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ORIGINAL ARTICLE

Cyclosporin-induced hypertension is associated with the up-regulation of Na^{+}-K^{+}-$2Cl^{-}$ cotransporter (NKCC2)

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ABSTRACT

Background. The use of cyclosporin A (CsA) is hampered by the development of nephrotoxicity including hypertension, which is partially dependent on renal sodium retention. To address this issue, we have investigated *in vivo* sodium reabsorption in different nephron segments of CsA-treated rats through micropuncture study coupled to expression analyses of sodium transporters. To translate the findings in rats to human, kidney-transplanted patients having CsA treatment were enrolled in the study.

Methods. Adult male Sprague–Dawley rats were treated with CsA (15 mg/kg/day) for 21 days, followed by micropuncture study and expression analyses of sodium transporters. CsA-treated kidney-transplanted patients with resistant hypertension were challenged with 50 mg furosemide.

Results. CsA-treated rats developed hypertension associated with reduced glomerular filtration rate. *In vivo* microperfusion study demonstrated a significant decrease in rate of absolute fluid reabsorption in the proximal tubule but enhanced sodium reabsorption in the thick ascending limb of Henle's loop (TAL). Expression analyses of sodium transporters at the same nephron segments further revealed a reduction in Na^{+}-H^{+} exchanger isoform 3 (NHE3) in the renal cortex, while TAL-specific, furosemide-sensitive Na^{+}-K^{+}-$2Cl^{-}$ cotransporter (NKCC2) and NHE3 were significantly upregulated in the inner stripe of outer medulla. CsA-treated patients had a larger excretion of urinary NKCC2 protein at basal condition, and higher diuretic response to furosemide, showing increased $FeNa^{+}$, $FeCl^{-}$ and $FeCa^{2+}$ compared with both healthy controls and FK506-treated transplanted patients.

Conclusion. Altogether, these findings suggest that up-regulation of NKCC2 along the TAL facilitates sodium retention and contributes to the development of CsA-induced hypertension.

Keywords: Cyclosporin A, hypertension, kidney transplant, micropuncture, NKCC2

INTRODUCTION

Cyclosporin A (CsA) is one of the most successful anti-rejection agents. It is widely used in solid organ transplant recipients and for the treatment of autoimmune diseases [1–3]. The administration of CsA is burdened by acute and chronic kidney toxicity as the development of resistant arterial hypertension [4]. Curtis *et al.* demonstrated that hypertension in CsA-treated renal transplant recipients was linked to defective sodium excretion, pointing to a direct correlation between blood pressure (BP) and renal salt handling [5]. Changes in sodium transporters abundances along the nephron may occur during the administration of calcineurin inhibitors [6]. Some evidence suggests that several sodium transporters are regulated by CsA in kidney-derived cultured cellular lines [7]. Sprague–Dawley rats treated with CsA for 7 days showed an increased furosemide-sensitive Na^{+}-K^{+}-$2Cl^{-}$ cotransporter type 2 (NKCC2) expression along the thick ascending limb of Henle's loop (TAL), but not the abundance of Na^{+}-H^{+} exchanger isoform 3 (NHE3) along the proximal tubules (PT) [8]. Na^{+}-Cl^{-} cotransporter (NCC) and epithelial sodium channel (ENaC) have a pivotal role in the regulation of sodium reabsorption and BP [9]. Wang *et al.* have shown that CsA increases ENaC sodium channels open probability in the A6 distal nephron cells [10], likely involving the modulation of the ATP-binding cassette A1 transporter. Moreover, there is evidence that CsA regulates the phosphorylation of NKCC2 and enhances chloride affinity and transport activity of NKCC2 in mice [11]. At variance, the inhibition of calcineurin by FK506, another calcineurin inhibitor, increases NCC phosphorylation and expression in distal convoluted tubules (DCT) in both animal models and humans [12, 13]. At present, only scattered data are available on the specific effect of CsA on sodium transporters in the pathogenesis of hypertension, and data in humans are poor. The aim of this study is to investigate the mechanisms underlying the development of hypertension under chronic treatment with CsA in rats and in transplanted patients, in an attempt to clarify the current understanding of CsA-induced hypertension and identify potential therapeutic targets. In particular, we performed *in vivo* micropuncture study together with a molecular biology approach and confirmed a pivotal role of NKCC2 in CsA-induced renal sodium retention specifically in TAL. This was corroborated by

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KEY LEARNING POINTS

What was known:

- While cyclosporin A (CsA) is a widely used anti-rejection agent, CsA induces kidney toxicity including hypertension.
- Current data on the molecular mechanisms of CsA-induced hypertension are so far scattered and insufficient for clinical relevance.

This study adds:

- *In vivo* micropuncture studies on rats demonstrated the thick ascending limb of Henle's loop as the site of sodium retention at least in part sustained by upregulation of Na^+-K^+-$2Cl^-$ cotransporter (NKCC2).
- Hypertensive CsA-treated transplanted patients showed high basal urinary NKCC2 excretion and enhanced diuretic response to furosemide.

