Ciclorsporin-induced hypertension is associated with increased sodium transporter of the loop of Henle (NKCC2)

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Abstract

Background. Hypertension induced by cyclosporine is associated with renal sodium and water retention. Using immunoblotting of kidney homogenates, we investigated the regulation of sodium and water transport proteins in a rat model of cyclosporine-induced hypertension.

Methods. Rats were treated with cyclosporine (25 mg/kg/day intraperitoneally) during 7 days. Control rats received vehicle.

Results. Cyclosporine-treated rats had an increase in blood pressure with a decrease in renal sodium excretion compared with control rats. There were no differences either in sodium intake or in plasma creatinine levels between the two groups of rats. These data suggest that the decrease in sodium excretion in the cyclosporine-treated rats was due to an increase in renal sodium absorption. The densitometric analysis of the renal immunoblot showed an increase in the Na-K-2Cl cotransporter of the loop of Henle (NKCC2) in cyclosporine-treated rats (178% ± 36) compared with control rats (100% ± 18; P < 0.05*). This protein rise was associated with an increase in the NKCC2 mRNA pointing to a transcriptional regulation of this sodium transporter.

There were no statistically significant changes in the sodium proton exchange (NHE-3) of the proximal tubule although in this renal segment, aquaporin-1 was increased in cyclosporine-treated rats compared with control rats (control 100% ± 18 vs cyclosporine 119% ± 6; P < 0.05*).

Conclusions. Our results pointed to the thick ascending limb of the loop of Henle as an important site of sodium retention in cyclosporine-induced hypertension. This data may have potential clinical implications for the treatment of hypertension induced by cyclosporine.

Keywords: aquaporins; cyclosporine; hypertension; NHE-3; NKCC2; renal sodium transporters

Introduction

Anticalcineurinics drugs are among the most widely used immunosuppressants for preventing graft rejection and autoimmune diseases. Since the introduction of cyclosporine for solid organ transplants, long-term survival after transplantation has significantly improved; however, cyclosporine treatment produces important side effects like high blood pressure. The pathogenesis of this hypertension is not completely clear. There is evidence suggesting that sodium and water retention is associated with the development of cyclosporine-induced hypertension [1].

The kidney plays a central role in the regulation of body salt and water balance. Disordered regulation of transport in the kidney is responsible for altered salt and water balance in several pathophysiological states, including hypertension. Regulation of salt and water excretion depends on an array of solute and water transporters in renal tubules and of vascular elements in the various regions of the kidney. Renal sodium absorption in the proximal tubule and in the thick ascending limb is mediated by sodium transporters. In the proximal tubule, the main protein that facilitates sodium transport in the apical or luminal plasma membrane is the sodium/hydrogen exchanger isoform 3 (NHE-3) [2]. In the thick ascending limb of
the loop of Henle, apical sodium transport is facilitated mainly by the sodium two-chloride potassium cotransporter (NKCC2) [2]. The transport of sodium in the basolateral plasma membrane along the renal tubules is facilitated by sodium potassium ATPase (NaKATPase) [2]. Aquaporins are a family of transmembrane proteins that function as molecular water channels [3]. Several aquaporins are known to be expressed in the kidney where they facilitate osmotic water transport across water-permeable epithelia and play critical roles in the urinary concentrating and diluting process. Aquaporin-1 is expressed in the apical and basolateral membrane of the proximal tubule and descending limb of Henle’s loop cells, while aquaporin-2 is expressed in the apical membrane of the collecting duct cells.

Over the past decade, these sodium transporters and aquaporins have been cloned and sequenced. This has allowed the possibility of obtaining polyclonal antibodies against these proteins and the development of approaches based on protein analysis of renal sodium and water transport in pathophysiological conditions [4]. This approach assesses the abundance of each renal tubule transporter protein as an estimation of the degree of sodium and water reabsorption in the different nephron segments.

In the current study, this specific type of approach was used for the first time to investigate the pathogenesis of cyclosporine-induced hypertension. Specifically, this work mainly focuses on proximal tubule and thick ascending limb sodium transporters, since preliminary data using ‘in vitro’ studies point to these renal segments as important parts of the effect of cyclosporine [5].

