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Localization of cyclophilin A and cyclophilin C mRNA in murine kidney using RT-PCR

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Localization of cyclophilin A and cyclophilin C mRNA in murine kidney using RT-PCR. Cyclosporin A (CsA), which is widely used as an immunosuppressant, has a nephrotoxic side effect. The mechanism of this nephrotoxicity is not well understood; however, recent studies suggest that cyclophilin (cyp) is responsible for mediating the immunosuppressive action of CsA through the interaction with the Ca^{2+}- and calmodulin-dependent phosphatase, calcineurin. While cyp A mRNA is expressed ubiquitously, cyp C mRNA has been shown to be topically expressed, including in the kidney. We examined: (1) distribution of cyp A and cyp C mRNA in microdissected murine nephron segments, using a combination of reverse transcription and polymerase chain reaction (RT-PCR) techniques, and (2) the effect of CsA administration on cyp C mRNA expression in proximal convoluted tubule. Among the nephron segments examined, large signals for cyp C PCR product were detected in proximal convoluted tubule and proximal straight tubule. Our data showed that the distribution of cyp C mRNA was uneven, and it mainly existed in segments that are relatively sensitive to CsA toxicity. In contrast, cyp A mRNA was found to be distributed almost equally along the nephron segments examined. By CsA administration, the signal for cyp C mRNA PCR product was increased. These results suggest that cyp C may play some role in the renal tubular disorder observed in CsA nephrotoxicity.

Cyclophilin (cyp) was originally identified as a specific binding protein for cyclosporin A (CsA) [1, 2]. Several isoforms of cyp have been discovered in human and other species. CsA is an undecapeptide fungal product, which is widely used to prevent allograft rejection [3] and has been used in the treatment of a variety of autoimmune diseases [4]. Cyp, as the receptor for CsA, is supposed to be the mediator of the immunosuppressive action of CsA.

The major limitation to the clinical use of CsA is its nephrotoxicity. The mechanism of this toxicity is obscure. Friedman and Weissman reported that mRNA encoding cyp C, a newly identified cyp, is topically expressed, including in the kidney [5]. Therefore, it appears that cyp C may be a candidate for involvement in CsA-induced renal damage.

Recently, Moriyama et al [6] introduced a new method for measurement of relative levels of specific mRNA in single microdissected renal tubules, using the polymerase chain reaction (PCR) coupled to reverse transcription (RT-PCR).

In the present study, we examined: (1) the difference in the microdistributions of cyp A and cyp C mRNA in nephron tubules using RT-PCR, and (2) the effect of CsA administration on cyp C mRNA expression in proximal convoluted tubule (PCT).

Methods

Renal tubule microdissection

Male ICR mice aged eight weeks were sacrificed by decapitation. The left kidney was perfused initially with 10 ml of ice-cold dissection solution (solution 1), and then with 10 ml of the same solution containing 1 mg/ml collagenase (collagenase solution) (type I, 300 U/mg Sigma Chemical, St. Louis, Missouri, USA) and 1 mg/ml bovine serum albumin (Sigma Chemical Co.). The dissection solution (solution 1) contained the following (in mM): 135 NaCl, 1 Na_2SO_4, 1.2 $MgSO_4$, 5 KCl, 2 $CaCl_2$, 5.5 glucose, and 5 HEPES (pH 7.4). The left kidney was removed and a coronal section containing the entire corticopapillary axis was made. This section was cut into three pieces: cortex, outer medulla, and inner medulla. These pieces were transferred into individual tubes containing 1 ml of the same collagenase solution used to perfuse the kidney. The tubes were incubated for 40 minutes (cortex and outer medulla) or 60 minutes (inner medulla) at 37°C in a shaking water bath. The solutions were bubbled with 100% oxygen during these incubations. Then tissues were transferred to the dissection solution containing 10 mM vanadyl ribonucleotide complex (Life Technologies, Inc., Gaithersburg, Maryland, USA), a potent RNase inhibitor, and they were placed on ice until microdissection.

