Aus dem Institut für Funktionelle Anatomie
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DISSERTATION

Calcineurin inhibitor-associated impairment of COX-2 signaling in kidney cortex is restored by angiotensin II receptor blocker

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Abbreviation

Ang II	angiotensin II
AT1R	angiotensin II receptor type 1
Cand	candesartan
CD	collecting duct
CnAα	calcineurin Aα
CnAβ	calcineurin Aβ
CNI	calcineurin inhibitors
CNT	connecting tubule
COX-2	cyclooxygenase 2
CsA	cyclosporine A
cTAL	cortical thick ascending limb
CXB	celecoxib
DAPI	4',6-diamidino-2-phenylindole
DCT	distal convoluted tubule
FE_{Na}	fractional sodium excretion
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFR	glomerular filtration rate
I.P.	intraperitoneal injection
MD	macula densa
NCC	Na^+-Cl^- cotransporter
NFAT	nuclear factor of activated T-cells
NF-kB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NKCC2	Na^+-K^+-$2Cl^-$ cotransporter

NOS1	neuronal nitric oxide synthase
NSAIDs	non-steroidal anti-inflammatory drugs
p38 MAPK	p38 mitogen-activated protein kinase
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PGE2	prostaglandin E2
RAS	renin-angiotensin system
TGF	tubuloglomerular feedback

1. Abstract

1.1 Abstract (English)

Immunosuppression based on calcineurin inhibitors (CNI) such as cyclosporine A (CsA) is the current standard for patients undergoing organ transplantation. Nephrotoxic side effects of CsA include reduction of renal cortical cyclooxygenase 2 (COX-2) expression along with pathophysiological alterations of glomerular filtration rate and sodium balance. The underlying molecular mechanisms are poorly understood. Since CsA stimulates the renin-angiotensin system (RAS), we hypothesized that suppression of COX-2 relates to enhanced RAS activity. Inhibition of calcineurin in cultured macula densa (MD) cells using CsA or siRNA technology increased COX-2 expression and activity via stimulation of p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-kB). Concomitant application of angiotensin II abolished these effects suggesting a dominant role for RAS. In rats, short (3 days) as well as chronic CsA treatment (3 weeks) led to increased renin biosynthesis, decreased COX-2 expression in the kidney cortex, reduced creatinine clearance, and sodium retention due to activation of major distal salt transporters, Na^+-K^+-$2Cl^-$ cotransporter (NKCC2) and Na^+-Cl^- cotransporter (NCC). Simultaneous administration of a RAS inhibitor candesartan during short term CsA partially restored COX-2 expression and creatinine clearance, diminished sodium retention and prevented NKCC2 and NCC activation. These parameters were completely normalized in the chronic experiment after three weeks of concomitant candesartan treatment. Single administration of the selective COX-2 inhibitor, celecoxib, during three days largely recapitulated the effects of CsA and significantly reduced the beneficial effects of candesartan by concomitant drug application**.** These results suggest that suppression of COX-2 is a major signal that contributes to CsA nephrotoxicity.

In summary, the present study has established calcineurin as an endogenous inhibitor of tubular COX-2, acting via suppression of p38 MAPK and NF-kB activity. CsA-induced RAS activation critically reduces cortical COX-2 activity, thus overriding local stimulatory effects of calcineurin inhibition. Our data support the use of RAS inhibitors for alleviation of CsA nephrotoxicity.

1.2 Abstract (Deutsch)

Calcineurin Inhibitoren (CNI) bilden aktuell die Basis für immunsuppressive Strategien nach Organtransplantation. Allerdings führt die Anwendung von CNI zur Hemmung der Cyclooxygenase-2 (COX-2) in der Nierenrinde mit assoziierten pathophysiologischen Veränderungen der glomerulären Filtrationsrate (GFR) und des Salzhaushalts. Die dafür verantwortlichen pathogenetischen Mechanismen sind nicht ausreichend charakterisiert. Es ist bekannt, dass CNI das Renin-Angiotensin-System (RAS) stimulieren. In der vorliegenden Arbeit wird die Hypothese verfolgt, dass die gesteigerte RAS-Aktivität als Ursache für die COX-2 Suppression bei CNI Gabe fungiert. Um diese Hypothese zu testen, wurden zunächst Effekte des CNI Cyclosporin A (CsA) in kultivierten Macula densa (MD) Zellen und in Rattennieren charakterisiert. Im Gegensatz zur COX-2 Suppression in der intakten Niere zeigten kultivierte MD Zellen eine Steigerung der COX-2 Expression nach CsA-Behandlung bzw. nach Calcineurin-Suppression mittels siRNA. Weitere Experimente in MD Zellen zeigten, dass die CsA-abhängige Steigerung der COX-2 Expression sich hier durch Enthemmung der *p38 mitogen-activated protein kinase* (p38 MAPK) und Aktivierung des *nuclear factor 'kappa-light-chain-enhancer' of activated B-cells* (NF-kB) erklärt. Diese lokalen Effekte der Calcineurin-Inhibition konnten durch gleichzeitige Applikation von Angiotensin II (Ang II) vollständig aufgehoben werden. Im Gegenzug konnte die CsA-abhängige COX-2 Suppression in der intakten Rattenniere durch gleichzeitige akute (3 Tage) oder chronische (3 Wochen) Verabreichung eines Ang II-Rezeptor Typ 1 (AT1R) Antagonisten teilweise bzw. komplett aufgehoben werden. Die bei CsA-Gabe auftretende GFR-Reduktion und renale Natriumretention wurden durch den AT1R Antagonisten gleichfalls teilweise oder komplett verhindert. Diese Wirkungen des AT1R Antagonisten wurden durch die gleichzeitige Applikation des selektiven COX-2 Inhibitors Celecoxib deutlich abgeschwächt. Insgesamt zeigen diese Daten, dass die Suppression der renalen COX-2 bei CNI durch die gesteigerte RAS Aktivität bedingt ist, welche über die lokalen, COX-2-stimulierenden Effekte der Calcineurin-Inhibition in MD-Zellen dominiert. AT1R Blockade normalisiert die COX-2 Aktivität und verbessert damit die Nierenfunktion. Diese Studie liefert neue Einblicke in die Pathogenese der CNI Nephrotoxizität und unterstreicht die protektive Wirkung von RAS Inhibitoren.

2. Introduction

2.1 Calcineurin inhibitors and renal side effects

Calcineurin inhibitors (CNI) such as cyclosporine A (CsA) are routinely used in patients undergoing organ transplantation to achieve optimal immunosuppression. Despite a generally positive outcome, renal side effects of these drugs such as reduction of glomerular filtration rate (GFR), salt retention and hypertension remain a major concern in their long-term usage.[1] Among the pathological mechanisms, CNI-induced regulatory dissociation between juxtaglomerular cyclooxygenase 2 abundance (COX-2; downregulated) and renin biosynthesis (upregulated) has been implicated. Resulting impairment in juxtaglomerular function may contribute to the renal side effects.[2]

2.2 Juxtaglomerular COX-2 expression and renal protective function

In the kidney, COX-2 is detected to express in macula densa (MD) cells, neighboring cortical thick ascending limb (cTAL) cells, and inner medullary interstitial cells.[3] Renal responses to changes in dietary salt content, endocrine stimulation, ischemia and inflammation are related to juxtaglomerular COX-2 expression.[4,5] COX-2 modulates the tubuloglomerular feedback (TGF) and renin release via prostanoids.[6,7] Activation of COX-2 in MD cells helps maintain the GFR in the state of volume depletion.[8] COX-2 further exerts inhibitory effects on salt reabsorption along the distal nephron.[8] COX-2 abundance in MD cells is inversely correlated with luminal Cl^- concentration sensed by the apical Na^+-K^+-$2Cl^-$ cotransporter (NKCC2).[9] In the distal convoluted tubule (DCT), function of the local Na^+-Cl^- cotransporter (NCC) is related to the effects of COX-2 as well.[10]

2.3 Juxtaglomerular COX-2 expression and related transcription factors

The immunosuppressive action of CNI is mediated by inhibition of nuclear factor of activated T-cells transcription factors (NFAT), which are substrates of calcineurin.[11] Suppression of NFAT signaling reduces the expression of key genes involved in T-lymphocyte activation including COX-2,[11] which led to the assumption that the same mechanism may explain the CNI-induced inhibition of renal cortical COX-2. In view of the fact that NFAT abundance and activity are limited in kidney and largely restricted to the renal medulla.[12] the role of NFAT on COX-2 expression in MD cells still need to be verified. Calcineurin-dependent modulation of mitogen-activated protein kinases (MAPK) and nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-kB) provides an

alternative pathway linking calcineurin and COX-2.[13] It has been shown that activation of NF-kB in kidney is associated with phosphorylation of p38 MAPK.[14] Activation of p38 MAPK promotes COX-2 biosynthesis in MD cells.[13] Calcineurin interferes with p38 MAPK activity, thus may regulate NF-kB and COX-2 via this signaling in kidney. Assuming this mechanism, the expected action of CNI in MD cells would be a stimulation of COX-2 expression.

2.4 Juxtaglomerular COX-2 expression and renin-angiotensin system

The relationship between COX-2 and renin-angiotensin system (RAS) is still controversial. In the MD region, COX-2 expression increases in high renin states induced by salt restriction, angiotensin converting enzyme (ACE) inhibition or renovascular hypertension.[15] The RAS effector peptide angiotensin II (Ang II) is an important regulator in kidney. Ang II has been shown to blunt the expression of renal cortical COX-2.[16] These findings suggest that in the kidney, cortical COX-2 expression is regulated at least in part by alterations in activity of the RAS. CsA enhances the production and secretion of renin.[17,18] Since RAS is critically involved in COX-2 regulation, induction of RAS upon CNI application may play a causative role in the suppression of COX-2.[2]

2.5 Objective and perspective of this study

To resolve the causes whereby CsA treatment leads to the suppression of COX-2 abundance and consequent impairment of renal function, we have reevaluated the effects of CsA on juxtaglomerular COX-2 biosynthesis and action with respect to their local and systemic functional consequences.[19] To this end, *in vivo* and *in vitro* models were adopted. We have followed the hypothesis that (1) RAS hyperactivity induced by CsA plays a dominant role in the inhibition of juxtaglomerular COX-2 expression and (2) the restoration COX-2 functions may alleviate the related renal side effects.[19]

3. Materials and Methods

3.1 Animals

Adult Wistar rats (10 to 12 week-old males, approximately 200g) were separated into different groups. They received vehicle, CsA (25 mg/kg/d, Novartis, Nürnberg, Germany), Candesartan (Cand, 5 mg/kg/d, HEXAL, Holzkirchen, Germany), Celecoxib (50mg/kg/d, Micro Labs GmbH, Frankfurt am Main, Germany) or combined administration for acute- (3 days) or chronic effects (3 weeks) (Table 1).[19] With free access to water and food, rats were housed in metabolic cages for 24 h to collect their urine on the prelast day of each experiment.[19] On the last day, rats were anesthetized with Nembutal (Sigma-Aldrich), then blood samples were taken from the inferior vena cava. One kidney was then clamped and removed for biochemical investigation.[19] The other kidney was *in vivo*-fixed by retrograde perfusion through the abdominal aorta with 3% paraformaldehyde (PFA) in PBS and prepared for morphological investigation.[19] All animal experiments were agreed and permitted by the German Animal Welfare Regulation Authorities.

3.2 Clinical parameters

Blood from rats was taken via the inferior vena cava using a heparinized syringe. Blood was allowed to clot at room temperature (RT) for 30 min and then centrifuged at 2000 x g for 10 min at 4°C to separate supernatant serum.[19] Concentration of creatinine and sodium in the urine and serum were measured by IMD Labor Berlin. Creatinine clearance (CCL) and Fractional excretion of sodium (FE_{Na}) were calculated with standard formula. CCL (ml/min) = [urinary creatinine (mg/dL) × urine flow (ml/min)]/serum creatinine (mg/dL).[19] FE_{Na} (%) = 100 × [urinary Na (mmol/L) × urine flow (ml/min)]/[CCL (ml/min) × serum Na (mmol/L)].[19] For the measurements of plasma renin and Ang II, serum of vehicle or CsA treated rats (3 days) was collected (Table 1, cohort 4). ELISA kits for rat renin (RAB1162, from Sigma-Aldrich) and Ang II (RAB0010, from Sigma-Aldrich) were used to test their concentration.[19]

Table 1. Treatment groups of animal cohorts

	Groups and Administration	entered	terminated
Cohort 1	Vehicle (sham operation)	5	5
3 weeks	CsA (Sandimmun; by mini-pumps)	5	5
	Cand (drinking water)	5	5
	CsA (Sandimmun; by mini-pumps) + Cand (drinking water)	5	5
Cohort 2	Vehicle (Cremophor; i.p.)	5	5
3 days	CsA (Sandimmun; i.p.)	5	5
	Cand (gavage)	5	5
	CsA (Sandimmun; i.p.) + Cand (gavage)	5	5
Cohort 3	Vehicle (Cremophor; i.p.)	4	4
3 days	CsA (Sandimmun; i.p.)	4	4
	CXB (gavage)	4	4
	CsA (Sandimmun; i.p.) + Cand (gavage) + CXB (gavage)	4	4
Cohort 4	Vehicle (Cremophor; i.p.)	6	6
3 days	CsA (Sandimmun; i.p.)	6	6

Table 1 was obtained and modified with permission from Junda Hu et al. 2020, Acta Physiol (Oxf), article PMID 33377278.[19]

3.3 Cell culture experiment

MD cells were cultured in DMEM medium (PAN-Biotech, Aidenbach, Germany) containing 5% fetal calf serum and 1% streptavidin at 37°C, 95% humidity, and 5% CO_2.[19] According to experiment design, cells were treated alone with CsA (Sigma-Aldrich; 1 to 40 µM; 24 h), or combined with Ang II (Abcam; 5 µM; 24 h), SB203580 (Cell Signaling Technology; 10 µM; 24 h) or Bay 11-7082 (Cell Signaling Technology; 3 µM; 24 h).[19] Cells were collected for biochemical study. Mouse distal convoluted tubule (DCT) cells, human renal medullary fibroblasts (TK173 cell line) and human embryonic kidney (HEK293) cells were applied in the present study for control.[19-21]

3.4 Immunofluorescence and Immunohistochemistry

Perfusion-fixed and paraffin-embedded rat kidneys were sectioned and the sections placed on glass slides. Frozen blocks from fixed kidneys were sectioned in a cryostat and

placed on glass slides. Paraffin sections were dewaxed, rehydrated and boiled in citrate buffer (pH 6.0) for 6 min to retrieve antigens.[19] Frozen sections were incubated with 0.5% Triton-X100 for 30 min at RT for antigen retrieval.[19] 5% bovine serum albumin in PBS was used to block unspecific binding sites for 30 min at RT. Sections were later followed by incubation with different primary antibodies (Table 2) diluted in the blocking solution at 4°C overnight.[19] Signals were detected using Cy2- or Cy3-labelled fluorescent (Dianova, Hamburg, Germany) or HRP-conjugated (Dako, Hamburg, Germany) secondary antibodies (RT, 1 h). The results were valued by LSM 5 Exciter confocal light microscope (Zeiss, Jena, Germany).[19] COX-2 or renin abundances in the renal cortex were quantified by counting immuno-positive MD or granular cells, respectively, and the numbers normalized to the total numbers of glomeruli, using earlier described methodology.[22]

3.5 Immunoblotting

MD cells or whole kidney tissue were first homogenized in RIPA buffer containing phosphatase inhibitor (Roche Diagnostics).[19] After sonication for 10 s, samples were centrifuged at 14,000 x*g* for 15 min at 4°C. Supernatants were withdrawn and their protein concentration measured by bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Bonn, Germany).[19] Samples were loaded and separated in polyacrylamide minigels (10%) and then transferred to nitrocellulose membranes. Membranes were later blocked by 5% bovine serum albumin in PBST (30 min at RT) and followed by incubation with different primary antibodies overnight at 4°C. Then HRP-conjugated secondary antibody was applied for 1 h at RT.[19] Signals were visualized by chemiluminescence using ECL solution and ChemoCam Imager ECL (Intas, Göttingen, Germany). GAPDH or β-actin in the present study was used as loading controls and to normalize data.[19]

Table 2 | Primary antibodies applied

Antibody	Provider	Location	Catalogue
COX-2	Santa Cruz Biotechnology	Dallas, USA	sc-1747
NFATc1	Santa Cruz Biotechnology	Dallas, USA	sc-7294
NFATc2	Santa Cruz Biotechnology	Dallas, USA	sc-514929
NFATc3	Santa Cruz Biotechnology	Dallas, USA	sc-8405
NFATc4	Santa Cruz Biotechnology	Dallas, USA	sc-271597
β-actin	Sigma-Aldrich	Darmstadt, Germany	A2228
CnAα	Pineda Services Corporate	Berlin, Germany	NA
CnAβ	Pineda Services Corporate	Berlin, Germany	NA
p38 MAPK	Cell Signaling Technology	Frankfurt a.M, Germany	9212
phospho-p38 MAPK	Cell Signaling Technology	Frankfurt a.M, Germany	4511
phospho-NF-kB	Cell Signaling Technology	Frankfurt a.M, Germany	3033
GAPDH	Abcam	Cambridge, UK	ab181602
Renin	Acris	Herford, Germany	AP00945PU-N
phospho-NKCC2	Pineda Services Corporate	Berlin, Germany	NA
NKCC2	Pineda Services Corporate	Berlin, Germany	NA
phospho-NCC	Pineda Services Corporate	Berlin, Germany	NA
NCC	Sigma-Aldrich	Darmstadt, Germany	AB3553
NOS1	Abcam	Cambridge, UK	ab76067

Table 2 was obtained and modified with permission from Junda Hu et al. 2020, Acta Physiol (Oxf), article PMID 33377278.[19]

3.6 siRNA transfection

For cellular knockdown of calcineurin isoforms, siRNAs targeting CnAα or CnAβ isoforms (Life Technologies) were transfected into cultured MD cells (30% confluence) for 72 h by INTERFERin (Polyplus transfection, Illkirch, France).[19] The following double strand siRNAs were used to silence CnAα and CnAβ in MD cells: CnAα, sense 5´-CAU UGA GAA UAA UAA CAG ATT-3´ and antisense 5´-UCU GUU AUU AUU CUC AAU GCA-3´; CnAβ, sense 5´-GGA UGA UAU UAG GAG AUU ATT-3´ and antisense 5´-UAA UCU CCU AAU AUC ACU CAG-3´.[19]

3.7 Quantitative real-time PCR

TRIzol reagent was used to extract total RNA from MD cells.[19] After harvesting RNA, cDNA was generated by reverse transcription. Specific forward and reverse primers for qPCR assays were designed (COX-2: 5'-AGC CAG GCA GCA AAT CCT T-3' and 5'-CAG TCC GGG TAC AGT CAC AC-3'; GAPDH: 5'-TGC ACC ACC AAC TGC TTA GC-3' and 5'-GGC ATG GAC TGT GGT CAT GAG-3').[19] DNA was amplified using the 7500 Fast Real-Time PCR system (Applied Biosystems, Darmstadt, Germany) and HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia).[19] Expression levels of GAPDH were used as controls. CT values of all samples were less than 38 throughout. Data were processed by the $\Delta\Delta$CT method and mean values of $\log_2$ relative quantification were compared among groups.[19]

3.8 Statistical analysis

Deduced from the experimental design, results were analyzed using routine parametric statistics for normal distribution. Two-group experiments were compared by unpaired t-test. Multiple experimental groups were compared by ANOVA with post hoc test.[19] ImageJ software (NIH, Maryland, USA) was applied to detect signal intensity. Data was analyzed using GraphPad Prism7 (San Diego, USA). In this study, $P<0.05$ was established as significant. Data are presented as means ± SD.