Potential impact:

- The use of furosemide in CsA-treated patients may provide beneficial effects on blood pressure control.

higher basal urinary NKCC2 excretion and increased diuretic response to furosemide in kidneys of transplanted patients treated with CsA.

MATERIALS AND METHODS

Ethical statement

This study was carried out in accordance with the Guide of the National Institutes of Health (National law: D.L. 26/2014; European law: Directive 2010/63/EU) and approved by the Italian Ministry of Health (Number: 2008/0 137 000).

Experimental protocol

Experiments were performed on a total of 30 adult male Sprague–Dawley rats weighing 200–230 g. They were housed under constant environmental conditions (temperature 22°C and a 12 h light–dark cycle). Animals were fed with a standard diet and water *ad libitum*. The rats were treated for 21 days as follows: (i) the CsA (Sigma-Aldrich) group ($n = 15$) received CsA 15 mg/kg/day intraperitoneally (i.p.), dissolved in castor oil; and (ii) the control group ($n = 15$) received a daily equivalent volume of vehicle for CsA, castor oil, i.p. The dose of CsA is known to induce acute or chronic nephrotoxicity depending on the time-length of treatment [14]. BP was recorded in anesthetized rats during the inulin clearance and micropuncture experiments, through the carotid and femoral artery using a BP recorder (Pressure Monitor BP-1, Word Precision Instruments).

RNA extraction, cDNA synthesis and competitive polymerase chain reaction (PCR)

Tubule microdissection was performed using the protocol previously described in detail [15]. Isolated PT, TAL and DCT from control ($n = 5$) and CsA-treated rats ($n = 5$) were utilized to determine mRNA expression by competitive PCR analysis (Foster City, CA, USA). Briefly, total RNA (0.5–2 μg) was purified from isolated tubules. cDNA was synthesized from equal amounts of total RNA through reverse transcriptase. Competitive PCRs were performed starting from the same amounts of total RNA (200 ng). For each experiment the control and CsA-treated samples were studied in parallel. PCR analysis was performed with specific oligonucleotide primers as following: Sense primers: 5′-ACCACGTCCAGGATCCATACA-3′ for NHE3; 5′-GGCCTCATATGCGCTTATTA-3′ for NKCC2; 5′-AAGTCGGGTGGTACCTATTT-3′ for NCC. Antisense primers: 5′-CACGAAGAAGGACACTATGCC-3′ for NHE3; 5′ AGTGTTTGGCTTCATTCTCC-3′ for NKCC2; 5′-CAGAAAATGGCCATGAGTGT-3′ for NCC. Linker antisense: 5′-GGACACTATGCCGTCACCGCGTCATTAA-3′ for NHE3; 5′-GCTTCATTCTCCTGTTCTTCATCAGCC-3′ for NKCC2; 5′-GCCATGAGTGTTGGAGAACATCCCGAAGAA-3′ for NCC. The PCR products were analyzed by agarose gel electrophoresis (18 g/L) and they were recovered from the gel using the agarose gel DNA extraction kit (Clontech). The concentration and purity were determined by measuring the absorbance at 260 and 280 nm. The expected sizes of the PCR products were: 622 bp for NHE3, 603 bp for NKCC2 and 550 bp for NCC. The cDNA was additionally utilized for the internal standard synthesis. The expected sizes of internal standards were 546 bp for NHE3, 520 bp for NKCC2 and 453 bp for NCC. The addition of decreasing amounts of internal standards resulted in a corresponding increase of the wild-type template products. The amounts of wild-type mRNA were calculated when the intensity of PCR products of internal standards and wild-types were equated (ratio = 1). The same approach was used to quantify the transcripts of sodium transporters to examine whether expression of each transporter at mRNA level was different in the controls and CsA-treated animals.

Immunoblotting

The kidneys of control ($n = 5$) and CsA-treated rats ($n = 5$) were dissected in cortex (CTX)/outer stripe of outer medulla (OSOM) and inner stripe of outer medulla (ISOM), and protein samples were extracted as described previously [15]. The protein concentration was measured by Bradford assay (Biorad Protein Assay, Hercules, CA, USA). The protein samples were then separated by SDS–PAGE using NuPage Bis–Tris gel 4%–12% (Novex by Thermo Fisher Scientific, Waltham, MA, USA) with sample reducing buffer 10× (Invitrogen, Carlsbad, CA, USA) and 4× LDS NuPAGE Sample Buffer (Invitrogen, Carlsbad, CA, USA) after heating for 15 min at 70°C. Proteins were then transferred to PVDF membranes (Invitrolon PVDF, Invitrogen, Carlsbad, CA, USA). The membranes were probed with 1:1000 diluted antibodies against NHE3 and NKCC2 (OriGene, Rockville, MD, USA). Blots were incubated with horseradish peroxidase–conjugated secondary antibodies (Applied Biosystems Inc., Foster City, CA, USA), according to the species of the primary antibodies and then developed using Western Light Plus Kit, CSPD (Applied Biosystems, Bedford, MA, USA). The quantification of signal intensity was obtained by image densitometry using ImageJ software. Coomassie Blue staining was used to assess equal loading.