Following the previously described procedures [6, 7], we microdissected the following structures: PCT, proximal straight tubule (PST), medullary thick ascending limb (MTAL), cortical collecting duct (CCD), outer medullary collecting duct (OMCD), inner medullary collecting duct (IMCD), and arcuate artery.

The length of the dissected tubules was measured using a calibrated eyepiece micrometer. Generally, 2 mm lengths of renal tubule segment or arcuate artery were transferred to each assay tube, as indicated.

Microdissected structures were transferred to separate wash dishes containing 10 ml of the dissection solution. Microdissected tubules were washed free of contaminating debris and

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the vanadyl ribonucleotide complex. They were then transferred into the appropriate RT-PCR reaction tube, which contained 10 μl of ice-cold dissection solution containing 1 U/μl of human placental RNase inhibitor (Boehringer Mannheim, GmbH, Mannheim, Germany) and 5 mM dithiothreitol (DTT, Sigma Chemical Co.).

Reverse transcription

Reverse transcription (RT) was performed using a cDNA synthesis kit (Boehringer Mannheim GmbH). The RNase inhibitor solution was removed, and 9 μl of Triton X-100, containing > 1 U/μl RNase inhibitor and 5 mM DTT, was added to permeabilize the cells. RT components were added to the reaction tubes as described previously [6, 7]: 4 μl of buffer I, 1 μl of RNase inhibitor, 2 μl of deoxynucleotide mixture, 2 μl of random primer, and 2 μl of avian myeloblastoma virus reverse transcriptase. Reaction tubes were incubated at 42°C for 60 minutes in a Programmed Tempcontrol System (Astec, Tokyo, Japan). At the end of the incubation period, the reaction was stopped by heating at 90°C for five minutes. This heat treatment also denatures RNA-cDNA hybrids and inactivates the reverse transcriptase.

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using the GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer Cetus, Norwalk, Connecticut, USA), with mouse cyp C-specific primers prepared on a DNA synthesizer (Applied Biosystems, Inc., Tokyo, Japan). We designed specific primers with high calculated Tm (>75°C), which allows a stringent annealing temperature in the PCR cycle. Cyp C primer 1 (antisense) was defined by bases 662 to 681, and primer 2 (sense) encompassed bases 103 to 122 [5]. The sequence of primer 1 was 5′-GGGACCTCAACCACAAAGGG-3′; primer 2 was 5′-GAGCCCTGGTGTCTTCTTCG-3′. The predominant cDNA amplification product was predicted to be 578 bp in length (the distance between primers plus primer length). A third oligonucleotide was synthesized to serve as an amplification product-specific probe. This oligonucleotide (sense) included bases 636 to 655 of the cDNA, positioned between primer 1 and primer 2. The sequence of this oligonucleotide probe was 5′-ACAGTGGCAAGATAGATGTG-3′.

Cyp A primer 1 (antisense) was defined by bases 489 to 508, and primer 2 (sense) encompassed bases 99 to 118 [8]. The sequence of primer 1 was 5′-ATCTTCTTGCTGGTCTTGCC-3′; primer 2 was 5′-GTCTCCTTCGAGCTGTTTGC-3′. The predominant cDNA amplification product was predicted to be 410 bp in length. A third oligonucleotide was synthesized to serve as an amplification product-specific probe. This oligonucleotide (antisense) included bases 453 to 472 of the cDNA, positioned between primer 1 and primer 2. The sequence of this oligonucleotide probe was 5′-ATTGTGGAAGCCATGGAGCG-3′.

To carry out the PCR, 80 μl of a PCR Master mix was added to each tube directly. Fifty picomoles each of primers 1 and 2 were used per reaction. Deoxynucleotides were added to a final concentration of 0.20 mM each. Reaction buffer (10×) was diluted (1/10) to have a final composition of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, and 2.5 units of Taq DNA polymerase.

One hundred microliters of mineral oil were overlaid to prevent evaporation during the high temperature incubations. The tubes were placed in a Programmed Tempcontrol system as follows: incubation at 94°C for three minutes (initial melt); then, 33 cycles of the following sequential steps: 94°C for one minute (melt), 60°C for one minute (anneal), and 72°C for three minutes (extend); and finally, incubation at 72°C for seven minutes (final extension).