4. Results

4.1 Effects of CsA on epithelial localization of COX-2 and NFAT in the renal cortex

Treatment with CsA for 3 weeks induced a substantial suppression of COX-2 immunoreactivity in MD and nearby cortical TAL cells in rats (Figure 1A, D).[19] The immunosuppression of CsA is related to NFAT transcription factors NFATc1 through NFATc4 isoforms, which are regulated by calcium/calcineurin signaling.[11,23] In order to test the potential role of NFAT in the regulation of COX-2 expression in MD cells[11,24], we studied the localization of NFATc1 - NFATc4 in cortical part of rat kidney. *In vivo*, NFATc3 immunofluorescent signal was significantly established in TAL cells but spared MD cells in kidney cortex (Figure 1C, F).[19] In contrast to COX-2, NFATc3 immunoreactive signal was unaltered by CsA (Figure 1B, E).

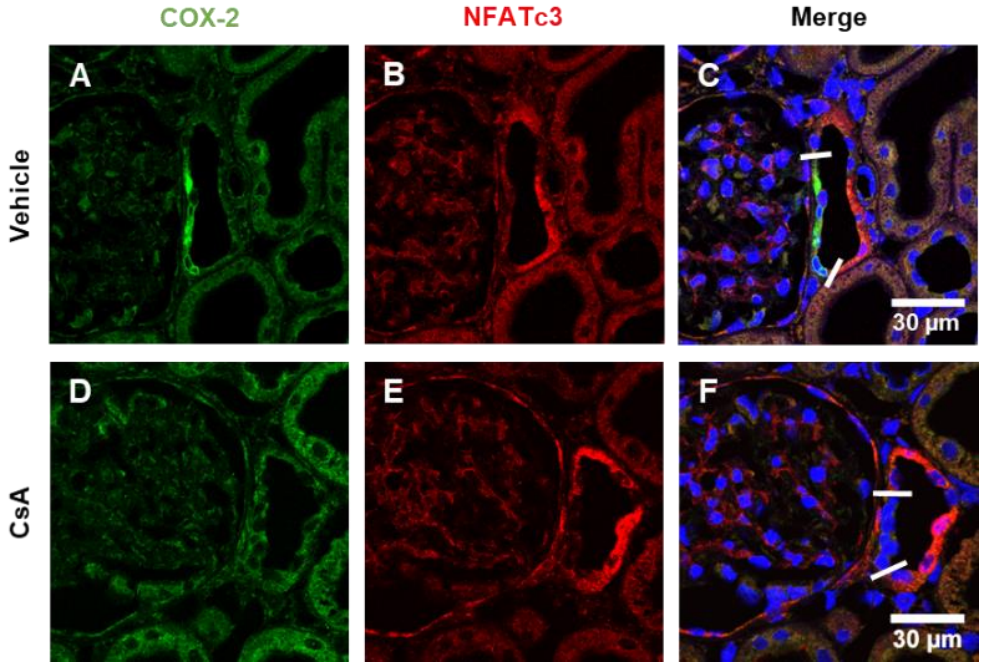

Figure 1. Effects of calcineurin inhibition by CsA on the expression of COX-2 and NFAT transcription factor isoforms in rat kidney cortex. A, D: Representative immunofluorescent images from vehicle- and CsA-treated rats (25 mg/kg/d for 3 weeks) show CsA-induced suppression of COX-2 expression (green fluorescence) in MD cells. C, F: Double staining of COX-2 (green) and NFATc3 (red) in kidneys of vehicle- or CsA-treated rats shows no substantial co-localization of them. CsA administration significantly decreases COX-2 expression but shows no influence on NFATc3 immunofluorescent intensity or distribution. Scale bars are indicated in corresponding images. Figure 1 was obtained and modified with permission from Junda Hu et al. 2020, Acta Physiol (Oxf), article PMID 33377278.[19]

Immunostaining for NFATc1, NFATc2, and NFATc4 was not detected in MD cells (Figure 2A-D), whereas immunofluorescent signals were observed in renal tumor tissue (Figure 2E-J).[19] Together, data confirmed that NFAT isoforms (NFATc1-c4) are absent from MD in rat kidney (Figure 2A-D). In consequence, results in the present study illustrate that effects of CsA on COX-2 biosynthesis in renal cortex are irrelevant to NFAT transcription factors.

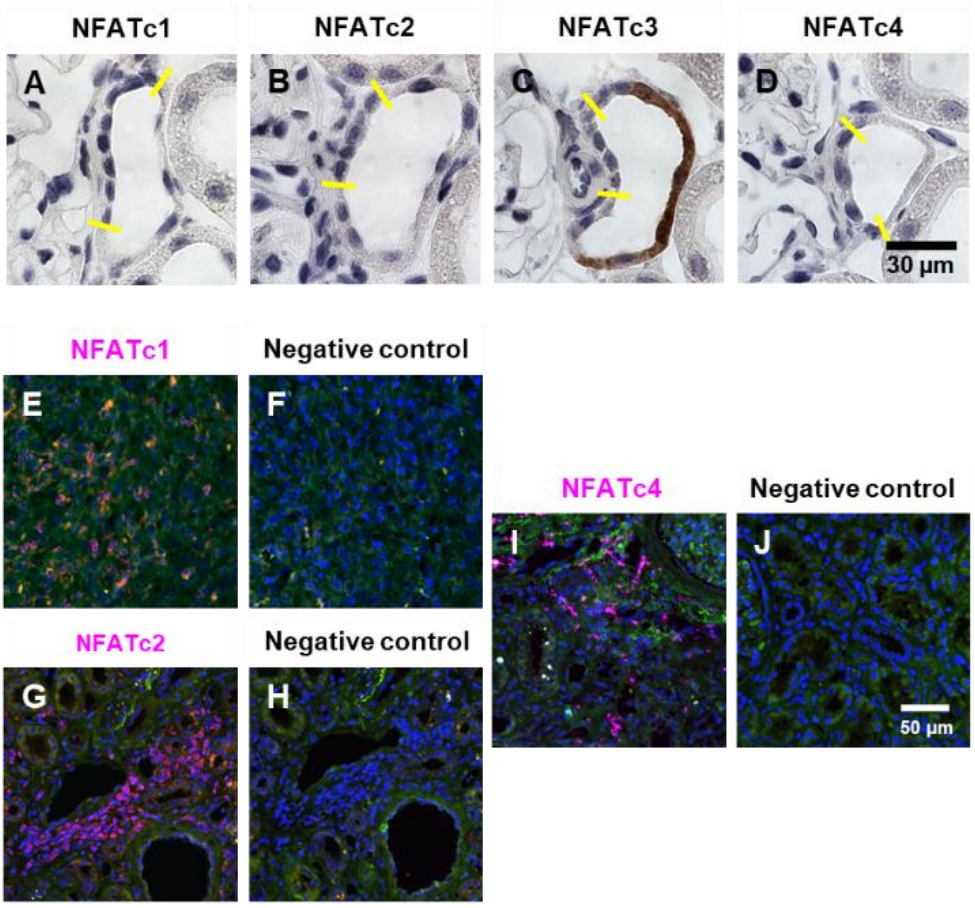

Figure 2. Localization of NFAT transcription factors in kidney. A-D: Representative images of HRP-staining of NFAT isoforms 1-4 (NFATc1-4) in the cortex of rat kidney. NFATc1, NFATc2 or NFATc4 immunostaining show no substantial signals, whereas NFATc3 is present in cTAL but absent from MD cells. E-J: Verification of antibodies to NFATc1, c2 and c4 in human kidney sections obtained after tumor nephrectomy. Signals show up in tumor parenchymal cells (E), immune cells (G), and interstitial fibroblasts (I), whereas omitting the primary antibodies for negative control did not produce detectable immunoreactivity. Scale bars are indicated in corresponding images. Figure 2 was obtained and modified with permission from Junda Hu et al. 2020, Acta Physiol (Oxf), article PMID 33377278.[19]

4.2 Effects of CsA on COX-2 biosynthesis in cultured MD cells

CsA was directly applied to cultured MD cells to elude CsA-induced systemic effects on juxtaglomerular COX-2 biosynthesis when administering the drug *in vivo*.[19] Applying CsA to MD cells for 24 h upregulated COX-2 mRNA levels (2- to 18 fold, $p<0.05$; Figure 3A), which were dose-dependent (5, 10 or 40 µM).[19] Immunoblotting revealed similar upregulation of COX-2 expression after CsA treatment ($p<0.01$; Figure 3B, C). Therefore, the direct effect of CsA on MD cells is to increase the expression of COX-2. DCT cells, renal fibroblasts and HEK293 cells did not show detectable COX-2 biosynthesis at baseline or with CsA treatment (Figure 3D, E), confirming that MD cells were suitable in the present study.

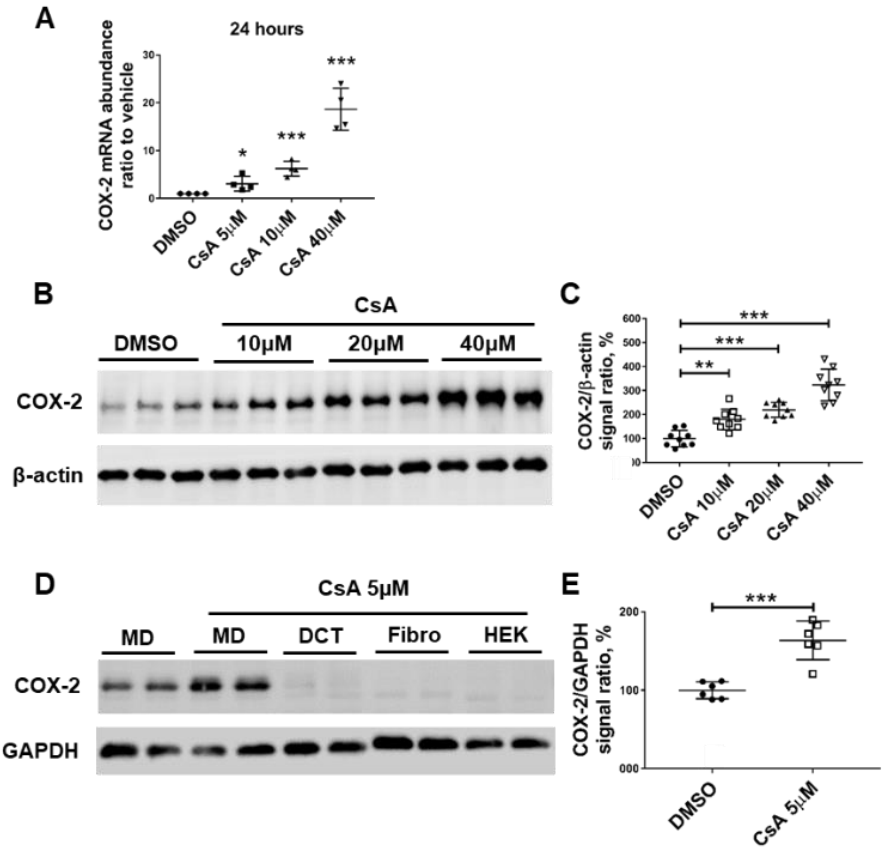

Figure 3. Effects of CsA on COX-2 expression *in vitro*. A: Quantitative PCR results show COX-2 mRNA levels in MD cells treated with vehicle (DMSO) or CsA for 24 h. B, C: Representative immunoblot results and quantification show increases of COX-2 expression in cell lysates of MD cells treated with vehicle or CsA for 24 h; ®-actin here serves as a loading control. D, E: Baseline and CsA-induced COX-2 expression was detected exclusively in cultured MD cells but not in DCT, Fibro or HEK cells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Dosages are indicated in corresponding images. Figure 3 was obtained and modified with permission from Junda Hu et al. 2020, Acta Physiol (Oxf), article PMID 33377278.[19]

4.3 Effects of calcineurin isoforms on COX-2 expression in cultured MD cells

Cyclophilin-drug complexes mediate CsA-induced calcineurin inhibition. To eliminate the effects of cyclophilin, here we used siRNA knockdown technology to inhibit α and β isoforms of calcineurin subunit A directly.[19] The specific siRNA probes successfully reduced the protein amount of respective CnA isoforms (-60% and -58%, respectively, $p<0.001$; Figure 4A-D). At the same time, COX-2 abundance was increased substantially (+63% and +69%, respectively, $p<0.001$; Figure 4A-D). The results of siRNA experiments show that suppression of calcineurin biosynthesis in MD cells upregulates COX-2 expression.

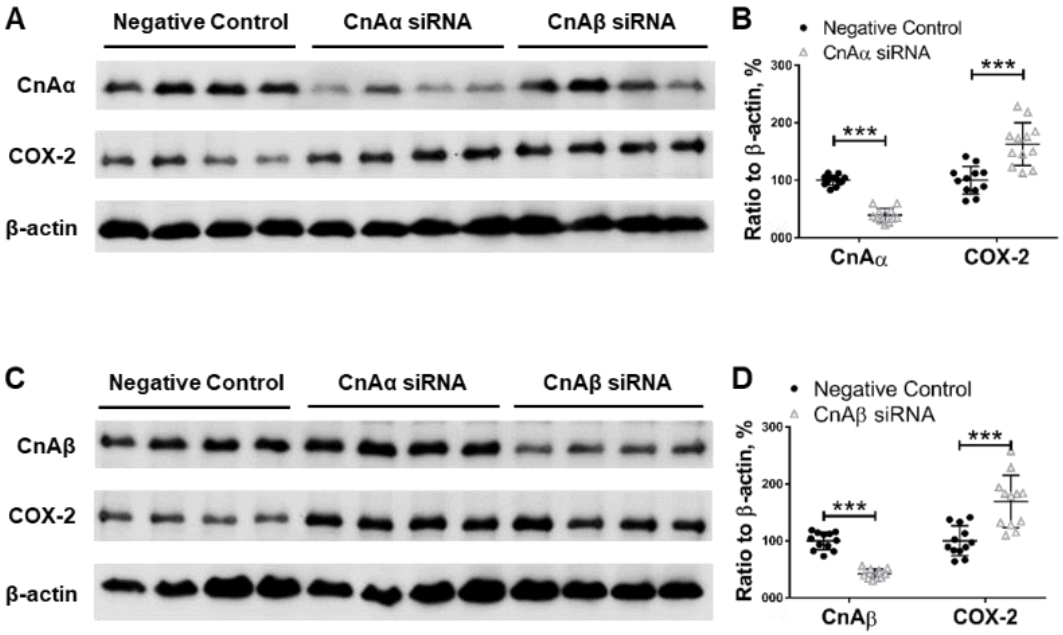

Figure 4. Effects of CnA subunits on COX-2 expression in cultured MD cells. A-D: Representative immunoblots show CnAα and CnAβ or COX-2 in cell lysates of cultured MD cells transfected with CnAα or CnAβ siRNA. Evaluation of bands shows significant suppression of calcineurin subunits A and parallel upregulation of COX-2 after CnAα- (A) or CnAβ knockdown (C). ***$p<0.001$. Figure 4 was obtained and modified with permission from Junda Hu et al. 2020, Acta Physiol (Oxf), article PMID 33377278.[19]

4.4 RAS mediates the CsA-induced COX-2 expression in cultured MD cells via p38 MAPK/NF-kB pathway

CsA induced opposite effects on COX-2 expression *in vivo* (downregulated) and *in vitro* (upregulated). One hypothesis of this study is that systemic activation of RAS induced by CsA suppresses COX-2 expression. To confirm it, we first investigated the effect of Ang II (5 μM, 24 h) on COX-2 expression in cultured MD cells under single treatment and its

combination with CsA (40 µM, 24 h). Ang II suppressed the baseline COX-2 biosynthesis and abolished the CsA-induced upregulation of COX-2 expression as evaluated by immunoblotting ($p<0.05$, Figure 5A, B).[19] Ang II further decreased baseline pp38 MAPK/pNF-kB abundance and prevented the CsA-dependent activation of pp38 MAPK/pNF-kB, as detected by immunoblotting (Figure 5C-F).[19]

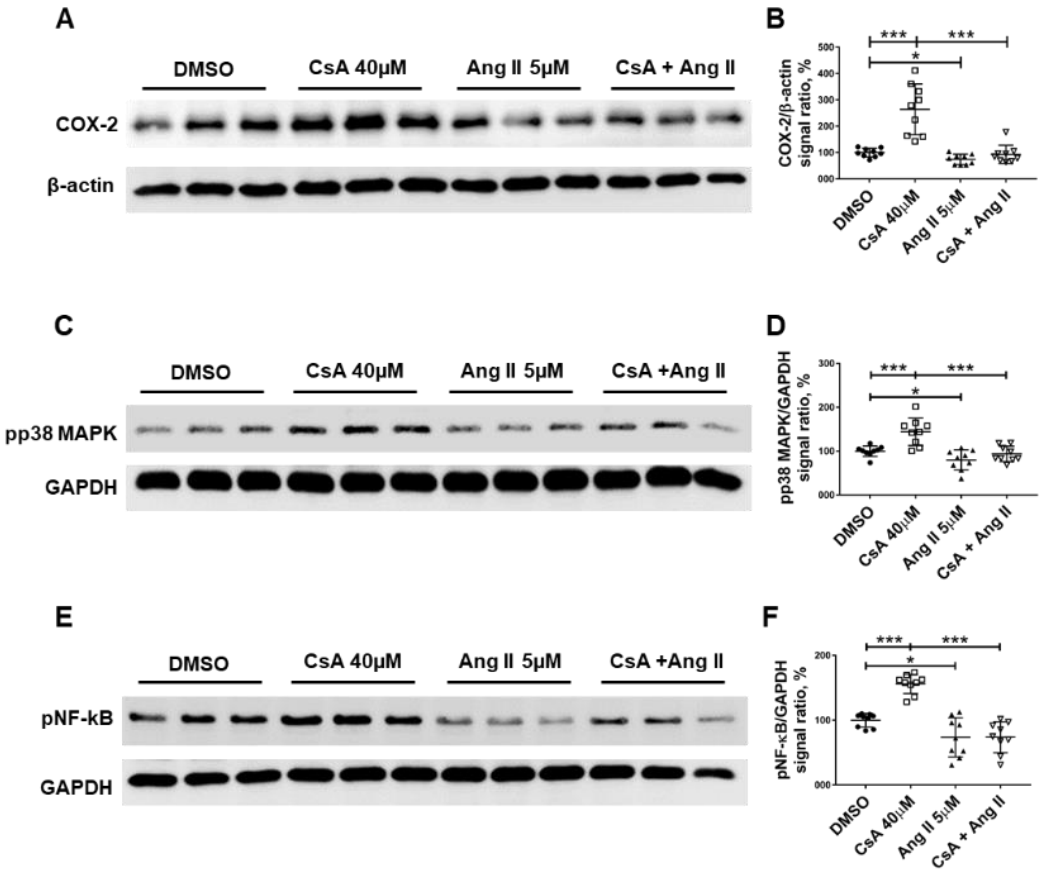

Figure 5. Effects of CsA and Ang II on COX-2, p38 MAPK and NF-kB in cell culture. A-F: Immunoblots present expression of COX-2 (A), phosphorylated (p) p38 MAPK (C) and pNF-kB (E) in cultured MD cells after treatment with vehicle (DMSO), CsA, Ang II, or their combination for 24 h. Quantification is showed on the right side of respective blots. Dosages are indicated in corresponding images. * $p<0.05$, *** $p<0.001$. Figure 5 was obtained and modified with permission from Junda Hu et al. 2020, Acta Physiol (Oxf), article PMID 33377278.[19]

The application of a p38 MAPK inhibitor SB203580 (10 µM, 24 h) or a NF-kB inhibitor Bay 11-7082 (3 µM, 24 h) upon CsA treatment blunted its stimulatory effect on COX-2 biosynthesis by MD cells (Figure 6A, C).[19] In addition, the increase in phosphorylation of NF-kB induced by CsA was abrogated under p38 MAPK suppression (Figure 6E).[19] These results suggested that the p38 MAPK–NF-kB signaling regulates COX-2

expression in MD cells. CsA activated this signaling pathway to enhance COX-2 biosynthesis, whereas Ang II exerted the opposite effect *in vitro*.

