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Expression of Endothelin-1 and Its Receptors in Cisplatin-Induced Acute Renal Failure in Mice

Seokwoo Lee and Dowhan Ahn

Department of Physiology, Kosin University College of Medicine, Busan 602-703, Korea

Endothelin-1 (ET-1) is unequivocally elevated in the kidney with ischemic acute renal failure (ARF), whereas ET receptors (ET_AR and ET_BR) are variably expressed. Although renal functional and structural changes are similar between ischemic and nephrotoxic ARF, there are few reports on the alteration in the ET system in nephrotoxic ARF. This study was, therefore, undertaken to investigate changes in renal expression of ET-1 and its receptors in nephrotoxic ARF induced by cisplatin. Mice were intraperitoneally injected with 16 mg of cisplatin/kg at a single dose, and the expression of mRNA and protein was then quantified by real-time RT-PCR and Western blot, respectively. Immunohistochemistry was conducted for localization. Three days after treatment, ET-1 transcript in cisplatin-treated mice was thirteen times higher than that in controls, whereas ET-1 peptide was increased by 1.5-fold. Cisplatin caused a 2-fold increase in the levels of ET_AR mRNA and protein. Most of the increased immunoreactive ET-1 and ET_AR were localized in damaged tubules. Neither the expression of ET_BR mRNA nor the abundance and immunoreactive level of ET_BR protein were changed. The findings suggest that the individual components of the renal ET system are differentially regulated in cisplatin-induced nephrotoxic ARF.

Key Words: Cisplatin, Acute renal failure, Endothelin, Endothelin receptor

INTRODUCTION

The endothelin (ET) system includes ET isotypes (ET-1, ET-2, ET-3), ET converting enzyme (ECE), and its receptors (ET_AR, ET_BR). ET exerts its effects by binding to the cell-surface ET receptors, which belong to G protein-coupled receptor. Vasoconstriction, mitogenesis, and anti-apoptosis are mediated through ET_AR, whereas vasodilation, Na and water balance, ET clearance, and inflammatory pain through ET_BR (Nelson et al, 2003). Evidence indicates that ET plays a role in the pathophysiology of various forms of renal disease, including renal hypertension (Hoffman et al, 1994; Ahn et al, 2004), renal fibrosis (Chatziantoniou & Dussaule, 2005), transplant rejection (Braun et al, 1999) and renal failure (Takaoka et al, 2000).

Acute renal failure (ARF) is characterized by an abrupt and reversible kidney dysfunction. Two spectra of ARF from intrarenal origin are ischemic and nephrotoxic. Ischemic ARF primarily affects renal hemodynamics with afferent arteriolar vasoconstriction. Reduced renal blood flow increases vasoconstrictors such as ET and angiotensin II, and decreases vasodilators such as nitric oxide and prostaglandin I-2 (Sheridan & Bonventre, 2001). The imbalance in intrarenal vasoactive hormones causes persistent intrarenal hypoxia, thereby exacerbating tubular injury (Sheridan & Bonventre, 2001). Nephrotoxic ARF usually causes primary tubular injuries, and an initial decrease in RBF is not necessary for the development of renal functional impairment as in ischemic ARF. Rather, a specific cellular insult may precede in this type of ARF. Depending on nephrotoxicants, injury sites may be glomeruli, proximal tubules, medulla, renal vessels, and interstitium. Tubular dysfunction by nephrotoxicants leads to reduced Na reabsorption, backleak of glomerular filtrate, and tubular obstruction. Both types of ARF result in reduced glomerular filtration, tubular cell necrosis and apoptosis (Stein et al, 1978; Schellmann & Kelly, 1999). Therefore, ischemic and nephrotoxic ARF are likely to show similar functional and histological changes.

Although renal expression of ET-1 in ischemic ARF is elevated in most cases, that of ET_AR and ET_BR varies depending on ischemic models and investigators (Roubert et al, 1994; Shimizu et al, 1998; Forbes et al, 2001). Prior to evaluating the usefulness of ET receptor antagonists, we examined the expressions of renal ET-1, ET_AR and ET_BR in nephrotoxic ARF. For that purpose we used cisplatin, which is well-known to cause nephrotoxic ARF (Stein et al, 1978; Ramesh & Reeves, 2002).