CsA administration

CsA was injected to the mice intraperitonealy for six days at a daily dosage of 50 mg/kg. On the seventh day, the mice were sacrificed and PCT was microdissected from the kidney with the method described in this paper. RT-PCR for cyp C was performed from PCT from CsA treated mice and control. In this experiment, 30 PCR cycles were performed to obtain the appropriate amount of PCR products and to preserve better linearity between the amount of starting material and the amplification products. This experiment was done with five pairs (CsA treated and control) of mice.

PCR product analysis

Ninety microliters of the total reaction volume were ethanol-precipitated [10]. The PCR products were size-fractionated by agarose gel electrophoresis. After electrophoresis and ethidium bromide staining, DNA bands were visualized with an ultraviolet transilluminator (Funakoshi, Tokyo, Japan).

For Southern blot analysis, gels were denatured and blotted onto a nitrocellulose filter (Funakoshi) essentially as described by Maniatis, Fritsch and Sambrook [10]. The synthetic oligonucleotide probes were end-labeled with ^{32}P as described previously [7]. Prehybridization/hybridization washes were also the same as previously described [7].

Results

Effect of reverse transcription on cyp C and cyp A mRNA amplification

With reverse transcription, we detected a clear single band, that was the predicted size of 578 bp for cyp C from PCT, and we also detected a clear single band that was the predicted size of 410 bp for cyp A from PCT (Fig. 1 A, C). When the PCR procedure was carried out in the absence of reverse transcriptase, the 578 bp band and the 410 bp band were not seen, and there was no other recognizable band. This indicated that the 578 and the 410 bp bands originated from mRNA, not from genomic DNA. The Southern blots of the gels demonstrated that cyp C-specific probe binds to the 578 bp product, and that cyp A-specific probe binds to the 410 bp product, confirming their identities (Fig. 1 B, D).

Distribution of cyp C mRNA in microdissected structures

Each reaction was performed using 2 mm of tubule length or arcuate artery. A single band of predicted size (578 bp) was consistently found from PCT, PST, MTAL, CCD, OMCD, IMCD, and arcuate artery (Fig. 2A). The Southern blots of the gel demonstrated specific binding of the oligonucleotide probe to the 578 bp product (Fig. 2B). A large signal was found in PCT and PST. Small but detectable signals were observed from MTAL, CCD, OMCD, IMCD, and arcuate artery.

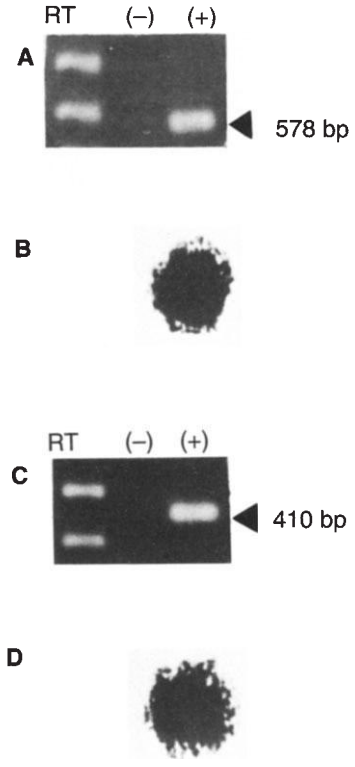

Fig. 1. *Effect of RT on cyp C and cyp A mRNAs amplification.* **A.** Ethidium-bromide-stained agarose gel for cyp C mRNA. The arrow indicates expected PCR product size (578 bp). **B.** Autoradiograms of corresponding Southern blots. The blots were probed with a ^{32}P-labeled oligonucleotide localized between the PCR primers. **C.** Ethidium-bromide-stained agarose gel for cyp A mRNA. The arrow indicates expected PCR product size (578 bp). **D.** Autoradiograms of corresponding Southern blots. The blots were probed with a ^{32}P-labeled oligonucleotide localized between the PCR primers. In each panel, the left lane is the PCR carried out without RT, the right lane is the PCR carried out with RT.

