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Increased urinary C-type natriuretic peptide excretion may be an early marker of renal tubulointerstitial fibrosis

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ABSTRACT

Although recent major advances have developed a much better understanding of the pathophysiological pathways, tubulointerstitial fibrosis (TIF) is still currently incurable. Therefore, early detection may mean that the condition is more manageable than it was in the past. C-type natriuretic peptide (CNP) has been found to be a potent vasodilator but a weak natriuretic factor. In addition, CNP has also been believed to be produced in tubular cells and presented as a local modulator with anti-inflammatory and anti-proliferative effects. Elimination of CNP occurs by three main mechanisms, neutral endopeptidase, natriuretic peptide receptor-C and urinary excretion. Among them, the status of urinary CNP excretion in nephropathies is not yet fully elucidated. In the present study, subgroups of rats were subjected to unilateral ureteral obstruction (UUO) or sham operation and observed for 24 h to 3 months. Urinary CNP excretion was significantly enhanced in UUO rats from 24 h to 1 month post-ligation compared to sham-operated rats. Urinary CNP excretion was also markedly higher than CNP concentrations both in abdominal aorta and in renal vein, and almost identical concentrations in these two vessels excluded major renal extraction of circulating CNP of systemic origin. Urinary CNP excretion was negatively correlated with urinary protein concentration, blood urea nitrogen and creatinine, while positively correlated with albumin. In conclusion, the increased urinary CNP excretion is strongly associated with TIF progression, and may serve as an early marker of TIF.

1. Introduction

Tubulointerstitial fibrosis (TIF) is a final common pathway leading to end-stage renal failure (ESRF), characterized by excess accumulation of extracellular matrix (ECM) and irreversible loss of renal function [18]. According to an epidemiological survey of 2,000,000 residents in Nanjing, China, TIF is the third-leading cause of ESRF [14]. Recent major advances have developed a much better understanding of the pathophysiological pathways involved in the initiation of TIF. Proteinuria, hypoxia, oxidative stress and many other factors which are induced during pathological conditions can stimulate pro-inflammatory and pro-fibrotic signaling in tubular cells [10,17]. However, TIF is still currently incurable, except for renal replacement. Therefore, early detection may mean that the condition is more manageable than it was in the past.

C-type natriuretic peptide (CNP), primarily isolated from central nervous tissues and endothelial cells, has only moderate natriuretic actions compared with the other natriuretic peptides and acts mainly as a vasodilating agent [24]. Currently, CNP is believed to be produced locally in tubular cells and glomeruli of normal human kidney [29]. CNP specifically binds to the transmembrane natriuretic peptide receptor-B, resulting in the synthesis of intracellular cyclic guanosine monophosphate. Elimination of CNP occurs by three main mechanisms, neutral endopeptidase, natriuretic peptide receptor-C and urinary excretion [13]. Among them, the status of urinary CNP excretion in nephropathies is not yet fully elucidated.

2. Materials and methods

2.1. Animals and treatment

Male Wistar rats weighting 190–250 g were used in the present study. All animal experimentation was performed at the animal facility within the Preclinical Medicine Institute of Anhui Medical University. The procedures and protocols were approved by the Institutional Animal Care and Use Committee. 96 rats were separated into 16 experimental groups: 8 groups undergoing left proximal unilateral ureteral obstruction (UUO) ($n = 6$) and 8 groups with sham-operated rats (SOR) ($n = 6$). The fasted animals were

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operated under intraperitoneal pentobarbital anesthesia (60 mg/kg body weight) and sterile conditions. UUO rats underwent left proximal ureteral ligation with 4–0 silk at the junction of the upper with the two lower thirds of its length. The ureter was cut between the ligatures to prevent retrograde urinary tract infection. SOR underwent a sham laparotomy with ureteric manipulation through a midline incision. No antibiotics were given. Animals were anesthetized by intraperitoneal pentobarbital injection and sacrificed by heart puncture at 24 h, 72 h, 1 w, 2 w, 3 w, 1 m, 2 m and 3 m post-ligation, respectively. The obstructed kidneys of UUO rats and the left kidneys of SOR were harvested.

2.2. Renal morphology

At harvest, each kidney was washed with saline, blotted dry on gauze, and weighed. Whole kidney weight was expressed as a percentage of body weight determined at the time when rats were euthanized. Estimation of cortical thickness was done by measuring the distance from renal capsule to corticomedullary junction. Midcoronal kidney sections were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin sections (4 μm thick) were stained with hematoxylin and eosin, and examined independently by two pathologists blinded to the experimental design.

2.3. Laboratory analysis

All rats were placed in metabolic cages and urine was collected for 24 h. Blood samples were taken from abdominal aorta on the same time points. Urinary protein concentration (UP) from 24 h urine sample was determined by biuret colorimetric method (Boehringer Mannheim, Italy). Serum total protein (TP), albumin (Alb), blood urea nitrogen (BUN), and creatinine (Cr) were measured by standard enzymatic method (Randox, UK).

