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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

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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

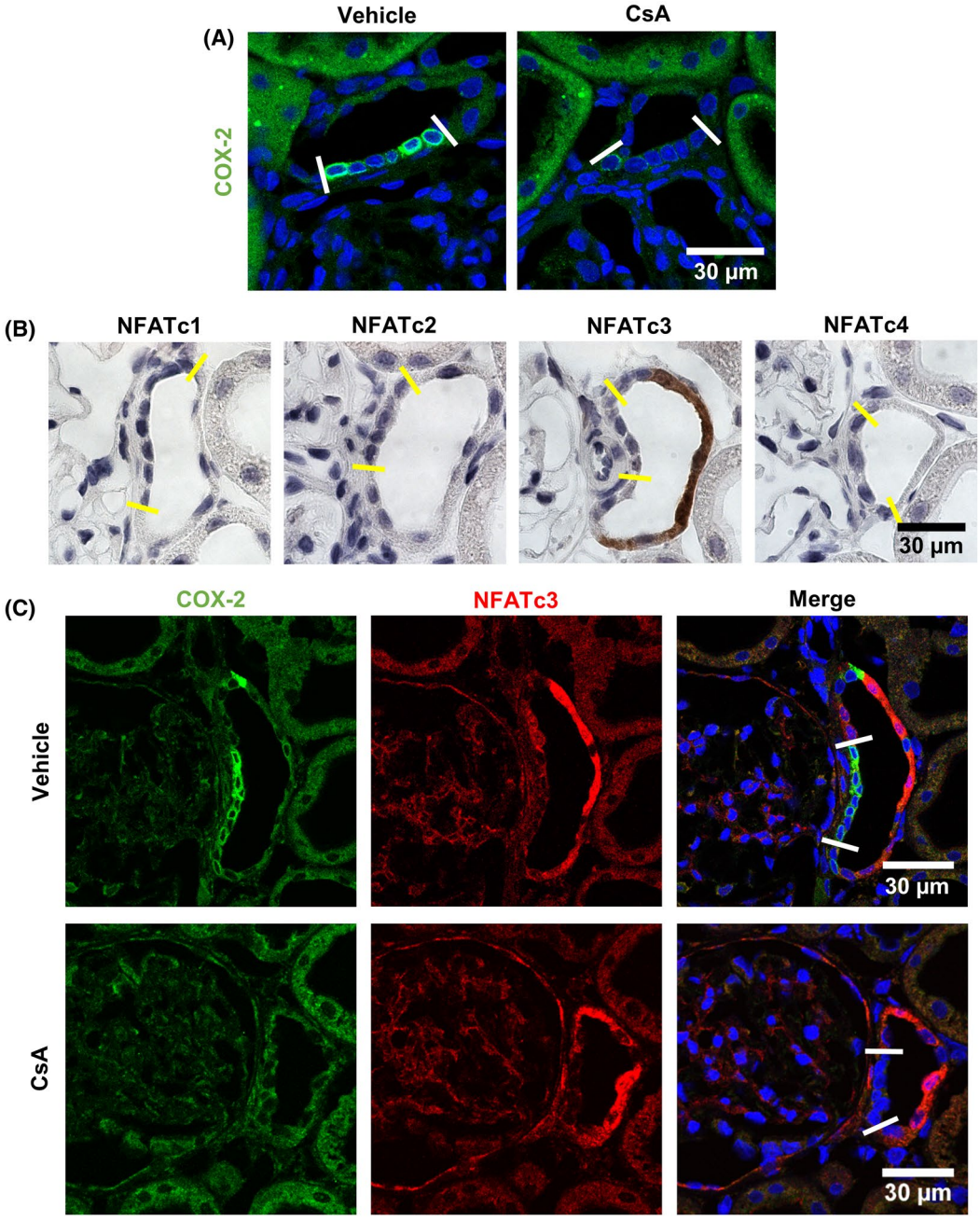


FIGURE 1 Distribution of nuclear factor of activated T cells (NFAT) transcription factor isoforms in rat kidney cortex. A, Representative confocal images of kidneys from vehicle- (n = 5) or cyclosporine A (CsA)-treated rats (25 mg/kg body weight*24 h for 3 wk; n = 5) document CsA-induced reduction in 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.