METHODS

Cisplatin administration and kidney preparation

C57BL/6 male mice of 10 to 11 week ages (20～25 g) were

Corresponding to: Dowhan Ahn, Department of Physiology, Kosin University College of Medicine, 35, Amnam-dong, Seo-gu, Busan 602-703, Korea. (Tel) 82-51-990-6415, (Fax) 82-51-990-3081, (E-mail) dwahn@kosin.ac.kr

ABBREVIATIONS: ET_AR, endothelin A receptor; ET_BR, endothelin B receptor; ECE, endothelin-converting enzyme; ARF, acute renal failure.

used and had free access to food and water. The animals received a single intraperitoneal injection of cisplatin (16 mg/kg, body weight) or saline. Seventy-two hrs after the treatment, mice were anesthetized with ketamine (50 mg/kg) and zylazine (16 mg/kg). Blood was drawn from cardiac puncture and then mice were sacrificed with cervical dislocation. Right kidneys and half of the left kidneys were frozen in liquid nitrogen and stored at −80°C for RNA or protein isolation. The rest of left kidneys were fixed in 10% buffered saline, embedded in paraffin, sectioned at 4 um, and stained with Periodic acid-Shiff (PAS). Plasma creatinine concentration and blood urea nitrogen (BUN) were measured by an enzymatic method using commercially available kits (Asanpharm Co., Seoul, Korea).

Tissue ET-1 level

One preweighed kidney was immersed in a 2 ml solution containing 0.2 M acetic acid and 0.5 M NaCl and then homogenized for 2×10 sec on ice. The homogenate was centrifuged at 30,000 rpm for 30 min., and the supernatant was saved. A BondElut C18 column (Varian, Harbor City, CA, USA) was pre-equilibrated with 3 ml of 60% acetonitrile in 0.1% trifluoroacetic acid (TFA) and washed four times with 0.1% TFA. After applying the supernatant to the column, the column was eluted with 3×3 ml of pre-equilibration solution. The eluant was kept at −70°C and then freeze-dried overnight. ET-1 was measured using EIA kit (IBL, Gunma, Japan) according to the supplier's instruction.

Real-time RT-PCR

Total RNA was extracted using RNAqueous-4PCR kit (Ambion, Austin, TX, USA) according to manufacturer's directions and treated with DNAse I included in the kit. After spectrophotometric determination of total RNA concentrations, reverse transcription was performed in a total volume of 40 ul containing 0.5 to 1 ug each of RNA and oligo (dT) using SuperScript II first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). PCR was run in a ABI Prism 7000 system with a 25 ul reaction which contained 5 ul of 5-fold diluted cDNA, 1 nM primer, and 2× SYBR II master buffer (Applied Biosystems, Foster City, CA, USA). Reaction conditions are: 95°C for 10 min followed by 45 cycles at 95°C for 15 sec and 60°C for 1 min. The relative mRNA expression was calculated using a comparative delta-delta method as described in ABI user bulletin #2. Relative PCR efficiencies of targets (ET-1, ET_AR, and ET_BR) and reference (β-actin) were approximately equal. Primers were generated using Primer 3 software (Rosen S and Skaletsky HJ, Whitehead Institute, MIT). All of them were designed to include one or two introns and their sequences are presented in Table 1.

Western blot

The frozen kidney was thawed, and the entire kidney except the papillary region was homogenized in mannitol buffer (300 mM mannitol, 10 mM HEPES/Tris, 1 mM EDTA, pH 7.4) with 1× protease inhibitor cocktail (Sigma, St Louis, MO, USA). Protein concentration was determined using Bio-Rad assay kit (Hercules, CA, USA). For Western blot, an aliquot of homogenates was electrophoresed and then transferred to PVDF membranes (Amersham Pharmacia, Alington Height, IL, USA). The blot membranes were incubated overnight at 4°C with either 1 : 200 dilution of rabbit anti-ET_AR antibody (Alomone labs, Jerusalem, Israel) and mouse monoclonal anti-ET-1 antibody (Sigma, St Louis, MO, USA) or 1 : 500 dilution of rabbit anti-ET_BR antibody (Alomone labs, Jerusalem, Israel). After washing the membranes, HRP-conjugated secondary antibody was added and signals were detected using ECL kit (Amersham Pharmacia, Alington Height, IL, USA). To confirm equal loading of sample proteins, blots were stripped for 30 min at 50°C in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) and then reprobed with primary antibody to β-actin. The intensity of bands was quantified and normalized to β-actin.