2.4. Radioimmunoassay

CNP immunoreactivities from samples of urine, abdominal aorta and renal vein were determined by a radioimmunoassay (cross reactivity to human ANP, BNP and DNP <1%; Phoenix Pharmaceuticals, USA) after extraction as previously described [6]. Blood samples were collected in disodium EDTA vacutainers containing aprotinin (500 KIU/mL of blood) and centrifuged at 1600 × *g* for 15 min at 4 °C. Plasma and urine samples were stored at −80 °C until assayed for CNP. 2 mL plasma or 5 mL urine were passed through Sep-Pack C18 cartridges and eluted with 5 mL 60% acetonitrile containing 0.1% trifluoracetic acid. The eluate was lyophilized and reconstituted for radioimmunoassay. Duplicate samples were tested for radioimmunoassay. Serial dilutions (1/1, 1/2, 1/4, 1/8, 1/16) of plasma and urine samples were subjected to CNP immunoreactivities. The correlation coefficients between the levels of CNP immunoreactivities and the degree of dilution were $r = 0.98$ and $r = 0.99$. The intra- and interassay coefficients of variation were 5.0% and 10.0% for plasma CNP immunoreactivities, and 6.0% and 11.0% for urine CNP immunoreactivities. The sensitivity and specificity were found to be 97.2% and 96.5%, respectively.

2.5. Statistical analyses

All values are expressed as mean ± SEM. Comparison of mean values between groups was made using one way ANOVA, and post hoc analysis was calculated using the Student–Newman–Keuls test. Correlations between variables were assessed by linear regression. A value of $P < 0.05$ was considered significant. Statistical analysis was performed using the statistical package for social studies SPSS version 11.5.

3. Results

Representative histology images of the kidneys obtained from SOR and UUO rats are presented in Fig. 1. All morphological lesions observed in the obstructed kidneys became more aggravated in time. After 24 h of UUO, glomerular and tubulointerstitial morphologies were almost normal. After 72 h of UUO, renal damage was limited to a reduction of peritubular capillaries, tubular atrophy and widen interstitial space, accompanied by an inflammatory cell infiltration in the interstitium. At 1, 2 and 3 weeks post-obstruction, all lesions observed at earlier time point worsened and increased ECM deposition became a prominent feature. Atrophic tubules were surrounded by a thickened, wrinkled basement membrane. Glomerular damage was limited to thickening of Bowman's capsule. At 1, 2 and 3 months after UUO, ECM deposition increased further and the interstitial space of the obstructed kidneys was populated by numerous fibroblasts. However, section derived from sham-operated kidneys had a normal appearance.

Measurement of renal anatomy is shown in Fig. 2. There were two intersections of the ratio of kidney weight to body weight (KW/BW) between SOR and UUO rats. KW/BW was significantly raised in UUO rats at 24 h, 72 h, 2 months, and 3 months post-ligation ($P < 0.05$), whereas decreased in UUO rats at 2 weeks post-ligation ($P < 0.05$), in comparison to their sham-operated counterparts. As compared with rats at 24 h after UUO, KW/BW was significantly lower in UUO rats at 1 week, 2 weeks, 3 weeks, and 1 month post-ligation respectively ($P < 0.05$). UUO rats exhibited significantly lower cortical thickness than those in SOR at 1 week, 2 weeks, 3 weeks, 1 month, 2 months, and 3 months post-ligation respectively ($P < 0.05$). In addition, the cortical thickness decreased time-dependently in the obstructed kidneys, and these differences reached statistical significance from 1 week to 3 months post-obstruction when comparing with 24 h after UUO ($P < 0.05$).

Biochemical parameters in all groups are shown in Table 1. At the time of sacrifice, UP levels were raised gradually, but significantly, in UUO rats from 72 h to 3 months post-ligation in comparison to their sham-operated counterparts ($P < 0.05$). Although no significant differences in serum TP were observed between SOR and UUO rats at all time points ($P > 0.05$), Alb was obviously decreased in UUO rats at 3 weeks, 1 month, 2 months, and 3 months post-ligation respectively ($P < 0.05$). UUO rats exhibited significant higher BUN than those in SOR from 2 weeks to 3 months post-ligation ($P < 0.05$), and significant higher Cr than those in SOR from 1 week to 3 months post-ligation ($P < 0.05$).

The mean CNP concentrations from urine, abdominal aorta and renal vein of SOR were 1.13 pmol/L, 1.11 pmol/L and 1.17 pmol/L at 24 h post-ligation, respectively. In comparison to SOR, urinary CNP excretion was significantly raised in UUO rats at 24 h, 72 h, 1 week, 2 weeks, 3 weeks and 1 month post-ligation ($P < 0.05$). CNP immunoreactivities from urine, abdominal aorta and renal vein of UUO rats are shown in Fig. 3. The mean urinary CNP excretion was markedly increased during the period from 24 h to 1 month post-ligation compared to CNP concentrations both in abdominal aorta and in renal vein ($P < 0.05$). At 24 h post-ligation, the mean urinary CNP excretion was significantly higher in UUO rats than that in SOR (3.96 pmol/L *vs.* 1.13 pmol/L, $P < 0.05$). In addition, urinary CNP excretion of UUO rats progressively declined over time and initially reached significance at 1 week after UUO ($P < 0.05$). To further investigate the origin of increased urinary CNP excretion, simultaneous determinations of CNP concentrations in abdominal aorta and renal vein were performed. Almost identical concentrations in these two vessels excluded major renal extraction of circulating CNP of systemic origin ($P > 0.05$). Urinary CNP excretion also significantly enhanced in UUO rats from 24 h to 1 month post-ligation compared to their sham-operated counterparts (data not shown,