Methods

Hypertension model and experimental protocol

Experiments were done in 10 adult Wistar rats (Charles River Breeding Laboratories, Saint Aubin les Elseuf, France). The hypertension model was induced by cyclosporine. Treated rats \( n = 5 \) received a daily intraperitoneal injection of cyclosporine at a dose of 25 mg/kg for a week, while control rats \( n = 5 \) received vehicle [6]. Rats were individually placed in metabolic cages in order to control water and sodium balance. All rats had free access to synthetic food (0.083 mmol/g Na, Formula A04, Panlab, Barcelona, Spain) and water. In order to calculate the intake of food and liquid we supplied a surplus of it everyday and we measured the remaining food and water every following day. Rats were weighed daily. Resting blood pressure was measured with a tail cuff at Day 1, 3 and Day 6.

Rats were euthanized by decapitation at Day 7. Blood was collected from the neck immediately after sacrifice and serum was separated by centrifugation. The kidneys were frozen at \(-80^\circ\text{C}\) for later processing. The protocols were performed according to the criteria of the Investigation and Ethics Committee of the Hospital Clinic in Barcelona.

Sodium and water balance

Sodium balance was calculated as the net difference between sodium intake and urine sodium excretion in relation to rats’ weight on Days 1, 3 and 6 (Figure 1). Water balance was estimated each day of the experiment by the difference between water intake and urine volume, corrected by the animals’ weight (Figure 2).

Blood and urine analysis

Serum and urine osmolality were determined by osmometric depression of the freezing point (Osmometer 3300; Advanced Instruments, Needham Heights, MA), and sodium by flame photometry (IL 943; Instrumentation Laboratory, Lexington, MA). Serum creatinine levels were determined by standard methods.

Polyclonal antibodies

Affinity-purified, peptide-derived polyclonal antibodies to sodium transporters and water channels were used for immunoblotting. The antibodies used were directed to NHE-3 [4], the thick ascending limb isoform of NKCC2 [4], the aquaporin-1 [7] and the aquaporin-2 [7]. The specificity of the antibodies was tested by competition experiments [4,7].
Preparation of kidney tissue for immunoblotting

The left kidneys from 10 rats were prepared to obtain whole-kidney homogenate. Protein preparation was performed as previously described [4].

Electrophoresis and immunoblotting of proteins

SDS–PAGE was done using 7.5–12% polyacrylamide minigels. In all cases, to confirm equality of loading among lanes, electrophoresis was initially run for the entire set of samples on a 12% gel which was then stained with Coomassie blue. Immunoblots were performed as previously described [4]. The densitometry values were normalized to the mean of the control to facilitate comparisons.

RNA isolation

Total RNA was extracted from the right kidneys of all rats. First, kidneys were pounded using pestle and mortar and, then, RNA was extracted using TRIZOL (Invitrogen-Life Technologies, Carlsbad, CA, USA) according to the specifications of the manufacturer. RNA purity and concentration were assessed spectrophotometrically (Ultrospec 3100 pro, Amersham Biosciences, Buckinghamshire, UK). RNA integrity was confirmed by inspection of 28S and 18S ribosomal RNA bands under UV illumination.

Northern blot

Probes synthesis. Two micrograms of total RNA were reverse transcribed (RT) with 450 ng random hexamers and 200 U SuperScript™ II Rnase H− Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in a 20-μl reaction volume, under conditions recommended by the manufacturer. This RT product was stored at −20°C until required. Then, digoxigenin-labelled specific probe NKCC2 was synthesized by PCR using the PCR DIG probe synthesis kit (Roche Applied Science, Penzberg, Germany). The sequences of NKCC2 primers have previously been reported [8].

Blotting, hybridization and immunological detection. Aliquots of 11 μg of total RNA were electrophoresed in 1% agarose gels, 40 mM morpholinopropane sulphonic acid (MOPS), 4 mM formaldehyde, 10 mM sodium acetate and 1 mM EDTA. Samples were run during 3 h in MOPS running buffer. Then, the gel was transferred overnight to a positively charged nylon membrane (Schleicher & Schuell, Dassel, Germany) by capillarity. Blotted membranes were probed using kits provided by the manufacturer (DIG wash and the block buffer set, Anti-Digoxigenin-AP and CSPD, Roche). Probing and detection of specific mRNA bands were performed as described previously [9]. Blots were exposed to X-ray film for 4 to 20 min to visualize the labelled bands. Ribosomic 28S subunit was used as endogen control to ensure equal loading (data not shown). Relative quantification of the band densities from the northern blots was carried out by densitometry using Gel Doc 2000 (Quantity One software, Bio-Rad).