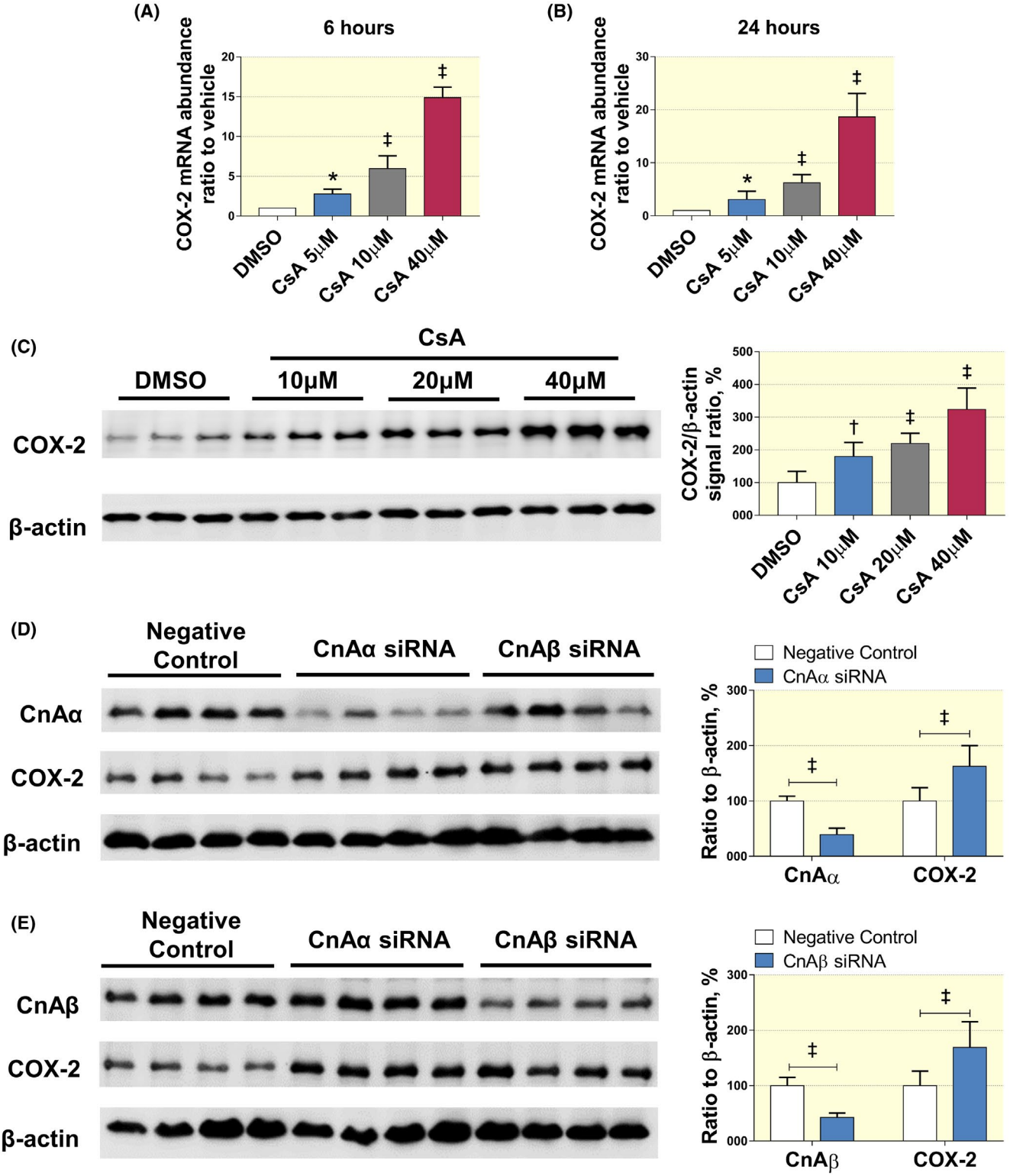