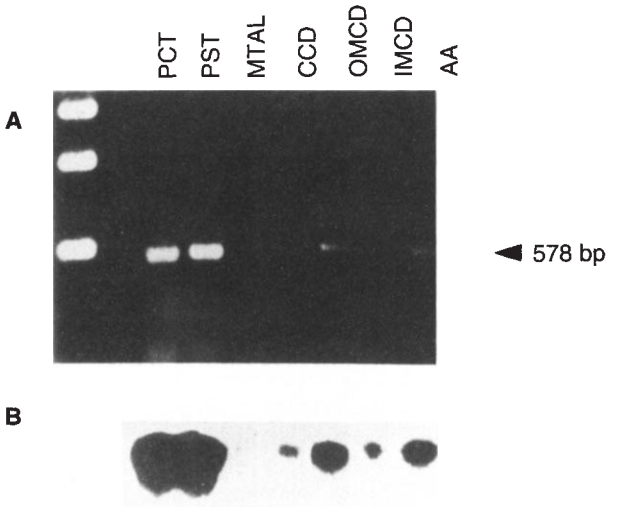

Fig. 2. *Detection of cyp C mRNA in microdissected renal structures by RT-PCR.* **A.** Ethidium-bromide-stained agarose gel for cyp C mRNA. The arrow indicates expected PCR product size (578 bp). **B.** Autoradiograms of corresponding Southern blots. The blots were probed with a ^{32}P-labeled oligonucleotide localized between the PCR primers. Abbreviations are: PCT, proximal convoluted tubule; PST, proximal straight tubule; MTAL, medullary thick ascending limb; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct; AA, arcuate artery.

Table 1. Distribution of cyp C mRNA in microdissected renal structures using RT-PCR

Experiment no.	Nephron segments						
	PCT	PST	MTAL	CCD	OMCD	IMCD	AA
1	++	++	±	+	+	+	+
2	+	+	±	±	+	±	+
3	++	+	±	+	+	+	ND
4	++	+	±	±	±	±	ND
5	+	++	±	−	+	+	ND
6	+	+	−	±	+	±	ND

Abbreviations are: PCT, proximal convoluted tubule; PST, proximal straight tubule; MTAL, medullary thick ascending limb; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct; AA, arcuate artery. The intensity of the autoradiogram spot for each segment is classified into: (++) strong; (+) intermediate; (±) weak; and (−) not detectable; ND, not done.

We performed these experiments six times. The results showed that large signals were consistently found in PCT and PST (Table 1).

Distribution of cyclophilin A mRNA in microdissected structures

The band for cyp A PCR product was detected almost equally in all of the nephron segments examined (Fig. 3A). The Southern blots of the gels demonstrated specific binding of the oligonucleotide probe to the 410 bp product (Fig. 3B). Almost equal signals were obtained from the segments.

Effect of CsA administration

The bands for cyp C PCR product were detected in the PCT of both the CsA treated mice and control (Fig. 4). The signal of PCT from the CsA treated mice was larger than that of the PCT from control. We performed this experiment five times and confirmed this result.

Discussion

Our study shows that mRNA encoding murine cyp C can be found throughout the nephron and arcuate artery. Among the structures examined, expression is high in PCT and PST. This mode of distribution is quite different from that of cyp A mRNA, which is almost equally expressed along the nephron segments examined. The cyp C mRNA expression is increased by CsA administration.

As will be discussed later, cyp is supposed to mediate the immunologic action of CsA. It is of interest to investigate whether cyp plays any role in the nephrotoxic action of CsA.