Table 1. Animal blood and urine data at Day 6

<table>
<thead>
<tr>
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<th>Control</th>
<th>Cyclosporine</th>
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<tbody>
<tr>
<td>Urine Na (mmol/l)</td>
<td>227 ± 32</td>
<td>77 ± 22*</td>
</tr>
<tr>
<td>Na (mmol/day)</td>
<td>1.9 ± 0.1</td>
<td>1.1 ± 0.1*</td>
</tr>
<tr>
<td>Osmolarity (mOsm/Kg)</td>
<td>1904 ± 356</td>
<td>792 ± 245*</td>
</tr>
<tr>
<td>Diuresis (ml/day)</td>
<td>9.8 ± 1.6</td>
<td>18.4 ± 4.9</td>
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<tr>
<td>Blood Creatinine (μmol/l)</td>
<td>62 ± 0.04</td>
<td>71 ± 0.04</td>
</tr>
<tr>
<td>Osmolarity (mOsm/Kg)</td>
<td>285 ± 1.4</td>
<td>287 ± 2.4</td>
</tr>
<tr>
<td>Na (mmol/l)</td>
<td>142 ± 0.6</td>
<td>139 ± 0.9*</td>
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Results are expressed as mean ± SEM.

*P < 0.05 vs control rats.

Presentation of data and statistical analyses

Quantitative data are presented as mean ± SEM. Statistical comparisons were accomplished by unpaired t-test (when variances were the same) or by Mann–Whitney rank-sum test (when variances were significantly different between groups). P-values < 0.05 were considered statistically significant.

Results

Characteristics of animal model

The first data from the animal model was that the rats treated with cyclosporine presented an increase in their mean arterial pressure (20 ± 8 mmHg) from Day 1 to Day 6 compared with control rats (8 ± 6 mmHg, P < 0.05), as has previously been shown in this experimental model [6]. Secondly, we confirmed that, at the end of the experimental protocol, cyclosporine-treated rats developed sodium retention (Table 1 and Figure 1). This retention was not secondary to differences in sodium intake, because there was no difference in food consumed by the control and cyclosporine-treated rats. At Day 6, the cyclosporine treated rats showed a positive sodium balance (Figure 1). The water balance was positive at Days 3, 4 and 5 in the cyclosporine-treated rats (Figure 2). Thirdly, there were no differences in the serum creatinine values between cyclosporine-treated rats and control rats, suggesting that the decrease in sodium excretion was predominantly due to an increase in renal tubule sodium absorption, and probably not to a decrease in glomerular filtration. However, in this study the glomerular filtration rate was not specifically measured. Finally, at the end of the experimental procedure, rats treated with cyclosporine showed a decrease in urine osmolarity compared with control rats (Table 1).

Immunoblot analysis

Proximal tubule and thick ascending limb sodium transporters’ protein abundances. There was no statistically significant difference in the abundance of NHE-3 protein in the kidneys of cyclosporine-treated rats compared with control rats (Figure 3, Table 2).
There was, however, a marked increase in the band density of the NKCC2 protein in the kidneys of rats treated with cyclosporine compared with control rats (Figure 3, Table 2).

**Proximal tubule and collecting duct aquaporins' protein abundances.** Immunoblots from aquaporins showed two bands representing the glycosylated and non-glycosylated forms of these water channels. We quantified the sum of both bands by densitometry and normalized the values by dividing by the mean of the control group. The abundance of aquaporin-1 was elevated in the kidney of cyclosporine-treated rats compared with control rats (Figure 4, Table 3). There was, however, a marked decrease in the abundance of aquaporin-2 protein in the kidneys of cyclosporine-treated rats compared with control rats (Figure 4, Table 3).
northern blot analysis, the expression level of NKCC2 mRNA in the kidney of both groups of rats. Northern blot data showed at least a 2-fold NKCC2 mRNA over-expression in the kidney of cyclosporine-treated rats compared with control rats (100±6 vs 218±20, respectively). *P < 0.05.