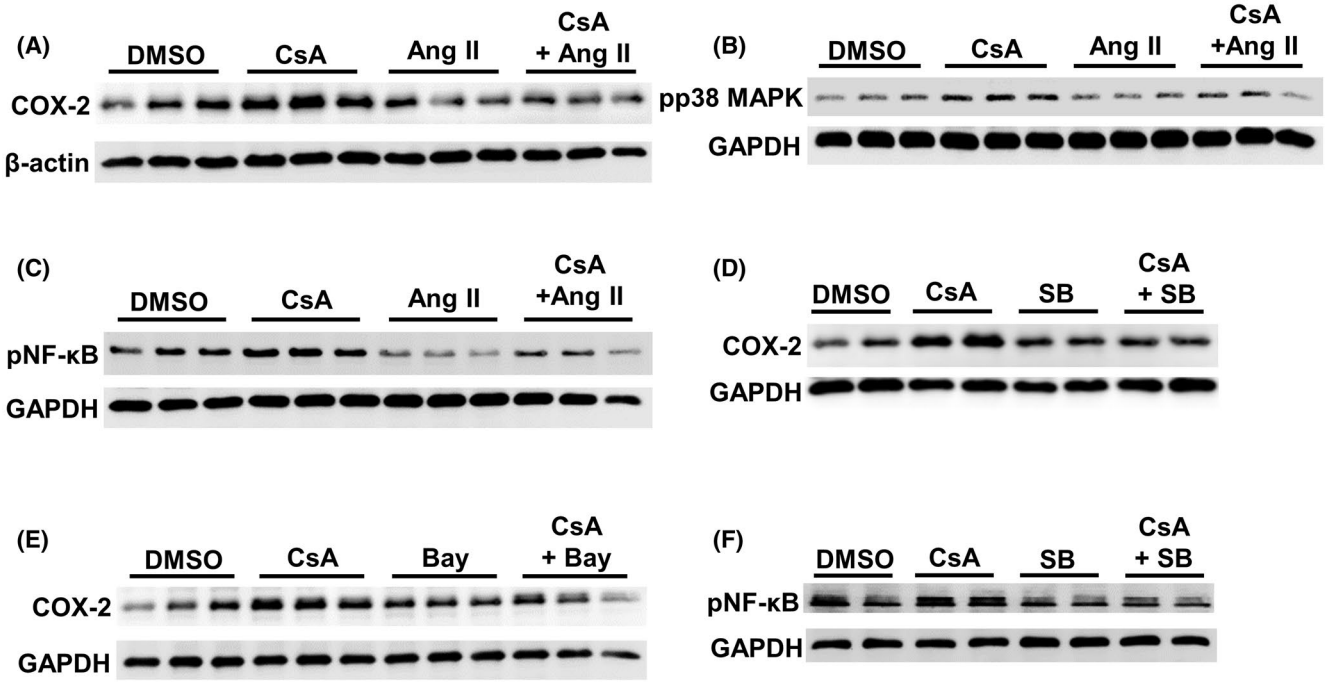


