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RAPID COMMUNICATION

# Site specific regulation in the kidney of endothelin and its receptor subtypes by cyclosporine

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**Site selective regulation in the kidney of endothelin and its receptor subtypes by cyclosporine.** Endothelin (Et) has been suggested by us and others to play a role in glomerular dysfunction that characterizes cyclosporine (Cs)-associated nephrotoxicity. Since Et exerts its actions through at least two receptor subtypes, and because these receptor subtypes have particular distributions in the renal parenchyma, we investigated changes in mRNA expression for Et and its receptor subtypes in glomeruli and medulla of rats treated with Cs. Polymerase chain reaction coupled with reverse transcription (RT-PCR) method was used to assess prepro-Et-1, type A (EtA) and type B (EtB) receptor mRNA at 1, 3, 6, and 24 hours after Cs (20 mg/kg body wt i.v.). Results were normalized to the expression of β-actin as an internal standard. Compared with control rats, glomerular mRNA expression for prepro-Et-1 was not affected by Cs. Similarly, Cs did not significantly change the glomerular mRNA expression of either EtA or EtB receptor subtypes. By contrast, in the medulla there was a marked and persistent increase in the expression for prepro-Et-1 and the EtB receptor subtype: prepro-Et-1 at 1, 3, 6, and 24 hours was 336 ± 61, 295 ± 65, 339 ± 73, 440 ± 123% of controls, respectively ($P < 0.05$ compared with controls at each time point). The EtB receptor mRNA at 1, 3, 6, 24 hours was 164 ± 22, 157 ± 15, 148 ± 14, 116 ± 18% (compared with controls, $P < 0.01$ at 3 hr and $P < 0.05$ at 1 and 6 hr), while the mRNA expression for EtA was not affected by Cs treatment. These results demonstrate that, *in vivo*, Cs selectively modulates renal mRNA expression for Et peptide and one of its receptor subtypes. Furthermore, the modulation is site specific. These changes are most conspicuous in the renal medulla, such that mRNA expression for prepro-Et-1 and EtB increases and remains elevated for at least six hours after Cs administration. These alterations may contribute to the vasospastic as well as proliferative abnormalities which characterize Cs nephrotoxicity.

Cyclosporine (Cs) has a well-recognized predilection to cause renal dysfunction including glomerular hypofiltration as well as cortical and medullary scarring, abnormalities that have recently been linked to the actions of endothelin (Et). We and others have shown that Cs stimulates Et production in both *in vivo* and *in vitro* settings, increases Et binding in renal tissue and increases urinary excretion of Et [1–5] . These findings support persistent activation of Et gene and/or receptor regulation within the kidney which contribute to Cs-associated injury. Et and its receptor subtypes have been found throughout the renal parenchyma. Thus, Et-1 has been localized within the medulla, especially inner medullary collecting duct [6, 7], while cultured glomerular cells, including mesangial [8], endothelial [9] and epithelial cells [10] all elaborate Et. Similarly, EtA and EtB receptor subtypes have been localized within the glomerulus [11] as well as the renal medulla [12].

To clarify the potentially distinct and separate modulation of Et and its receptor subtypes by Cs, we studied expression for Et, EtA and EtB in glomeruli and medulla of Cs treated rats using polymerase chain reaction coupled with reverse transcription (RT-PCR).

## Methods

### *Materials*

Random primer, ribonuclease H (RNAse H), dithiothreitol (DTT) and SuperScript™ reverse transcriptase with reaction buffer (5x) (20 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% NP-40, and 50% glycerol) were purchased from GIBCO/BRL (Gaithersburg, Maryland, USA). RNAsin (RNAse inhibitor), Taq DNA polymerase with reaction buffer (10x) (50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 50% glycerol, and 1.0% Triton X-100), deoxynucleotide mixture (dNTP) and $MgCl_2$ were purchased from Promega Corporation (Madison, Wisconsin, USA).

### *Experimental protocols*

Male Munich-Wistar rats weighing 200 to 220 g were used. The animals had free access to water and standard rat chow until the day of experiment. Each rat received Cs (Sandoz, East Hanover, New Jersey, USA; 20 mg/kg body wt) through the tail vein. Following pentobarbital sodium anesthesia (50 mg/kg i.p.), both kidneys were harvested 1, 3, 6 or 24 hours after Cs administration. Glomeruli were isolated by the sieving method. Total RNA was extracted from isolated glomeruli from four kidneys or medulla from one kidney; each of these constituted a single experiment.

