Intranephron Distribution and Regulation of Endothelin-Converting Enzyme-1 in Cyclosporin A-Induced Acute Renal Failure in Rats

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Abstract. Endothelin-1 (ET-1) is thought to play a significant role in acute renal failure induced by cyclosporin A (CsA). The cDNA sequence encoding endothelin-converting enzyme-1 (ECE-1), which produces the active form of ET-1 from big ET-1, was recently reported. To elucidate the role of ECE-1 in the glomerular and tubular dysfunction induced by CsA, the effects of CsA on mRNA and protein expression of ECE-1 in rat kidney and on mRNA expression of prepro-ET-1 and ET A- and B-type receptors in glomeruli were studied. ECE-1 mRNA was detected in glomeruli and in whole nephron segments. ECE-1 mRNA expression was downregulated in all nephron segments at 24 h after CsA injection. Protein levels were also downregulated in glomeruli and in the outer and inner medulla. CsA rapidly increased prepro-ET-1 mRNA expression in glomeruli at 30 to 60 min after injection; this rapid increase was followed by an increase in plasma ET-1 levels. These increases were followed by decreased expression of ECE-1, ET A-type receptor, and ET B-type receptor mRNA at 6 h after injection, and serum creatinine levels were increased at 24 h after CsA injection. It is suggested that downregulation of glomerular and tubular ECE-1 expression may be caused by increased ET-1 synthesis in CsA-induced acute renal failure.

Cyclosporin A (CsA) is widely used as an effective immunosuppressive agent to prevent allograft rejection and to treat several autoimmune diseases. However, the therapeutic potential of CsA is limited by a significant complication, i.e., nephrotoxicity. It is now established that CsA-induced renal dysfunction consists of two phases: the acute phase and the chronic phase. The acute phase is functional and reversible, whereas the chronic phase is irreversible (1). However, the precise mechanisms of CsA nephrotoxicity have not yet been clarified. Recent studies indicated that the renal dysfunction of the acute phase was caused mainly by vasoconstriction (2,3). It was suggested that some vasoconstrictive factors, including the renin-angiotensin system, the sympathetic nervous system, and thromboxane A2, were involved in this phenomenon (1). Studies have also revealed that endothelin-1 (ET-1), which is known to have strong vasoconstrictive effects, plays an important role in CsA-induced acute renal failure (ARF) (4,5). CsA injections in rats caused increases in plasma ET-1 levels and decreases in GFR (5,6). Anti-ET-1 antibody and specific ET receptor antagonists ameliorated glomerular dysfunction induced by CsA (5,7,8). Furthermore, other reports showed that CsA increased mRNA expression of prepro-ET-1 (ppET-1) and ET B-type receptor (ETBR) in the renal medulla (9) and that CsA increased ET A-type receptor (ETAR) mRNA expression in the rat aorta and mesenteric artery (10). Taken together, these studies suggest that CsA induces ET-1 and that this induced ET-1 produces vasoconstrictive effects through ETAR and/or ETBR in the kidney, resulting in ARF.

ET-1 is produced from big ET-1 by a unique endoproteolytic cleavage, between Trp21 and Val22, by endothelin-converting enzyme-1 (ECE-1) (11). Structural characterization of ECE-1 and its cDNA sequence has been recently performed (12–14). Some isoforms and subtypes of ECE were also found. These were identified as ECE-1α, ECE-1β, and ECE-2 (15,16). Northern blot analysis revealed that ECE-1 mRNA exists in many tissue types, such as lung, adrenal gland, ovary, testis, liver, and kidney (12,14). It was recently demonstrated that phosphoramidon, an ECE-1 inhibitor, protected rat kidneys from functional and structural deterioration in a model of ARF (17). The protective effect of phosphoramidon was more significant than that of an ETAR antagonist. The aforementioned studies prompted us to investigate the precise localization and regulation of the ECE-1 gene in the kidney, to explore the mechanisms of ARF induced by CsA.
Materials and Methods