**Fig. 5.** (A) Northern blot of total RNA of control and treated rats reacting with fluorescent NKCC2 probe, showing a 4.7-Kb band corresponding to the length of this mRNA. (B) Densitometric analysis revealed a significant over-expression of NKCC2 mRNA in treated rats compared with control rats (100±6 vs 218±20, respectively). *P < 0.05.

**Northern blot data of the thick ascending limb sodium transporter (NKCC2)**

To study NKCC2 regulation we investigated, using northern blot analysis, the expression level of NKCC2 mRNA in the kidney of both groups of rats. Northern blot analysis showed at least a 2-fold NKCC2 mRNA over-expression in the kidney of cyclosporine-treated rats (control rats 100% ± 6%; cyclosporine-treated rats 228% ± 20%; P < 0.05) (Figure 5).

**Discussion**

Hypertension is a risk factor for shortened allograft survival, progressive renal failure [1] and the development of cardiovascular diseases. Kasiske et al. [10] has already shown poor blood pressure control after organ transplantation. A better understanding of the pathogenesis of immunosuppressant-induced hypertension would allow specific treatment and reduced cardiovascular diseases, which nowadays is the main cause of death in this population.

The current article reports the first study assessing sodium and water balances in a model of hypertension induced by cyclosporine using the analysis of renal sodium transporters and water channels. This method offers various advantages over the techniques used so far to study renal sodium balance. It allows for exploring the entire tubule of both superficial and deep nephrons and offers more direct evidence of sodium and water balances than clearance techniques. On the other hand, since many factors influencing the tubular content of the different sodium transporters and water channels are well established, this approach provides not only data on segmental sodium reabsorption but also on the possible mechanisms influencing renal sodium metabolism.

Sodium chloride absorption in the thick ascending limb of the loop of Henle is facilitated by NKCC2 [2]. One of the main findings of the current study is that the amount of NKCC2 in the kidney of rats with hypertension induced by cyclosporine was markedly increased, suggesting exaggerated sodium reabsorption in the ascending limb of the loop of Henle. Approximately 25–30% of the filtered sodium is reabsorbed in the loop of Henle [2]. Therefore, this segment represents one of the major sites in sodium reabsorption and may be of great importance in the pathogenesis of sodium retention and the development of hypertension by cyclosporine. The conventional view is that thick ascending limb sodium chloride transport is mainly geared to regulation of water balance through regulation of concentrating and diluting ability. Normally, any changes in sodium transport would be buffered by the tubular glomerular feedback. However, our findings of a marked augment in NKCC2 proteins associated with increase in blood pressure are consistent with previous data both in animal models and humans. Hoagland et al. [11] recently reported in a model of salt-sensitive hypertension an increase in NKCC2 in the kidney. In two different models of genetic hypertension, an up-regulation of NKCC2 has been observed in the early phase of the disease [12,13]. Moreover, mutations on the NKCC2 gene have also been associated with essential hypertension in humans [14].

The gene of NKCC2 co-transporter in the thick ascending limb of the loop of Henle is regulated, at least in part, by prostaglandin E2 (PGE2) [15]. Intracellular signalling is mediated by the EP3 receptor via coupling to adenylyl cyclase through the inhibitory heterotrimeric G-protein G_i [16]. PGE2 would be expected to act on the thick ascending limb by decreasing intracellular cAMP levels [17]. The 5′-flanking region of the NKCC2 cotransporter gene contains a cyclic-AMP regulatory element which increases gene transcription [18]. It is well known that cyclosporine decreases the PGE2 formation in the kidney [19]. Therefore, it can be speculated that elimination of an EP3-receptor-mediated tonic inhibitory effect of PGE2 on cyclic AMP production will increase NKCC2 gene transcription. Moreover, the finding of NKCC2 mRNA over-expression in the kidney of cyclosporine-induced hypertension is consistent with this hypothesis. The observation that cyclosporine stimulates Na⁺-K⁺-Cl⁻ cotransporter activity in culture cells is also in keeping with our data [5].