The nephrotoxicity of CsA roughly consists of two different patterns. One is an acute hemodynamic and reversible pattern, which often occurs soon after onset of CsA treatment. The other pattern of toxicity is chronic and irreversible; it is seen with longer-term CsA treatment and is associated with morphologic changes. Several explanations have been offered to account for the CsA nephrotoxicity, including stimulation of the

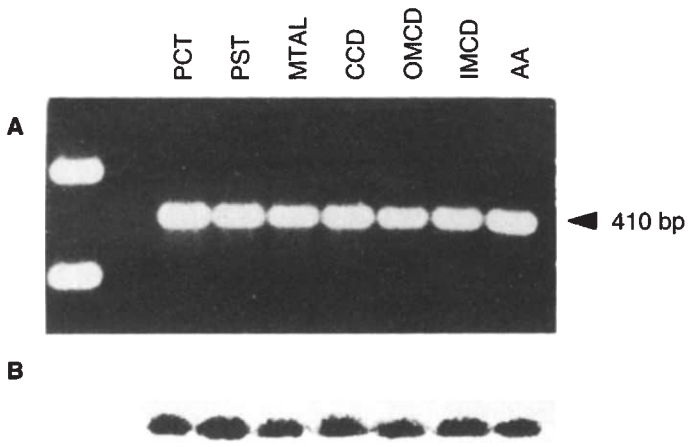

Fig. 3. *Detection of cyp A mRNA in microdissected renal structures by RT-PCR.* **A.** Ethidium-bromide-stained agarose gel for cyp A mRNA. The arrow indicates the expected PCR product size (410 bp). **B.** Autoradiograms of corresponding Southern blots. The blots were probed with a ^{32}P-labeled oligonucleotide localized between the PCR primers. Abbreviations are: PCT, proximal convoluted tubule; PST, proximal straight tubule; MTAL, medullary thick ascending limb; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct; AA, arcuate artery.

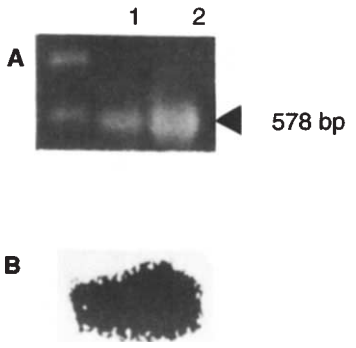

Fig. 4. *Effect of CsA administration on cyp C mRNA expression.* **A.** Ethidium-bromide-stained agarose gel for cyp C mRNA. The arrow indicates expected PCR product size (578 bp). **B.** Autoradiograms of corresponding Southern blots. The blots were probed with a ^{32}P-labeled oligonucleotide localized between the PCR primers. Lane 1: PCT from control mouse; Lane 2: PCT of CsA treated mouse.

renin-angiotensin system [11], activation of tubuloglomerular feedback [12], abnormalities in renal arachidonic acid metabolism [13, 14], and increased production of endothelin [15]. However, the mechanism(s) of the nephrotoxicity is not fully understood. Cyp C mRNA is topically expressed, including in the kidney [5]. This gives rise to the possibility that cyp C is involved in CsA nephrotoxicity. Meanwhile, it does not seem likely that cyp A plays a major role in this toxicity, because cyp A mRNA expression is abundant and ubiquitous [16].

There have been some reports about the morphology associated with CsA nephrotoxicity. Mihatsch et al [17] demonstrated tubular inclusion bodies corresponding to autolysosomes and giant mitochondria, tubular vacuolization due to dilation of endoplasmic reticulum, tubular microcalcification, and tubular regeneration limited to or predominating in the proximal tubule. Bertani et al [18] reported isometric vacuolization and loss of the brush border involving proximal tubular cells in the early phase, followed by a peculiar lesion in distal tubular cells due to glycogen accumulation. Thliveris et al [19] documented numerous vacuoles, lysosomal-like structures, and loss of cellular integrity in proximal and distal tubules. Finally, Wilson and Hartz [20] indicated that cultured cells from proximal convoluted tubule and proximal straight tubule epithelia were sensitive to the toxic effect of CsA. There are some dissimilarities among these reports, due presumably to the differences in animal models, CsA dose, and duration and route of administration. There is, however, the common feature of predominant proximal tubule injury. Our data indicate that cyp mRNA expression is high in PCT and PST, and this seems to be compatible with the idea that cyp C plays some role in CsA nephrotoxicity, taking into account this common morphologic feature. When CsA is administered, it may be bound and trapped more within PCT and PST, which are enriched with cyp content, and may be prevented from cellular elimination. This leads to increased toxic action of CsA on proximal tubule cells. Because CsA binds to cyp C, the amount of unbound cyp C, which plays some physiological roles, was depleted. Thus, the amount of cyp C mRNA expression could increase with some feedback mechanism(s). Our result from the CsA treated experiment is consistent with the idea of "CsA-cyp C linking." However, the possibility cannot be excluded that some other factor which is still unknown may also be responsible for the toxicity. With regard to this, Stillman et al [21] reported that CsA induces atrophic and hypertrophic changes in MTAL, a structure in which cyp C mRNA expression is low according to our results. Recently, CsA-induced inhibition of Na^+,K^+-ATPase activity in the CCD, MTAL, and OMCD from the outer stripe was reported [22]. This finding can well explain the clinical hyperkalemia and hypomagnesemia observed in patients treated with CsA [22].