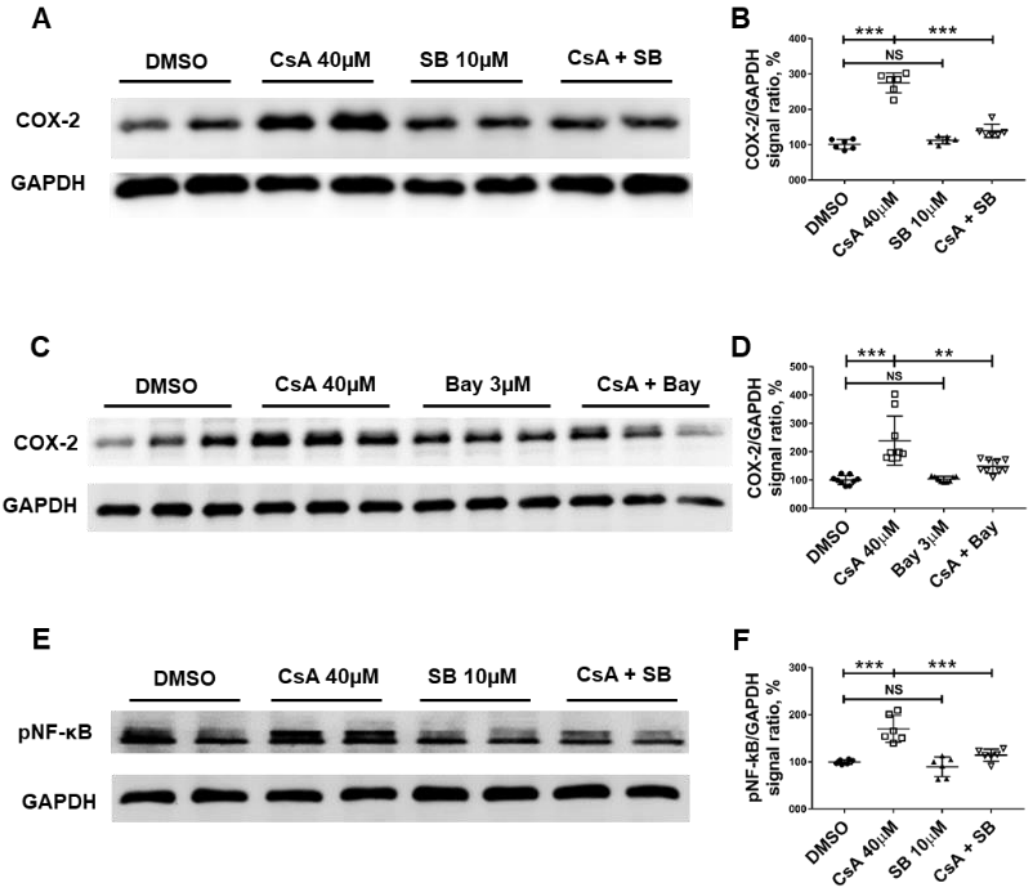

Figure 6. Role of p38 MAPK and NF-kB in mediating effects of CsA and Ang II on COX-2 *in vitro*. A: COX-2 expression in MD cells treated with vehicle (DMSO), CsA, p38 MAPK inhibitor SB203580 (SB), or their combination for 24 h shown by immunoblot. C: Immunoblot presents COX-2 biosynthesis by MD cells treated with DMSO, CsA, NF-kB inhibitor Bay 11-7082 (Bay) or their combination for 24 h. E: Phosphorylation of NF-kB in MD cells after treatment with DMSO, CsA, SB, or their combination for 24 h. Quantification is showed on the right side of respective blots (B, D, F). Dosages are indicated in corresponding images. * p<0.05, ** p<0.01, *** p<0.001, NS – not significant. Figure 6 was obtained and modified with permission from Junda Hu et al. 2020, Acta Physiol (Oxf), article PMID 33377278.[19]

4.5 Renin and Ang II increase after CsA administration *in vivo*

After confirming that CsA-induced COX-2 stimulation was prevented by Ang II *in vitro*, we then tested renin and Ang II expression after CsA administration *in vivo*.[19] Consistent with previous reports, CsA significantly increased renin expression in granular cells as detected by immunofluorescence in the present study. This trend was found after both 3 days and 3 weeks of CsA administration (+43% and +88% immunoreactivity, respectively,

p<0.01; Figure 7A-D). In addition, plasma renin and Ang II levels significantly rose upon CsA administration for 3 days (renin: +182%, p<0.001; Ang II: +163%, p<0.001; Figure 7E, F), indicating enhanced renin release from granular cells and activation of RAS.[19]

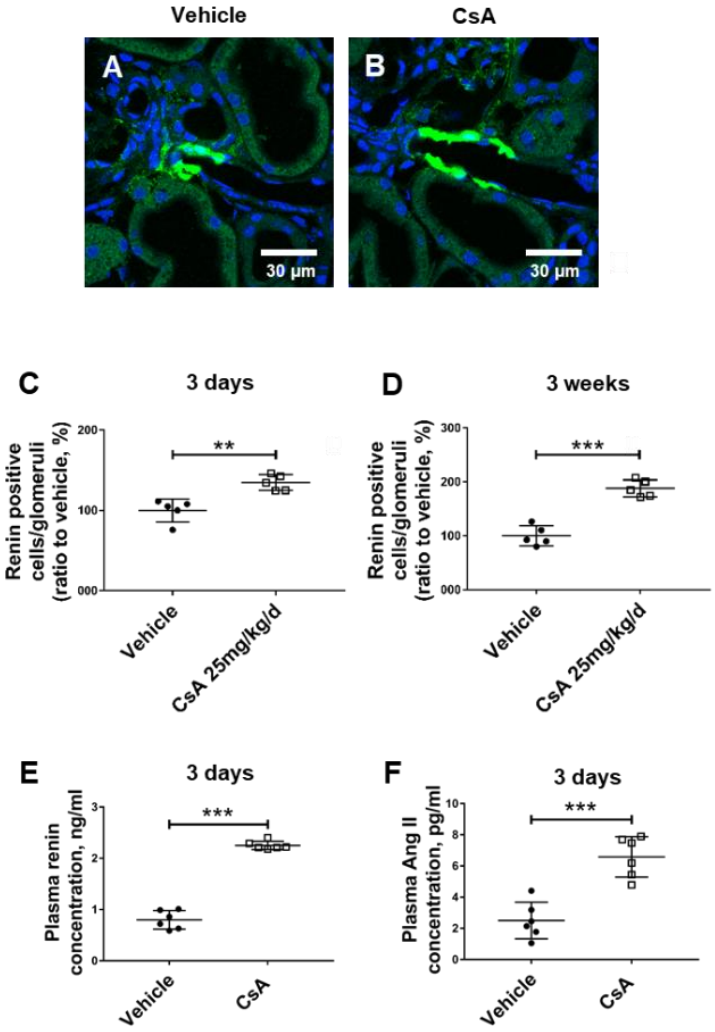

Figure 7. Effects of CsA on RAS *in vivo*. A, B: Representative immunofluorescent images of cortical part from rats treated with vehicle or CsA (25 mg/kg/d) for 3 weeks present renin biosynthesis in granular cells. C, D: Graphs show quantification of renin-positive cells in rats after vehicle or CsA treatment protocols. E, F: Quantification of plasma renin and Ang II levels in rats after 3 days of vehicle or CsA application. ** p<0.01, *** p<0.001. Scale bars are indicated in corresponding images. Figure 7 was obtained and modified with permission from Junda Hu et al. 2020, Acta Physiol (Oxf), article PMID 33377278.[19]

4.6 Ang II receptor blockade stimulates COX-2 expression in kidney cortex

To assess the functions of stimulated RAS *in vivo*, we applied the Ang II receptor type 1 (AT1R) antagonist, Cand, to CsA-treated rats as rescue experiments (3 days and 3 weeks).[19] As a result, Cand did not alter cortical COX-2 abundance in vehicle-treated rats

but significantly alleviated CsA-induced COX-2 suppression after concomitant administration for 3 days (-87% vs. -25% after CsA vs. CsA + Cand, respectively, p<0.001) and completely restored COX-2 expression after 3 weeks of treatment (Figure 8A-F).[19] These results support our hypothesis that RAS activation induced by CsA is responsible for inhibition of COX-2 expression in juxtaglomerular portion.

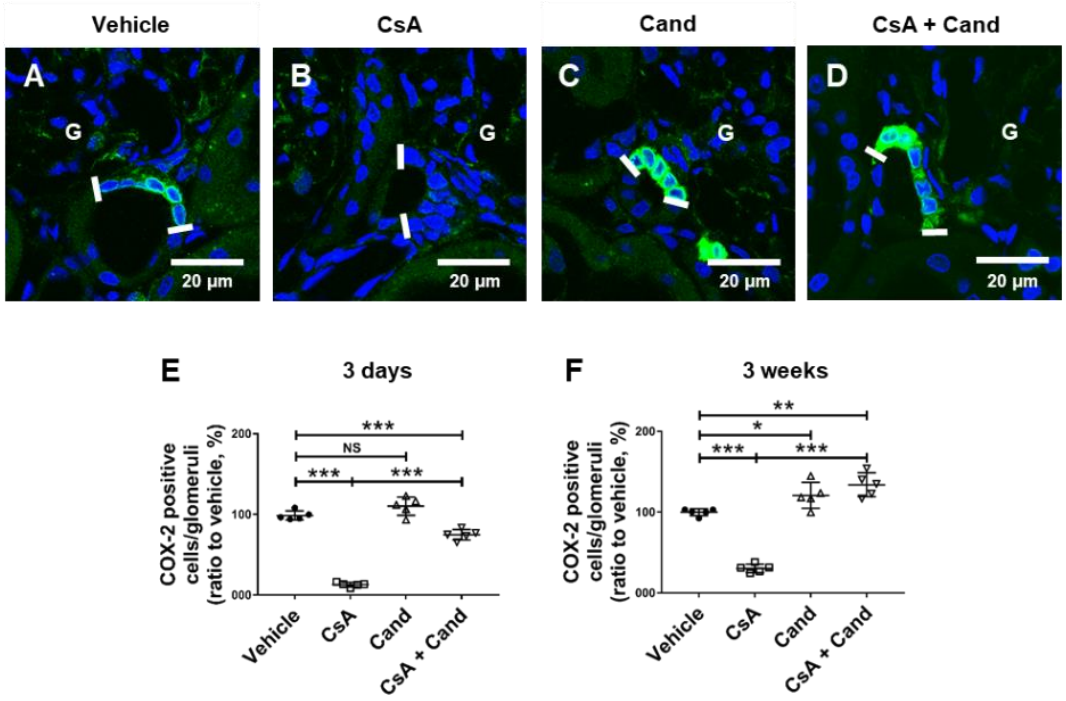

Figure 8. Rescue of CsA-induced COX-2 suppression by AT1 receptor antagonist Cand *in vivo*. A-D: Representative immunofluorescent images show COX-2 expression in macula densa portion from rats treated with vehicle, CsA (25 mg/kg/d), Cand (5 mg/kg/d) or their combination for 3 weeks; G = glomerulus. E, F: Graphs show quantifications of COX-2 signals in renal cortex from rats treated with vehicle, CsA, Cand, or their combination for 3 days (E) or 3 weeks (F). * p<0.05, ** p<0.01, *** p<0.001, NS – not significant. Scale bars are indicated in corresponding images. Figure 8 was obtained and modified with permission from Junda Hu et al. 2020, Acta Physiol (Oxf), article PMID 33377278.[19]

4.7 Impaired renal function upon CsA is improved by Ang II receptor blocker

In the present study, both 3 days and 3 weeks CsA treatment protocols induce significant decreases of creatinine clearance (both -34%, p<0.01; Figure 9A, G).[19] Concomitant administration of Cand in the acute setting diminished the decrease (from -34% to -17%, p<0.05) and completely restored this parameter in the chronic setting (Figure 9A, G).[19] Since COX-2 activity typically shows inverse correlation with distal salt reabsorption, we have evaluated the FE_{Na}. Compared to vehicle groups, CsA led to significant decreases of FE_{Na} in both acute and chronic settings (p<0.01; Figure 9B, H).[19] Cand significantly

attenuated CsA-induced sodium retention acutely and completely restored the FE_{Na} in the chronic treatment protocol (Figure 9B, H).[19] To investigate whether COX-2 plays a role in the Cand-induced restoration, we furthermore treated rats with celecoxib (a selective COX-2 inhibitor) for 3 days. Celecoxib did not show a substantial effect on creatinine clearance level, but significantly decreased FE_{Na} when applied alone (-20%, $p<0.05$; Figure 9D, E).[19] In combined treatment with CsA, Cand, and celecoxib, creatinine clearance and FE_{Na} were significantly reduced compared to the vehicle group, that is, celecoxib prevented improvement of these two parameters induced by Cand.[19] These results suggest that the favorable effects of Cand on renal function at least partially rely on COX-2 activity (Figure 9D, E). We evaluated urinary potassium as well, since CsA may induce hyperkalemia. Acute CsA treatment (3 days) led to a substantial impairment of potassium excretion (-52%, $p<0.01$).[19] The impairment was partially attenuated by concomitant Cand application (-31% vs. CsA alone, $p<0.05$, Figure 9C).[19] Celecoxib blunted the improvement of renal potassium handling induced by Cand (Figure 9F). In consequence, restoration of COX-2 activity by RAS blocker, Cand, has the capacity to alleviate impairments of renal function induced by CsA.[19]

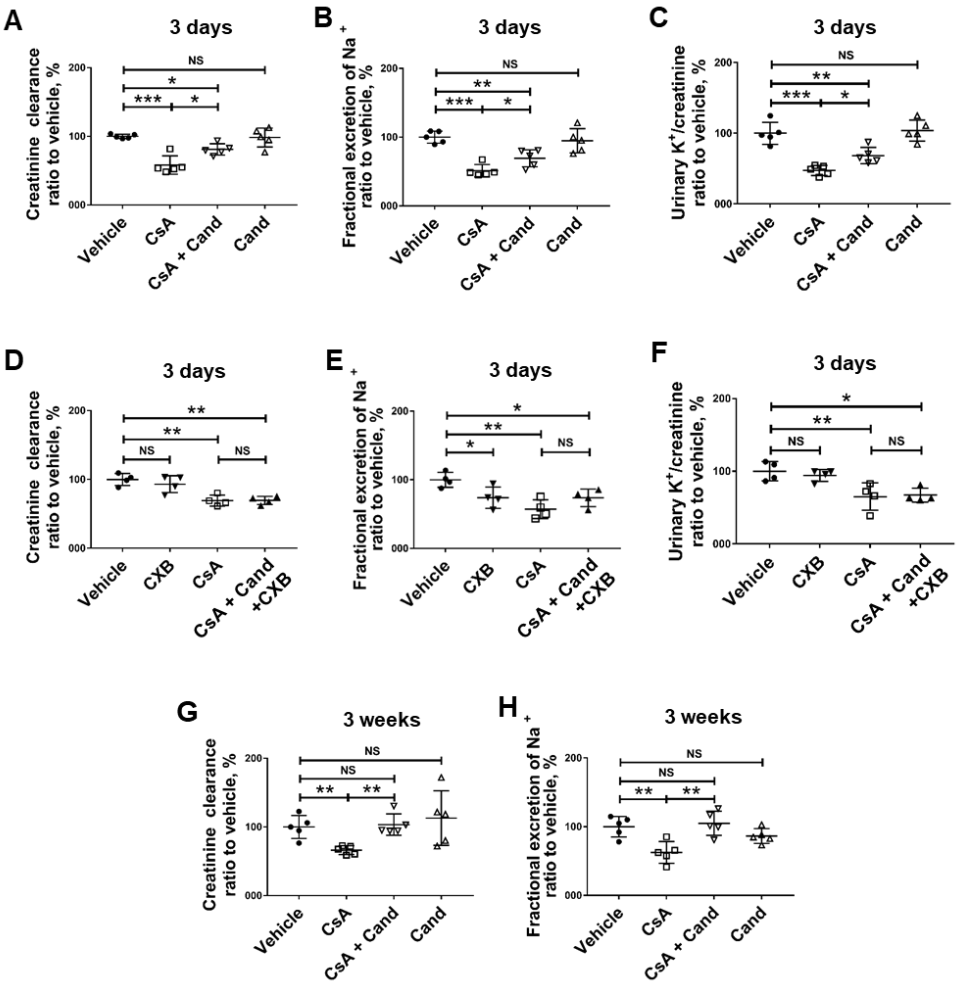

Figure 9. Effects of CsA, Cand and celecoxib (CXB) on renal function upon single or combined treatments. A-C: Graphs show creatinine clearance, FE_{Na} or urinary K+/creatinine in rats treated with vehicle, CsA (25 mg/kg/d), Cand (5 mg/kg/d) or their combination for 3 days. D-F: Graphs show creatinine clearance, FE_{Na} or urinary K+/creatinine in rats treated with vehicle, CXB (50 mg/kg/d), CsA, or CsA + Cand + CXB for 3 days. G, H: Graphs show creatinine clearance and FE_{Na} in rats treated with vehicle, CsA, Cand or their combination for 3 weeks. * p<0.05, ** p<0.01, *** p<0.001, NS – not significant. Figure 9 was obtained and modified with permission from Junda Hu et al. 2020, Acta Physiol (Oxf), article PMID 33377278.[19]

4.8 CsA-induced activation of NKCC2 and NCC are abolished by RAS inhibition

Chronic CsA treatment has been shown to increase phosphorylation levels of both NKCC2 and NCC.[25,26] Here, acute CsA administration caused significant increases in phospho-NKCC2 (T96/T101; +350%, p<0.01, Figure 10A) and phospho-NCC levels (S71; +209%, p<0.001, Figure 10D), whereas total NKCC2 and NCC abundances were not affected.[19] Concomitant application of Cand prevented the surges in NKCC2 and NCC phosphorylation levels induced by CsA.[19] The total amounts of these two transporters

were mildly increased after combined treatment (+17% [not significant] and +23% [p<0.05], Figure 10).[19] The favorable effects of Cand on sodium and potassium balance may be partially dependent on the inhibition of NKCC2 and NCC activation.