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Glomerular filtration rate (GFR) measurement (clearance of inulin)

At the end of the treatment, animals (control: $n = 5$, CsA-treated: $n = 5$) were anesthetized with 2% isoflurane (Isotec 4, Palermo Italy) and prepared for the assessment of renal clearance as previously described [16]. Briefly, the left jugular vein was cannulated with a PE-50 polyethylene tube and used for i.v. infusion by syringe pump of 0.74 mg • 100 g body weight/min of inulin in 10% saline solution. Arterial blood samples (100 μL) were taken at the beginning and end of each collection period. Urine samples were collected by catheterization with PE-50 tubing. After an equilibration period for 60 min, the first of four 30 min urine collections was initiated. Inulin concentration in plasma and urine was assessed by colorimetric method and the absorbance was read at 550 nm using a Glomax Multi+ detection system (Promega Corporation, Milan, Italy). The GFR was calculated using standard clearance formula [17] and normalized to total animal weight.

In vivo microperfusion study for determination of absolute fluid reabsorption (Jv) in PT and sodium reabsorption (JNa) in TAL

S2 segments of the PT in CsA-treated ($n = 5$) and control rats ($n = 5$) were identified by insertion of a "finding pipette" [8-μm outer diameter (OD)] containing artificial tubular perfusion fluids (ATF) stained with FD&C green dye (0.075%). A small volume of ATF was injected to identify direction of flow and approximate location. If the bolus injection passed three or more loops, the site was selected as S2. An immobile grease block (Apiezon T, Manchester, UK) was placed into the finding puncture site to stop flow. A perfusion pipette (6- to 8-μm OD) was inserted immediately downstream from the block, and connected to a nanoliter perfusion pump with flow rate as 20–24 nL/min. Samples with <95% and >105% of the microperfused inulin were discarded. A collection pipette (10-μm OD) containing dye-stained light mineral oil was inserted two to four loops downstream from the perfusion site. A bolus of oil was inserted and maintained just distal to the insertion site by light pressure. A timed collection (3–5 min) was initiated immediately after placement of the oil block. The following types of ATF have been used in the PT (in mM): 125 NaCl, 20 $NaHCO_3$, 5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 1 $NaHPO_4$, 5 glucose, 4 urea, 0.075% green FD&C, pH 7.4. Then, samples were transferred into a constant-bore capillary tube to measure the tubular fluid volume. Thereafter, FITC-labeled inulin was determined using a Spectra Max2 plate (Promega) reader with excitation and emission wavelengths of 490 and 520 nm, respectively. At the end of the perfusion, the segment was microperfused with Microfil solution (Flow Tech, Carver, MA, USA) to measure the length of the microperfused segment. The Microfil formed a cast within 20–30 min, which was dissected at the end of the experiment. The length of the cast was measured under a dissecting microscope [18]. Moreover, the loop of Henle was identified by inserting a micropipette into the late proximal tubule to inject a small bolus of stained ATF (in mM): 113 NaCl, 10 $NaHCO_3$, 5 KCl, 2 $CaCl_2$ and 7.5 urea, 0.075% green FD&C, pH 7.4. Then, a collecting pipette (7- to 9-μm tip) was inserted into the first accessible distal tubular loop to perform a timed collection of tubular fluid (8–10 min in duration) using a short mineral oil [19]. Sodium concentration was determined by atomic absorption with graphite furnace (THGA-AAS, Perkin Elmer AAnalyst 800) supplied by the Institute of Protein Biochemistry, National Research Council of Italy (CNR).

Furosemide administration to healthy control, and CsA- or FK506-treated patients

The study protocol was approved by the University of Campania "L. Vanvitelli" Ethics Committee (protocol number 64/2020) and conducted according to the Declaration of Helsinki and the European Good Clinical Practice guidelines. All participants signed an informed written consent. Adult kidney-transplanted patients chronically treated with CsA ($n = 3$) for at least a year were enrolled. Inclusion criteria were: (i) immune-suppressive regimen based on CsA, and (ii) presence of resistant hypertension defined as BP >140/80 mmHg, requiring more than two first-line antihypertensive agents (excluding furosemide and other diuretics). Exclusion criteria were: (i) estimated GFR (eGFR) <60 mL/min/1.73 m^2 (Chronic Kidney Disease Epidemiology Collaboration formula), and (ii) the presence of major comorbidity such as severe cardiovascular diseases, water-electrolyte imbalances or ongoing infections. Two control groups were included: (i) normotensive and untreated, healthy, age- and gender-matched volunteers (controls, $n = 3$), and (ii) kidney-transplanted patients treated with a CsA-free regimen but including FK506 ($n = 3$). All participants underwent furosemide sensitivity test as modified by Walsh *et al.* [20]. Urine and plasma electrolytes were measured by ion-selective electrodes and serum creatinine by the enzymatic method.