**Materials**

CsA was a kind gift from Sandoz (Tokyo, Japan). Cremophor was a gift from BASF Japan (Tokyo, Japan). The cDNA synthesis kit, the PCR master kit, PCR DIG (digoxigenin) probe synthesis kit and DIG luminescent detection kit were obtained from Boehringer Mannheim (Mannheim, Germany). Vanadyl ribonucleoside complex (VRC) was purchased from Life Technologies BRL (Gaithersburg, MD), collagenase (type I) from Sigma (St. Louis, MO), and agarose from Sea Kem (Rockland, ME). Nylon membranes were supplied by Funakoshi (Tokyo, Japan). Immobilon-P membranes were obtained from Millipore (Bedford, MA). The enhanced chemiluminescence Western blotting detection reagents were purchased from Amersham (Buckinghamshire, United Kingdom).

**Monitoring of Serum Creatinine, Plasma ET-1, and Blood CsA Levels**

Pathogen-free male Sprague Dawley rats (75 to 100 g body wt), which had free access to tap water and standard rat chow, received CsA (50 or 100 mg/kg) through the tail vein. The control group was given cremophor as the vehicle (same amount as for 100 mg/kg CsA). Serum creatinine and plasma ET-1 concentrations were measured using a creatinine assay kit (Wako, Tokyo, Japan) and RIA, respectively. Blood samples were collected at 0, 1, 6, 24, 72, and 168 h after CsA injections. The antibody for the ET-1 RIA cross-reacted 30% with ET(1-20) and, respectively. Blood concentrations of CsA were measured by fluorescence polarization immunoassays, using cyclosporine-SP-Dainabot (Dainabot, Tokyo, Japan) and a TDX analyzer (Abbott Diagnostics, North Chicago, IL). Whole blood was collected at 3 min and 1, 3, 6, and 24 h after injection (100 mg/kg CsA) and kept frozen at −30°C until analysis.

**Microdissection of Glomeruli and Nephron Segments**

Male Sprague Dawley rats received CsA (100 mg/kg) through the tail vein. In some experiments, 25, 50, or 100 mg/kg CsA or vehicle was injected through the tail vein. After induction of anesthesia with sodium pentobarbital (50 mg/kg), the rat aorta was cannulated and the left kidney was perfused with solutions A and B at 0, 1, 6, 24, and 72 h after CsA administration. The left kidney was rapidly microdissected in a Petri dish filled with solution A1 at 4°C, as described previously (19,20). The following glomeruli and nephron segments were isolated: superficial glomerulus (s-Glm), juxtamедullary glomerulus (j-Glm), proximal convoluted tubule (PCT), medullary thick ascending limb (MAL), cortical thick ascending limb (CMT), proximal straight tubule (PST), and outer medullary collecting duct (OMCD), and inner medullary collecting duct (IMCD). Three to eight glomeruli and 2-3mm-long nephron segments were transferred into a new Petri dish, which was filled with solution A, to wash out VRC. After washing, these segments were transferred into an Eppendorf tube, to which 10 μl of solution A2 was added. The composition of solution A was as follows: 130 mM NaCl, 5 mM KCl, 1 mM NaH2PO4, 1 mM MgSO4, 1 mM calcium lactate, 2 mM sodium acetate, 5.5 mM glucose, and 10 mM Hepes; the pH was adjusted to 7.4 with NaOH. Solution B was prepared by adding 1 mg/ml bovine serum albumin (Sigma) and 1 mg/ml collagenase (type I) to solution A. Solution A1 had the same composition as solution A except that 10 mM VRC was added. Solution A2 was also the same as solution A except that >1 U/μl RNase inhibitor (Boehringer Mannheim) and 1 M Dl-dithiothreitol (DTT) (Sigma) were added.