Immunohistochemistry

The kidneys were perfusion-fixed with 4% paraformaldehyde and postfixed overnight in the same solution. Paraffin sections were mounted on probe-on slides (Fisher Scientific Korea, Seoul, Korea), deparaffinized, hydrated, and treated with 3% H_2O_2 for 6 min to inhibit intrinsic H_2O_2 of the tissue. H_2O_2 was removed by extensive washing in immunoassay buffer (Biomeda, Fostar City, CA, USA). The tissue sections were then treated with blocking buffer (Zymed, South San Francisco, CA, USA) for 15 min and incubated with the primary antiserum of ET-1 (1 : 40), ET_AR (1 : 20), or ET_BR (1 : 40) as instructed by the manufacturer (HistoMouse-Max kit, Zymed, South San Francisco, CA, USA). The immunoreactive signals were observed through 3-amino-9-ethylcarbazole staining.

Table 1. Primer sequences for real time RT-PCR

Gene		Sequence	Size	GenBank
ET-1	F	5'-ACT TCT GCC ACC TGG GCA TC-3'	199	NM 010104
	R	5'-ACT TTG GGC CCT GAG TTC TT-3'		
ET_AR	F	5'-ACG GTC TTG AAC CTC TGT GC -3'	263	NM 010332
	R	5'-AGC CAC CAG TCC TTC ACA TC -3'		
ET_BR	F	5'-AGC TGG TGC CCT TCA TAC AG-3'	231	NM 007904
	R	5'-GGG GCT TTC CTT TGT AGT CC-3'		
β-Actin	F	5'-GAC GGC CAG GTC ATC ACT AT-3'	216	NM 007393
	R	5'-CTT CTG CAT CCT GTC AGC AA-3'		

ET-1, endothelin-1; ET_AR, endothelin A receptor; ET_BR, endothelin B receptor.

Statistical analysis

Data are expressed as mean±SD. Unpaired Student's t-test was performed and a p value less than 0.05 was considered statistically significant.

RESULTS

Induction of ARF

As shown in Table 2, plasma creatinine and BUN were significantly increased at 72 hr after cisplatin treatment, indicating deterioration of renal function. PAS staining showed that cisplatin administration caused necrosis of proximal tubules and produced tubular luminal casts, however, no effects on glomeruli, distal tubules, and medullary regions (data not shown).

Changes in transcript and protein levels

Quantitative real-time PCR was performed to follow changes in mRNA expression of the ET system. Fig. 1 depicts a relative renal expression of ET-1, ET_AR, and ET_BR mRNAs. Cisplatin administration upregulated ET-1 mRNA by 13-fold and ET_AR mRNA by 2-fold compared with controls. However, the expression of ET_BR mRNA was not different between cisplatin-treated mice and controls. To examine whether the changes in mRNA level are correlated with protein expression, ELISA and Western blot were conducted. Fig. 2 shows that the content of ET-1 peptide in kidney homogenates was increased by 1.5-fold after cis-

Table 2. Changes in BUN and plasma creatinine concentration

	Control	Cisplatin
BUN (mg/dl)	18.3±4.0	107±31.6*
$P_{creatinine}$ (mg/dl)	0.72±0.07	1.11±0.16*

Blood was obtained 72-hr after saline or cisplatin (16 mg/kg) injection. Values are mean±SD of 6 animals. *p < 0.05 vs. control