### *RNA extraction*

Total RNA was prepared by acid-guanidinium-phenol-chloroform (AGPC) method [13]. Briefly, tissue samples were lysed by addition of 10 ml of RNA zol B® (TEL-TEST Inc., Friendswood, Texas, USA). Chloroform (1.0 ml) was added to lysates, which was then vortexed vigorously for 30 seconds and iced for 15 minutes. After centrifugation at 12,000 g at 4°C for 30 minutes, RNA in an aqueous phase was precipitated with

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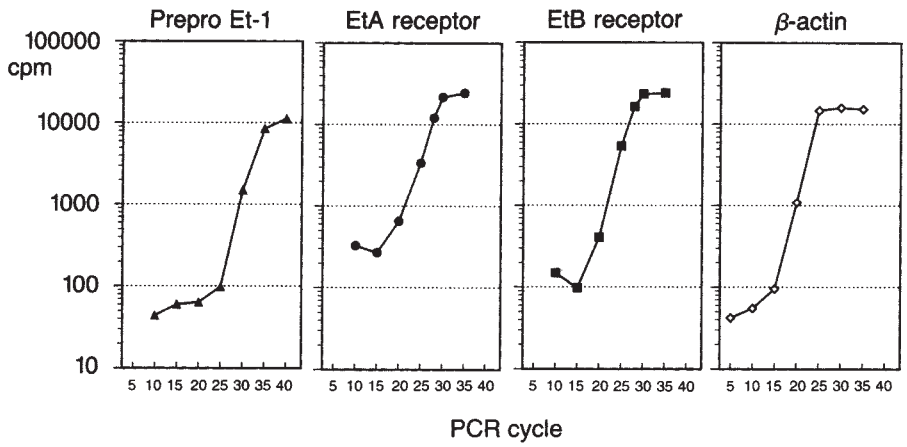


**Fig. 1.** *PCR amplification curve of prepro-Et-1, EtA and EtB receptor subtypes and β-actin.*

isopropanol for 30 minutes. Total RNA was pelleted by centrifugation at 12,000 g at 4°C for 15 minutes, washed with 75% ethanol and the resultant pellet dissolved in diethylpyrocarbonate-treated $H_2O$. Samples were stored at −70°C.

*Reverse transcription (RT)*

Total RNA (2 μg) from isolated glomeruli or renal medulla was used in a RT reaction. The samples were heated to 70°C for 10 minutes, iced. Nineteen μl of the RT reaction mixture containing 50 ng random primer, 40 units RNAsin, 10 mM DTT, 1 mM dNTP, 5x reaction buffer, and 200 units reverse transcriptase were added. The reaction mixture was incubated at 37°C for 60 minutes. At the end of the incubation, the reaction mixture was heated to 95°C for five minutes to inactivate the reverse transcriptase activity and to denature RNA-cDNA hybrids. The samples were treated with RNAseH at 37°C for 30 minutes. Two μl of cDNA templates were used for each polymerase chain reaction (PCR) reaction.

