Cyclophilin B Expression in Renal Proximal Tubules of Hypertensive Rats

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Abstract—Rat cyclophilin-like protein (Cy-LP) is a candidate hypertension gene initially identified by differential hybridization and implicated in renal mechanisms of salt retention and high blood pressure. We report the molecular characterization of rat cyclophilin B (CypB) and demonstrate, through sequence analysis and an allele-specific polymerase chain reaction primer assay, that CypB but not Cy-LP is expressed in rat kidney. CypB is an endoplasmic reticulum–localized prolyl-isomerase that interacts with elongation initiation factor 2-β, an important regulator of protein translation and a central component of the endoplasmic reticulum stress response to hypoxia or ATP depletion. Active renal transport of sodium is increased in the spontaneously hypertensive rat (SHR), and there is evidence that this coincides with hypoxia and ATP depletion in the renal cortex. In the present studies we have examined expression of CypB in rat proximal tubules, which contributes to the increased renal sodium reabsorption in this model of hypertension. We report that CypB transcript abundance is significantly elevated in proximal convoluted tubules from SHR compared with the control Wistar-Kyoto strain. This upregulation occurs in weanling animals and precedes the development of hypertension, indicating that it is not a simple response to hypertension in SHR. Further, CypB expression is also higher in a proximal tubule cell line derived from SHR compared with a similar line derived from Wistar-Kyoto rats, indicating that this difference is genetically determined. No sequence differences were observed in the CypB cDNA from these 2 strains. These observations suggest that a genetically determined alteration in proximal tubules from SHR occurs that leads to increased expression of CypB. In view of evidence linking CypB to the regulation of elongation initiation factor-2, the upregulation of CypB may result from metabolic stress. (Hypertension. 2000;35:958-964.)

Key Words: rats, spontaneously hypertensive ● blood pressure ● gene expression ● polymerase chain reaction

In the spontaneously hypertensive rat (SHR), elevated blood pressure appears to depend on genetic mechanisms operating through genes that are expressed in the kidney.1 Several avenues of investigation have been developed in an effort to identify which genes contribute to the disease phenotype. One avenue is to compare the patterns of expression of genes in kidneys from hypertensive and normotensive rat strains. Differential hybridization has been useful in identifying renal hypertension genes. For example, the Sa gene was identified because it is upregulated in the kidney of SHR,2 and restriction fragment length polymorphism analysis of the human homologue of this gene implicates Sa gene sequence variation in hypertension in some human populations.3 Rat cyclophilin-like protein is an atypical member of the family of cyclophilin peptidyl-prolyl isomerases with no homologue known in other species. This gene has also been identified as differentially expressed in the kidneys of normotensive rats during sodium depletion and upregulated in the kidneys of SHR, suggesting that it might participate in renal sodium retention mechanisms contributing to hypertension in SHR.4 In the present study, we have examined the sequence of rat cyclophilin-like protein to shed further light on this unusual gene. These studies indicate that this gene is, in fact, rat cyclophilin B (CypB). We have used a novel, accurate, sensitive, and precise competitive reverse transcription–polymerase chain reaction (RT-PCR) method5,6 to evaluate expression of CypB in microdissected renal proximal tubules of SHR and Wistar-Kyoto rats (WKY) at 4 to 5 and 15 to 16 weeks of age. These ages correspond to the earliest emergence of hypertension and to its full establishment in SHR. We have also examined whether the phenotype of altered CypB expression in SHR kidney is preserved in immortalized proximal tubule cell cultures originated from these 2 rat strains.7 Furthermore, angiotensin II (Ang II) was tested for its ability to regulate CypB transcript abundance and was shown to upregulate expression. Finally, we have compared the cDNA sequences of CypB in SHR and WKY to determine whether altered function of this gene may be attributable to a primary variation in the encoded protein. The results of our experiments, along with recent information indicating that...
CypB interacts with key proteins of the endoplasmic reticulum stress response, suggest that CypB upregulation in the SHR kidney is an adaptation to metabolic stress.

**Methods**

**Animals**

Sprague Dawley (SD), spontaneously hypertensive (SHR), and matched WKY control rats were obtained from Harlan (Indianapolis, Ind.). Animals were housed in a room maintained at 22° to 25°C on a 12-hour:12-hour light/dark cycle and were fed a standard Purina laboratory rodent diet and deionized water ad libitum. Studies in SHR and WKY were performed on animals between 4 to 5 weeks of age and in another group 15 to 16 weeks of age. SD animals were studied at ~12 weeks of age. All animals were held in the animal facility for ~5 days before tissue collection. The presence of the hypertensive phenotype was confirmed by tail-cuff plethysmography after warming (mean systolic blood pressures in mm Hg±SEM: SHR 207.7±5.5, WKY 135.7±6.7).