Sodium reabsorption in the proximal tubule is facilitated mainly by NHE-3 [2]. Our results show changes without statistical significance in the content of this sodium transporter in the kidneys of cyclosporine-treated rats. In contrast, these rats showed
an increase of renal aquaporin-1. This water channel is located in the apical and basolateral plasma membrane of proximal tubule cells. Tubular fluid osmolality at the end of the proximal tubule is similar to the plasma indicating that the fluid reabsorption through the proximal tubule cells is more or less isosmotic. An increase in aquaporin-1 expression at this level would be expected to have direct effect on proximal water absorption because there is normally enough aquaporin-1 present to permit water to follow NaCl absorption virtually isosmotically. However, a further decrease of the osmotic gradient needed to drive water absorption through the proximal tubule cells could allow NaCl absorption to occur somewhat more efficiently, by reducing NaCl back-leak in the proximal tubule, and could potentially account for greater net NaCl absorption in the proximal tubule. Therefore, an increase in aquaporin-1 in cyclosporine-treated rats could contribute to an increase in proximal fluid absorption.

In a normal state, urine is concentrated as a result of the combined functions of the loop of Henle and the collecting duct. The loop of Henle generates a high osmolality in the renal medulla by driving the counter-current multiplication process. The collecting duct, when vasopressin and aquaporin-2 are present, permits osmotic equilibrium between the urine and the hypertonic medullary interstitium. The development of high osmolality in the renal medulla is dependent on net NaCl absorption mainly through the NKCC2 cotransporter of the thick ascending limb. Increased expression of NKCC2 in the cyclosporine-treated rats would be expected to result in increased counter-current multiplication. However, cyclosporine rats showed diluted urine compared with control rats. A possible explanation of this effect is the decrease in aquaporin-2 observed in the kidney of cyclosporine-treated rats. The substantial decline in aquaporin-2 protein abundance would be indicative of a significant impairment in the water permeability of the collecting duct and in the ability of the collecting duct fluid to osmotically equilibrate with the hypertonic medullary interstitium. The significance for urinary concentration ability produced by a reduction in aquaporin-2 has been shown in previous studies [20]. In the present study, we did not directly investigate the mechanism for the decrease in aquaporin-2 expression. In cyclosporine-treated rats with an increase in NKCC2 and AQP-1 in the kidney, a possible decrease in the water permeability of the collecting duct could represent a compensatory response of the renal tubules to diminish water reabsorption and, therefore, counteract the increase in sodium and water reabsorption and also the elevation of the blood pressure.

**Limitations of the study**

The number of rats included in the study was relatively small. It would be important to perform further studies including a large number of rats. Second, animals were studied at a single point in time; it would be interesting to study rats at several time points to find out whether renal sodium transporters expression change over time and also investigate the role of distal tubule and collecting duct sodium transporters. Third, and most important, this study has been done in animals. Therefore, future studies will be needed to assess the pathogenic role of NKCC2 in the hypertension induced by anticalcineurinic drugs in humans. These future studies may have potential therapeutic implications pointing to the loop diuretic as a specific treatment of this type of hypertension.

In conclusion, the current article shows that in rats with hypertension induced by cyclosporine there are marked changes in several of the most relevant renal tubular sodium transporters and water channels. The total amount of the most important sodium transport protein of the proximal tubule (NHE-3) was not significantly modified although we cannot rule out a cyclosporine effect in the expression of this transporter. On the contrary, the transport protein of the thick ascending limb of the loop of Henle (NKCC2) was markedly increased. These data point to the loop of Henle as a possible site of sodium retention in cyclosporine-induced hypertension.

**Acknowledgements.** Funding for this study was received from the intramural budget of the National Heart Lung and Blood Institute (National Institutes of Health, M.A.K.), Fondo de Investigación Sanitaria (FIS-21547 to P.F.L.L.), Fundación Marató TV3 (N-2000-TV-0210 to P.F.-L.) and from Dirección General de Investigación Científica y Técnica (SAF03-02597 to W.J.).

**Conflict of interest statement.** None declared.

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Received for publication: 7.9.06
Accepted in revised form: 24.5.07