Reverse Transcription Coupled with PCR

Reverse transcription (RT)-PCR was performed using a cDNA synthesis kit and a PCR master kit, as described previously (21,22). Each sample was centrifuged at 15,000 rpm for 30 s, and the supernatant was discarded. A solution (3.5 μl) of 2% Triton X-100 with RNase inhibitor and DTT was added, and samples were incubated for 15 min on ice. After incubation, a RT mixture containing random primers was added and samples were incubated at 42°C for 60 min. RT was stopped by heating the sample to 90°C for 5 min, and samples were stored at 4°C until PCR. Specific primers for ppET-1, ECE-1, ETAR, ETBR, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed in different exons.

The ppET-1 sense primer was defined by bases 344 to 363 (5′-TCTTGCTCCTCCTCGTGA-GGA-3′) and the antisense primer by bases 795 to 815 (5′-AAACACCCAGGGGCTCTGTA-A′). The cDNA PCR amplification product was predicted to be 471 bp in length (23).

The ECE-1 sense primer was defined by bases 676 to 695 (5′-AAATACAGGACCTGTGGG-3′) and the antisense primer by bases 1045 to 1064 (5′-AGCTCAGCACGCTGACTTT-3′). The cDNA PCR amplification product was predicted to be 384 bp in length (12).

The ETAR sense primer was defined by bases 241 to 260 (5′-TGCTATTGCCCACAGCAGA-3′) and the antisense primer by bases 687 to 706 (5′-TGCTCTGGGGATGCGCAAGA-3′). The cDNA PCR amplification product was predicted to be 466 bp in length (24).

The ETBR sense primer was defined by bases 264 to 283 (5′-TTGTGGGGGATGGGAGA-3′) and the antisense primer by bases 717 to 736 (5′-AGGGCAACGCTTCCTGACAT-3′). The cDNA PCR amplification product was predicted to be 473 bp in length (25).

The GAPDH sense primer was defined by bases 492 to 511 (5′-TCCCTCAAGATTGTCAGCA-3′) and the antisense primer by bases 780 to 799 (5′-AGATCCACACGGGATACATT-3′). The cDNA PCR amplification product was predicted to be 308 bp in length (26).

Each primer was used at 0.2 pmol/μl, and the total PCR mixture was adjusted to 100 μl. The annealing temperature for ppET-1, ECE-1, ETAR, ETBR, and GAPDH was 62°C. PCR cycles for ppET-1, ECE-1, ETAR, ETBR, and GAPDH were 28, 28, 30, and 28 cycles, respectively.

Competitive PCR

To quantify ECE-1 mRNA expression, competitive PCR was performed (27). The competitor was synthesized in *vitro* overlap-extension PCR, according to the protocol described by Higuchi et al. (28,29). Inner primers (sense and antisense) were designed on the basis of the ECE-1 cDNA sequence of the PCR product, as shown in Figure 1. The inner antisense primer was designed to anneal to ECE-1 cDNA from base 691 to base 710 and to have a 5′ tail complementary to the annealing site of the inner sense primer. The inner sense primer was designed to anneal to ECE-1 cDNA from base 898 to base 917 and to have a 5′ tail complementary to the annealing site of the inner antisense primer. Two sets of PCR were performed sequentially. Short fragments were produced with the first PCR, using a combination of outer sense and inner antisense primers and a combination of outer antisense and inner sense primers. The sequence of the inner antisense primer was 5′-TTCCTCCAGCTGGAACATGTATGGAAAGTT-GTCTTGGCACA-3′, and the first PCR product from the combination of outer sense and inner antisense primers was predicted to be 55_vars inserted...
The sequence of the inner sense primer was 5′-TGGGCCAAGGA-CAACTTCCATACATGGTCCAGCTGGGGAA-3′, and the first PCR product from the combination of outer antisense and inner sense primers was predicted to be 187 bp. After the first PCR, the two PCR products were mixed and the second PCR was performed, using outer sense and outer antisense primers. In this step, short fragments that had an overlapping region were recombined, and a DNA competitor of 202 bp, with a deletion from the ECE-1 cDNA, was produced. The second PCR product (the DNA competitor) was purified by removal of all primers using Centricon 30 cartridges (Amicon, Beverly, MA). RT-competitive PCR was performed by adding appropriate concentrations of the competitor to the PCR mixture. All PCR products are shown in Figure 2. After PCR, ECE-1 mRNA expression was quantified by comparison with that of the competitor, in ethidium bromide-stained gels, using a densitometer (Atto, Tokyo, Japan). The ratio of ECE-1 cDNA to competitor cDNA was corrected for differences in molecular weight. To determine the amount of ECE-1 mRNA, a standard curve was produced by coamplification of the competitor with a series of dilutions of known amounts of ECE-1 cDNA.