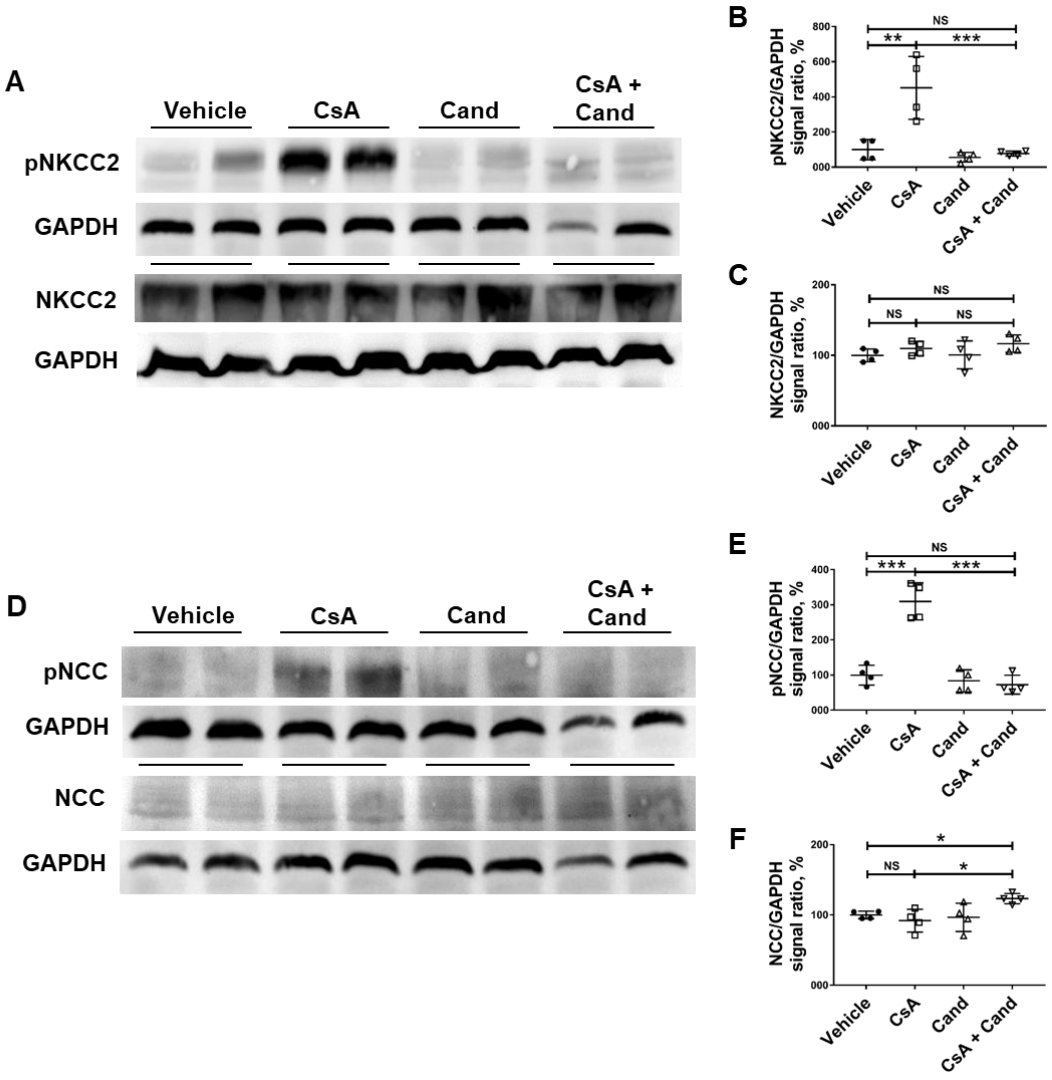

Figure 10. Effects of CsA, Cand, or combined treatment on NKCC2 and NCC. A, D: Immunoblots of kidney lysates from rats treated with vehicle, CsA (25 mg/kg/d), Cand (5 mg/kg/d), or their combination for 3 days present expression of phosphorylated (p) and total protein abundance of NKCC2 (A) or NCC (D). Quantification of signals is displayed close to the corresponding immunoblots. * p<0.05, ** p<0.01, *** p<0.001, NS – not significant. Figure 10 was obtained and modified with permission from Junda Hu et al. 2020, Acta Physiol (Oxf), article PMID 33377278.[19]

4.9 NOS1 expression and activity are not influenced by CsA

Neuronal nitric oxide synthase (NOS1) expression in MD cells may be regulated by COX-2 and RAS as well. Therefore, in the present study, we furthermore detected NOS1 biosynthesis and activity in rats administrated with vehicle, CsA, Cand, or their combination for 3 weeks.[19] Investigation of NOS1 using immunofluorescence or immunoblotting techniques showed no substantial differences among different treatment protocols.[19] Additionally, NOS1 activity indicated by NADPH-diaphorase reaction was similar in different groups (Figure 11A-J).[19] In consequence, no obvious evidence was established for regulation of NOS1 activity in MD cells by CsA alone or combined treatment with Cand.

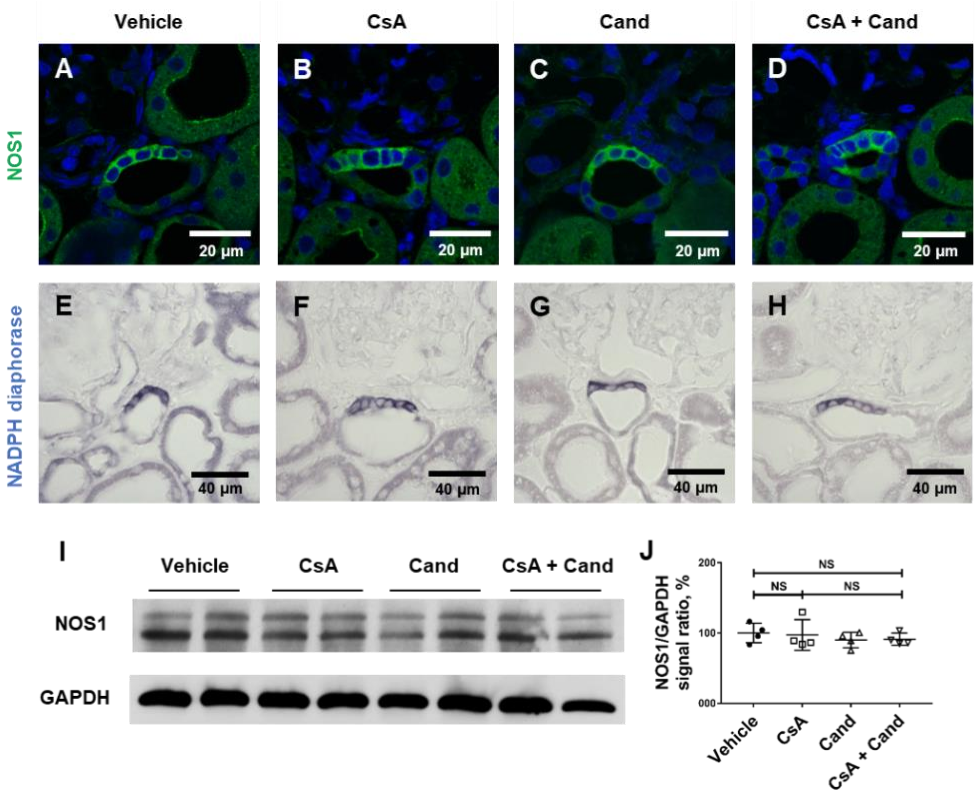

Figure 11. Effects of CsA, Cand, or their combination on cortical NOS1 and NADPH-diaphorase reaction in rats. Analysis of NOS1 in macula densa cells of rats treated with vehicle, CsA, Cand or CsA + Cand for three weeks using immunofluorescence (A-D), NADPH-diaphorase reaction (E-H) or immunoblotting (I) revealed no differences between the treatment groups. Figure 11 was obtained and modified with permission from Junda Hu et al. 2020, Acta Physiol (Oxf), article PMID 33377278.[19]

5. Discussion

Stimulation of RAS and inhibition of renal cortical COX-2 are major side effects of CsA, contributing to reduction in GFR as well as to salt retention and associated hypertension.[1] Although the suppression of renal COX-2 as a consequence of CsA administration has been well documented *in vivo*,[18] little information was available on the immediate effects of CsA in COX-2 expressing cells when systemic factors were absent. Unlike the *in vivo* situation, the present *in vitro* experiments in cultured MD cells have demonstrated that pharmacological or siRNA-mediated inhibition of calcineurin activity actually upregulates COX-2 expression in this cell type.[19] The underlying molecular pathways involve MAPK signaling, since CsA enhanced activating p38 MAPK phosphorylation. Other stimuli of COX-2 activity such as extracellular Cl^- depletion have been shown to increase p38 MAPK phosphorylation as well.[9] Calcineurin has been proposed to act as an endogenous inhibitor of p38 MAPK.[27] Activation of p38 MAPK upon calcineurin inhibition may then promote COX-2 expression.[7] The key role of p38 MAPK in stimulation of COX-2 in response to CsA has been further corroborated by pharmacological inhibition of the kinase, which completely prevented the CsA-induced COX-2 upregulation in the present study. Molecules acting downstream of p38 MAPK to increase COX-2 expression may involve various transcription factors, among which NFAT and NF-kB have been considered as major regulators of the PTGS2 gene encoding COX-2.[12,23] Since earlier studies and our present experiments did not detect significant expression of NFAT isoforms in MD, effects of CsA may rather depend on NF-kB or alternative pathways of transcriptional regulation.[12] CsA may affect NF-kB activity via different mechanisms including regulation of c-Jun-N-terminal kinases, NF-kB-inducing kinase, CCAAT-enhancer-binding proteins or MAPK, likely depending on the cell type and concomitant local or systemic stimuli.[28] In our *in vitro* experiment, NF-kB was shown to be activated by CsA and to participate in the expression of COX-2 in MD cells.[19] CsA activated NF-kB in MD cells via stimulation of p38 MAPK, since p38 MAPK inhibitor prevented the CsA-induced increase of phospho-NF-kB.[19]

To explain the opposing effects of CsA on COX-2 expression *in vivo* vs. *in vitro*, we tested the hypothesis that CsA-induced RAS activation may interfere with the local COX-2 regulation in MD cells. The effect of concomitant Ang II and CsA application in cultured MD cells was a decrease in p38 MAPK and NF-kB phosphorylation as well as an abolishment of CsA-induced COX-2 stimulation, suggesting that Ang II exerts a dominant

inhibitory effect on the p38 MAPK/NF-kB/COX-2 pathway, independent of calcineurin activity.[19] Therefore, Ang II may act downstream of calcineurin to inhibit p38 MAPK/NF-kB and suppress COX-2 biosynthesis upon CsA.[19] These conclusions are consistent with RAS activation and decreased COX-2 expression in rats receiving CsA.[2,29] The present rescue experiments using AT1R inhibitor Cand not only restored the COX-2 expression but also blunted the CsA-induced GFR reduction, which may be related with effects of COX-2-derived vasoactive prostanoids.[30]

Notably, animal models of high-renin state such as dietary salt depletion typically exhibit increased COX-2 expression in MD, which has been proposed to contribute to RAS activation.[31] In contrast, a clear dissociation between the COX-2 and renin expression patterns was observed in CsA-treated rats suggesting that COX-2 activity is not critical to renin expression in this setting. In fact, induction of renin biosynthesis may as well be related with increased sympathetic activity upon CsA or direct inhibition of calcineurin activity within renin-producing granular cells.[17,32] In aggregate, our results suggest that CsA enhances RAS activity, which leads to suppression of the juxtaglomerular COX-2 expression via an Ang II-mediated negative feedback.[16]

Intact COX-2 function is critical to the capacity of the kidney to excrete sodium, since COX-2-deficient mice acquired hypertension in response to high salt diet.[33] Moreover, diuretic effects of furosemide (the NKCC2 inhibitor) and hydrochlorothiazide (the NCC inhibitor) were found to be partially dependent on COX-2 stimulation, suggesting that COX-2-derived prostanoids interfere with salt reabsorption along the distal nephron.[34] In line with this, furosemide has been shown to increase the COX-2 expression in MD cells.[35] We speculate that reduced availability of COX-2-derived prostanoids stimulates NKCC2 and NCC and thereby aggravates renal salt retention and hypertension.[36,37] In support of this assumption, increased levels of activating NKCC2 and NCC phosphorylation and reduced FE_{Na} in CsA-treated rats were significantly normalized by Cand in the present study.[19]

Treating patients with CsA bears the risk of hyperkalemia.[1] One previous study demonstrated that patients receiving CNI benefit from concomitant AT1R inhibitors administration without more vulnerability to hyperkalemia.[38] This concept has been further supported by our present analysis of urinary K+ excretion, which showed that Cand improved rather than deteriorated the CsA-induced potassium retention. Remarkably,

celecoxib abrogated the increase of potassium excretion induced by Cand in CsA-treated rats, which demonstrated that COX-2 participates in renal potassium management.[19]

The effects of RAS inhibitors in alleviating nephrotoxic side effects of CsA have been revealed by earlier studies in patients and animal models; beneficial effects on renal hemodynamics, kidney morphology, and blood pressure have been reported.[39] The underlying mechanisms were involved in ameliorative vasoconstriction and inhibition of transforming growth factor signaling.[40] The present data suggest that restoration of COX-2 expression in MD is also a positive impact of RAS inhibition in improving renal hemodynamics and alleviating the sodium and potassium retention.[19]

In conclusion, our results demonstrate that calcineurin functions as an endogenous inhibitor of COX-2 expression in MD cells.[19] The systemic regulation of COX-2 by RAS overrides calcineurin-dependent mechanisms in the cell autonomous level.[19] As a result, CsA exerted an inhibitory effect on COX-2 expression in MD *in vivo*. AT1R inhibitor Cand rescues COX-2 biosynthesis and improves renal hemodynamics indicated by creatinine clearance.[19] Furthermore, blocking AT1R by Cand performs favorable effects on sodium and potassium homeostasis.[19] These findings support its clinical application to alleviate CsA-induced nephrotoxicity. Enhanced understanding of COX-2 regulation in the kidney, as provided here, may have clinical indications concerning therapeutic regimens that involve the administration of CsA, NSAIDs, or RAS inhibition.[19]

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7. Statutory Declaration

“I, Junda Hu, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic [Calcineurin inhibitor-associated impairment of COX-2 signaling in kidney cortex is restored by angiotensin II receptor blocker], independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

8. Declaration of individual contribution to publication

Junda Hu has contributed to this publication:

Hu Junda, Xu Yan, Bachmann Sebastian, Mutig Kerim. Angiotensin II receptor blockade alleviates calcineurin inhibitor nephrotoxicity by restoring cyclooxygenase 2 expression in kidney cortex. Acta Physiol (Oxf). 2020 Dec 29:e13612. doi: 10.1111/apha.13612.

He is the sole first author. He conceived and designed research with the help of Prof. Sebastian Bachmann and Dr. Kerim Mutig. Junda performed animal experiments with the help of Yan Xu. He collected all blood and urine samples, analyzed all data, and interpreted the results of the experiments. He further prepared all Figures (Figure 1-8, supplemental Figure 1-5) and Tables (Table 1-3). He also wrote the methods and results sections and revised the final draft.