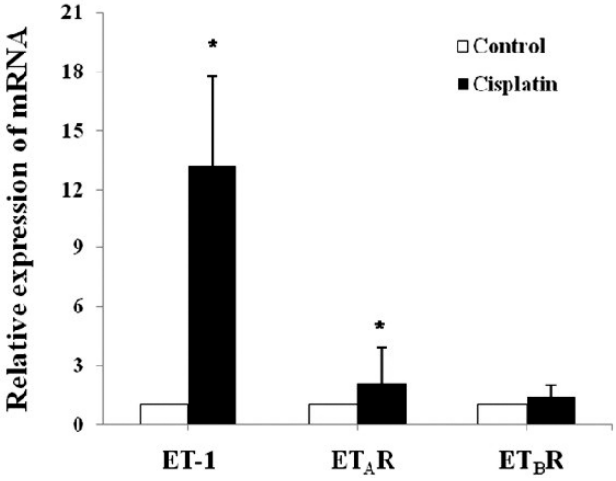

Fig. 1. Relative expression of ET-1, ET_AR, and ET_BR genes in the kidney of the control and cisplatin-treated mice. Real-time RT-PCR was performed as described in Materials and Methods. Relative expression was calculated by a comparative C_T method. Values are mean±SD of 3 separate experiments. *p < 0.05 vs. matched control.

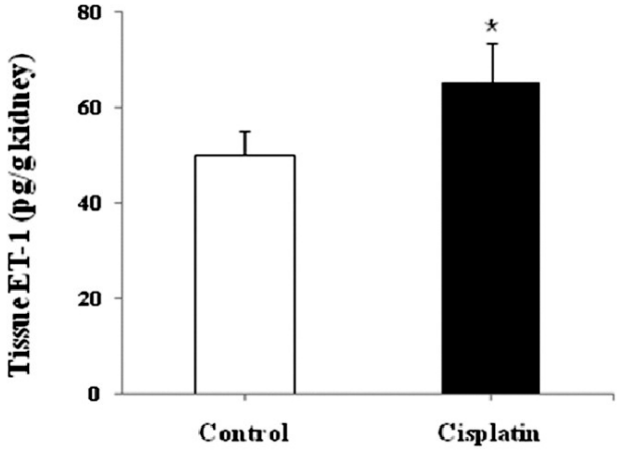

Fig. 2. ET-1 contents in kidney homogenates of the control and cisplatin-treated mice. Values are mean±SD of 6 animals. *p < 0.05 vs. control.

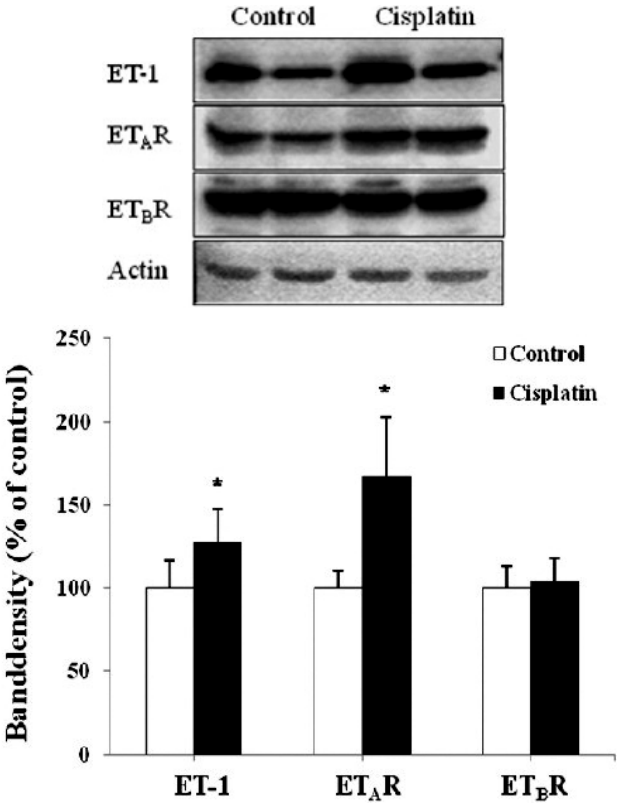

Fig. 3. Representative immunoblots (upper panel) and densitometric analysis (lower panel) of ET-1, ET_AR, and ET_BR proteins. Each blot was loaded with 50 ug of protein. Band density was normalized to β-actin. Values are mean±SD of 3 separate experiments. *p < 0.05 vs. respective control.