Immunoblotting of urine samples from human

Spot urine samples (15 mL) collected from human subjects, were first centrifuged at 10 000 r.p.m. for 10 min at 4°C in a RP-5B Refrigerated Superspeed Centrifuge to eliminate all debris and cells. Afterwards 16 mL of methanol (Sigma-Aldrich), 4 mL of chloroform (Sigma-Aldrich) and 12 mL of ddH_2O were added to 4 mL of cleared urine. The solution was further centrifuged at 6500 r.p.m. for 20 min at 10°C. Protein content in the lower phase was isolated and resuspended in 12 mL of methanol. After centrifugation at 65 000 r.p.m. for 50 min the supernatant was discarded and the pellet was completely air-dried and resuspended in 150 µL of lysis buffer containing 0.3 M sucrose (Sigma-Aldrich), 2 µM leupeptin (Sigma-Aldrich), 1 mM phenylmethanesulphonyl fluoride (Sigma-Aldrich), 25 mM imidazole (Sigma-Aldrich) and 1 mM EDTA (Sigma-Aldrich). Urine samples were diluted with the loading buffer and normalized to urinary creatinine. Immunoblotting was carried out as described above.

Statistical analysis

Statistical analyses were performed using the GraphPad software using unpaired Student's *t*-test or one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. Statistical threshold was considered at $P < .05$.

RESULTS

Chronic CsA treatment increased BP associated with enhanced sodium reabsorption in TAL

Chronic CsA treatment (21 days) in Sprague–Dawley rats significantly increased systolic BP (SBP) by 17% (CsA group: 135 ± 6.02 mmHg vs control group:115 ± 2 mmHg, $P < .05$, unpaired *t*-test) and diastolic BP (DBP) by 23% (CsA group: 121 ± 7 mmHg vs control group: 98 ± 5 mmHg, $P < .05$, unpaired *t*-test). In order to identify the major site of sodium retention in the nephron, we performed micropuncture studies. As shown in Fig. 1A, GFR measured by inulin clearance showed significant reduction in CsA-treated rats. Together with the remarkable

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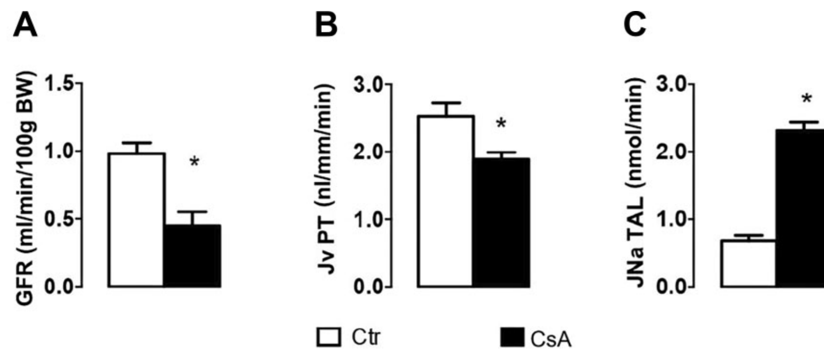

Figure 1: Effects of CsA on GFR and tubular reabsorption. (**A**) Effects of 21-day treatment with CsA (black bar) on rat GFR, (**B**) Jv in PT and (**C**) JNa in TAL. White bar stands for control rats. Data are expressed as mean ± standard deviation. *P-value <.05.

increase in BP, these results confirmed the recapitulation of two major effects of chronic CsA administration in rats. Jv in the PT averaged 2.52 ± 0.2 nL/mm/min in control rats, while chronic CsA administration considerably decreased Jv by 25% (1.89 ± 0.08 nL/mm/min, *P < .05, Fig. 1B). In contrast, JNa was significantly increased in the TAL of CsA-treated rats compared with controls (CsA group: 2.320 ± 0.12 nmol/min; control group: 0.680 ± 0.08 nmol/min, *P < .05, Fig. 1C).

CsA-treated rats showed an increased sodium transporters expression in TAL

Furthermore, in order to corroborate the micropuncture results, we performed mRNA and protein expression analyses mainly focusing on NHE3 and NKCC2, the main apical sodium transporters in PT and TAL, respectively. Competitive PCR revealed that NHE3 mRNA abundance was down-regulated along the PT of CsA-treated animals compared with controls (Fig. 2A). Conversely, NHE3 and NKCC2 mRNA levels in the TAL were remarkably enhanced (Fig. 2B and C). At variance, mRNA expression of NCC in DCT was significantly decreased in CsA-treated rats compared with controls (Fig. 2D). Moreover, immunoblotting of NHE3 and NKCC2 in CTX/OSOM and ISOM demonstrated that NHE3 abundance in CsA-treated rats was considerably decreased in CTX/OSOM where PT is predominantly enriched (Fig. 3). However, CsA-treated rats showed significantly enhanced protein levels of NHE3 and NKCC2 in ISOM, in which the abundance of these two transporters largely reflects the expression in medullary TAL. These results suggest that upregulation of sodium transporters in TAL are, at least in part, responsible for increased BP induced by chronic CsA treatment. Since NKCC2 serves as a major sodium transporter at this nephron segment, CsA-induced hypertension in rats is presumably associated with enhanced sodium reabsorption in TAL mainly acquired through upregulation of NKCC2.