*Polymerase chain reaction*

PCR was performed with rat prepro-Et-1, EtA and EtB specific oligonucleotide primers prepared on a DNA synthesizer (Applied Dept. of Molecular Physiology in Vanderbilt University, Nashville, TN, USA). Prepro-Et-1 primer 1 (anti-sense) was defined by bases 341-360, sequence 5′-TGCTCCTGCTCCTCCTTGAT-3′; primer 2 (sense), bases 792-811, sequence 5′-CACCACGGGGCTCTGTAGTC-3′ [14]. The cDNA amplification product was predicted to be 471 bp in length. EtA primer 1 (anti-sense) was defined by bases 538-557, sequence 5′-GAAGTCGTCCGTGGGCATCA-3′; primer 2 (sense), bases 734-753, sequence 5′-CTGTGCTGCTCGCCCTTGTA-3′ [15]. The cDNA amplification product was predicted to be 216 bp in length. EtB primer 1 (anti-sense) was defined by bases 1004-1023, sequence 5′-TTACAAGACAGCCAAAGACT-3′; primer 2 (sense), bases 1549-1568, sequence 5′-CACGATAGAGGACAATGAAGAT-3′ [16]. The PCR product was predicted to be 565 bp in length. Twenty-five picomoles of each of primers 1 and 2 were used per reaction for prepro-Et-1, EtA, EtB and β-actin. 5.0 U of Taq DNA polymerase were used in each PCR reaction. The reaction mixture (100 μl) was overlaid with 50 μl mineral oil. The tubes were placed in a Programmable Thermal Controller, PTC-100 (MJ Research Inc., Watertown, Massachusetts, USA) programmed as follows: incubation at 92°C for three minutes (initial melt); then, (a) prepro Et-1: 35 cycles of the following sequential steps: 92°C for 30 seconds (melt); 60°C for 20 seconds (anneal); 72°C for one minute (extension); (b) EtA, EtB and β-actin: 27 cycles (EtA), 25 cycles (EtB) or 22 cycles (β-actin) of the following sequential steps: 92°C for one minute; 60°C for one minute; 72°C for one minute. Preliminary experiments which determined the number of amplification cycles for each mRNA species are shown in Figure 1. Final incubation was performed at 72°C for five minutes. We performed RT-PCR of β-actin as an internal standard [17]. Beta-actin primer 1 (anti-sense), bases 3055-3079, sequence 5′-ACCTTCAACACCCCAGCCATGTACG-3′; primer 2 (sense), bases 2170-2194, sequence 5′-CTGATCCACATCTGCTGGAAGGTGG-3′. Beta-actin primers spanned two introns and resulted in a 703 bp product. PCR amplified products in 90 μl of the total reaction mixture were saved, and kept at −20°C until Southern blot analysis.

*Southern blot analysis of PCR products*

The identity of PCR products was confirmed by Southern hybridization. The PCR products were size-fractionated with 2% agarose gel electrophoresis and the DNA bands, visualized with ethidium bromide staining, were photographed. PCR products were blotted to Gene Screen (DuPont New England Nuclear, Boston, Massachusetts, USA) in 10xSSC solution. The blotted DNA was fixed by baking at 80°C for two hours. Oligoprobes (antisense DNA) positioned between 5′ and 3′ primers were synthesized to hybridize PCR products. The sequence of each 20 oligonucleotide was 5′-CAAAGAACTCCGAGCCCAAA-3′ (prepro-Et-1), 5′-CCCCTTGATTACCGCCATTTG-3′ (EtA), 5′-TGTGCTGCTGGTGCCAAACG-3′ (EtB) and 5′-CTGCGTCTGGACCTGGCTGGCCGGG-3′ (β-actin), respectively [14–17]. The synthetic oligonucleotide for hybridization was end-labeled with [$\gamma$-$^{32}$P] ATP (6,000 Ci/mmol, DuPont, New England Nuclear, Boston, Massachusetts, USA) using a 5′-end oligonucleotide labeling kit (DuPont New England Nuclear Research Products). Prehybridization (3 hrs) and hybridization (overnight) were carried out at 50°C (prepro-Et-1, β-actin) or at 65°C. After washing once (EtA, EtB) with 2xSSC, 0.5% SDS for 20 minutes, autoradiography was performed at