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Signature, date and stamp of first supervising university professor / lecturer

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Signature of doctoral candidate

9. Journal Data Filtered By: Selected JCR Year: 2019 Selected Editions: SCIE Selected Categories: 'PHYSIOLOGY' Selected Category Scheme: WoS

Journal Data Filtered By: **Selected JCR Year: 2019** Selected Editions: SCIE,SSCI
Selected Categories: **"PHYSIOLOGY"** Selected Category Scheme: WoS
Gesamtanzahl: 81 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	PHYSIOLOGICAL REVIEWS	28,712	25.588	0.024010
2	Annual Review of Physiology	9,466	19.556	0.010190
3	JOURNAL OF PINEAL RESEARCH	10,537	14.528	0.009430
4	PHYSIOLOGY	3,583	7.212	0.005380
5	International Journal of Behavioral Nutrition and Physical Activity	11,154	6.714	0.018870
6	Comprehensive Physiology	4,877	6.604	0.009170
7	JOURNAL OF CELLULAR PHYSIOLOGY	26,456	5.546	0.024290
8	Acta Physiologica	5,106	5.542	0.008320
9	EXERCISE AND SPORT SCIENCES REVIEWS	3,290	4.915	0.002720
10	Reviews of Physiology Biochemistry and Pharmacology	805	4.700	0.000670
11	JOURNAL OF PHYSIOLOGY-LONDON	50,045	4.547	0.037090
12	AMERICAN JOURNAL OF PHYSIOLOGY-LUNG CELLULAR AND MOLECULAR PHYSIOLOGY	13,085	4.406	0.015510
13	AMERICAN JOURNAL OF PHYSIOLOGY-HEART AND CIRCULATORY PHYSIOLOGY	26,114	3.864	0.020400
14	AMERICAN JOURNAL OF PHYSIOLOGY-GASTROINTESTINAL AND LIVER PHYSIOLOGY	14,186	3.725	0.012280
15	PSYCHOPHYSIOLOGY	14,586	3.692	0.012670
16	JOURNAL OF GENERAL PHYSIOLOGY	7,476	3.628	0.007380
17	International Journal of Sports Physiology and Performance	5,072	3.528	0.009760

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
18	AMERICAN JOURNAL OF PHYSIOLOGY-CELL PHYSIOLOGY	15,502	3.485	0.010450
19	AMERICAN JOURNAL OF PHYSIOLOGY-ENDOCRINOLOGY AND METABOLISM	18,917	3.469	0.013710
20	Frontiers in Physiology	21,190	3.367	0.052500
21	JOURNAL OF MAMMARY GLAND BIOLOGY AND NEOPLASIA	1,951	3.293	0.001080
22	CLINICAL JOURNAL OF SPORT MEDICINE	4,242	3.165	0.005100
23	PFLUGERS ARCHIV-EUROPEAN JOURNAL OF PHYSIOLOGY	9,355	3.158	0.009810
24	AMERICAN JOURNAL OF PHYSIOLOGY-RENAL PHYSIOLOGY	16,035	3.144	0.017010
25	JOURNAL OF BIOLOGICAL RHYTHMS	3,258	3.122	0.003220
26	JOURNAL OF APPLIED PHYSIOLOGY	43,194	3.044	0.020180
27	AMERICAN JOURNAL OF PHYSIOLOGY-REGULATORY INTEGRATIVE AND COMPARATIVE PHYSIOLOGY	17,896	2.992	0.013690
28	Journal of Physiological Sciences	1,380	2.955	0.002160
29	JOURNAL OF PHYSIOLOGY AND BIOCHEMISTRY	1,854	2.952	0.002340
30	PESTICIDE BIOCHEMISTRY AND PHYSIOLOGY	5,930	2.751	0.005660
31	PHYSIOLOGICAL GENOMICS	4,535	2.749	0.004520
32	INTERNATIONAL JOURNAL OF BIOMETEOROLOGY	6,418	2.680	0.007220
33	JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY	3,342	2.644	0.002740
34	INTERNATIONAL JOURNAL OF PSYCHOPHYSIOLOGY	8,822	2.631	0.009440
35	EUROPEAN JOURNAL OF APPLIED PHYSIOLOGY	16,418	2.580	0.012130

Selected JCR Year: 2019; Selected Categories: “PHYSIOLOGY”

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
36	ARCHIVES OF PHYSIOLOGY AND BIOCHEMISTRY	1,104	2.575	0.001010
37	Conservation Physiology	1,342	2.570	0.004180
38	NEUROPHYSIOLOGIE CLINIQUE-CLINICAL NEUROPHYSIOLOGY	1,385	2.553	0.001770
39	Applied Physiology Nutrition and Metabolism	5,955	2.522	0.010250
40	CHRONOBIOLOGY INTERNATIONAL	5,708	2.486	0.006600
41	CLINICAL AND EXPERIMENTAL PHARMACOLOGY AND PHYSIOLOGY	5,813	2.456	0.004650
42	EXPERIMENTAL PHYSIOLOGY	5,710	2.431	0.006580
43	PHYSIOLOGICAL MEASUREMENT	6,066	2.309	0.006240
44	CRYOBIOLOGY	4,661	2.283	0.003850
45	CHEMICAL SENSES	4,553	2.261	0.003220
46	PHYSIOLOGICAL AND BIOCHEMICAL ZOOLOGY	3,033	2.250	0.002650
47	JOURNAL OF INSECT PHYSIOLOGY	9,006	2.246	0.006520
48	FISH PHYSIOLOGY AND BIOCHEMISTRY	4,825	2.242	0.004130
49	JOURNAL OF NEUROPHYSIOLOGY	40,570	2.225	0.032060
50	QUARTERLY JOURNAL OF EXPERIMENTAL PSYCHOLOGY	5,922	2.077	0.010080
51	Journal of Comparative Physiology B-Biochemical Systems and Environmental Physiology	3,768	2.042	0.002830
52	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY A-MOLECULAR & INTEGRATIVE PHYSIOLOGY	10,391	1.966	0.005190
53	CANADIAN JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY	4,900	1.946	0.003990
54	KIDNEY & BLOOD PRESSURE RESEARCH	1,903	1.898	0.003130

Selected JCR Year: 2019; Selected Categories: “PHYSIOLOGY”

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
55	JOURNAL OF MEMBRANE BIOLOGY	3,765	1.877	0.002400
56	KOREAN JOURNAL OF PHYSIOLOGY & PHARMACOLOGY	1,076	1.805	0.001780
57	HYPERTENSION IN PREGNANCY	1,314	1.787	0.001450
58	JOURNAL OF ELECTROMYOGRAPHY AND KINESIOLOGY	5,312	1.740	0.004060
59	Journal of Physiological Anthropology	826	1.730	0.000980
60	JOURNAL OF VASCULAR RESEARCH	1,551	1.725	0.001150
61	CLINICAL PHYSIOLOGY AND FUNCTIONAL IMAGING	2,409	1.704	0.003340
62	JOURNAL OF MUSCULOSKELETAL & NEURONAL INTERACTIONS	1,611	1.660	0.001490
63	PHYSIOLOGICAL RESEARCH	3,598	1.655	0.003680
64	RESPIRATORY PHYSIOLOGY & NEUROBIOLOGY	6,495	1.591	0.004210
65	ARCHIVES OF INSECT BIOCHEMISTRY AND PHYSIOLOGY	1,973	1.536	0.001230
66	ADVANCES IN PHYSIOLOGY EDUCATION	1,634	1.534	0.001620
67	JOURNAL OF COMPARATIVE PHYSIOLOGY A-NEUROETHOLOGY SENSORY NEURAL AND BEHAVIORAL PHYSIOLOGY	5,007	1.516	0.003390
68	JOURNAL OF BIOLOGICAL REGULATORS AND HOMEOSTATIC AGENTS	1,924	1.506	0.002820
69	Lymphatic Research and Biology	860	1.492	0.001130
70	PEDIATRIC EXERCISE SCIENCE	1,987	1.489	0.002220
71	Physiology International	137	1.410	0.000280
72	CHINESE JOURNAL OF PHYSIOLOGY	575	1.151	0.000480

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
73	GENERAL PHYSIOLOGY AND BIOPHYSICS	958	1.070	0.000890
74	BIOLOGICAL RHYTHM RESEARCH	816	0.826	0.000860
75	CRYOLETTERS	925	0.702	0.000610
76	ZHURNAL VYSSHEI NERVNOI DEYATELNOSTI IMENI I P PAVLOVA	305	0.432	0.000110
77	JOURNAL OF EVOLUTIONARY BIOCHEMISTRY AND PHYSIOLOGY	371	0.382	0.000230
78	NEUROPHYSIOLOGY	232	0.322	0.000200
79	Revista Brasileira de Medicina do Esporte	792	0.309	0.000290
80	LYMPHOLOGY	791	0.233	0.000490
81	KLINISCHE NEUROPHYSIOLOGIE	54	0.111	0.000010

10. Manuscript of the publication

Hu J, Xu Y, Bachmann S, Mutig K. Angiotensin II receptor blockade alleviates calcineurin inhibitor nephrotoxicity by restoring cyclooxygenase 2 expression in kidney cortex. Acta Physiol (Oxf). 2020 Dec 29:e13612. doi: 10.1111/apha.13612. Epub ahead of print. PMID: 33377278.

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REGULAR PAPER

ACTA PHYSIOLOGICA

Angiotensin II receptor blockade alleviates calcineurin inhibitor nephrotoxicity by restoring cyclooxygenase 2 expression in kidney cortex

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Abstract

Aim: The use of calcineurin inhibitors such as cyclosporine A (CsA) for immunosuppression after solid organ transplantation is commonly limited by renal side effects. CsA-induced deterioration of glomerular filtration rate and sodium retention may be related to juxtaglomerular dysregulation as a result of suppressed cyclooxygenase 2 (COX-2) and stimulated renin biosynthesis. We tested whether CsA-induced COX-2 suppression is caused by hyperactive renin-angiotensin system (RAS) and whether RAS inhibition may alleviate the related side effects.

Methods: Rats received CsA, the RAS inhibitor candesartan, or the COX-2 inhibitor celecoxib acutely (3 days) or chronically (3 weeks). Molecular pathways mediating effects of CsA and RAS on COX-2 were studied in cultured macula densa cells.

Results: Pharmacological or siRNA-mediated calcineurin inhibition in cultured cells enhanced COX-2 expression via p38 mitogen-activated protein kinase and NF-kB signalling, whereas angiotensin II abolished these effects. Acute and chronic CsA administration to rats led to RAS activation along with reduced cortical COX-2 expression, creatinine clearance and fractional sodium excretion. Evaluation of major distal salt transporters, NKCC2 and NCC, showed increased levels of their activating phosphorylation upon CsA. Concomitant candesartan treatment blunted these effects acutely and completely normalized the COX-2 expression and renal functional parameters at long term. Celecoxib prevented the candesartan-induced improvements of creatinine clearance and sodium excretion.

Conclusion: Suppression of juxtaglomerular COX-2 upon CsA results from RAS activation, which overrides the cell-autonomous, COX-2-stimulatory effects of calcineurin inhibition. Angiotensin II antagonism alleviates CsA nephrotoxicity via the COX-2-dependent normalization of creatinine clearance and sodium excretion.

KEYWORDS
calcineurin inhibitors, cyclooxygenase 2, macula densa, nephrotoxicity, renin-angiotensin system

1 | INTRODUCTION

Calcineurin inhibitors (CNI), cyclosporine A (CsA) or tacrolimus, maintain adequate immunosuppression levels in organ transplant patients; however, toxic renal effects remain a concern.[1-3] CNI-induced suppression of cyclooxygenase 2 (COX-2) and stimulation of renin biosynthesis impair juxtaglomerular regulation. As a result of this dissociation, reduced glomerular filtration rate (GFR) and sodium retention may occur.[3-5] The COX isoforms, COX-1 and COX-2, catalyse oxidation of arachidonic acid to prostaglandin H (PGH) as the rate-limiting step for synthesis of bioactive prostanoids, such as prostaglandin E_2 (PGE_2).[6-8] Renal adaptive responses include modulation of juxtaglomerular COX-2 expression, whereas distinctly localized COX-1 displays a constitutive expression pattern.[9-12] COX-2 is expressed in macula densa (MD) and neighbouring cortical thick ascending limb (cTAL) cells, as well as in inner medullary interstitial cells.[13,14] COX-2-derived prostanoids modulate the tubuloglomerular feedback mechanism and help maintain GFR, in part via functional interaction with neuronal nitric oxide synthase type 1 (NOS1).[15-18] Prostanoids further promote renin release from juxtaglomerular granular cells and exert inhibitory effects on salt reabsorption along the distal nephron.[19,20] COX-2 expression in MD and adjacent cTAL cells shows an inverse correlation with luminal Cl^- concentration sensed by the apical Na^+-K^+-$2Cl^-$ cotransporter (NKCC2).[21] Loss-of-function mutations in the *SLC12A1* gene encoding for NKCC2 lead to increased production of COX-2-derived PGE_2 and pronounced salt wasting in patients with antenatal Bartter's syndrome, also termed hyperprostaglandin E syndrome.[22] Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX activity and alleviate the clinical symptoms of this syndrome, likely by reducing GFR and enhancing tubular salt reabsorption.[22,23] Similar to CNI, long-term use of NSAIDs is associated with nephrotoxic side effects.[3,4,7,10,24]

Little is known about the signalling pathways linking calcineurin phosphatase to COX-2. Immunosuppressive effects of CNI are mediated by inhibition of nuclear factor of activated T-cells transcription factors (NFAT), followed by suppression of key genes mediating T-lymphocyte activation including COX-2.[1,25,26] In the kidney, expression of juxtaglomerular COX-2 responds to the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-kB) rather than to NFAT.[27] CNI stimulate the p38 mitogen-activated protein kinase (p38 MAPK), which may promote the NF-kB activity and COX-2 expression in juxtaglomerular MD and cTAL cells.[28-30] Yet, parallel, CNI-induced activation of the renin-angiotensin system (RAS), induced by CNI, may interfere with cell-autonomous mechanisms, resulting in net inhibition of COX-2.[4,5,31]

We follow the hypothesis that CNI-induced RAS hyperactivity is crucial in mediating the suppression of juxtaglomerular COX-2 biosynthesis. Using CsA, we addressed cell-autonomous and RAS-dependent mechanisms of COX-2 regulation in rats and cultured macula densa cells, as well as the nephrotoxic impact of COX-2 suppression. Our data support the use of RAS inhibitors for alleviation of CNI nephrotoxicity.

2 | RESULTS

2.1 | CNI and epithelial localization of COX-2 and NFAT

Chronic treatment of rats with the calcineurin inhibitor cyclosporine A (CsA; 25 mg/kg body weight) for 3 weeks resulted in numerical reduction in COX-2 immunoreactive cells located in MD and adjacent cTAL portion (Figure 1A). To address the previously assumed role of NFAT signalling in the regulation of COX-2 in MD cells,[32] we re-evaluated the distribution of NFAT isoforms in rat kidney cortex. Antibodies to NFATc1, NFATc2, and NFATc4 produced no significant signals in MD or adjacent cTAL, whereas clear immunostaining of these isoforms was obtained elsewhere in renal tissue (Figure 1B, Figure S1A-C). NFATc3 signal was detectable in TAL but spared the COX-2 expressing MD and adjacent cTAL cells in both vehicle and CsA-treated rats (Figure 1B,C). These results suggest that effects of CsA on cortical COX-2 expression are unrelated to NFAT signalling, at least at the cell-autonomous level.

2.2 | CNI and COX-2 expression in cultured MD cells

To avoid CNI-induced systemic or paracrine factors affecting juxtaglomerular COX-2 expression, we applied CsA directly in cultured MD cells. Adding CsA to the culture medium (5, 10, or 40 μmol/L) for 6 hours or 24 hours produced dose-dependent increases in COX-2 mRNA levels ranging from 2- to 18-fold at both time points ($P < .05$; Figure 2A,B). Immunoblotting revealed similar, dose-dependent increases in COX-2 protein abundance after CsA application for 24 hours (+80%, +120% and +220% with 10, 20 and 40 μmol/L CsA, respectively, $P < .01$; Figure 2C). Testing several other cell lines did not reveal detectable COX-2 abundance at baseline or upon CsA administration, confirming that MD cells were the adequate model (Figure S2A). CsA-induced calcineurin inhibition is mediated by cyclophilin-drug complexes.[33] To exclude a bias of cyclophilin-dependent effects, we applied siRNA technology to directly suppress Aα or Aβ isoforms of the catalytic

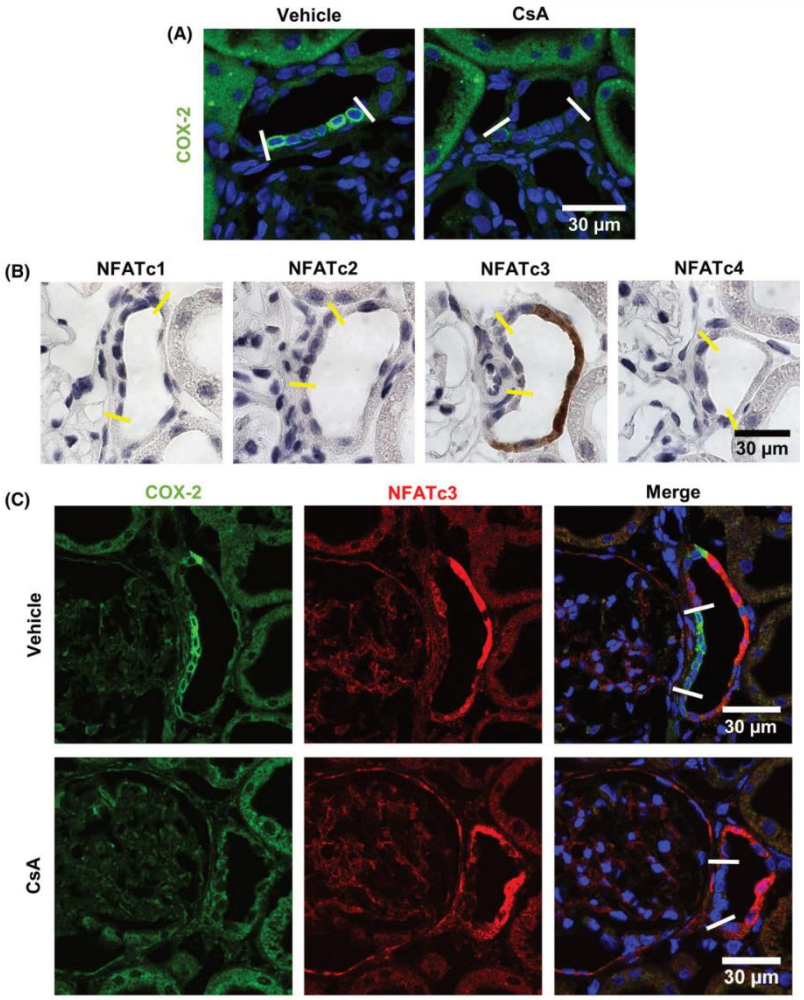

FIGURE 1 Distribution of nuclear factor of activated T cells (NFAT) transcription factor isoforms in rat kidney cortex. A, Representative confocal images of kidneys from vehicle- (n = 5) or cyclosporine A (CsA)-treated rats (25 mg/kg body weight*24 h for 3 wk; n = 5) document CsA-induced reduction in cyclooxygenase 2 (COX-2) abundance (green immunofluorescence) in macula densa (MD) and neighbouring cortical thick ascending limb (cTAL) cells; nuclei are counterstained with DAPI (blue signal). B, Representative images of rat kidney cortex (n = 5) show immunoperoxidase staining of NFAT cytoplasmic isoforms 1-4 (NFATc1-4). Antibodies to NFATc1, NFATc2 or NFATc4 produce no significant signals, whereas NFATc3 (brown staining) is present in cTAL but absent from MD and adjacent cTAL cells. C, Double-immunofluorescence labelling of COX-2 and NFATc3 in kidneys from vehicle- or CsA-treated rats shows no significant co-localization of these products; COX-2 signal is present in MD and neighbouring cTAL cells, whereas NFATc3 is expressed in COX-2-negative cTAL cells. CsA treatment markedly suppresses COX-2 signal but has no significant effects on NFATc3 signal intensity or distribution; nuclei are counterstained with DAPI (blue signal in the merge images). Scale bar = 30 µm; MD cells are flanked by lines

calcineurin subunit. The respective siRNA probes significantly decreased the protein levels of calcineurin Aα and Aβ (−60% and −58%, respectively, $P < .001$; Figure 2D,E). Under these conditions, COX-2 levels were enhanced to a comparable extent (+63% and +69%, respectively, $P < .001$; Figure 2D,E). These results demonstrate that local inhibition of calcineurin or suppression of its biosynthesis in MD cells increases COX-2 expression.