FIGURE 2 Effects of calcineurin inhibition on cyclooxygenase 2 (COX-2) expression and function in cell culture. A and B, Quantitative PCR shows dose-dependent increases in COX-2 mRNA levels in cultured mouse macula densa (MD) cells treated with CsA for 6 h (A) or 24 h (B) as compared to vehicle (DMSO); 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



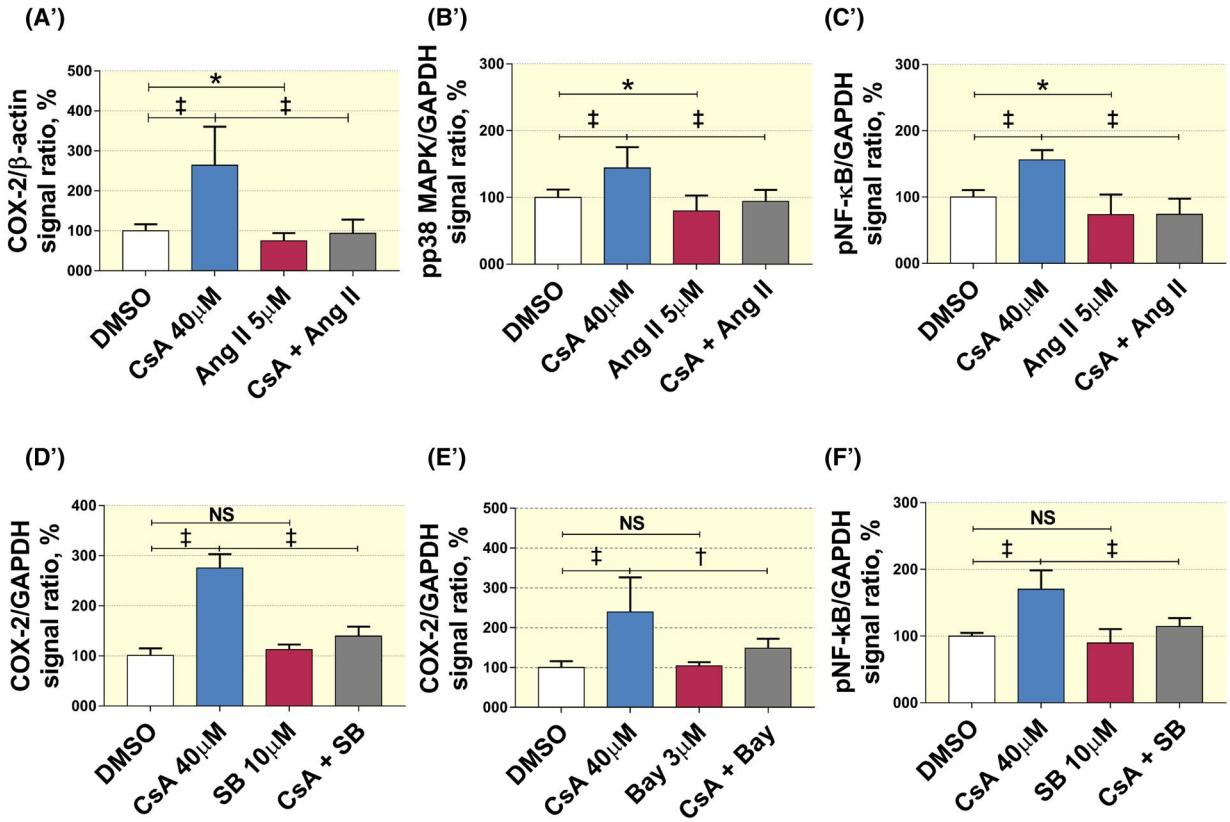


FIGURE 3 Role of p38 mitogen-activated protein kinase (p38 MAPK) and nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-kB) in mediating effects of cyclosporine A (CsA) and angiotensin II (Ang II) on cyclooxygenase 2 (COX-2) in cell culture. A-C, Representative immunoblots show detection 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; *$P < .05$, †$P < .01$, ‡$P < .001$, NS, not significant