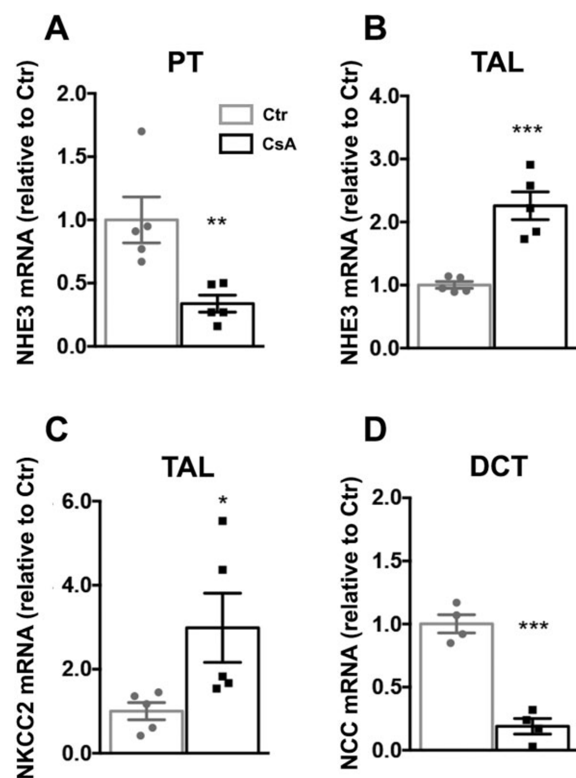

Figure 2: mRNA abundance of the main sodium transporters after CsA treatment. Competitive PCR analysis of (**A**) NHE3 in PT, (**B**) NHE3 in TAL, (**C**) NKCC2 in TAL and (**D**) NCC in DCT on rats treated with CsA for 21 days (black squares) or controls (grey circles). Data are reported as ratio relative to control with individual points (mean ± standard error of the mean). *P-value <.05; **P-value <.01; ***P-value <.001.

CsA-treated kidney-transplanted patients showed higher basal urinary NKCC2 excretion and increased sensibility to the NKCC2-blocker furosemide

Finally, to translate these findings in rats to humans, kidney-transplanted patients under chronic treatment with CsA or FK506, as well as healthy controls were enrolled for further studies. Clinical data of participants are reported in Table 1. While all subjects presented no major differences in body mass index (BMI), age or eGFR, basal SBP values were remarkably higher in CsA-treated patients compared with both healthy subjects and FK506-treated kidney-transplanted patients. Kidney-transplanted patients had higher DBP at baseline compared with healthy control subjects (Table 1). Notably, urinary excretion of NKCC2 at basal condition was significantly enhanced in the CsA-treated group, implying the up-regulation of NKCC2 in these patients (Fig. 4A). As previously reported for hypertension in humans [21], overexpression of NKCC2 may be associated with hypertension in CsA-treated patients. To address this issue, all subjects were challenged with oral administration of 50 mg NKCC2 blocker furosemide, followed by time-course analyses in urinary excretion fraction of sodium ($FeNa^+$, Fig. 4B), chloride ($FeCl^-$, Fig. 4C), calcium ($FeCa^{2+}$, Fig. 4D) and BP measurement. After 3 and 4 h from furosemide administration, the CsA group showed remarkable increases in $FeNa^+$, $FeCl^-$ and $FeCa^{2+}$ compared with the other groups, paralleled by nearly 20% reduction in SBP (baseline: 161 ± 3 mmHg, 4 h after furosemide: 130 ± 1 mmHg, *P < .05). BP decrease in FK506-treated

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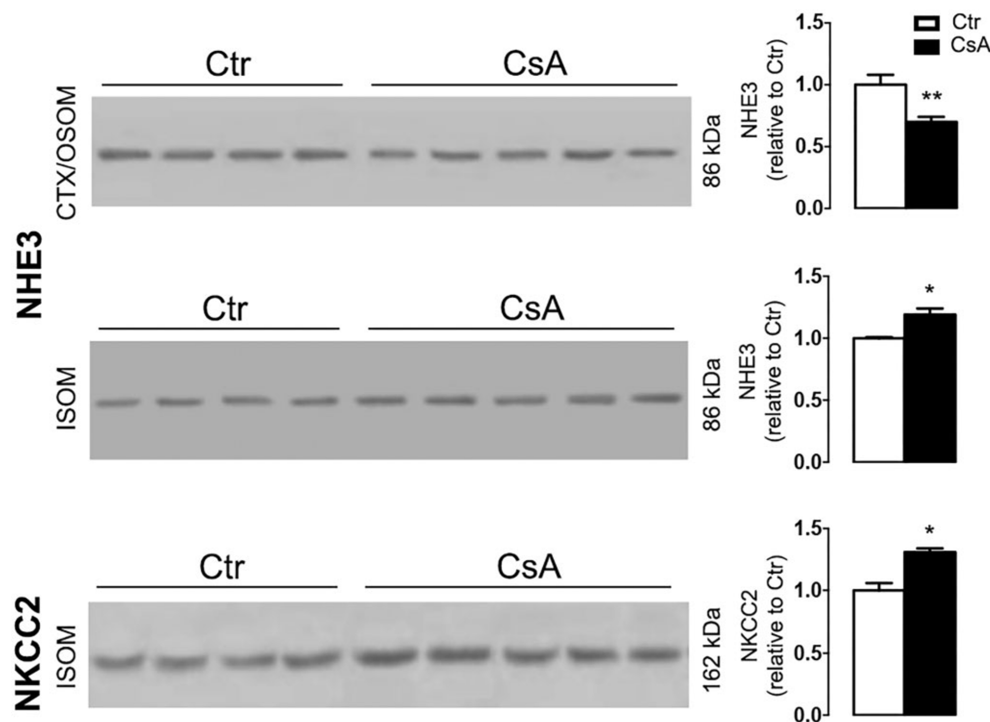