**Preliminary Characterization of Rat Cyclophilin-Like Protein**

Specific primers were designed to amplify rat cyclophilin-like protein (Cy-LP) on the basis of the published sequence of this gene. Kidney total RNA was prepared from an adult rat, and RT-PCR was performed with the use of Moloney murine leukemia virus RT, random hexamer and reverse primer priming, and Perkin-Elmer AmpliTaq. Reaction products were cloned, and cloned cDNA was sequenced (see below).

**Sequencing of Rat CypB**

We used a forward primer (5'-TACCACTACTATGGATG-3') corresponding to nt 757 to 774 of the pGAD10 vector (Clontech) and a reverse primer that corresponds to nt 693 to 712 of the Cy-LP coding sequence (5'-TTTTGACGCTGCTTTAC-3'). To obtain the remaining 186 nucleotides of the Cy-LP coding sequence, a primer corresponding to nt 509 to 528 in the Cy-LP coding sequence (5'-TTGGTAGCGGACTGTTCA-3') was used with a primer corresponding to nt 862 to 894 of the pGAD10 sequence (5'-ATCGTAGATACTGAAAACCCCGCAAGTTCA-3'). PCR products made with these primer combinations were incubated with exonuclease I (Exo I; Boehringer Mannheim) and shrimp alkaline phosphatase (Boehringer Mannheim), purified with Centri-Sep spin columns (Promega), and sequenced by cycle-sequencing with “Big Dye” (Perkin Elmer) dye terminator reactions, with fluorescent detection on an ABI Prism 377 DNA sequencer (Perkin Elmer).

**Nephron Microdissection and RNA Preparation**

Nephrons were dissected from rats deeply anesthetized with pentobarbital (40 mg/kg IP). The abdominal cavity was opened and the aorta clipped above the bifurcation of the renal artery as previously described. A catheter was inserted into the aorta for perfusion. One kidney was then perfused with 20 mL ice-cold dissection solution containing 1.0 mg/mL collagenase (Sigma Chemical Co) and albumin in a Tris-Cl buffered physiological salt solution. This kidney was removed, sliced into thin sections along the corticomedullary axis, and incubated in collagenase dissection solution bubbled with compressed air for 15 minutes at 37°C. These slices were then rinsed to remove collagenase and placed for dissection in a Petri dish containing ice-cold dissection medium and the RNAse inhibitor. They were then lysed directly in the well with RNA extraction solution (RNeasy B), and the lysate was transferred to a microcentrifuge tube. The coprecipitants yeast transfer RNA (100 ng/μL) and linear acrylamide (Ambion) were added to increase yield. Precipitated nephron RNA was dried and redissolved in yeast transfer RNA (100 ng/μL). The RNA was diluted for storage in order that RNA from 0.25-mm nephron was present per microliter of RNA preparation. Storage of RNA extracts was at −80°C. Integrity of RNA was validated by extraction of RNA from a slice of kidney after dissection. Intact 18S and 28S bands were evident on examination of the RNA by denaturing agarose electrophoresis.

**Proximal Tubule Cell Culture**

Proximal tubule cell lines derived from SHR and WKY rats were the generous gift of Dr. Ulrich Hopfer, Case Western Reserve University. Cell cultures were grown at 37°C in a 5% CO2 humidified atmosphere. Cell lines were routinely cultured in D-MEM:F12 (1:1), supplemented with 15 mmol/L HEPES, 1.2 mg/mL NaHCO3, 10 ng/mL epidermal growth factor, 5 μg/mL insulin, 4 μg/mL dexamethasone, 5 μg/mL transferrin, 5% fetal bovine serum, and 100 μg/mL streptomycin sulfate/100 IU penicillin G. Because viability and attachment was extremely low when grown on plastic, cultures were grown on collagen I–coated 100-mm dishes. Collagen-coated plates were purchased (Becton Dickinson) or made by wetting plates with a 20% rat tail collagen type I (Becton Dickinson) dispersion in 60% ethanol. These plates were allowed to air dry in a laminar flow hood irradiated with a UV light to ensure sterility. Cultures were treated with Ang II by removal of medium followed by replacement with growth medium (with or without serum supplementation), to which various concentrations of Ang II had been added with incubation for 4 hours followed by harvesting of cells and RNA extraction.