platin treatment ($p < 0.05$). Similar increase was also observed in Western blot (Fig. 3). The primary antibody to ET-1 recognized the band at about 21 kDa, which is the expected size of unprocessed preproET-1 (Gumz et al, 2003). ET_AR expression was increased by 1.7-fold, but ET_BR expression was not different between cisplatin- treated mice and controls.

Localization of immunoreactivity

To examine the localization of protein expression of the ET system, immunohistochemistry was applied (Fig. 4). Immunoreactive ET-1 was strongly detected in some of proximal tubules and vessels, but not in glomeruli. Although it was not as intense as in controls, ET-1 expression was more diffuse in cisplatin-treated mice than in controls. The ET_AR immunoreactivity showed a distribution similar to ET-1 and tended to increase in cisplatin-treated mice. It was predominantly localized in damaged proximal tubules, but was sparsely detected in peritubular capillaries and arteries. In spite of its relatively high background, ET_BR immunoreactivity was not changed by cisplatin treatment.

DISCUSSION

Nephrotoxic ARF is usually caused by heavy metals, anticancer agents, and aminoglycoside antibiotics. There are few reports on the expression of the ET system in nephrotoxic ARF. In cisplatin-induced nephrotoxic ARF, we found that renal damages were confined to the cortex, and ET-1 and ET_AR were up-regulated without change of ET_BR. Yanagisawa et al (1993) showed that $HgCl_2$ treatment increased ET-1 mRNA in the kidney. Elevated ET-1 expression has been also found in other renal diseases such as ischemic ARF (Roubert et al, 1994; Shimizu et al, 1998; Forbes et al, 2001), chronic transplant rejection (Deng et al, 2000), polycystic kidney disease (Ong et al, 2003), mesangial proliferative nephritis (Yoshimura et al, 1995), and aminonucleoside-induced nephrosis (Nakamura et al, 1995). ET-1 directly inhibits Na-K-ATPase of renal tubular cells (Garvin & Sander, 1991) and modulates a variety of signal transduction pathways through its receptors as well. Therefore, while there is little doubt that ET-1 plays a certain role in the pathogenesis of renal diseases, it is not yet clear whether ET-1 acts as a causative factor in various renal diseases including ARF. An unusual finding in the present study was that ET-1 mRNA was increased in cis-