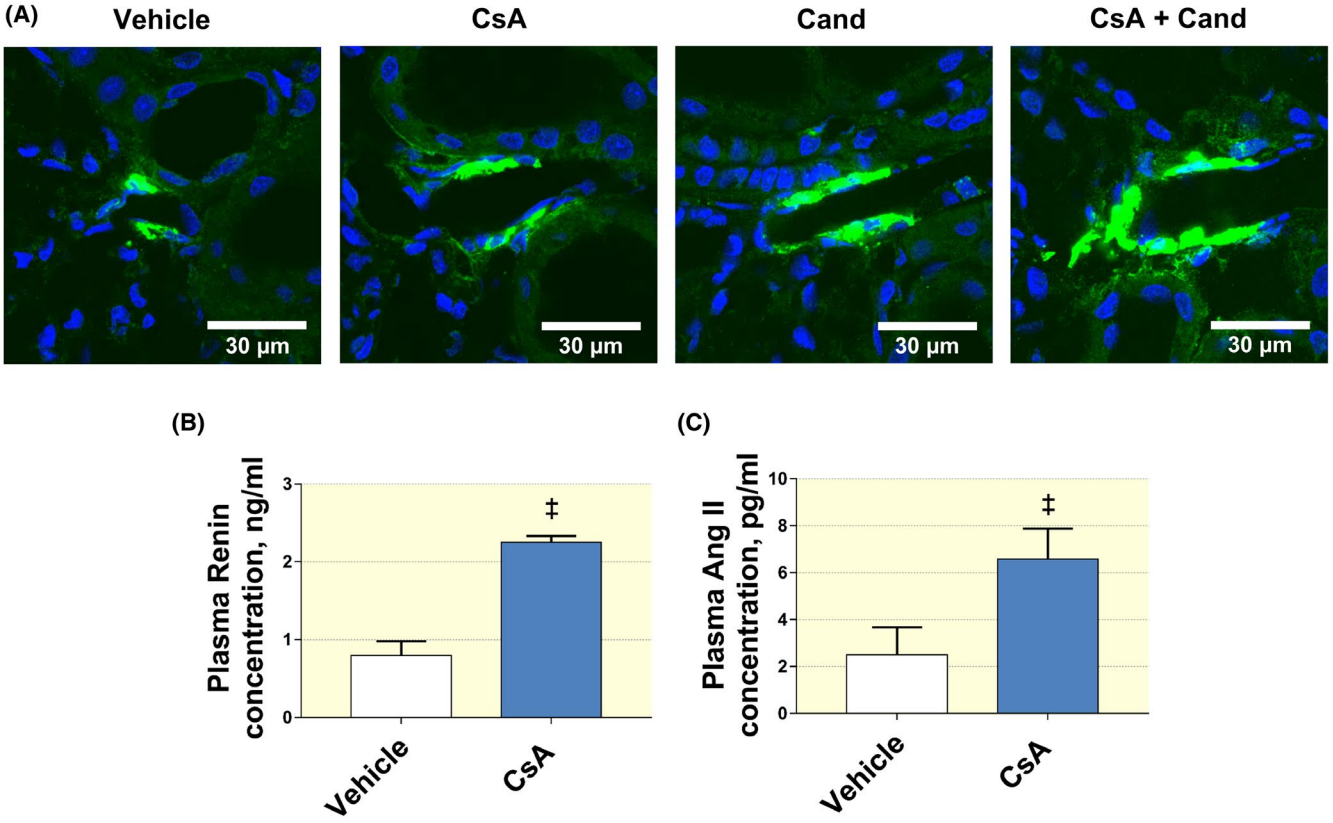


FIGURE 4 Effects of cyclosporine A (CsA), candesartan (Cand) or their combination on RAS in vivo. A, Representative confocal images of juxtaglomerular arteriolar portions in kidneys from rats treated with vehicle, CsA (25 mg/kg body weight*24 h), Cand (5 mg/kg/d) or combined CsA + Cand for 3 days show 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; ‡$P < .001$. 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

