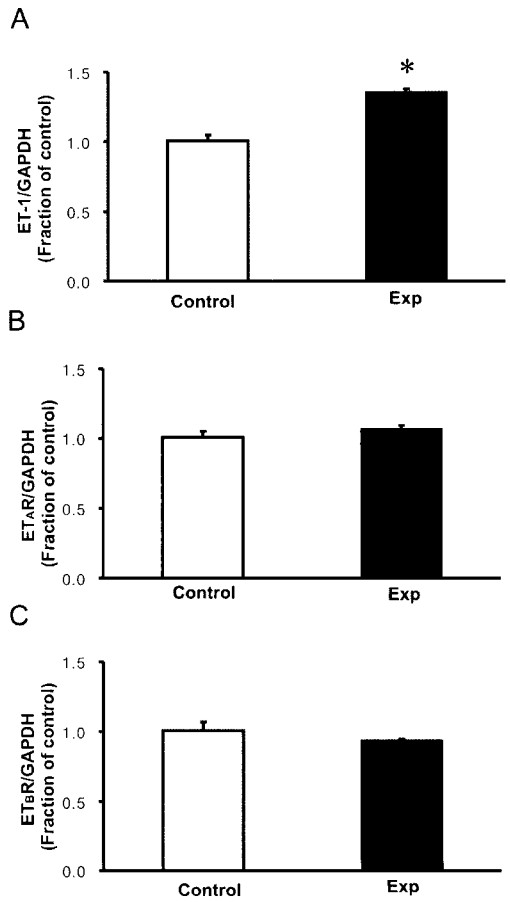

Fig. 2. Expressions of ET-1, ET_AR and ET_BR mRNA in the glomeruli of rat kidney. *p<0.05 vs. control.

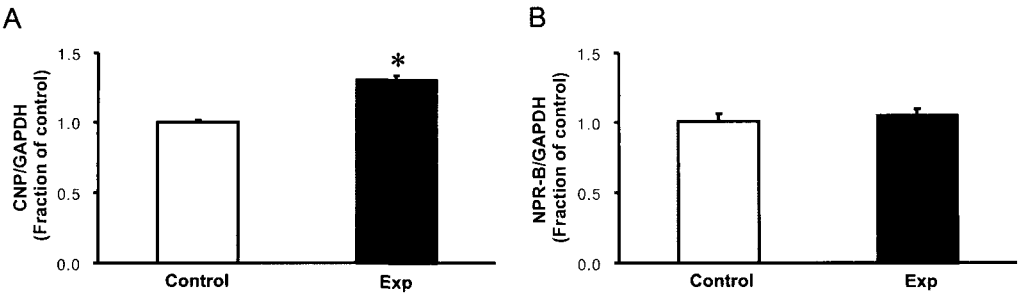

Fig. 3. Expressions of CNP and NPR-B mRNA in the glomeruli of rat kidney. *$p<0.05$ vs. control.

mRNA expression of NP system

Fig. 3 shows the expressions of CNP and natriuretic peptide receptor-B (NPR-B) mRNA in the glomerulus. The mRNA expression of CNP was increased in BUO, whereas the expression of NPR-B was not changed.