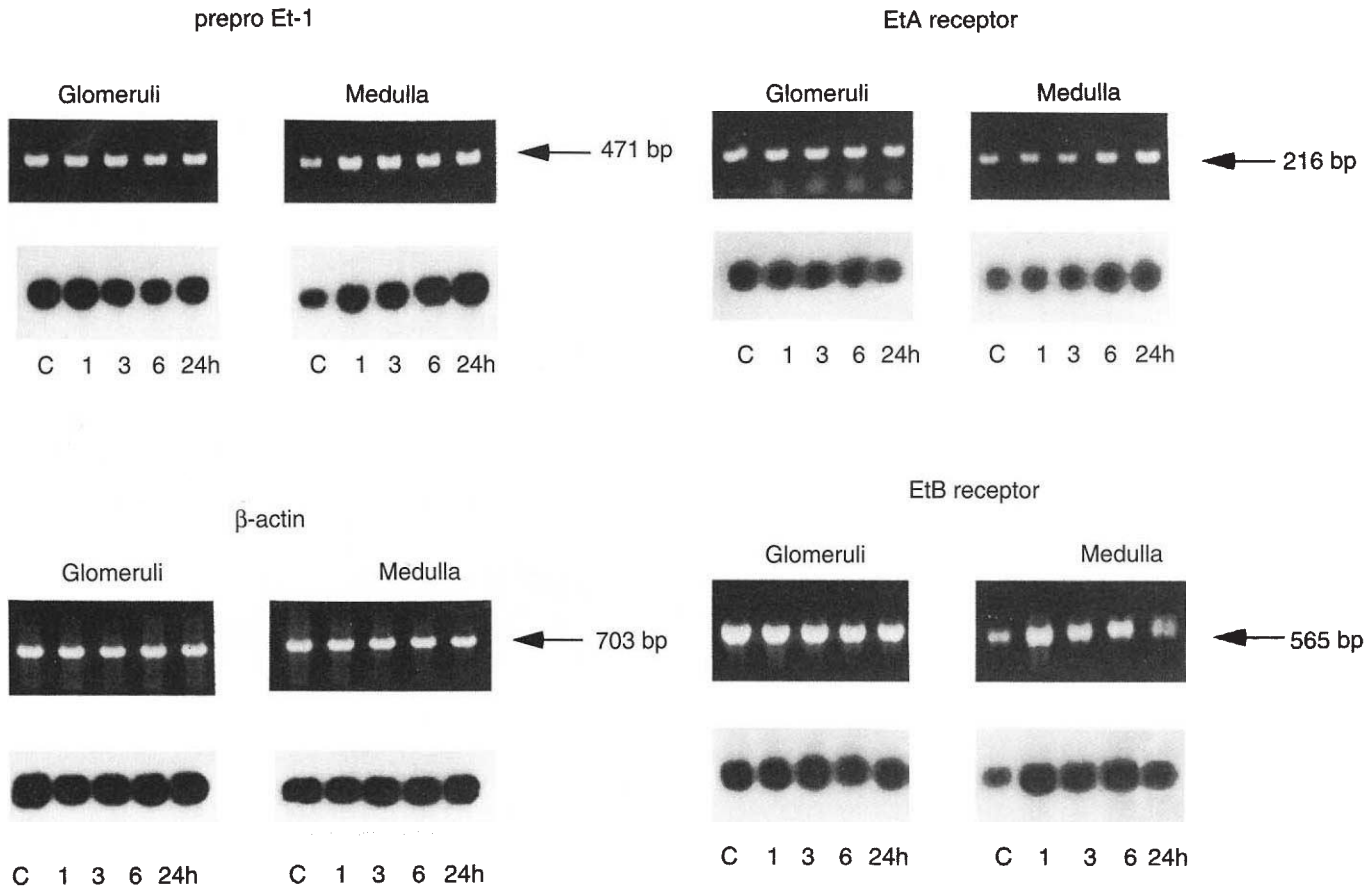


**Fig. 2.** *RT-PCR analysis of the time course for prepro-Et-1 and β-actin as well as EtA and EtB receptor subtypes in glomeruli and medulla of kidneys from control and Cs-treated rats.* The upper panel of each set demonstrates the ethidium bromide stained PCR products in 2% agarose gel. Arrows indicate the expected size for each PCR product. The lower panel shows the corresponding Southern blot probed with $^{32}$P-labeled oligonucleotides that localized each PCR primer. In each set (glomeruli or medulla), lane 1 denotes control; lane 2 is 1 hr after Cs; lane 3 is 3 hr after Cs; lane 4 is 6 hr after Cs; lane 5 is 24 hr after Cs.

room temperature for 30 minutes, 50 minutes, 4 hours and overnight for β-actin, EtB, prepro-Et-1, and EtA, respectively.

*Relative quantitation of mRNA level from autoradiographs*

The optical density (OD) of mRNA for PCR products of prepro-Et-1, EtA, EtB and β-actin on the autoradiograph was determined by Videodensitometry (Bio-Rad Laboratories, Richmond, California, USA). Experimental OD value was divided by the control OD value in each experiment and expressed as a percent of control.