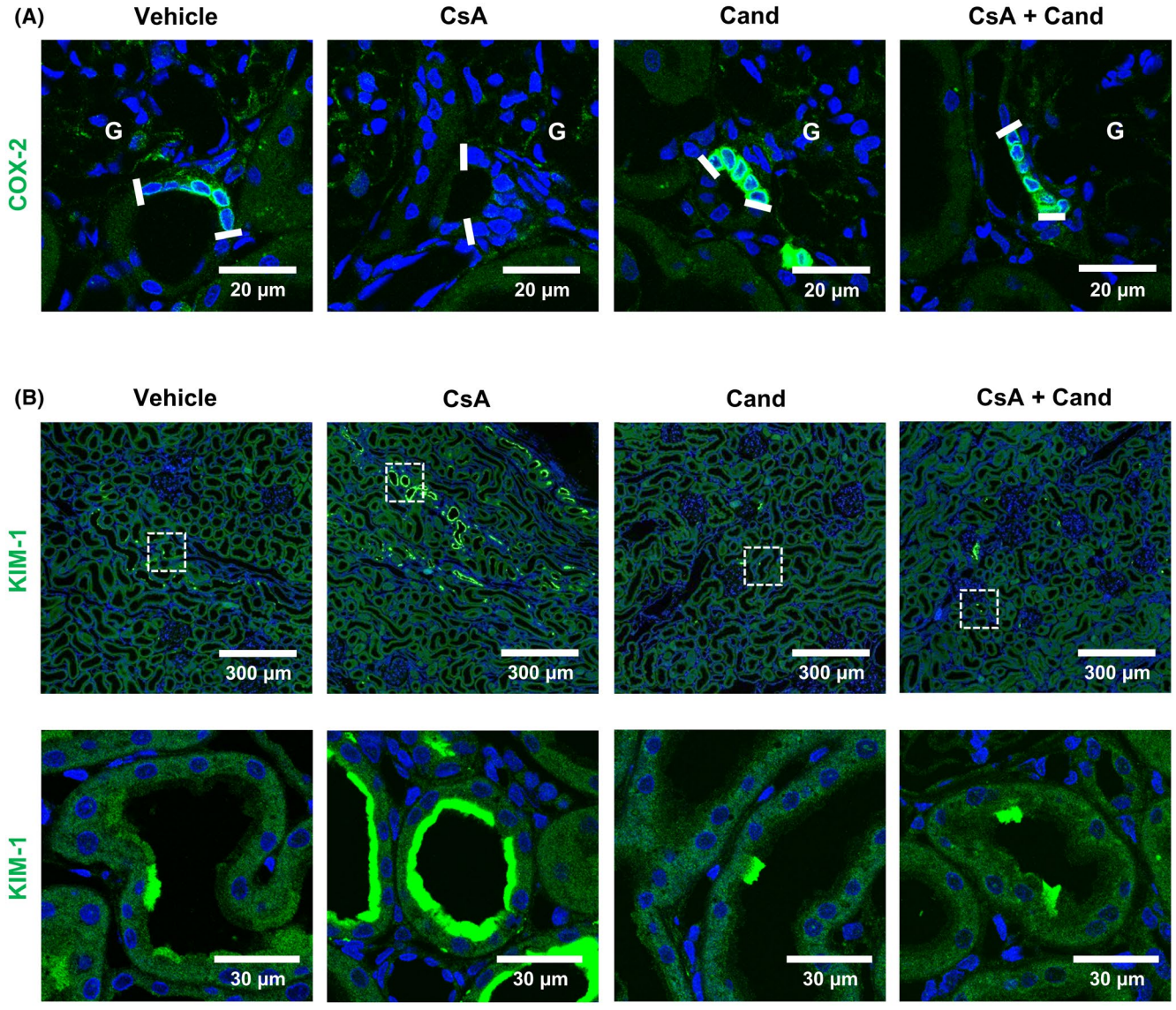


FIGURE 5 Effects of angiotensin II receptor antagonist candesartan (Cand) on cyclooxygenase 2 (COX-2) and kidney injury molecule-1 (KIM-1) expression in vehicle and cyclosporine A (CsA)-treated rats. A, Representative confocal images of macula densa regions (flanked by lines) show COX-2 (green signal) 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.