**Incubation Study**

Twelve to 20 microdissected glomeruli were transferred into Eppendorf tubes filled with solution A containing CsA or vehicle (same amount of cremophor as for CsA). The samples were incubated for 6 h at 37°C and centrifuged at 15,000 rpm for 5 min. The supernatant was discarded, and RT-PCR was performed as described above. Incubation with solution A was considered the control reaction.

**Ethidium Bromide Staining**

After PCR, 90 μl of the PCR product was transferred into a dish filled with 20× SSC, which was left overnight. After DNA transfer, the membrane was baked in an ultraviolet baking system (M&S Instruments Trading, Vallee, France) and stored at 4°C until Southern hybridization. A nonisotopic DIG-nucleotide detection system was used for Southern Blotting.

**Southern Blotting**

After ethidium bromide staining, each gel was denatured twice in denaturing solution for 15 min, washed with distilled water, and neutralized twice in neutralizing solution for 15 min. A nylon membrane (Hybond-N++; Amersham, Buckinghamshire, United Kingdom) was soaked in distilled water for 20 s and in 20× SSC (3 M NaCl, 0.3 M sodium citrate) for 30 to 60 min, until the gel was formed. PCR products were transferred to a nylon membrane in a dish filled with 20× SSC, which was left overnight. After DNA transfer, the membrane was baked in an ultraviolet baking system (M&S Instruments Trading, Vallee, France) and stored at 4°C until Southern hybridization. A nonisotopic DIG-nucleotide detection system was used for....
Preparation of Membrane Fractions for Western Blot Analysis

Membrane fractions were prepared from the cortex, the outer medulla, and the inner medulla of rat kidney as described previously (31). Rats were euthanized at 0, 48, and 96 h after CsA injection. Kidneys were rapidly removed and transferred into phosphate-buffered saline on ice. Kidneys were sliced and separated into blocks of cortex, outer medulla, and inner medulla with a razor blade. Each block was minced and homogenized with five strokes at 1000 rpm, using a glass homogenizer, in lysis buffer (0.5 mM Na3VO4, 1 mM ethylenediaminetetraacetate, 5 mM ethylene glycol bis(β-aminopropyl ether)-N,N′,N′′,N′′′-tetraaceteate, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 5 mM β-glycerophosphate, 20 mM Hepes, pH 7.4) at 4°C. The supernatant was collected at centrifugation of that supernatant at 15,000 rpm for 30 min at 4°C. After measurement of protein contents, the membrane fractions were stored at −30°C until use. In some experiments, the kidneys were perfused with solutions A and B, and glomeruli were microdissected. One hundred and fifty glomeruli were collected, transferred into 10 µl of lysis buffer, and stored at −30°C.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed using the buffers described previously (32). Membrane fractions were mixed with sample buffer (final concentrations of 10% glycerol, 2.3% SDS, 5% β-mercaptoethanol, 65 mM DTT, 0.005% bromphenol blue, and 62.5 mM Tris-HCl, pH 6.8) and running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Samples were denatured for 5 min at 95°C, electrophoresed on polyacrylamide plate gels (4 to 20% gradation; Daiichi Pure Chemicals, Tokyo, Japan), and electrophoretically transferred to membranes (Immobilon-P; Millipore). After being blocked with Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) with polyoxyethylenesorbitan monolaurate (Tween 20) (TBS-T) containing 5% milk and washed with TBS-T, the membranes were incubated overnight with primary antibody to ECE-1 (AEC 27-121; 800× in TBS-T with 5% milk), at 4°C (33). After hybridization, the membranes were washed with TBS-T. Horseradish peroxidase-linked anti-mouse Ig F(ab′)2 fragment (from sheep) was then hybridized for 1 h at room temperature. Membranes were washed again with TBS-T, and specific protein expression was detected using enhanced chemiluminescence Western blotting detection reagents. The samples of isolated glomeruli were precipitated with ethanol and resuspended in running buffer and sample buffer. Western blot analysis was then performed as described above.