*Statistics*

Data are presented as mean ± SE. Wilcoxon signed rank test was used to evaluate statistical significance. $P < 0.05$ was considered to be significant.

## Results

Figure 2 shows the ethidium bromide staining of agarose gels and corresponding Southern blots for the PCR products for prepro-Et-1, EtA, EtB and β-actin mRNA in glomeruli and medulla from kidneys of rats treated with Cs as well as controls. The expected size of each PCR product is apparent: prepro-Et-1 (471 bp), EtA (216 bp), EtB (565 bp), and β-actin (703 bp). Southern hybridization using the specific oligoprobes confirmed the identity of each of these PCR products. When PCR was carried out in the absence of reverse transcriptase, these bands were not seen, indicating that each band was derived from mRNA, and not from the genomic DNA. The amplification product of β-actin served as an internal standard for the RT-PCR reaction. The optimum number of amplification cycles used for quantitative RT-PCR was chosen on the basis of pilot experiments that established the linear range of the reaction (Fig. 1).

Figures 3 and 4 graphically summarize the time course of changes in mRNA expression for prepro-Et-1, EtA and EtB in glomeruli and medulla from kidneys of Cs treated rats. Each data point represents results from four to nine independent experiments and is expressed as percent of the values obtained in control rats. When compared with controls, glomerular mRNA expression for prepro-Et-1 was not significantly affected by Cs. Thus, prepro-Et-1 at 1, 3, 6 and 24 hours was 77 ± 20, 84 ± 18, 60 ± 18, and 84 ± 21%, respectively (Fig. 3, open triangles). By contrast, in the medulla, there were marked and persistent increases in expression for prepro-Et-1 and the EtB receptor subtype. Compared with controls, prepro-Et-1 mRNA at 1, 3, 6 and 24 hours was 336 ± 61, 295 ± 65, 339 ± 73, and

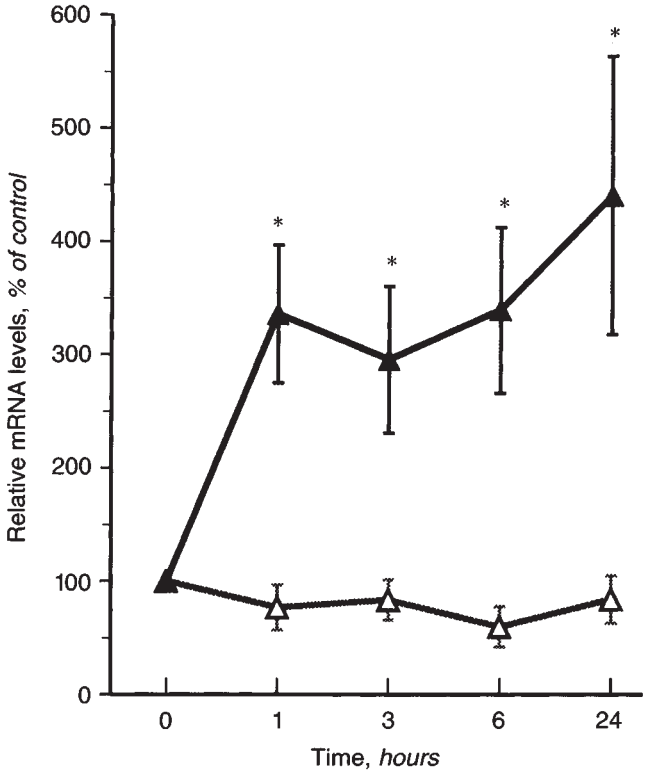


**Fig. 3.** *The time course for prepro-Et-1 mRNA in glomeruli (–△–△–) and medulla (–▲–▲–) of Cs-treated rats.* Expression was analyzed by using the RT-PCR method. $^{32}$P-labeled oligonucleotide probes for prepro-Et-1 was hybridized with the PCR product and autoradiography performed. Bands were quantified by densitometry. Each point was normalized to the expression of β-actin as internal standard. Values are expressed as percent of control values. * Statistically significant difference versus control animals.