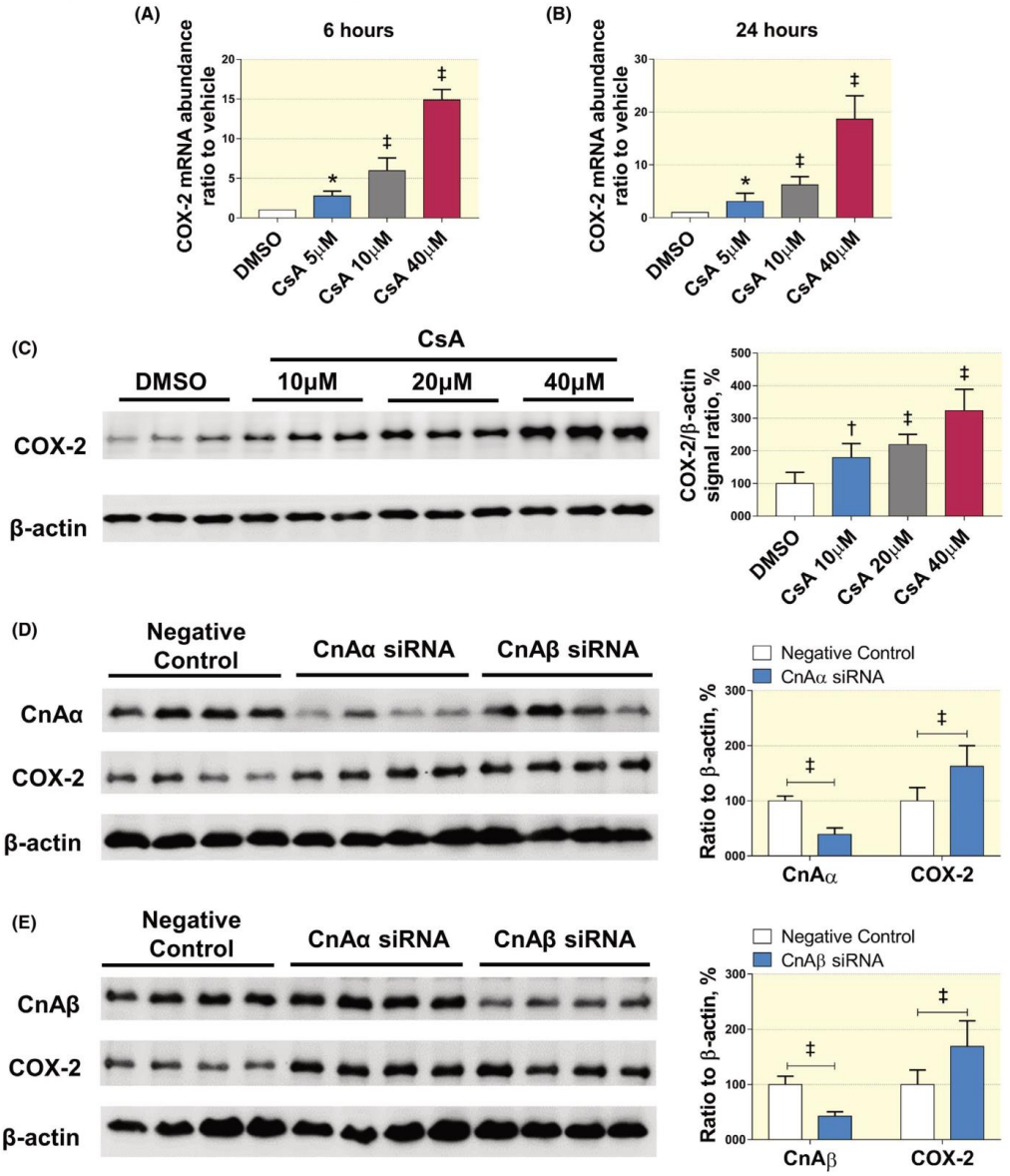

FIGURE 2 Effects of calcineurin inhibition on cyclooxygenase 2 (COX-2) expression and function in cell culture. A and B, Quantitative PCR shows dose-dependent increases in COX-2 mRNA levels in cultured mouse macula densa (MD) cells treated with CsA for 6 h (A) or 24 h (B) as compared to vehicle (DMSO); n = 4 independent experiments for each time point. C, Representative immunoblot and densitometric evaluation show dose-dependent increases in COX-2 immunoreactivity (approximately 74 kDa) in cell lysates of MD cells treated with CsA for 24 h; β-actin serves as loading control (approximately 42 kDa); n = 3 independent experiments. D and E, Representative immunoblots show detection of CnAα and CnAβ (both at approximately 59 kDa) or COX-2 in cell lysates of cultured MD cells transfected with either CnAα or CnAβ siRNA; for negative control cells were treated with transfection reagents only. Densitometric evaluation of signals obtained after CnAα- (D) or CnAβ knockdown (E) shows significant decreases in calcineurin catalytic subunits along with increased abundance of COX-2. Data are means ± SD; n = 3 independent experiments with four biological replicates each. CsA doses are provided in the figure for each experiment. Data are means ± SD; $^{*}P < .05$, $^{\dagger}P < .01$, $^{\ddagger}P < .001$

2.3 | Angiotensin II suppresses p38 MAPK, NF-kB, and COX-2 in cultured MD cells

We aimed to resolve the discrepancy between the CsA-induced COX-2 suppression in vivo and its stimulation in cultured MD cells. We tested the hypothesis that systemic activation of RAS in response to CsA may inhibit COX-2, thus overriding the local stimulation. To this end, we studied the effect of angiotensin II (Ang II; 1 μmol/L and 5 μmol/L for 24 hours) on COX-2 abundance and activity in cultured MD cells with or without concomitant CsA administration. Ang II suppressed baseline COX-2 abundance at both doses

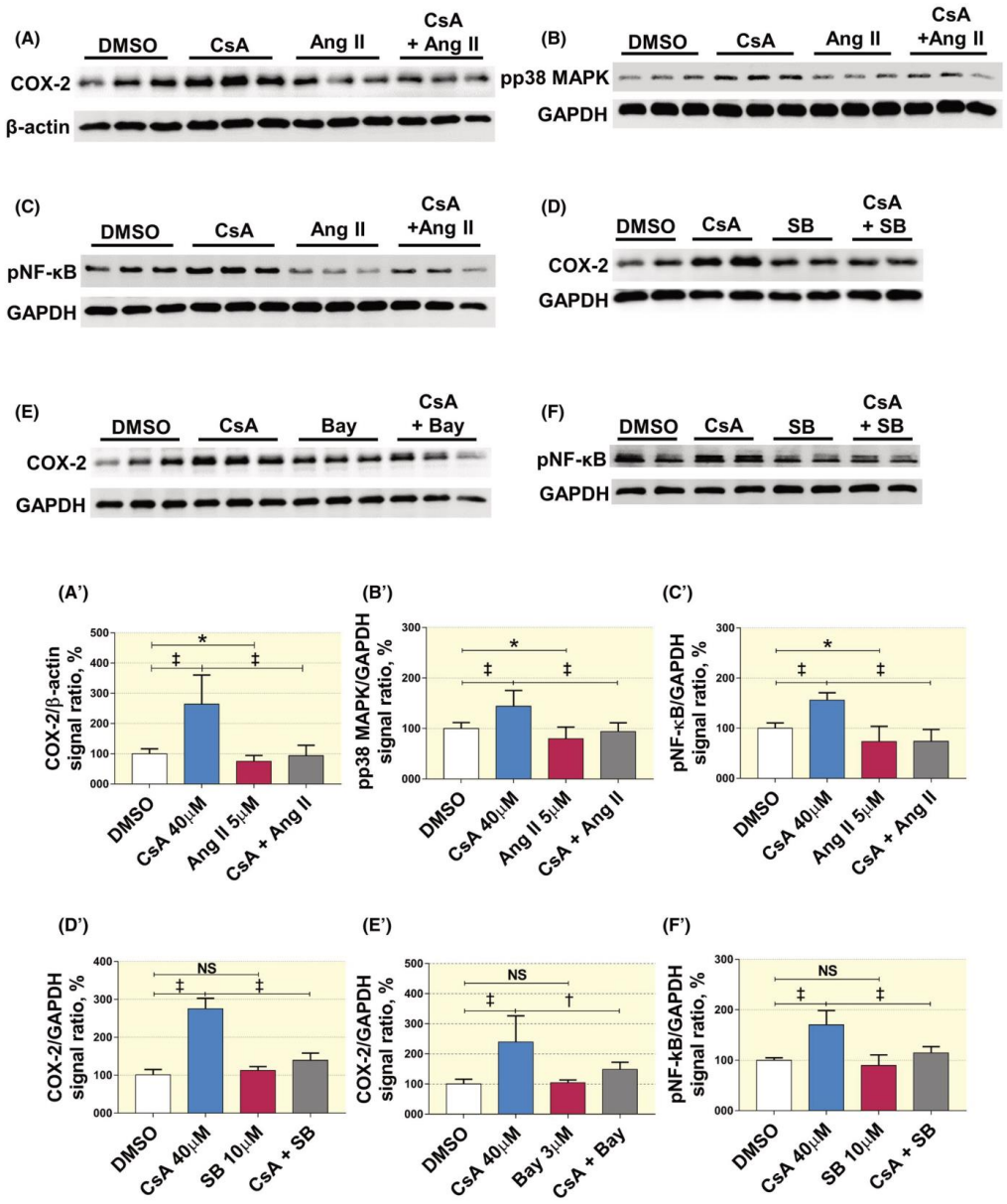

FIGURE 3 Role of p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-kB) in mediating effects of cyclosporine A (CsA) and angiotensin II (Ang II) on cyclooxygenase 2 (COX-2) in cell culture. A-C, Representative immunoblots show detection of COX-2 (A, approximately 74 kDa), phosphorylated (p) p38 MAPK (B, approximately 43 kDa) and pNF-kB (C, approximately 65 kDa) in lysates of cultured MD cells treated with DMSO, CsA, Ang II or CsA + Ang II for 24 h each. D, Immunoblot shows COX-2 detection in lysates of cultured MD cells treated with DMSO, CsA, p38 MAPK inhibitor SB203580 (SB; 10 μM) or CsA + SB for 24 h each. E, Immunoblot shows COX-2 detection in lysates of cultured MD cells treated with DMSO, CsA, NF-kB inhibitor Bay 11-7082 (Bay; 3 μM) or CsA + Bay for 24 h each. F, Immunoblot shows pNF-kB detection in lysates of cultured MD cells treated with DMSO, CsA, SB (10 μM) or CsA + SB for 24 h; β-actin (approximately 42 kDa) or GAPDH (approximately 37 kDa) serve as loading control. Densitometric evaluation is presented below the blots. Data are means ± SD; n = 3 independent experiments; $^{*}P < .05$, $^{\dagger}P < .01$, $^{\ddagger}P < .001$, NS, not significant

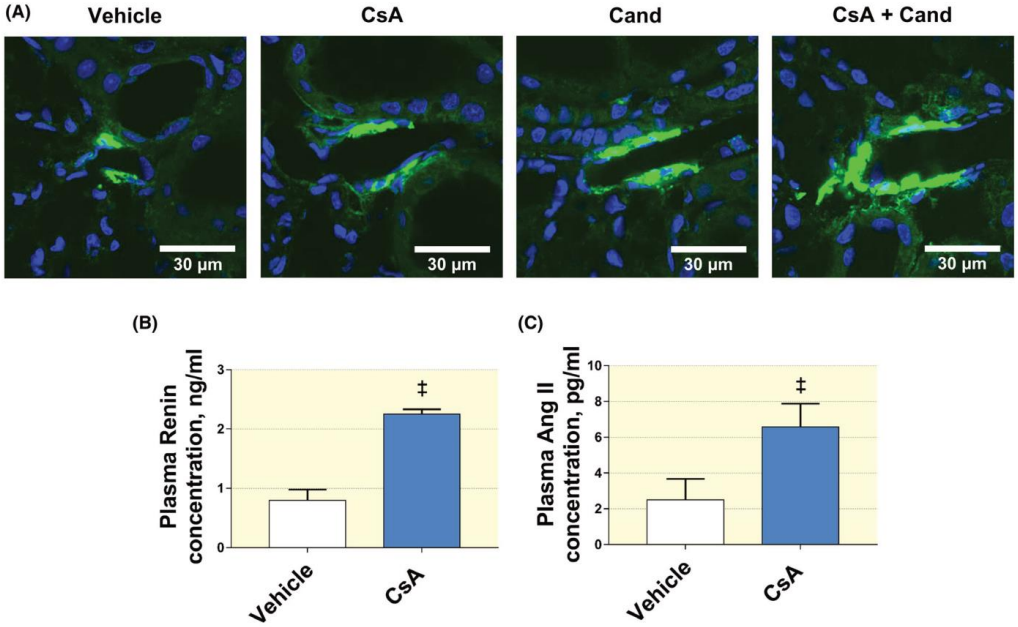

FIGURE 4 Effects of cyclosporine A (CsA), candesartan (Cand) or their combination on RAS in vivo. A, Representative confocal images of juxtaglomerular arteriolar portions in kidneys from rats treated with vehicle, CsA (25 mg/kg body weight*24 h), Cand (5 mg/kg/d) or combined CsA + Cand for 3 days show renin abundance by immunofluorescence (green signal). B and C, Graphs show quantification of the renin (B) and Ang II (C) concentration in plasma after vehicle or CsA administered for 3 days. Data are means ± SD; n = 5-6 animals in each group; $^{\ddagger}P < .001$. Scale bar = 30 μm

(1 μmol/L: −24%, $P < .05$; 5 μmol/L: −30%, $P < .05$) and completely prevented the CsA-induced increase in COX-2 biosynthesis (Figure 3A,A′ and Figure S2B). Parallel evaluation of PGE_2 levels in culture medium, used as a correlate of COX-2 activity, revealed dose-dependent increases in PGE_2 concentrations upon CsA, which were abolished by simultaneous application of Ang II (Figure S2C).

CNI are known to suppress the expression of angiotensin II receptor type 1 (AT1R).[34] In our experiments, AT1R mRNA was reduced in CsA-treated MD cells as well (−45%, $P < .001$; Figure S2D). Despite this fact, Ang II still exerted significant effects suggesting that the residual AT1R expression was sufficient to mediate Ang II signalling.

Previous work identified p38 MAPK as a COX-2 activator acting via NF-kB.[29,30] We, therefore, evaluated effects of CsA (5 and 40 μmol/L for 24 hours) on activating p38 MAPK phosphorylation at T180 and Y182 (pp38 MAPK) in cultured MD cells using a phospho-specific antibody. Immunoblotting of cell lysates revealed dose-dependent increases in pp38 MAPK levels upon CsA (+42% [$P < .05$] and +160% [$P < .001$], respectively) without concomitant changes in total p38 MAPK abundance (Figure 3B,B′, Figure S3A,B). Analysis of nuclear

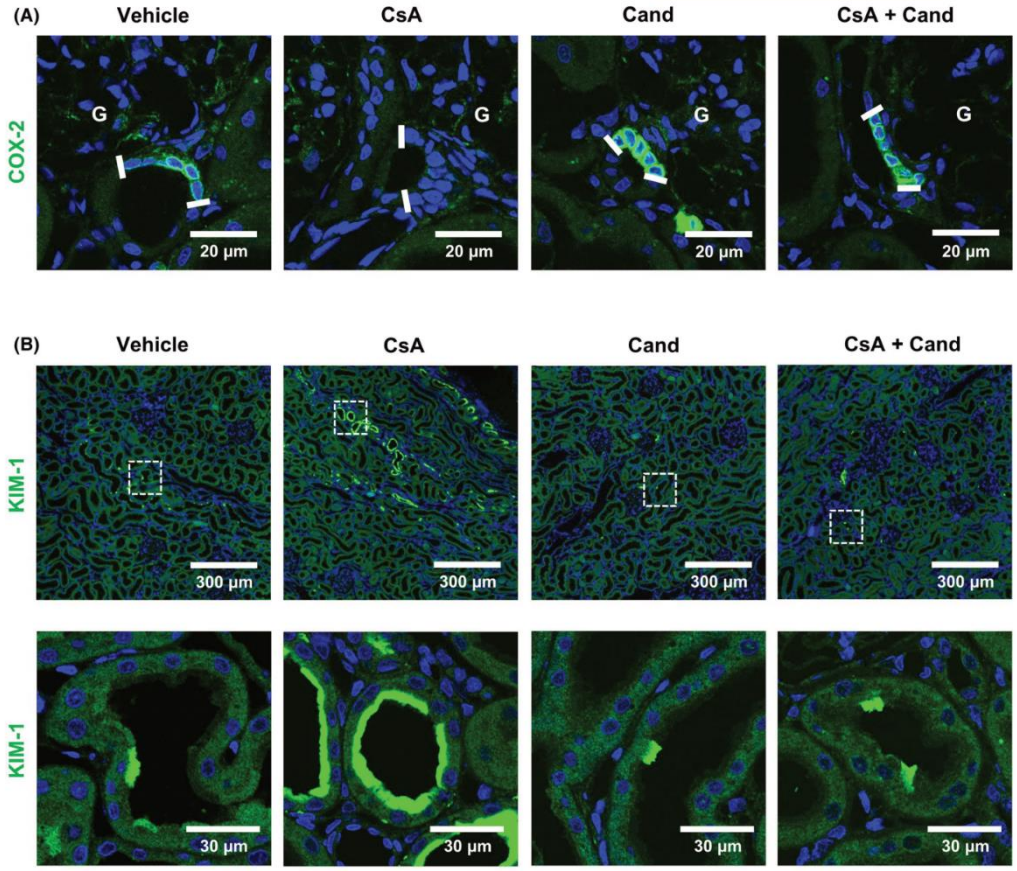

FIGURE 5 Effects of angiotensin II receptor antagonist candesartan (Cand) on cyclooxygenase 2 (COX-2) and kidney injury molecule-1 (KIM-1) expression in vehicle and cyclosporine A (CsA)-treated rats. A, Representative confocal images of macula densa regions (flanked by lines) show COX-2 (green signal) in kidneys from rats treated with vehicle, CsA (25 mg/kg body weight × 24 h), Cand (5 mg/kg/d) or CsA + Cand for 3 wk; G = glomerulus. Scale bar = 20 μm. B, Representative overview (upper panel) and high-resolution images (lower panel) of KIM-1 (green signal) in kidneys from rats treated with vehicle, CsA, Cand or CsA + Cand for 3 wk. Inserts in the overview panel correspond to high-resolution images below. Scale bar = 300 μm or 30 μm, as indicated. n = 5 animals in each group

vs cytoplasmic cell fractions showed that CsA (40 μmol/L for 24 hours) enhanced the pp38 MAPK signal chiefly in the nuclear fractions (+264% [$P < .001$] in nuclear and +70% [$P < .001$] in cytoplasmic fractions by immunoblot); these results were structurally confirmed by immunofluorescence (Figure S3C-E). In line with the observed CsA-induced p38 MAPK activation, parallel evaluation of NF-kB revealed increased levels of its activating phosphorylation at S536 in lysates from CsA-treated cells (+56%, $P < .001$; Figure 3C,C'). Ang II moderately decreased baseline phosphorylation levels of both p38 MAPK and NF-kB (−26% and −20%, respectively; $P < .05$) and abolished the CsA-induced increases in their phosphorylation levels as evaluated by immunoblotting (Figure 3B,B′,C,C′). Application of a p38 MAPK inhibitor SB203580 (10 μmol/L) or a NF-kB inhibitor Bay 11-7082 (3 μmol/L) concomitantly with CsA (40 μmol/L for 24 hours) prevented stimulation of COX-2 in both cases (Figure 3D,D′,E,E′). Moreover, inhibition of p38 MAPK abolished the CsA-induced increase in activating NF-kB phosphorylation levels suggesting that NF-kB acts downstream of p38 MAPK (−56% for CsA+SB203580 vs CsA treatment, $P < .001$, Figure 3F,F'). These results show that in cultured MD cells CsA stimulates, whereas Ang II suppresses COX-2 biosynthesis via the p38 MAPK–NF-kB signalling.