Statistical Analyses

Results are expressed as mean ± SEM. Statistical analyses were performed using the t test, ANOVA, or Kruskal–Wallis analysis, followed by Dunnett or Fisher multiple comparison, as appropriate. A value of P < 0.05 was considered significant.

Results

Serum Creatinine Levels

After CsA injection, serum creatinine levels were increased significantly in the 100 mg/kg CsA-treated group at 24 to 72 h but not in the 50 mg/kg CsA-treated group (Figure 3). In the control (vehicle-treated) group, serum creatinine levels did not change. These data revealed that a bolus injection of CsA at 100 mg/kg induced ARF. No remarkable changes in body weight were observed for these groups.

Plasma ET-1 Levels

In the 100 mg/kg CsA-treated group, plasma ET-1 concentrations were significantly increased, from 3.3 ± 0.3 to 7.2 ± 0.2 pg/ml (P < 0.05, n = 3 to 6), at 1 h after CsA injection and returned to basal levels by 24 h (Figure 4). No significant change was observed in the control group.

Blood Concentrations of CsA

Blood concentrations of CsA in the 100 mg/kg CsA-treated group were 256 ± 19, 88 ± 6, 30 ± 3, 18 ± 6, and 10 ± 0.9 µM at 3 min, 1 h, 3 h, 6 h, and 24 h, respectively. Data are given as mean ± SEM (n = 3).

Figure 3. Serum creatinine (S-Cr.) levels after cyclosporin A (CsA) injection (CYA). A high dose of CsA (100 mg/kg) caused increases in serum creatinine levels at 24 to 72 h. ●, vehicle; ▲, CsA at 50 mg/kg; ■, CsA at 100 mg/kg. Values are mean ± SEM (n = 3 to 6). *P < 0.05 versus the vehicle-treated group.
Quantification of ECE-1 mRNA by Competitive PCR

For quantification of ECE-1 mRNA, a constant amount of ECE-1 cDNA was coamplified with a series of dilutions of a known concentration of the competitor. There was a linear relationship between the competitor/ECE-1 cDNA ratio and the known concentration of input competitor (Figure 5). To quantify the ECE-1 cDNA in each sample, a series of dilutions of a known concentration of ECE-1 cDNA were coamplified with 3 fg of the competitor. The competitor was added to the PCR mixture at 3 fg/three glomeruli or 2-mm tubule/100 μl of PCR mixture, in a RT-competitive PCR study. As shown in Figures 2 and 5, the competitor was detected to be 202 bp, which was smaller than the ECE-1 cDNA by 187 bp. The ECE-1/competitor cDNA ratio was measured by densitometry, to estimate ECE-1 mRNA expression in the samples. Mean intra- and interassay coefficients of variation for ECE-1 mRNA determinations were 5.1 and 9.5%, respectively.

Effects of CsA on ECE-1 mRNA Expression in Glomeruli

The effects of three doses of CsA on ECE-1 mRNA expression in glomeruli were examined. CsA at 50 and 100 mg/kg caused significant decreases in ECE-1 mRNA expression, whereas 25 mg/kg CsA did not (Figure 6). Because 50 mg/kg CsA did not increase serum creatinine levels (Figure 3), we used 100 mg/kg CsA in the following study.