440 ± 123%, respectively; $P < 0.05$ compared to control at each time point (Fig. 3, closed triangles). Cs did not change the glomerular mRNA expression of either EtA or EtB receptor subtypes; as shown in Figure 4A, glomerular mRNA expression for EtA at 1, 3, 6 and 24 hours was 110 ± 12, 106 ± 14, 141 ± 24, and 116 ± 12%, respectively. Glomerular mRNA expression for EtB at 1, 3, 6, 24 hours was 101.1 ± 9.6, 119.0 ± 10.3, 87.5 ± 16.3, and 92.3 ± 19.1% of the corresponding control values (Fig. 4A). Medullary mRNA expression for the EtB receptor subtype at 1, 3, 6 and 24 hours was 164 ± 22, 157 ± 15, 148 ± 14, and 116 ± 18%; $P < 0.01$ at 3 hours, $P < 0.05$ at 1 and 6 hours (Fig. 4B). The mRNA expression for EtA in the medulla was only not significantly different from control values: EtA at 1, 3, 6 and 24 hours was 118 ± 11, 119 ± 13, 112 ± 18, and 129 ± 20%, respectively (Fig. 4B).

## Discussion

These studies show that Cs exerts a unique and distinct modulation on the individual components of the endothelin system, namely, the Et peptide and its receptor subtypes. In addition, this modulation is site specific. Thus, glomeruli isolated from kidneys of rats treated with Cs showed little changes in the mRNA expression for prepro-Et-1, EtA or EtB over the time period studied, while in the medulla, the expression of prepro-Et-1 and EtB was markedly and persistently increased. Previous studies showed that Cs stimulates Et production both in *in vivo* and *in vitro* settings, and that the associated functional changes such as glomerular hypoperfusion/hypofiltration or cellular proliferation can be abrogated with Et antibody [1, 3, 5, 18]. These observations suggested that the Et peptide plays a central role in Cs-related glomerular dysfunction, and that antagonism of the peptide attenuates actions of Cs. The observations of the current study that glomerular prepro-Et-1 mRNA expression changes little following Cs suggests that the glomerulus is not a primary source for enhanced Et production in this setting. Instead, the current study demonstrates that the renal medulla exhibits a striking and persistent increase in prepro-Et-1 mRNA expression. This suggests that vascular and/or tubule epithelial cells in the medulla are important sites for increased Et synthesis which in turn may induce glomerular hemodynamic dysfunction as well as impact morphological changes occurring in this region of the kidney chronically exposed to Cs. This observation is in good agreement with previous findings that epithelial cells, including the proximal tubule-like LLC-PK$_1$ cells [19, 20] and distal tubule-like MDCK cells, are prolific producers of Et [21]. Further, the inner medullary collecting duct cells (IMCD) contain the highest concentration of immunoreactive Et [22] and have been recently shown to be the greatest producers of Et among tubule cells [23, 24]. It should be noted that while the *in vivo* studies emphasize the renal medulla as a primary current source of Et production, higher doses of Cs, or more continuous exposure to Cs (typical of *in vitro* experiments) may alter prepro-Et-1 expression in glomerular cells.

In addition to effects on the Et peptide mRNA expression, Cs also modulates the EtB receptor subtype. Thus, glomerular receptor gene expression was not significantly affected by Cs in the current study, whereas the renal medulla showed distinct changes: mRNA expression for the EtB receptor subtype was markedly and persistently above that seen in medulla of control kidneys. As in the above discussion of prepro-Et-1, the enhanced medullary expression of EtB may reflect increases in either vasa recta which express both receptor subtypes [11] and/or in tubule epithelial cells. In this regard, IMCD cells have been demonstrated to express either predominantly [12] or exclusively EtB receptor subtype [11] and appear to bind more Et than the other tubule cells [12]. Taken together, it appears that previous observations of increased Et binding to medullary tissue may reflect activity of the EtB receptor subtype. The current findings are also in agreement with a preliminary study in ischemic kidneys which found persistently increased mRNA expression for Et and the EtB receptor subtype [25]. The concurrent increase in the renal medullary mRNA expression for both prepro-Et-1 and EtB receptor subtype raises the intriguing possibility that Et may up-regulate one of its receptor subtypes, at least in the renal medulla. Similar up-regulation of a receptor by its ligand was recently described for the angiotensin system in rabbit proximal tubule cells where angiotensin up-regulated the mRNA for the type 1 angiotensin receptor both in *in vitro* and *in vivo* systems [26]. It is also possible that Cs itself, independent of Et, directly modulates mRNA for both the prepro-Et-1 and EtB receptor subtype.