Cyp was proved to be identical to peptidyl-prolyl *cis-trans* isomerase (PPIase) [23, 24]. This PPIase activity is inhibited when cyp binds to CsA. Cyp-CsA complex competitively binds to and inhibits the Ca^{2+}- and calmodulin-dependent phosphatase calcineurin [25]. Therefore, the hypothesis has been forwarded that cyp may play some role in the pharmacological action of CsA, that is, its immunosuppressive effect and nephrotoxicity, through the PPIase activity of cyp and/or phosphatase activity of calcineurin. The speculation that the action of CsA develops through the inhibition of PPIase is challenged by several findings, however. First, cyps were found intracellularly at concentrations far above the concentration of CsA required for immunosuppression [26, 27]. Second, several CsA analogues were reported with cyp-binding affinities and immunosuppressive activities that did not correlate [28]. Third, FKBP, the binding protein for another immunosuppressant, FK506, also has PPIase, and even when PPIase activity of cyp is inhibited by binding to CsA, its enzyme activity is intact [29]. On the contrary, several studies have supported the hypothesis that calcineurin is the second target of CsA, which acts through its primary cyp target. Transfection of a calcineurin catalytic subunit increases the 50% inhibitory concentration (IC_{50}) of CsA [30]. Calcineurin activates the interleukin-2 promoter in a CsA-sensitive manner [30]. CsA prevents T cell proliferation by

inhibiting the induction of interleukin-2 transcription [31]. Furthermore, overexpression of calcineurin in the T cell line Jurkat increases resistance to CsA [32]. And finally, at low concentration, CsA enhances the phosphorylation of endogenous protein substrates in rat brain tissue and PC12 cells (a neuronal-like cell line) [33]. Therefore, it appears that calcineurin may interact with CsA and Cyp, and reduce the Ca^{2+}-dependent phosphorylation of endogenous protein substrates. The inhibition of phosphatase activity of calcineurin by CsA-cyp complex reduces the dephosphorylation of substrate proteins, some of which must be essential to maintaining cell function, and this effect may lead to cell injury. Cyp C, which is largely expressed in PCT and PST, may produce the toxic effect of CsA by inhibiting the phosphatase activity of calcineurin in these segments.

We chose a simple approach, RT-PCR, to relative quantitation rather than any of the complicated alternatives for the following reason. The value of knowing the absolute number of mRNA molecules present in a given cell may be extremely limited for most physiological questions, because there is presently no way to relate this number to the ultimate functional expression of the gene product. We believe that our method gives a relative measure of the amount of cyp A and cyp C mRNA initially present in the cells. This conclusion is based on the observation that there is an approximately linear relationship between the tubular length used for the assay (that is, the amount of starting material) and the value obtained from densitometry of the amplification product band [34].

In summary, mRNA encoding murine cyp C can be found from PCT to IMCD along the nephron and arcuate artery. Among the structures examined, cyp C mRNA expression is uneven, and it exists mainly in PCT and PST, which are relatively sensitive to CsA toxicity, particularly its chronic form. Cyp A mRNA expression is almost equal along the renal tubule segments examined. Cyp C mRNA expression in PCT from the CsA treated mice was increased. These results suggest that cyp C may play some role in the renal tubular disorder observed in CsA nephrotoxicity.

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