2.4 | RAS mediates CsA-induced COX-2 suppression in vivo

To test the hypothesis that stimulated RAS mediates the CsA-induced COX-2 suppression in vivo, we performed a rescue experiment using a AT1R antagonist, candesartan, in CsA-treated rats. Acute (3 days) vs chronic (3 weeks) treatment protocols were compared in order to evaluate the dynamics of CsA effects upon concomitant administration of candesartan. CsA significantly increased juxtaglomerular renin abundance both in acute and chronic settings as detected immunofluorescence (Figure 4A; Figure S4A,B). CsA further enhanced plasma renin and Ang II levels significantly, as determined in the 3 days treatment groups (+182% for renin, $P < .001$ and +163% for Ang II, $P < .001$; Figure 4B,C). Candesartan also increased juxtaglomerular renin abundance both alone and in combination with CsA which may reflect a compensatory feedback to AT1R blockade (Figure 4A). Parallel analysis of juxtaglomerular COX-2 showed significant CsA-induced decreases after acute and chronic treatments (Figure 5A). Baseline COX-2 abundance was unaltered after acute and moderately increased after chronic candesartan administration (Figure 5A). Candesartan completely normalized COX-2 expression after 3 weeks of combined administration, supporting the hypothesis that CsA-induced RAS activation is responsible for suppression of juxtaglomerular COX-2 (Figure 5A).

2.5 | RAS inhibition prevents the CsA-induced increase in KIM-1 expression

Kidney injury molecule-1 (KIM-1) has been recognized as an early biomarker of renal tubular injury responding to various types of kidney diseases including allograft nephropathy in patients receiving CNI.[35,36] Therefore, we evaluated KIM-1 expression in our rat model using immunofluorescence. Compared to vehicle-treated rats, kidneys from CsA-treated rats showed a markedly increased proportion of KIM-1-positive proximal tubules. Concomitant candesartan administration prevented the CsA-induced increase in KIM-1 expression (Figure 5B). These results suggest that CsA-induced kidney injury is partially mediated by RAS activity.

2.6 | Rescue of CsA-induced COX-2 downregulation with AT1R blocker improves renal function

Functionally, both acute and chronic CsA protocols led to similar decreases in creatinine clearance (−34% and −34%, respectively, $P < .01$) and fractional sodium excretion (FE_{Na}: −52% in the acute and −37% in the chronic setting, $P < .01$; Figure 6A-D). Candesartan administration did not affect creatinine clearance in vehicle-treated animals, but blunted the CsA-induced reduction after 3 days (from −34% to −17%, $P < .05$) and completely restored this parameter after 3 weeks of combined treatment (Figure 6A,B). Candesartan did not affect FE_{Na} in vehicle-treated animals but blunted the CsA-induced FE_{Na} decrease acutely (FE_{Na}: −52% vs −30% for CsA vs combined treatment, $P < .05$) and completely prevented the FE_{Na} reduction in the chronic setting (Figure 6C,D). To illustrate the role of COX-2 in the candesartan-induced rescue context, we additionally applied the selective COX-2 inhibitor celecoxib for 3 days. Celecoxib did not significantly affect creatinine clearance but reduced FE_{Na} when administered alone (−20%, $P < .05$; Figure 6E,F). When combined with CsA and candesartan, celecoxib abolished the candesartan-induced improvements of creatinine clearance and FE_{Na} suggesting that these beneficial effects of candesartan depend on COX-2 activity (Figure 6E,F). Since CNI may provoke hyperkalaemia, we also evaluated urinary potassium levels. Acute CsA treatment led to a significant reduction in potassium excretion (−52%, $P < .01$), which was alleviated by concomitant candesartan administration (−31%, $P < .05$ vs CsA alone; Figure 6G). Celecoxib abolished the candesartan-dependent improvement of renal potassium handling as well (Figure 6H). Together these results suggest that normalization of COX-2 activity by antagonizing AT1R may attenuate CsA-induced deteriorations of renal function.

2.7 | RAS inhibition abolishes CsA-induced activation of NKCC2 and NCC

COX-2-derived prostanoids are known to decrease the transport activities of NKCC2 and NCC, suggesting that CsA-induced COX-2 inhibition may promote their functions.[20,37] In line with this, we have reported increased levels of activating NKCC2 and NCC phosphorylation upon chronic CsA treatment.[5] To extend our previous results, we have now evaluated the effects of acute CsA treatment and found significant increases in phospho-NKCC2 (T96/T101; +350%, $P < .01$) and phospho-NCC levels (S71; +209%, $P < .001$) without concomitant increases in their total protein levels (Figure 7A,B). Concomitant administration of candesartan abolished the increases in phosphorylation of both transporters, whereas their protein abundances were moderately enhanced in this setting (+17% [n.s.] and +23% [$P < .05$], respectively; Figure 7A,B). Prevention of NKCC2 and NCC activation may thus be partially responsible for beneficial effects of candesartan on sodium balance in CsA-treated rats.

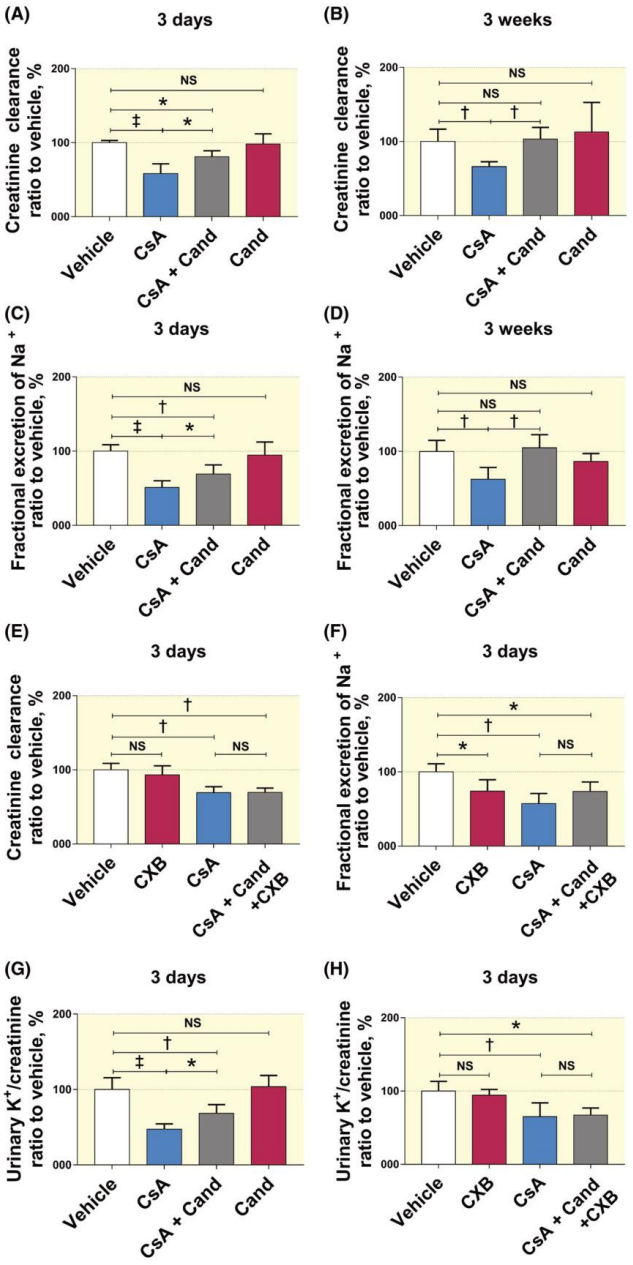

FIGURE 6 Effects of mono- or combined cyclosporine A (CsA), candesartan (Cand) and celecoxib (CXB) treatments on the renal physiology in vivo. A-D, The graphs show evaluation of creatinine clearance (A, B) or fractional sodium excretion (FE_{Na}; C, D) in rats treated with vehicle, CsA (25 mg/kg body weight*24 h), CsA + Cand (5 mg/kg body weight × 24 h) or Cand for 3 d (A, C) or 3 wk (B, D). E, F, The graphs show evaluation of creatinine clearance (E) or FE_{Na} (F) in rats treated with vehicle, CXB (50 mg/kg body weight × 24 h), CsA, or CsA + Cand + CXB. G, H, The graphs show evaluation of urinary K^+/creatinine in rats with mono- or combined CsA, Cand and CXB treatments. Data are means ± SD; n = 4-5 animals in each group; $^*P < .05$, $^\dagger P < .01$, $^\ddagger P < .001$, NS, not significant

2.8 | CsA does not affect NOS1 expression and activity

Since both COX-2 and RAS may regulate NOS1 expression in MD cells, thereby affecting GFR,[38,39] we have additionally evaluated NOS1 abundance and activity in MD cells of rats either treated with vehicle, CsA, candesartan, or the combination of CsA and candesartan for 3 weeks. Detection of NOS1 using immunofluorescence or immunoblotting revealed no significant differences between the treatment

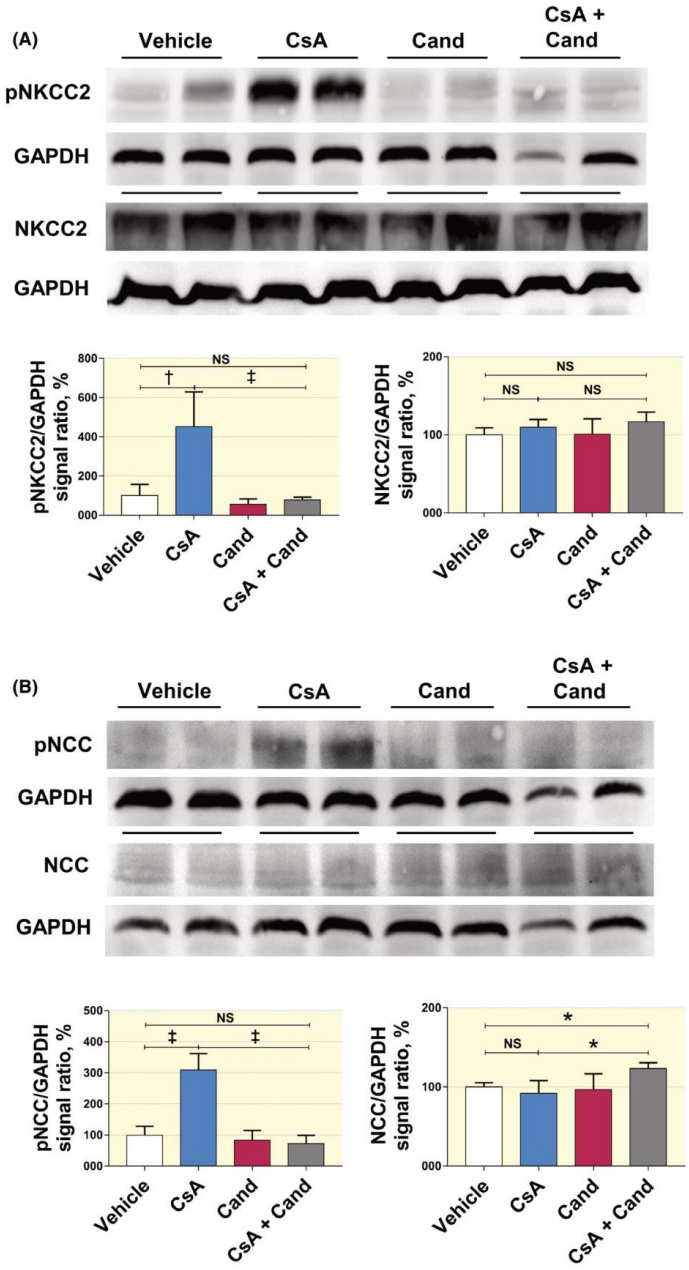

FIGURE 7 Effects of cyclosporine A (CsA), candesartan (Cand) or their combination on Na^+-K^+-$2Cl^-$ cotransporter (NKCC2) and Na^+-Cl^- cotransporter (NCC). A, B, Representative immunoblots of kidney lysates from rats treated with vehicle, CsA (25 mg/kg/d), Cand (5 mg/kg/d), or CsA + Cand for 3 d show detection of phosphorylated (p) and total protein levels of NKCC2 (A) or NCC (B; all approximately at 160 kDa). GAPDH serves as loading control (approximately at 37 kDa). Densitometric evaluation of signals is shown below the corresponding immunoblots. Data are means ± SD; n = 4 animals per group; * $P < .05$, † $P < .01$, ‡ $P < .001$, NS, not significant

groups. Furthermore, NADPH-diaphorase reaction, an indicator of NOS1 activity, produced similar results at the MD in all groups (Figure S5A-C). Hence, no evidence for modulation of NOS1 activity in MD cells by CsA alone or combined with candesartan was obtained.

3 | DISCUSSION

RAS stimulation and renal cortical COX-2 inhibition are major CNI-related pathophysiological effects, contributing to reduced GFR, salt retention, hypertension, and hyperkalaemia.[3,4,31] Although CNI-induced suppression of renal cortical COX-2 has been well documented in vivo,[4,5] little information has been available on direct effects of CNI in MD cells in the absence of systemic stimuli. The present experiments demonstrate that, unlike the in vivo situation, pharmacological or siRNA-mediated suppression of calcineurin activity in cultured MD cells increases COX-2 expression via activation of the p38 MAPK-NF-kB pathway. Calcineurin has earlier been implicated in dephosphorylation and inactivation of p38 MAPK.[28] CsA-induced nuclear accumulation of pp38 MAPK observed in the present study suggests its role in COX-2 stimulation.[17,21,29,40] Pathways acting downstream of p38 MAPK to promote COX-2 expression were shown to involve NFAT and NF-kB.[27,41] Previous and the present localization studies, however, failed to detect NFAT isoforms in MD, whereas NF-kB activity has been formerly implicated in the regulation of cortical COX-2.[42] In line with this, the present experiments in cell culture revealed CsA-induced NF-kB activation along with increased COX-2 expression, which was prevented by pharmacological inhibition of either p38 MAPK or NF-kB. Apart from MAPK signalling, previous work implicated c-jun N-terminal kinases, NF-kB-inducing kinase, and CCAAT enhancer-binding protein in CNI-induced modulation of NF-kB activity, but the reported effects were controversial among the respective studies.[28,30,43-45]

The opposing effects of CsA on COX-2 expression in vivo vs ex vivo may be related with systemic effects of calcineurin inhibition overriding cell-autonomous COX-2 regulation. Among the systemic pathophysiological effects of CsA, RAS activation has been recognized as a major factor contributing to renal salt retention, vasoconstriction, and hypertension.[2] Since RAS interferes with juxtaglomerular COX-2 expression,[46] we investigated whether CNI-induced RAS activation may underlie the suppression of juxtaglomerular COX-2 in vivo. The stimulating effects of CsA on the p38 MAPK-NF-kB-COX-2 cascade in cultured MD cells were completely abolished by concomitant Ang II treatment. Hence, Ang II likely acted downstream of calcineurin, thus overriding effects of CNI. CNI-induced RAS activation has been well documented in previous and the present studies.[4,5,31] COX-2 expression typically harmonizes with renin biosynthesis and release.[19,46,47] However, CNI-treated rats exhibited a clear dissociation between COX-2 and renin expression suggesting that stimulation of renin in this model was independent of COX-2.[4,5,31] Effects of CsA on renin may be mediated by direct inhibition of calcineurin in renin-producing granular cells or, indirectly, through sympathetic activation.[31,48-50] In this context, decreased COX-2 expression might reflect a negative feedback effect of high Ang II plasma levels, overriding the cell-autonomous, stimulating effects of calcineurin inhibition (Figure 8A).[46]

COX-2 expression is inversely correlated with NKCC2 activity and may consequently be stimulated by furosemide.[51,52] CsA normally increases NKCC2 function, but this effect is probably irrelevant for COX-2 regulation, since concomitant administration of furosemide failed to restore COX-2 expression.[5,51-54] Local mechanisms of COX-2 regulation also include paracrine effects of nitric oxide released by NOS1,[17] which, however, was unaltered upon CsA in our hands. This result corroborates our earlier observation that NOS1 activity is not critical to the juxtaglomerular COX-2 expression.[55] Therefore, activated RAS appears to play the dominant role in COX-2 downregulation. Accordingly, CsA-induced COX-2 suppression was prevented by concomitant candesartan administration in the present study. Candesartan affects major systemic parameters such as blood pressure, vascular tone and electrolyte balance, which may indirectly modulate the juxtaglomerular COX-2 expression.[56,57] However, our data from cell culture have shown that AT1R signalling inhibits COX-2 expression independently of other systemic effects. We, therefore, believe that candesartan-induced normalization of juxtaglomerular COX-2 expression in CsA-treated rats is, at least in part, mediated by inhibition of AT1R signalling in macula densa and adjacent TAL cells. Parallel normalization of creatinine clearance and FE_{Na} observed in the present study required COX-2 activity, since these candesartan-induced functional improvements were reversed by additional celecoxib administration (Figure 8B).[58,59] Improvement of the sodium balance upon candesartan observed in the present study may result from increased GFR and attenuated activity of distal salt transport proteins. NKCC2 and NCC have been identified as key mediators of the CNI-induced salt retention.[5,53,60,61] Candesartan prevented their activation by CsA, which may be mediated by local AT1R inhibition in TAL and DCT, increased COX-2 activity or changes in plasma aldosterone levels.[54,56] The present results suggest that the CNI-induced hyperactivity of NKCC2 and NCC may be a consequence of stimulated RAS as well.[31]

The impact of COX-2 in renal sodium handling has been demonstrated using COX-2-deficient mice, which developed marked hypertension in response to high salt diet.[62] A previous study showed that diuretic effects of furosemide and hydrochlorothiazide are partially mediated by COX-2 activation.[20]

Therefore, intact COX-2 function is critical to the renal sodium handling and its normalization by candesartan observed in the present study may have significantly contributed the improvement of the sodium balance in our rat model.