Lack of change in glomerular or medullary mRNA expression for the EtA receptor subtype is of interest since previous

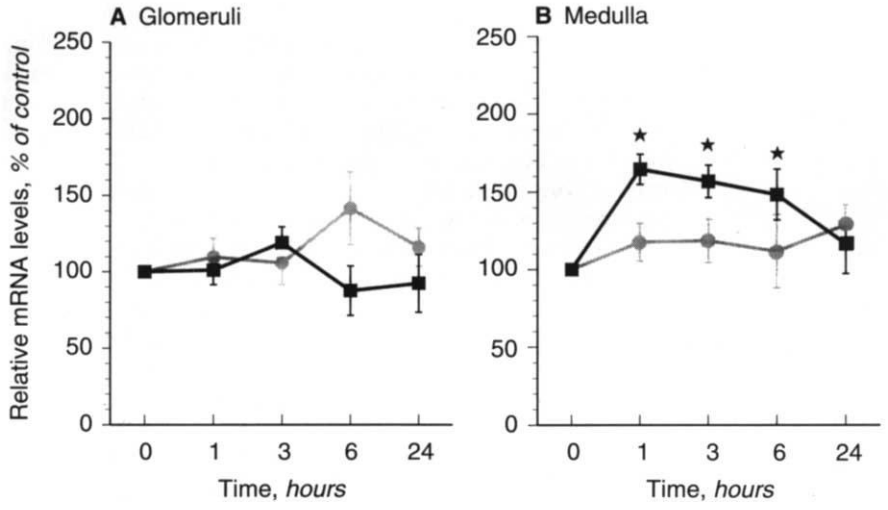


**Fig. 4. A.** *The time course for mRNA for EtA (–●–●–) and EtB (–▲–▲–) receptor subtypes in glomeruli from Cs-treated rats.* **B.** The time course of mRNA for EtA and EtB receptor subtypes in the renal medulla of the same rats. Expression was analyzed by using the RT-PCR method. $^{32}$P-labeled oligonucleotide probes for rat EtA and EtB receptors were hybridized with each PCR product and autoradiography performed. Bands were quantified by a densitometer. Each point was normalized to the expression of β-actin as internal standard. Values are expressed as percent of control values. ★ Statistically significant difference versus control animals.

studies have shown that EtA receptor antagonist can ameliorate glomerular hypofiltration associated with acute Cs administration and lessen the increase in myosin light chain phosphorylation in cultured mesangial cells exposed to Cs [18, 27]. Moreover, it has been demonstrated that Et binding to glomerular membranes is increased in kidneys of animals given Cs [2]. These findings together with the present observations suggest that Et exerts its actions through the EtA receptor in the absence of discernible changes in the mRNA level of this receptor subtype. Of great interest in this regard are the recent observations that the endothelin-receptor complex is rapidly internalized [28], followed by a slow reappearance of the receptor sites [29, 30]. In one study [29], an EtA receptor antagonist, BQ-123, attenuated Et-1 induced contraction in cardiac myocytes either before or after addition of endothelin. These authors believe that efficacy of the receptor antagonist even after addition of the ligand suggests appearance of new receptors and propose functional coupling of the recycling EtA receptors. These findings support the notion that EtA-mediated functions, such as contraction may not require up-regulation of the receptor mRNA expression, instead, recycling of internalized receptors (EtA) contribute to the powerful, long-lasting effects of Et. Therefore, the increased Et binding to glomerular membrane occurring in the absence of increased EtA receptor mRNA expression may be the result of stimulated externalization of these Et receptors.

In summary, the present study shows that administration of Cs modulates renal mRNA expression for prepro-Et-1 and the EtB receptor subtype and demonstrates that the modulation is site selective within the kidney. The renal medulla appears most susceptible to Cs-induced changes in gene expression, such that mRNA for prepro-Et-1 and EtB remains persistantly elevated after Cs administration. We hypothesize that, through paracrine mechanisms, Et contributes to Cs-associated vasospasm, while autocrine mechanisms are involved in the scarring which often affects the renal medulla of Cs-exposed kidney.

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