Figure 3: Immunoblotting of NHE3 and NKCC2 in renal CTX/OSOM and ISOM dissected from control (white bar) or CsA-treated rats (black bar). Equal loading was assessed by Coomassie Blue staining. Data are reported as ratio relative to control (mean ± standard deviation). *P-value <.05; **P-value <.01.

Table 1: Physiological parameter of kidney-transplanted patients treated with CsA or FK506 or healthy control subjects (Ctr).

Parameters	Ctr	CsA	FK506
Number	3	3	3
Age (years)	38 ± 2	44 ± 3	40 ± 2
BMI (kg/m^2)	23.8 ± 1.2	24.5 ± 3.1	25.2 ± 2.3
Serum creatinine (mg/dL)	0.82 ± 0.2	1.12 ± 0.5	1.07 ± 0.4
eGFR (mL/min/1.73 m^2)	97.6 ± 18	79.3 ± 13	79 ± 8
SBP (mmHg)	120 ± 3	161 ± 3***	141 ± 13***/###
DBP (mmHg)	76 ± 2	98 ± 4***	95 ± 5***

Values are reported as mean ± standard deviation.
***P < .001 Ctr vs CsA or FK506; ###P < .001 CsA vs FK506 (one-way ANOVA).

subjects after furosemide was only 10% (baseline 141 ± 13 mmHg, 4 h after furosemide: 126 ± 9 mmHg) and, similarly, about 12% reduction in healthy controls (baseline: 120 ± 3 mmHg, 4 h after furosemide: 105 ± 5 mmHg).

DISCUSSION

The present study suggests that chronic treatment with CsA induces hypertension by increasing renal tubular sodium reabsorption along the TAL through the up-regulation of NKCC2. CsA radically improved the chance of survival for transplant recipients, at the price of a severe nephrotoxicity, including hypertension [22, 23]. The pathogenesis of CsA-induced hypertension has been related to multiple mechanisms. Chronic nephrotoxicity of CsA derives in large part from afferent arteriolar vasoconstriction and a decrease in GFR, endothelial and vascular injury, and finally tubular-interstitial damage which can lead to non-reversible and dose-independent structural changes [24–26]. All these functional and structural modifications have been pointed to as causes of the increasing incidence of hypertension. Conversely, CsA-induced hypertension potentially increases the risk of long-term allograft failure [27]. In addition, high sodium diet has been demonstrated to aggravate CsA-induced hypertension [28]. Moreover, it is well demonstrated that a major factor contributing to renal salt retention and hypertension is related to the renin–angiotensin system [29] and several data in the literature have demonstrated that the effects of CsA on renin may be mediated by direct inhibition of calcineurin in renin-producing granular cells or by sympathetic activation [30]. Finally, oral potassium and magnesium supplementation have beneficial effects on CsA nephrotoxicity [31].

Our experiments confirmed that long-term CsA administration causes GFR decline in rats. This is in line with previous findings by Thomson and colleagues [32] showing a significant reduction in single-nephron GFR already just 8 days after CsA administration. In addition, we also confirmed that CsA-treated rats developed both systolic and diastolic hypertension as previously demonstrated [14]. Although some data suggest that oxidative stress plays a role [14, 33], a primary role of sodium retention in CsA-induced hypertension has been prompted by several lines of evidences [7, 8, 29]. With regard to this, by *in vivo* micropuncture studies we identified that the site for primary sodium reabsorption was the TAL. Indeed, we found a reduction in Jv along the PT. Since at this level, sodium and water reabsorption are almost equal, the Jv data represent a valid readout of sodium reabsorption along the PT. In normal conditions, the kidney has the capability to restore renal hemodynamics through the adjustment of the GFR in response to changes in NaCl handling along the nephron. As demonstrated in mice, deficiencies of NHE3 and of the water channel aquaporin 1 (AQP1) lead to decreased proximal fluid absorption along with proportionate reductions in GFR. This compensatory mechanism for the transport deficiency acquired through a reduced level in filtered load represents a crucial physiological role, since neither NHE3- nor AQP1-deficient mice develop