Microlocalization of ECE-1 mRNA along the Nephron

Microdissection and RT-competitive PCR revealed the expression of ECE-1 mRNA along the nephron segments (Figure 7). In control rats, ECE-1 mRNA expression was detected in all tested nephron segments, i.e., superficial glomerulus, juxtamedullary glomerulus, PCT, PST, medullary thick ascending limb, cortical thick ascending limb, cortical collecting duct, OMCD, and IMCD. High levels of expression of ECE-1 mRNA were detected in the superficial glomerulus, juxtamedullary glomerulus, and PST, moderate levels of expression were observed in distal tubules (from the medullary thick ascending limb to the IMCD), and low levels of expression were detected in PCT. At 24 h after injection of 100 mg/kg CsA, ECE-1 mRNA levels were significantly decreased in these nephron segments, except in PCT and OMCD. The

Figure 5. Coamplification of ECE-1 cDNA with a series of dilutions of the competitor. A constant amount of ECE-1 cDNA was coamplified with various concentrations of the competitor. The competitor concentrations added were 0, 0.6, 1.2, 1.8, 3.0, 6.0, and 6.0 fg/PCR tube in lanes 1 to 7 (left to right), respectively. ECE-1 cDNA was not added in lane 7. This graph shows the linear relationship between the competitor/ECE-1 mRNA ratio and the concentration of the competitor.

Figure 6. Effects of CsA (CyA) on ECE-1 mRNA expression in glomeruli. ECE-1 mRNA expression in glomeruli was examined 24 h after CsA injection. CsA at 50 and 100 mg/kg but not 25 mg/kg caused a significant decrease in ECE-1 mRNA expression in glomeruli. *P < 0.05 versus without CsA.
largest reduction of ECE-1 mRNA (by 86%) was observed in PST. ECE-1 mRNA expression was decreased by one-half in other segments. GAPDH mRNA did not change significantly in these segments.

**Time-Course Study**

The time course of the effect of CsA injection on ppET-1, ECE-1, ETAR, and ETBR mRNA expression in glomeruli was examined. ppET-1 mRNA levels were increased at 30 to 60 min and returned to control levels after 6 h (Figure 8). ECE-1 and ETAR mRNA levels were decreased at 1 to 24 h and returned to control levels at 72 h. ETBR mRNA levels were decreased at 6 h and recovered to control levels at 72 h. GAPDH mRNA levels did not change during the experimental period. These data showed that the rapid and transient increase in ppET-1 levels was followed by decreases in ECE-1, ETAR, and ETBR mRNA levels.

The time course of ECE-1 mRNA expression after CsA injection was also investigated by RT-competitive PCR. This study showed that ECE-1 mRNA levels were significantly decreased (by 64%) in glomeruli at 6 h after CsA injection (Figure 9).

**Incubation Study**

To reveal the direct effect of CsA on ECE-1 mRNA expression in glomeruli, microdissected glomeruli were incubated with CsA or vehicle for 6 h at 37°C. Because the blood concentration of CsA decreased rapidly after injection (see above), isolated glomeruli were incubated with low concentrations of CsA (0 to 20 μM) or vehicle. ECE-1 mRNA levels were significantly decreased by incubation with 20 μM CsA. GAPDH mRNA levels did not change after incubation with CsA or cremophor (Figure 10).

**Western Blot Analysis**

A specific monoclonal antibody against ECE-1 was used to demonstrate ECE-1 protein expression. As shown in Figure 11A, ECE-1 protein was expressed in the renal cortex, the outer medulla, and the inner medulla under basal conditions. The ECE-1 protein levels in the cortex did not change after CsA injection but those in the outer and inner medulla were decreased at 48 and 96 h after the injection. Although ECE-1
protein levels did not change in the cortex, levels were markedly decreased in isolated glomeruli at 48 and 96 h after CsA injection (Figure 11B).