**Determination of Cyclophilin B mRNA Abundance**

Quantification of gene expression was performed by a competitive RT-PCR method that we have described fully elsewhere. Briefly, the method used in vitro transcribed size-mutant RNA competitor that shared high sequence homology with the amplified native transcript. Similar competitors have been previously demonstrated to share identical RT and PCR amplification efficiency and result in estimates of nephron gene expression that have a coefficient of variance of <10%. Measurements are performed in replicate, and the resulting estimate is reported as molecules of specific mRNA expressed per unit length of nephron.

**Competitive RT-PCR Product Analysis**

A critical component in the accurate and precise quantification of nephron gene expression is the method used to analyze the products of competitive RT-PCR reactions. As described in detail previously, reaction products were analyzed by a denaturing high-performance liquid chromatography technique (DNAsep column, Transgenic, Inc.). Product detection was by UV absorbance (254 nm).

**Sequencing**

RT-PCR products corresponding to the entire coding sequence and available 5’ and 3’ UTRs were produced. These products were incubated with exonuclease I (Boehringer) and shrimp alkaline phosphatase (Boehringer) to remove unincorporated primers and nucleotides from the RT-PCR reaction. Products were then purified further with Centri-Sep spin columns (Promega) and sequenced by cycle-sequencing with Thermosequenase (Amersham) in the presence of “Big Dye” terminators followed by sequence analysis on an ABI Prism 377 DNA sequencer (Perkin Elmer).

**Statistical Analysis**

Differences between strains at each age group were tested by use of the Student’s t test with the null hypothesis rejected at the 95% confidence interval.
Results

We used primers directed toward the peptidyl-prolyl isomerase domain of Cy-LP to amplify and clone RT-PCR products from rat total RNA. The cloned cDNAs from 11 individual clones were sequenced. In each case, consistent single base alterations from the published rat Cy-LP sequence (indicated by arrows in Figure 1) were detected. This suggested to us that the original rat Cy-LP may contain sequencing errors. To verify that CypB rather than Cy-LP is expressed in rat kidney, we screened a rat kidney cDNA library by using primers directed against the 5′ and 3′ termini of the published Cy-LP sequence. We generated a clone that was 840 nucleotides long, included an open reading frame between nucleotides 23 and 649 flanked by initiation (ATG) and termination (TAG) codons, had a short, 23-bp 5′ untranslated region, and a 191-bp 3′ untranslated region (Figure 1). The coding sequence encoded a 208–amino acid polypeptide that had a derived molecular mass of 18 kDa that included the consen-
sus peptidyl-prolyl isomerase (PPIase) domain shared by all cyclophilins. Moreover, it contained a C-terminal endoplasmic reticulum–retention motif, an N-terminal signal peptide domain, and a motif displaying characteristics of a nuclear localization sequence (Figure 2).

Further analysis (Figures 1 and 2) revealed that the nonhomologous region of Cy-LP (aas 46 to 62) resulted from an upstream, single nucleotide deletion that shifts the gene out of frame with respect to the CypB sequence. Furthermore, a compensatory insertion 54 nucleotides downstream restores the original frame, resulting in a hypothetical protein exhibiting 100% amino acid identity to mouse CypB from amino acids 1 to 45 and 98.6% identity from amino acids 63 to 209 but 6% identity (1 of 17 amino acids) in the region encoded by the sequence between the frameshifts (Figure 2).

Despite the repeated sequencing of multiple RT-PCR reaction products from rat kidney total RNA, we were unable to demonstrate the expression of a gene with identical sequence to Cy-LP. Instead, each attempt resulted in products with sequences corresponding to that of CypB, suggesting again that only CypB and not Cy-LP was expressed in rat kidney (Figure 3). To determine whether both CypB and Cy-LP were expressed in the kidney cDNA library, we performed an allele-specific primer amplification assay by designing primers with 3′ nucleotides corresponding to those nucleotides displayed both in the presence and the absence of the aforementioned frameshifts and testing these primers for amplification at progressively increasing annealing temperatures (Figure 4). When PCR reaction cycle annealing temperature was 53°C, products resulting from reactions with primers specific for Cy-LP were observed. However, increasing annealing temperatures during the PCR reactions designed to specifically produce either CypB or Cy-LP resulted in reduction or disappearance of Cy-LP but not CypB product accumulation. These observations further indicate that only CypB and not Cy-LP is expressed in rat kidney tissue.