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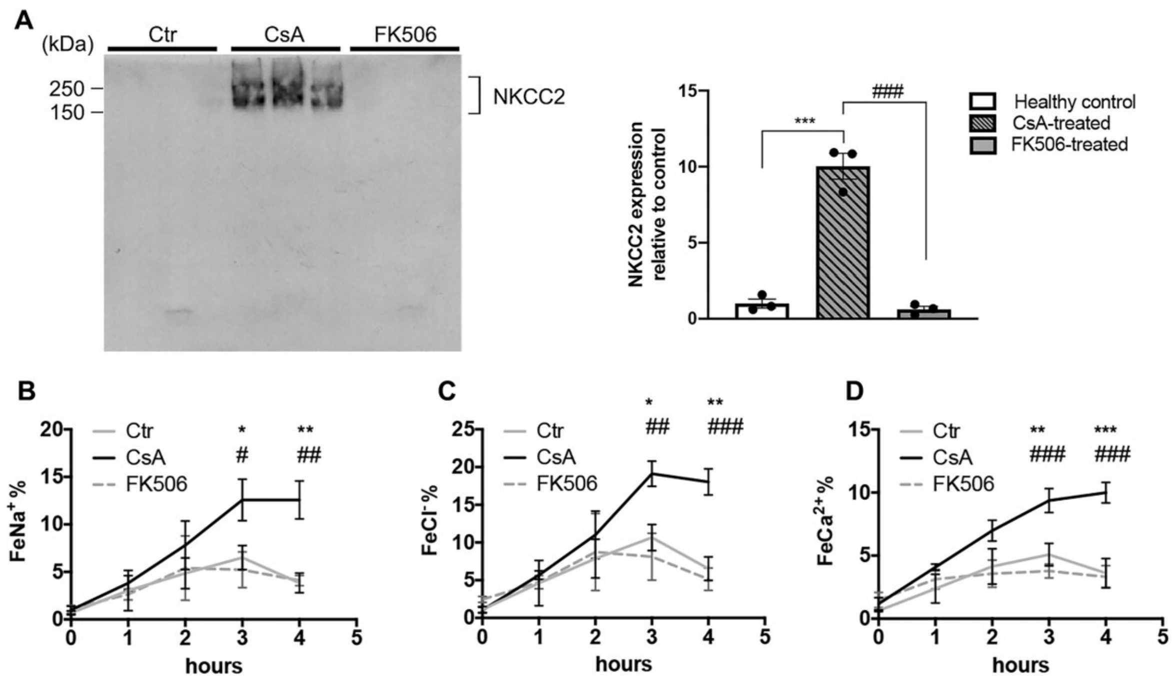

Figure 4: Furosemide challenge in kidney-transplanted patients and healthy subjects. (**A**) Immunoblotting of urinary NKCC2 expression in CsA-treated (CsA, gray dashed bar, $n = 3$) or FK506-treated patients (FK506, gray bar, $n = 3$) and healthy control subjects (Ctr, white bar, $n = 3$). Values are reported as ratio relative to healthy control. (**B**) Time course changes in $FeNa^+$, (**C**) $FeCl^-$ and (**D**) $FeCa^{2+}$ in kidney-transplanted patients receiving CsA (CsA, black line, $n = 3$) or FK506 (grey dashed line, n = 3) and healthy control subjects (Ctr, grey line, $n = 3$) challenged with 50 mg furosemide per os. Values are reported as mean ± standard error of the mean. A * compares Ctr vs CsA; $^{\#}$ compares CsA vs FK506 (one-way ANOVA). (D) *** compares Ctr vs CsA; $^{\#\#\#}$ compares CsA vs FK506 (one-way ANOVA). *P*-values: * and $^{\#}$*P*-value <.05; ** and $^{\#\#}$*P*-value <.01; *** and $^{\#\#\#}$*P*-value < .001.

clinically symptomatic sodium losses [34]. In our model, Jv reduction at PT level is associated with down-regulation of NHE3 both at mRNA and protein level at PT and GFR decline, supporting the close relation of these two events in the physiology of PT represented by glomerulo-tubular balance.