Functional impairment of renal allograft in patients typically correlates with morphological damage. CNI may cause or aggravate allograft nephropathy.[3] Although a relatively short CsA-treatment protocol applied in the present study

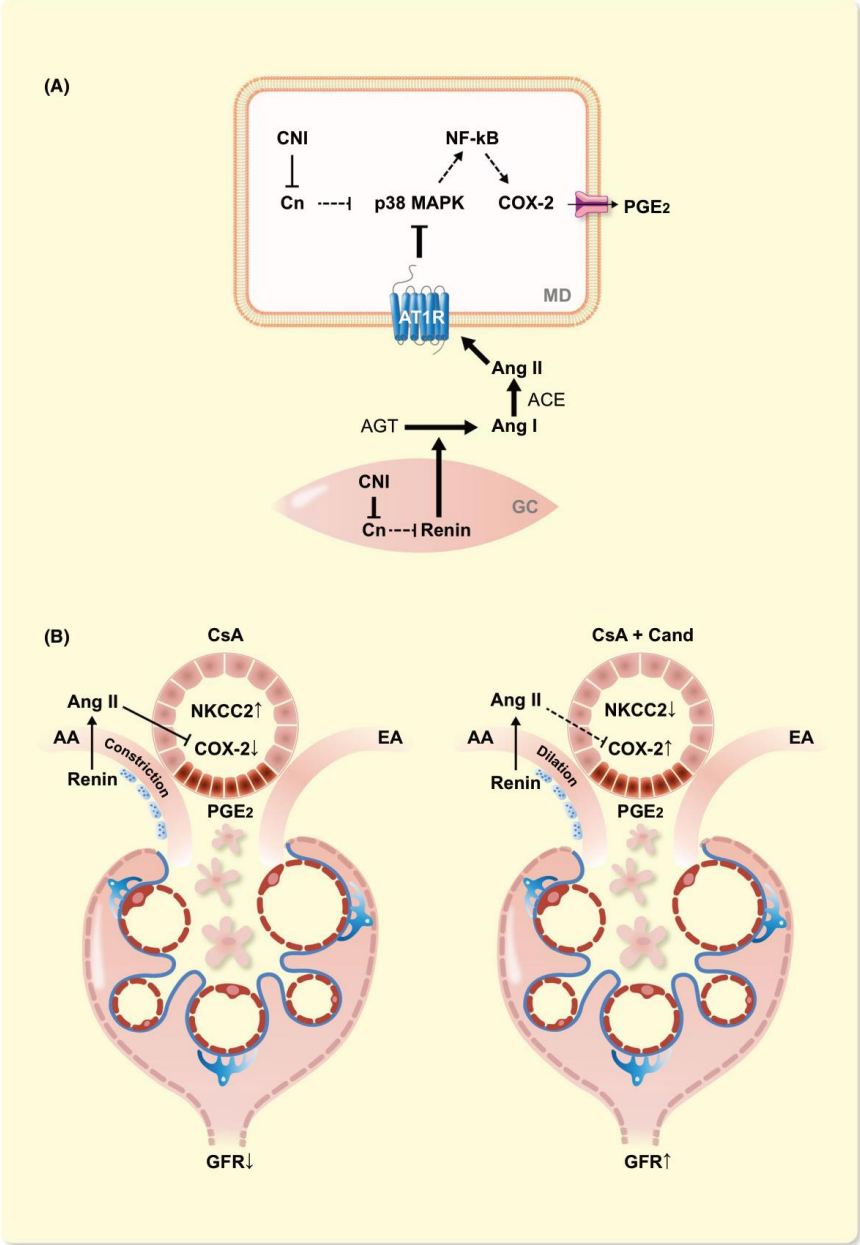

FIGURE 8 Schematic summary of cell-autonomous and systemic effects of calcineurin inhibitors (CNI) affecting expression of the juxtaglomerular cyclooxygenase 2 (COX-2). A, Calcineurin (Cn) inhibits p38 MAPK and NF-kB pathway to suppress COX-2 expression in macula densa (MD) cells. In contrast, CNI increases the COX-2 expression via activation of the p38 MAPK-NF-kB pathway. In renin-producing granular cells (GC), CNI promote renin expression leading to activation of the renin-angiotensin system. Angiotensin II (Ang II) elicits a negative feedback in MD cells overriding the cell-autonomous COX-2 regulation and leading to reduced COX-2 expression and decreased prostaglandin E2 (PGE2) levels. B, Functional consequences of CsA-induced COX-2 suppression include decreased glomerular filtration rate (GFR) because of constriction of the afferent arteriole, as well as activation of NKCC2. These effects can be abolished by concomitant Ang II antagonism using candesartan. Arrows show stimulation, whereas T-shaped bars indicate inhibitory effects. Dashed lines show blunted effects

was not expected to produce major structural kidney damage, we were able to detect increased KIM-1 expression as an evidence for early CsA-induced pathomorphological changes.[35,36] Notably, concomitant candesartan treatment prevented the CsA-induced stimulation of KIM-1 suggesting that patients receiving CNI may benefit from AT1R antagonism.

The potential of RAS inhibitors to alleviate nephrotoxic effects of CNI has been addressed in previous studies in animal models and patients, demonstrating beneficial effects on renal haemodynamics, kidney morphology and blood pressure.[63-67] The underlying mechanisms were attributed to reduced vasoconstriction and suppression of transforming growth factor β signaling.[63-67] Our present results suggest that maintenance of COX-2 expression is another positive effect of RAS inhibition to improve renal haemodynamics and prevent NaCl retention. The choice of RAS inhibitors may play a role, since inhibition of angiotensin-converting enzyme using ramipril failed to abolish effects of CsA on COX-2 expression,[4] whereas in our hands, AT1R inhibition by candesartan was efficient herein. Mechanisms underlying this discrepancy deserve further characterization.

Both CNI and RAS inhibitors bear the risk of hyperkalaemia, which may complicate their combined use in patients.[3,68] Nevertheless, an early clinical study suggested that renal transplant recipients receiving CNI may profit from AT1R inhibitors without increased risk of hyperkalaemia.[69] Indeed, in our hands candesartan alleviated rather than aggravated the CsA-induced potassium retention as judged from urinary K^+ excretion. This may be related with inhibition of NKCC2 and NCC, causing increased Na^+ delivery to the CNT/CD and the consequent, Na^+-coupled K^+ secretion in these segments. Notably, celecoxib abolished the candesartan-induced increases in K^+ excretion in CsA-treated rats, which suggested a role for COX-2 activity in renal potassium handling as well.

Tacrolimus is currently used at least as broadly as CsA in patients undergoing organ transplantation. Previous work demonstrated that both CsA and tacrolimus suppress the juxtaglomerular COX-2 expression to a comparable extent.[4] Therefore, patients receiving tacrolimus may profit from AT1R inhibitors as well. Nevertheless, further studies in animal models will be helpful to compare between juxtaglomerular effects of CsA vs tacrolimus and evaluate the rescue potential of candesartan.

In summary, calcineurin acts as an endogenous COX-2 suppressor rather than activator in MD cells. However, the endocrine regulation of COX-2 by Ang II has priority over cell-autonomous, calcineurin-dependent mechanisms. Therefore, the net effect of CNI on COX-2 expression is the inhibitory as a result of CNI-dependent RAS activation. AT1R inhibition rescues COX-2 expression, thus improving renal haemodynamics assessed by creatinine clearance. Moreover, AT1R antagonism exerts beneficial effects on the sodium homeostasis, which further supports the use of this strategy for alleviation of CNI nephrotoxicity. MAPK-NF-kB signalling appears to mediate both local and RAS-dependent effects of CNI on COX-2, bearing the potential for its pharmacological targeting in addressing CNI nephrotoxicity. Improved understanding of COX-2 regulation in MD cells may have clinical implications for several therapeutic regimens including CNI, NSAIDs, or RAS inhibitors.

4 | METHODS

4.1 | Animals

Animal experiments were approved by the German Animal Welfare Regulation Authorities on the protection of animals used for scientific purpose (G0148/18). Adult (10 to12 weeks) male Wistar rats were divided into groups receiving vehicle, cyclosporine A (CsA; 25 mg/kg × 24 hours, Sandimmun, Novartis, Nürnberg, Germany), candesartan-cilexetil (5 mg/kg × 24 hours, HEXAL, Holzkirchen, Germany), celecoxib (50mg/kg × 24 hours, Micro Labs GmbH, Germany) or their combination treatments for 3 days (i.p.) or 3 weeks (subcutaneous osmotic mini-pumps, Alzet, 2ML4, Cupertino, California) (Table 1). For mini-pump implantation, rats were anesthetized with isoflurane inhalation. An incision of the neck skin was performed and the subcutaneous tissue dilated to create a pocket for the pump. The filled pump was then inserted into the pocket and the wound closed with clips. On the second last day of each experiment, rats were placed in metabolic cages for 24 hours with water and chow ad libitum to collect urine. At the end of the experiments, rats were anesthetized using Nembutal (Sigma-Aldrich) to obtain blood samples from the inferior vena cava. Urine and blood samples were analysed by a commercial laboratory (IMD Labor Berlin, Germany). One of the two kidneys was clamped and

removed for biochemical analysis. The remaining kidney was fixed by retrograde in vivo perfusion with 3% paraformaldehyde (PFA) in PBS via the abdominal aorta and prepared for morphological analysis.

4.2 | Blood and urine analysis

Rats were anesthetized using Nembutal solution. Blood was taken from the vena cava using heparinized syringes, decanted into Eppendorf tubes, left for 30 minutes at room temperature for clotting and centrifuged at 2000× *g* for 10 minutes at 4°C to obtain serum as the supernatant. Creatinine measurement was conducted using the enzymatic method (IMD Labor Berlin). Creatinine clearance (CCL) was calculated with the following formula: CCL (mL/min) = [C_U (mg/dL) × urine flow (mL/min)]/C_S (mg/dL), where C_U is the concentration of creatinine in the urine and C_S is the creatinine concentration in the serum. Fractional excretion of sodium (FE_{Na}) was calculated using the following formula: FE_{Na} (%) = 100 × [Na_U (mmol/L) × urine flow (ml/min)]/[CCL (mL/min) × Na_S (mmol/L)], where Na_U is the sodium concentration in the urine and Na_S is the sodium concentration in the serum. For plasma renin and angiotensin II concentration measurements, serum samples were collected from rats treated with vehicle or CsA for 3 days and tested by rat renin ELISA Kit (RAB1162, Sigma-Aldrich) or angiotensin II EIA Kit (RAB0010, Sigma-Aldrich).

TABLE 1 Animal cohorts and treatment groups

	Groups and Administration	Entered	Terminated
Cohort 1 3 wk	Vehicle (sham operation)	5	5
	CsA (sandimmun by mini-pumps)	5	5
	Cand (drinking water)	5	5
	CsA + Cand (as indicated)	5	5
Cohort 2 3 d	Vehicle (cremophor by IP)	5	5
	CsA (sandimmun by IP)	5	5
	Cand (gavage)	5	5
	CsA + Cand (as indicated)	5	5
Cohort 3 3 d	Vehicle (cremophor by IP)	4	4
	CsA (sandimmun by IP)	4	4
	CXB (gavage)	4	4
	CsA + Cand + CXB (as indicated)	4	4
Cohort 4 3 d	Vehicle (cremophor by IP)	6	6
	CsA (sandimmun by IP)	6	6

Abbreviations: Cand, candesartan; CsA, cyclosporine A; CXB, celecoxib; IP, intraperitoneal injection.

4.3 | Cell culture

Immortalized mouse MD cells (J. Schnermann, Bethesda, MD)[21,70] were cultivated in DMEM medium (PAN-Biotech) supplemented with 5% foetal calf serum and 1% penicillin/streptavidin at 37°C, 95% humidity and 5% CO_2. Cells were grown to 70% confluence and treated with CsA (1-40 µmol/L for 6 hours or 24 hours, respectively; Sigma-Aldrich), Ang II (1 and 5 µmol/L for 24 hours; Abcam), SB203580 (10 µmol/L for 24 hours; Cell Signaling Technology) or Bay 11-7082 (3 µmol/L for 24 hours; Cell Signaling Technology) alone or in different combinations. For a knockdown of calcineurin isoforms, specific siRNAs targeting CnAα or CnAβ (Table 2) were transfected into MD cells at 30% cell

TABLE 2 cDNA and siRNA primers applied

cDNA primers		
Product	**Forward primer**	**Reverse primer**
COX-2	5′-AGC CAG GCA GCA AAT CCT T-3′	5′-CAG TCC GGG TAC AGT CAC AC-3′
AT1R	5′-CTC TTT CCT ACC GCC CCT CAG-3′	5′-GGA TCA TGT CAC TAG CAG GC-3′
GAPDH	5′-TGC ACC ACC AAC TGC TTA GC-3′	5′-GGC ATG GAC TGT GGT CAT GAG-3′
siRNA		
Target	**Sense**	**Antisense**
CnAα	5′-CAU UGA GAA UAA UAA CAG ATT-3′	5′-UCU GUU AUU AUU CUC AAU GCA-3′
CnAβ	5′-GGA UGA UAU UAG GAG AUU ATT-3′	5′-UAA UCU CCU AAU AUC ACU CAG-3′

Abbreviations: AT1R, angiotensin II type 1 receptor; CnA, calcineurin A; COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

confluence for 72 hours using INTERFERin (Polyplus transfection). Finally, cells were harvested for biochemical analysis or fixed with 4% PFA in PBS for immunofluorescence; culture medium was collected for measurements of extracellular PGE_2 release (Lipidomix, Berlin, Germany). Mouse distal convoluted tubule (DCT) cells, human renal medullary fibroblasts (TK173 cell line) and human embryonic kidney (HEK293) cells were used as control cell lines.[71,72]

4.4 | Immunofluorescence and immunohistochemistry

Primary antibodies are listed in the Table 3. Immunofluorescence and immunohistochemistry were performed as described previously.[60] In brief, sections from paraffin-embedded rat and human kidneys were dewaxed, rehydrated and boiled in citrate buffer (pH 6) for antigen retrieval. Human renal biopsy specimens were obtained from Department of Pathology of Charité-Universitätsmedizin, Berlin, which was classified as part of an ongoing pathological investigation with no requirement of informed consent and was undertaken in accordance with the Declaration of Helsinki. Cryostat rat kidney sections or fixed coverslips with cultured cells were incubated with 0.5% Triton-X100 for 30 minutes to retrieve antigens. Unspecific binding sites were blocked with 5% skim milk or 5% bovine serum albumin in PBS or TBS buffer for 30 minutes followed by incubation with primary antibodies diluted in blocking medium for 60 minutes at room temperature and then overnight at 4°C. For multiple staining, primary antibodies were applied sequentially, separated by washing steps. Signals were generated using Cy2- or Cy3-labelled fluorescent (Dianova) or HRP-conjugated (Dako) secondary antibodies and evaluated by an LSM 5 Exciter confocal microscope (Zeiss).

TABLE 3 Primary antibodies applied

Antibody	Provider	Location	Catalogue number	Dilution
COX-2	Santa Cruz Biotechnology	Dallas, Texas	sc-1747	1:300 IF 1:300 WB
NFATc1	Santa Cruz Biotechnology	Dallas, Texas	sc-7294	1:300 IF
NFATc2	Santa Cruz Biotechnology	Dallas, Texas	sc-514929	1:300 IF
NFATc3	Santa Cruz Biotechnology	Dallas, Texas	sc-8405	1:300 IF
NFATc4	Santa Cruz Biotechnology	Dallas, Texas	sc-271597	1:300 IF
β-actin	Sigma-Aldrich	Darmstadt, Germany	A2228	1:5000 WB
CnAα	Pineda Antibody Services Corporate	Berlin, Germany	NA	1:1000 WB
CnAβ	Pineda Antibody Services Corporate	Berlin, Germany	NA	1:1000 WB
p38 MAPK	Cell Signaling Technology	Frankfurt am Main, Germany	9212	1:1000 WB
phospho-p38 MAPK	Cell Signaling Technology	Frankfurt am Main, Germany	4511	1:500 IF 1:300 WB
phospho-NF-kB	Cell Signaling Technology	Frankfurt am Main, Germany	3033	1:300 WB
Histone H3	Cell Signaling Technology	Frankfurt am Main, Germany	4499	1:300 WB
GAPDH	Abcam	Cambridge, UK	ab181602	1:5000 WB
Tubulin	Santa Cruz Biotechnology	Dallas, Texas	sc-53029	1:500 IF
Renin	Acris	Herford, Germany	AP00945PU-N	1:5000 IF
KIM-1	R&D Systems	Abingdon, UK	AF3689	1:200 IF
phospho-NKCC2 T96/T101	Pineda Antibody Services Corporate	Berlin, Germany	NA	1:200 WB
NKCC2	Pineda Antibody Services Corporate	Berlin, Germany	NA	1:200 WB
phospho-NCC S71	Pineda Antibody Services Corporate	Berlin, Germany	NA	1:200 WB
NCC	Sigma-Aldrich	Darmstadt, Germany	AB3553	1:1000 WB
NOS1	Abcam	Cambridge, UK	ab76067	1:500 IF 1:300 WB

Abbreviations: CnA, calcineurin A; COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IF, immunofluorescence; KIM-1, kidney injury molecule-1; MAPK, mitogen-activated protein kinase; NA, not available; NCC, Na^+-Cl^- cotransporter; NFAT, nuclear factor of activated T cells; NF-kB, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells; NKCC2, Na^+-K^+-$2Cl^-$ cotransporter; NOS1, neuronal nitric oxide synthase isoform; WB, Western blotting.

4.5 | Immunoblotting

For immunoblotting, whole kidney tissue or MD cells were homogenized in RIPA buffer containing protease inhibitor (Cell Signaling Technology) and phosphatase inhibitor (Roche Diagnostics). After sonication for 10 seconds, supernatants were harvested by centrifugation at 14 000× *g* for 15 minutes at 4°C. Alternatively, cell fractionation was performed as described elsewhere.[73] Then, protein concentration of the supernatant was measured by bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Samples were separated in 10% polyacrylamide minigels, and then electrophoretically transferred to nitrocellulose membranes. The membranes were subsequently subjected to blocking with 5% bovine serum albumin in PBST (RT, 30 minutes), followed by incubation with primary antibodies overnight at 4°C and HRP-conjugated secondary antibody for 1 hours at RT. Signals were generated by chemiluminescence using ECL and ChemoCam Imager ECL (Intas). Each experiment was repeated at least 3 times. GAPDH or β-actin detection served for the loading control and data normalization.

4.6 | Quantitative real-time PCR

Total RNA was extracted from MD cells using TRIzol Reagent and cDNA generated by reverse transcription (Promega). Specific forward and reverse primers were designed (Table 2). Amplification was performed using the 7500 Fast Real-Time PCR system (Applied Biosystems) and the HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne). GAPDH served as the housekeeping gene. Data were processed according to the ΔΔCT method and mean values of $\log_2^{\text{relative quantification}}$ were compared between the treatment groups.

4.7 | In situ hybridization

Renin mRNA expression was evaluated by in situ hybridization. Generation of the riboprobe and in situ hybridization procedure were performed as described previously and signals were detected using a Leica DMRB microscope.[74]

4.8 | Statistical analysis

Results were done in an observer blinded way and were analysed using routine parametric statistics for normal distribution, as assumed from the experimental design. Comparative analysis between two groups was performed by unpaired *t* test. Comparative evaluation of multiple groups was performed using ANOVA with post hoc test. GraphPad Prism7 (San Diego, USA) was used to analyse parameters. A probability level of $P < .05$ was accepted as significant.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

JH, YX, SB and KM conceived and designed research; JH and YX performed experiments; JH and YX analysed data; JH, YX and KM interpreted results of experiments; JH, YX, SB and KM designed figures; JH and KM drafted manuscript; JH, YX, SB and KM approved final version of manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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12. Complete list of publications

1. **Hu J**, Xu Y, Bachmann S, Mutig K. Angiotensin II receptor blockade alleviates calcineurin inhibitor nephrotoxicity by restoring cyclooxygenase 2 expression in kidney cortex. Acta Physiol (Oxf). 2020 Dec 29:e13612. doi: 10.1111/apha.13612. Epub ahead of print. PMID: 33377278. *Impact Factors 2019: 5.542*

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