A quantitative tissue survey that used competitive RT-PCR revealed that CypB is differentially expressed in various tissues, with kidney demonstrating the highest transcript abundance of all tissues surveyed (Figure 5). Similarly, a quantitative nephron segment survey demonstrated that proximal tubule expresses CypB at a greater level than any of the other nephron regions examined (Figure 6). Many gene quantification systems allow only for relative comparisons in expression levels between different genes or gene products. However, we have shown that our techniques for measure-
ment of transcript abundance by competitive RT-PCR are quantitatively accurate, therefore it is possible to compare the abundance of transcripts from different genes. Quantification of proximal tubule CypB revealed that it is expressed at a level similar to that of the α-subunit of sodium-potassium-ATPase (NKA).

The high sensitivity of competitive RT-PCR allowed us to examine the level of CypB transcript abundance in individual, functionally and morphologically distinct nephron regions and to make comparisons across multiple individuals from each strain. We focused these studies on the proximal convoluted and straight tubules in which approximately two thirds of renal sodium reabsorption occurs. Our results (Figure 7) indicate greater CypB transcript abundance in the proximal convoluted tubules from SHR compared with WKY kidneys. This difference is apparent at 5 weeks of age, suggesting that CypB upregulation is not a simple response to the high level of blood pressure that subsequently develops in this strain. CypB transcript abundance was also greater in proximal straight tubule from SHR, though the difference in this nephron segment was not statistically significant.

The same pattern of CypB transcript abundance was also observed in RNA from immortalized proximal tubule cell lines derived from SHR and WKY. These cultures provide an opportunity to examine proximal tubule CypB expression in the absence of varying extrarenal influences such as circulating hormones or autonomic innervation. Figure 8 demonstrates that CypB transcript abundance was also significantly

Figure 4. CypB but not Cy-LP is expressed in rat kidney. Allele-specific, primer-based PCR assay was performed with upstream primers shown in Figure 3 in combination with single downstream primer that was fully complementary to sequence common to both CypB and Cy-LP. Annealing temperatures of 53°, 60°, 63°, and 66°C were used. Top panel, Upstream primer complementary to sequences shared by both CypB and Cy-LP (positive control). Second panel, Upstream primer CypB FS1 primer designed to complement CypB fully at location of first possible frameshift site (FS1 in Figure 3). Third panel, Upstream primer Cy-LP FS1 designed to complement Cy-LP fully at first frameshift site; note that at highest annealing temperature PCR product accumulation was reduced. Fourth panel, Upstream primer CypB FS2 designed to complement CypB fully at location of second frameshift site (FS2 in Figure 3). Bottom panel, Upstream primer Cy-LP FS2 designed to complement Cy-LP fully at second frameshift site; note disappearance of PCR products at higher annealing temperatures.
higher in cultured proximal tubule cells derived from SHR than WKY. Furthermore, treatment of cell cultures with Ang II was associated with significantly increased expression of CypB. This was particularly pronounced in serum-starved cultures in which a linear dose-response relation was observed between $10^{-9}$ and $10^{-7}$ mol/L Ang II (data not shown).

No difference in response to Ang II was observed between the 2 cell lines.

The link between CypB expression and hypertension in SHR led us to investigate whether the CypB message expressed by SHR exhibited any sequence differences from WKY. No such differences were detected between the 2 strains, either in the coding or small amount of noncoding sequence examined. Limited 5' and 3' rat CypB sequences have thus far been obtained, thereby hindering the evaluation of mechanisms involving differences between SHR and WKY enhancers or other distal cis-acting elements capable of producing strain-specific differences in CypB expression.

**Discussion**

Iwai and Inagamit used differential hybridization to isolate a clone termed “cyclophilin-like protein” (Cy-LP) and showed that its expression in kidney was increased in genetically hypertensive rats. Sodium depletion also altered renal expression, suggesting that this protein might be involved in abnormal renal sodium retention occurring in this genetic model of hypertension. Sequence analysis revealed that the Cy-LP gene was highly homologous to CypB, an endoplasmic reticulum-localized member of the cyclophilin protein family. Only a short sequence of the Cy-LP gene encodes amino acids that bear no homology to CypB, whereas the remainder is nearly identical. Both mouse and human tissues have been shown to express CypB, but no such demonstration had been made in rat tissues. We hypothesized that Cy-LP was the rat CypB ortholog that may be involved in renal mechanisms of sodium balance and hypertension. We report the rat CypB cDNA sequence and show that it differs from the previously reported Cy-LP sequence but is highly homologous to mouse and human CypB. We were unable to find evidence for the renal expression of transcripts corresponding to Cy-LP.