The main finding of our *in vivo* study is that CsA treatment stimulates sodium reabsorption along the TAL, likely by promoting NKCC2 expression both at mRNA and protein levels. Under normal condition, 20% of filtered sodium is reabsorbed along the TAL [35]. The apical furosemide-sensitive transporter NKCC2 is the principal protein for sodium reabsorption at this level and its over-expression has been related to the early phase of some genetic forms of hypertension [17]. Our results are in agreement with a previous report by Esteva-Font *et al.* which demonstrates that the abundance of NKCC2 protein increases in CsA-treated rats compared with untreated rats [36]. In addition, Ferrer-Martínez and colleagues [37] showed that CsA at two single doses (0.6 mg/L and 2.5 mg/L) inhibited Na^+/K^+ ATPase activity and expression, and increased NKCC2 activity in the bovine renal epithelial cell line NBL-1. Altogether these data are in line with our findings, showing that CsA stimulates NKCC2, resulting in increased sodium reabsorption and a rise in BP. On the other hand, we observed down-regulation of NCC in distal nephron at mRNA level, which is in contrast to the data from Hoorn and colleagues demonstrating a primary role of NCC in the development of FK506-induced hypertension [13]. This is likely due to a different calcineurin inhibition pattern promoted by CsA and FK506 [4]. Although these two drugs are among the most widely used immunosuppressants, they exert their inhibitory effects on phosphatase calcineurin through binding to distinct immunophilins; CsA with cyclophilin A (CypA), and FK506 with FK506-binding protein 12 (FKBP12). Accordingly, the effects of each drug may be dependent on several factors including the distribution patterns and expression levels of immunophilins, and binding mode of drug-immunophilin complex to carcineurin. A proteomic study on protein abundances in microdissected male Sprague–Dawley rat kidney tubule segments reported by Limbutara *et al.* [38] showed that protein expressions of CypA and FKBP12 are observed throughout the nephron, but the expressions of CypA are higher than those of FKBP12 in all segments. On the other hand, the crystal structure of the calcineurin–cyclophilin–cyclosporin complex demonstrated that, unlike the FKBP–FK506 complex, the cyclophilin–cyclosporin complex interacts with active site of calcineurin, indicating a direct involvement of cyclophilin–cyclosporin in the regulation of calcineurin catalysis [39]. In addition, CypA may directly interact with cullin-3 (CUL3) [40, 41], which is one of the genes implicated in Gordon's syndrome. CUL3 forms E3 ligase complex with Kelch Like Family Member 3 (KLHL3), which is exclusively expressed in DCT. Although further investigation is required, differences in the binding mode to calcineurin, as well as the diverse interactors between CypA and FKBP12, may at least in part explain the molecular mechanisms in which two distinct immunosuppressants target the same calcineurin but leading to different regulations on sodium transport.

Post-translational regulation by CsA through phosphorylation/dephosphorylation on NKCC2 as well as NCC via WNK/SPAK/OSR1 regulatory axis was also documented by Blankenstein *et al.* [42]. They demonstrated that acute (4 h) and long-term (14 days) CsA administration in Wister rats increased phosphorylated levels of NKCC2 and NCC, but there were no increases in mRNA levels at both durations and total protein levels at 4 h. They showed an increased urinary sodium excretion in response to furosemide after 14 days CsA treatment in animal model. Their results are somewhat different from our data such as the lack of mRNA effects and increased NCC activation, which are likely to be related to time and dose of CsA used in the rat animal model. In addition, our

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results are in contrast to the previous finding by Lim *et al.*, showing the reduced abundances of NKCC2 and NCC after 28-day treatment with CsA on Sprague–Dawley rats [43]. However, their CsA treatment was in combination with low-salt diet in order to exacerbate CsA nephropathy [44], while our models are generated under standard diet. It should be noted that dietary salt intake modulates the expressions of renal sodium transporters involving the activation of renin–angiotensin–aldosterone system [45]. Thus, together with the complex interaction between CsA and renin–angiotensin–aldosterone system [46], the CsA model in the study by Lim *et al.* would be incompatible with our present study. Furthermore, our findings on immunoblotting have been supported by *in vivo* micropuncture study demonstrating a reduced Jv in PT and enhanced JNa in TAL. To our knowledge, this is the first report on *in vivo* evaluation of fluid and sodium reabsorption ability in different nephron segments in CsA-treated rats by means of micropuncture. These findings on rats were corroborated by a clinical correlate. Indeed, CsA-treated kidney-transplanted patients were more sensitive to the diuretic action of furosemide (both sodium and chloride excretion and BP reduction) compared with FK506-treated patients and healthy volunteers. Finally, this was related to significantly higher urinary excretion of NKCC2 in CsA-treated recipients compared with the FK506 group or normal subjects, in agreement with previous findings [36]. All these results indicate that CsA-induced hypertension is at least in part mediated by NKCC2 up-regulation and suggest that the use of furosemide in these patients may provide benefit in terms of BP control.

The study has some limitations. First, the rat model is not the transplant model thus these animals are not immunocompromised and may have diverse immune responses compared with transplant animals. Second, the human study was conducted on a low number of patients based on the indications of the ethics committee.

In conclusions, our study demonstrates that chronic CsA treatment induces a salt-sensitive form of hypertension, due to an increased Na^+ reabsorption along the TAL likely sustained by up-regulation of NKCC2. Further studies are needed to clarify the mechanism through which CsA up-regulates NKCC2 expression (possibly via the CypA–NKCC2 pathway) and the clinical impact of loop-diuretics treatment on CsA-induced hypertension.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are not available due to privacy of research participants, but are available from the corresponding author (G.Capasso, gb.capasso@biogem.it) upon reasonable request.

AUTHORS' CONTRIBUTIONS

G.Capolongo, R.M.P., M.Z and M.S. performed studies on patients, S.D., M.R., E.Z. and R.C. collected the data on animals, Y.S., G.Capolongo and F.T wrote and revised the manuscript, and G.Capasso supervised the study.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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