Discussion

In this study, we demonstrated the microlocalization of ECE-1 mRNA along the nephron segments. ECE-1 mRNA was detected in glomeruli and whole nephron segments. Administration of 100 mg/kg CsA caused a significant decrease in ECE-1 mRNA expression in all nephron segments except the PCT and OMCD after 24 h. The time-course study in glomeruli revealed that CsA decreased ECE-1 mRNA expression at 1 to 24 h and expression returned to control levels by 72 h after the injection. In glomeruli, CsA caused a rapid increase in ppET-1 mRNA expression at 30 to 60 min, and an increase in plasma ET-1 concentrations ensued 1 to 6 h after CsA administration. The levels of ECE-1, ETAR, and ETBR mRNA then decreased. Although plasma ET-1 concentrations returned to control levels 24 h after CsA injection, the increase in serum creatinine levels that occurred at 24 h lasted until 72 h after the injection. These data showed that 100 mg/kg CsA caused rapid increases in ppET-1 mRNA, plasma ET-1, and serum creatinine levels, which were followed by decreases in ECE-1, ETAR, and ETBR mRNA expression.

We previously reported substantial synthesis of ET-1 and ppET-1 mRNA expression in rat glomeruli and IMCD (34). The data presented here showed that ECE-1 mRNA was detected not only in glomeruli and IMCD but also in other proximal and distal nephron segments. Quantitative PCR revealed that ECE-1 mRNA expression was more abundant in glomeruli and PST than in the other nephron segments. Therefore, the localization of ECE-1 mRNA expression in nephron segments is slightly different from that of ET-1 synthesis, suggesting the possibility that cell surface ECE-1 may also produce ET-1, using exogenous big ET-1. There is also the possibility that there is a small amount of ET-1 synthesis, which could not be detected in the previous study, in nephron segments other than glomeruli and IMCD.

It is known that CsA causes increases in circulating plasma ET-1 levels and renal dysfunction (1,3,5,35,36). It was also reported that ET-1 plays an important role in several models of acute renal dysfunction (5–8,37–40). However, those reports documented only early hemodynamic changes, and it is questionable whether such changes caused sustained renal failure. In this study, 100 mg/kg but not 50 mg/kg CsA increased serum creatinine levels at 24 h after the injection, although both 50 and 100 mg/kg CsA caused significant decreases in ECE-1 mRNA expression in glomeruli. Therefore, we used 100 mg/kg CsA to investigate ARF caused by CsA in this study. CsA at 100 mg/kg caused an increase in circulating plasma ET-1 levels at 1 to 6 h after the administration. Subsequently, serum creatinine levels were increased at 24 h and

Figure 9. Time course of ECE-1 mRNA expression in glomeruli (Glm) after CsA injection (CYA). ECE-1 mRNA expression was examined using RT-competitive PCR. (Top) Typical example of ethidium bromide-stained gel. (Bottom) Changes in ECE-1 mRNA expression, quantified using a standard curve. ECE-1 mRNA expression in glomeruli showed significant downregulation at 6 to 24 h after CsA injection. Values are mean ± SEM (n = 5). *P < 0.05 versus zero time.

Figure 10. Effects of CsA (CYA) on ECE-1 mRNA expression in glomeruli in vitro. Twelve to 20 glomeruli were incubated with CsA or vehicle (cremophor) for 6 h at 37°C. (Top) Typical example of ethidium bromide-stained gel. Lane 1, control; lane 2, vehicle at 10 μM; lane 3, vehicle at 20 μM; lane 4, CsA at 10 μM; lane 5, CsA at 20 μM. (Bottom) Bar graph showing the ratio of ECE-1 mRNA expression to the control level. ■, with cremophor (vehicle); □, with CsA; 0, control (with solution A only); 10 and 20, concentrations of CsA or vehicle (μM). Values are mean ± SEM (n = 3). *P < 0.05 versus vehicle.
remained at high levels throughout the experimental period. Therefore, an increase in ET-1 synthesis is thought to be crucial for the production of acute renal dysfunction by CsA in our model, as also noted in other reports.