The present studies indicate that expression of the endoplasmic reticulum–located peptidyl-prolyl isomerase, cyclophilin B, is increased in renal tubules from SHR compared with its genetically related normotensive control strain. This difference has emerged at a time (5 weeks) when renal sodium reabsorption is increased in SHR compared with control but before substantial elevation of blood pressure has occurred. This indicates that the difference in CypB expression is not secondary to hypertension but is more likely to reflect the pathogenetic processes that lead to blood pressure elevation. Our results also indicate that this difference in CypB expression is preserved in immortalized renal proximal tubule cell lines derived from these strains.

We have shown by yeast 2-hybrid analysis that CypB interacts with elongation initiation factor (eIF)2-β. eIF2-β is
part of a ternary complex protein with initiator methionine-tRNA and GTP. The eIF2 protein complex is the principal regulator of ribosomal translation and plays a central role in the cellular response to stress. eIF2 activity is regulated by phosphorylation. Cyclosporine A, an immunosuppressant drug for which CypB has high binding affinity, is able to alter eIF2 phosphorylation through mechanisms that may involve CypB and thereby inhibit protein translation. The present observations suggest that altered regulation of CypB in SHR may reflect a cellular stress response in the renal epithelium. This response can be induced by a number of signals that result in accumulation of unfolded proteins in the endoplasmic reticulum lumen, including ATP depletion and oxidative stress.

In SHR, renal epithelial stress may arise from a genetically determined increase in renal ATP consumption resulting from increased active sodium reabsorption. The energizing mechanism for renal sodium reabsorption is the renal NKA. In the rat nephron, this enzyme contains an additional $\gamma$-subunit not expressed in other tissues. The $\gamma$-subunit increases the affinity of the enzyme for ATP and has been proposed to protect renal tubular epithelial sodium reabsorption from ATP depletion. The renal cortex and adjacent medulla are vulnerable to hypoxic injury, in part because the presence of an arteriovenous shunt results in perfusion of the renal proximal nephrons with poorly oxygenated blood and because of the high energy consumption required to sustain renal active sodium transport.

Increased sodium reabsorption occurs in SHR kidney tubules and results in levels of oxygen consumption that have been estimated to be 15% to 25% greater than in normotensive controls. The $\mathrm{P}_2$ in SHR kidney cortex is significantly less than in WKY, with the difference attributed to higher $\mathrm{O}_2$ consumption in sodium reabsorption. The resulting increase in ATP use may produce metabolic stress and relative energy starvation in SHR nephrons. Susceptibility of the renal proximal tubules to metabolic injury is reflected in experimental and genetic models from both rat strains showing that reduction of cellular ATP levels in cultured mouse proximal tubule cells to only $\sim 70\%$ of control was associated with an apopotic cell loss of $\sim 25\%$.

The persistence of increased CypB expression in immortalized proximal tubule cell lines from SHR suggests that this phenomenon is determined by genetically influenced renal epithelial abnormalities in SHR and is not the result of alteration in mechanisms arising outside the kidney (e.g., circulating hormones, autonomic nerve activity). Cell cultures from both rat strains showed positive modulation of CypB expression in response to treatment with Ang II. Because this hormone has been shown to increase sodium transport and NKA activity, it is possible that upregulation of CypB expression in response to Ang II is an adaptation to the increased metabolic activity resulting from Ang II stimulation of sodium transport in these cell lines. There was no difference in response to Ang II between cell lines from the 2 strains. The primary abnormality responsible for altered CypB expression does not appear to be in the coding sequence of CypB itself because no sequence variation between the rats strains was detected.

Our observations of altered CypB gene expression in SHR, its appearance before the onset of hypertension, and its persistence in immortalized proximal tubule cells is evidence to suggest that CypB may participate in abnormal function in renal transport epithelium, which contributes to arterial hypertension in SHR. These results also suggest that persistent metabolic stress resulting from genetic alterations leading to hypertension may provide a link to metabolic renal injury and therefore should be considered as a mechanism underlying the association of hypertension with chronic kidney failure.

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