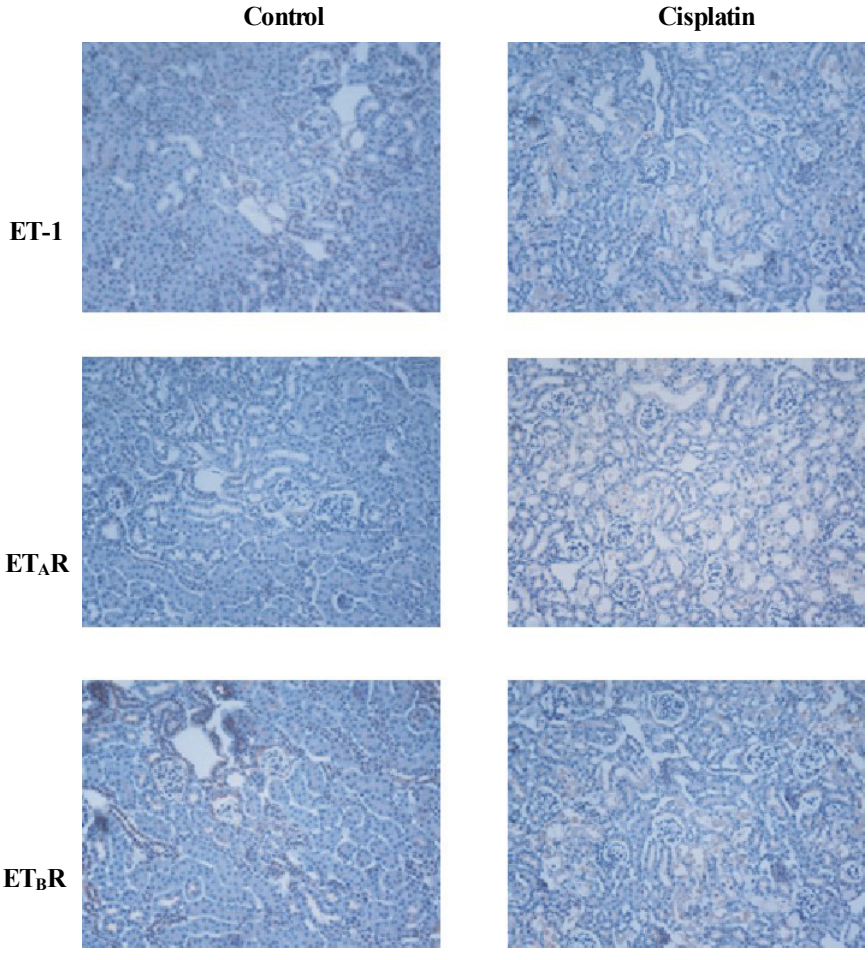

Fig. 4. Immunostaining for ET-1, ET_AR, and ET_BR in the renal cortex of control and cisplatin-treated mice. ET-1, ET_AR, and ET_BR are diffusely expressed in tubular segments. Note that intense immunostaining for ET-1 and ET_AR is found in the cisplatin- treated group. Magnification: ×100.

platin-treated mice by 13-fold compared with controls, whereas ET-1 peptide was increased by less than 2-fold (Figs. 1 and 2). Since ET-1 gene is known to be transcriptionally regulated, such a discrepancy remains to be clarified.

Although ET-1 exerts its action through ET_AR and/or ET_BR, expression of ET receptors varies depending on ischemic ARF models used. In ischemic ARF induced by renal artery occlusion, Nambi et al (1993) showed that the affinity of ET receptor to ET-1 increased, accompanied with no change in the number of ET_AR, whereas Forbes et al (2001) demonstrated that the level of ET_AR decreased in the cortex. In glycerol-induced ischemic ARF, in which the pronounced fall of renal blood flow has been considered to be an important pathogenic event, ET_AR mRNA in the cortex was up-regulated in one study but not in the other study (Roubert et al, 1994; Shimizu et al, 1998). In our model of nephrotoxic ARF, ET_AR mRNA and protein was up-regulated by 1.5 to 2.0-fold compared with controls. Considering that ET_AR mediates vasoconstrictive action of ET-1, an increase in ET_AR expression could have reduced blood supply to the cortex, thus leading to aggravate the renal injury. It is, however, interesting to note that administration of selective ET_AR antagonists has partly ameliorated renal tubular injuries and functions, in spite of decreased expression of ET_AR in ischemic ARF (Gellai et al, 1994; Birck et al, 1998; Kuro et al, 2000).

ET_BR level has been shown to be reduced or increased in ischemic ARF (Roubert et al, 1994; Shimizu et al, 1998; Forbes et al, 2001). The present study showed that both ET_BR mRNA and protein expression levels were not different between controls and cisplatin-treated mice. Using a similar ET_BR antibody that we have used, Francis et al (2004) reported that ET_BR was detected mainly in tubular structures and was preferably expressed in the inner medulla. We found that the immunoreactivity of ET_BR was much greater in the inner medulla than in the cortex and its immunoreactivity in the medulla seemed to be not different between the two groups (data not shown). Because renal damages by cisplatin administration was mainly confined to proximal tubules of the cortex, we speculate that ET_BR expression would be least affected, if any.

In conclusion, this study demonstrated that the expressions of ET-1 and ET_AR, but not ET_BR, were increased, suggesting that the individual components of the renal ET system are differentially regulated in cisplatin-induced nephrotoxic ARF. Therefore, drug targeting to ET_AR could help lessen nephrotoxic ARF induced by cisplatin.

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