DICUSSION

The RAAS axis is involved in virtually all forms of progressive renal diseases (Remuzzi & Bertani, 1998). Regardless of the nature of the primary disease, Ang II accelerates renal scarring by mediating changes in glomerular hemodynamics and by directly affecting on renal cells. Recently, Ang II is thought to be a true cytokine and modulate cell growth and extracellular matrix synthesis and degradation (Wolf, 1993; Ruiz-Ortega & Egido, 1997; Ruiz-Ortega et al, 2001). The net result is the stimulation of synthesis and activity of profibrotic cytokine, TGF-β1, and the accumulation of extracellular matrix materials in glomeruli and renal interstitium (Ketteler et al, 1995). The present study showed that the expression of renin and ACE1 mRNA was increased, whereas other components of RAAS, including ACE2 and CYP11B2, were not changed. Ang-(1-7), which is produced by ACE2, is thought to be a peptide with anti-angiogenic and vasodilatory actions thus antagonizing the effect of Ang II (Ferrario & Chappell, 2004). Therefore, ACE2 is a critical enzyme to play a balancing role in the control of vasoactive and growth promoting activities of the RAS. CYP11B2 is the enzyme responsible for aldosterone synthesis mainly in the adrenal gland, however it has recently been reported that CYP11B2 is expressed in kidney and can produce aldosterone locally (Xue & Siragy, 2005). Aldosterone also stimulates cellular hypertrophy, matrix formation and cell death, therefore, it is highly possible that it plays an important role in glomerulosclerosis (Xue & Siragy, 2005). In the present study, we demonstrated the expression of CYP11B2 in the glomerulus, however, it was not affected by ureteral obstruction. Thus, changes of ACE2 and CYP11B2 gene transcription rate appear to be not implicated in the glomerulopathy in bilateral obstructive uropathy. Nevertheless, it should be noted that the present study was not carried out for long enough to examine changes during entire time course in the glomerulopathy of BUO. Thus, it is quite possible that changes of ACE2 or CYP11B2 activity may play a role in the glomerulopathy at a later stage. In the present study, CYP11B2 mRNA expression was found to be not changed. Nevertheless, this study can still be valuable, since expression of CYP11B2 in the glomerulus, which was previously demonstrated by a single study (Xue & Siragy, 2005), was confirmed.

Ang II stimulates directly renal ET-1 expression, at least in mesangial and glomerular endothelial cells (Bakris & Re, 1993; Wolf et al, 1996). Thus, there appears to be a close interaction between the RAS and ET-1. In the present study, ET-1 expression was found to increase. Therefore, it is possible that Ang II stimulates renal ET-1, in agreement with a previous study (Bakris & Re, 1993; Wolf et al, 1996). Renal vessels are peculiarly sensitive to the vasoconstrictive effect of ET-1, which is infused in the kidney, and decreases renal blood flow and glomerular filtration rate. This effect, together with ET-1 to induce contraction and proliferation of mesangial cells as well as accumulation of mesangial matrix proteins, suggest that ET-1 may participate in the renal events that lead to renal disease progression. However, the effect of ET-1 on TGF-β1 expression in renal cells is not known. Because ET-1 is known to regulate TGF-β1 in extrarenal cells, such as fetal skin-derived cultured mast cells (Matsushima et al, 2004) and cardiac cells (Ammarguellat et al, 2001; Shimojo et al, 2006), it is likely that ET-1 also regulates TGF-β1 expression in renal cells. However, the present study showed that the expression of TGF-β1 was unchanged, even though expressions of Ang II and ET-1 mRNA were increased. This suggests that TGF-β1 expression was not changed at a transcriptional level, however, it is still possible that the translation rate was involved or 24-hours of experiment was too short to induce the change of TGF-β1 expression. Increased activity of CNP may also contribute, as discussed below.

CNP is produced mainly in the endothelial cells (Barr et al, 1996), and exerts it's biological actions via the NPR-B and induction of the second messenger cyclic guanosine monophosphate (cGMP). In vitro, NPR-B receptors as well as CNP-inducible cGMP production were demonstrated in glomerular mesangial cells and epithelial cells (Zhao et al, 1994) and is thought to act in a local, paracrine fashion to regulate vascular smooth muscle tone and proliferation. Natriuretic peptides can inhibit the secretion of Ang II (Zhao et al, 1994) and ET-1 (Motwani et al, 1993) in heart, therefore, it has been suggested that CNP inhibits Ang II and ET-1 mediated mRNA expression of TGF-β during acute stage of BUO, as in heart. In addition, CNP substantially inhibits vasoconstriction of the renal vascular bed which is usually observed after ureteral obstruction, and

reduces the glomerular filtration rate and the effective renal plasma flow (Hsu et al, 1977; Purkerson & Klahr, 1989). Locally synthesized CNP in the vasculature may then compensate for the hypertension induced by fluid volume retention. Vasodilators, such as atrial natriuretic peptide, have early been shown to inhibit tubular transport, matrix synthesis, and cell growth (Wolf, 1993), therefore, CNP could be viewed as antagonists of vasoconstrictors such as Ang II and ETs, even for the non-hemodynamic effects on tubular function.

In summary, mRNA expressions of renin, Ang II and ET-1 were increased, but TGF-β1 expression was not changed in the glomerulus of rats with BUO, which may play a role in the glomerulopathy. Increased mRNA expression of CNP may play a compensatory role in the acute phase of progressive glomerulopathy.

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