CsA has also been shown to regulate ligand and receptor genes. CsA selectively increased ppET-1 and ETBR mRNA expression in the renal medulla (9). CsA administered for 4 wk increased ETAR mRNA expression in the rat aorta and mesenteric artery (10). In our study, ppET-1 mRNA was significantly increased, whereas both ETAR and ETBR mRNA expression in glomeruli was decreased after CsA injection. The increase in plasma ET-1 levels was preceded by the increase in ppET-1 mRNA levels in glomeruli, whereas ECE-1 mRNA and protein expression in glomeruli and nephron segments was downregulated by CsA injection. Therefore, the increase in plasma ET-1 levels seems to be caused by activation of the ppET-1 gene but not by the ECE-1 gene or protein. It is not clear whether this upregulation of ppET-1 mRNA was induced by CsA itself or other factors, such as hypoxia caused by vasoconstriction (41).

Our data suggest either that the increase in ET-1 synthesis induced by CsA caused the vasoconstriction or that CsA itself caused the vasoconstriction, which subsequently increased ET-1 synthesis. Therefore, to determine whether vasoconstriction is necessary for the downregulation of ECE-1, the effect of CsA on ECE-1 mRNA expression was investigated using in vitro incubations of isolated glomeruli. Incubation of glomeruli with 20 μM CsA significantly decreased ECE-1 mRNA expression in the absence of vasoconstriction. It is of great interest that ECE-1 mRNA and protein levels were decreased 6 to 24 h and 48 to 96 h after CsA injection, respectively, although ppET-1 mRNA expression in glomeruli and plasma ET-1 levels were rapidly increased. Naomi et al. (42) reported that ET-1 reduced ECE-1 mRNA and protein expression through ETBR in pulmonary endothelial cells. Taken together, these data suggest that CsA reduced ECE-1 mRNA expression probably through the induction of ET-1 by some direct or indirect mechanisms other than vasoconstriction. ET-1 is known to cause autoinduction via ETBR in mesangial and endothelial cells (43–45), leading to prolonged deterioration of renal function produced by increased levels of ET-1 itself. In our study, ECE-1, ETAR, and ETBR mRNA expression was downregulated after increases in ppET-1 expression and plasma ET-1 levels. Without downregulation of ECE-1 expression, autoinduction of ET-1 and further deterioration of renal function might have occurred.

In summary, microdissection coupled with RT-competitive PCR revealed the microlocalization of ECE-1 mRNA in rat nephron segments, including glomeruli, proximal tubules, medullary and cortical thick ascending limbs, cortical collecting ducts, OMCD, and IMCD. An early increase in ppET-1 mRNA expression in glomeruli after CsA injection was followed by an increase in plasma ET-1 levels and then by an increase in serum creatinine levels. ECE-1, ETAR, and ETBR mRNA and ECE-1 protein levels were downregulated in glomeruli after injection of CsA. ECE-1 mRNA expression was also downregulated in all nephron segments except PCT.

Figure 11. (A) ECE-1 protein expression in the cortex (CX), the outer medulla (OM), and the inner medulla (IM) after CsA injection. Membrane fraction protein (33 μg) was loaded in each lane for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis. (Top) Typical example of ethidium bromide-stained gel. (Bottom) Results from four experiments. (B) ECE-1 protein expression in glomeruli. One hundred-fifty isolated glomeruli were used for each lane. (Top) Typical example of ethidium bromide-stained gel. (Bottom) Results from three experiments. *P < 0.05 versus time 0.
and OMCD. These data suggest that downregulation of glomerular and tubular ECE-1 expression may be caused by increased ET-1 synthesis, rather than vasoconstriction, in CsA-induced ARF.

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References