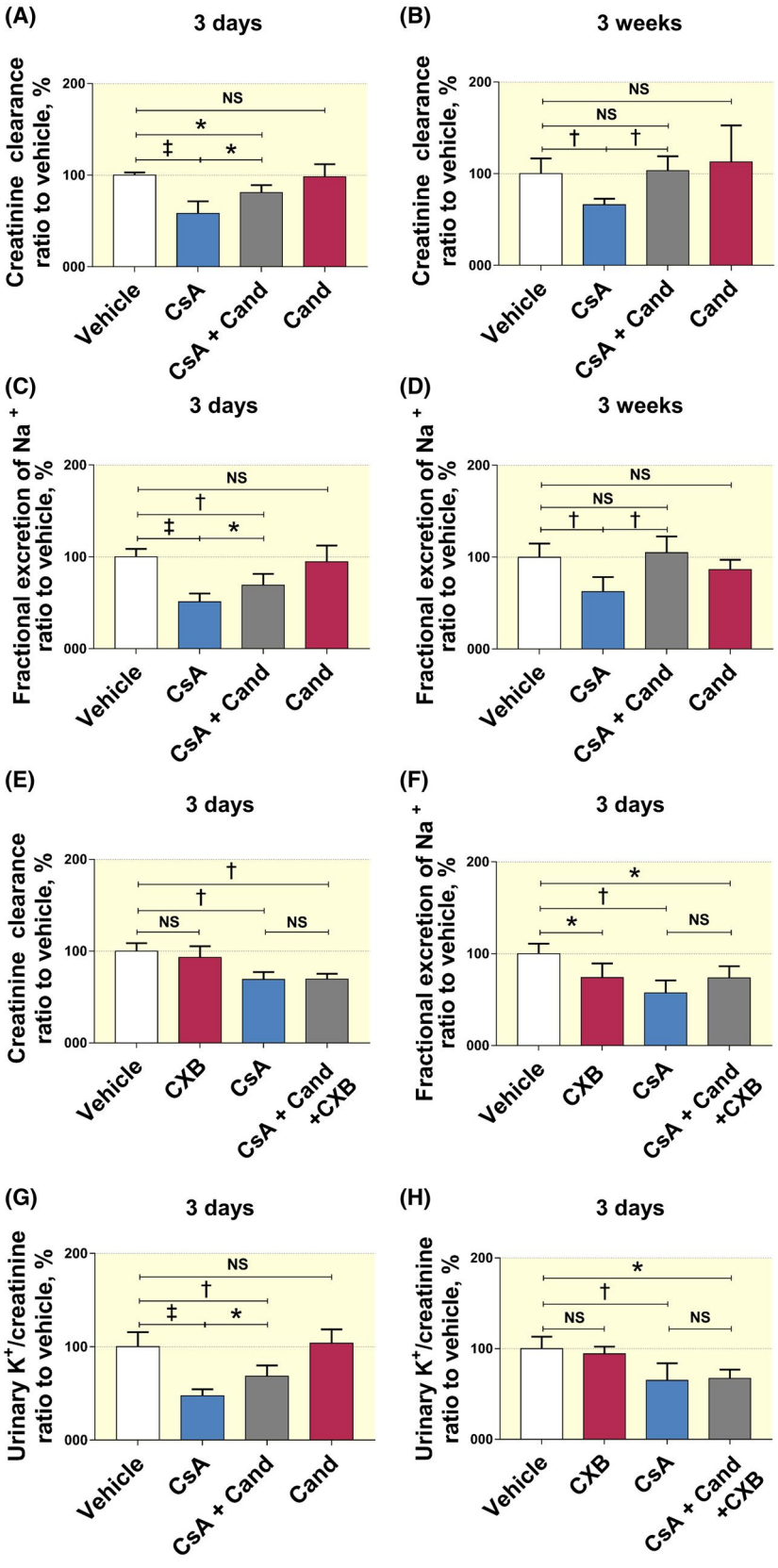


FIGURE 6 Effects of mono- or combined cyclosporine A (CsA), candesartan (Cand) and celecoxib (CXB) treatments on the renal physiology in vivo. A-D, The graphs show evaluation of creatinine clearance (A, B) or fractional sodium excretion (FE_{Na}; C, D) in rats treated with vehicle, CsA (25 mg/kg body 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

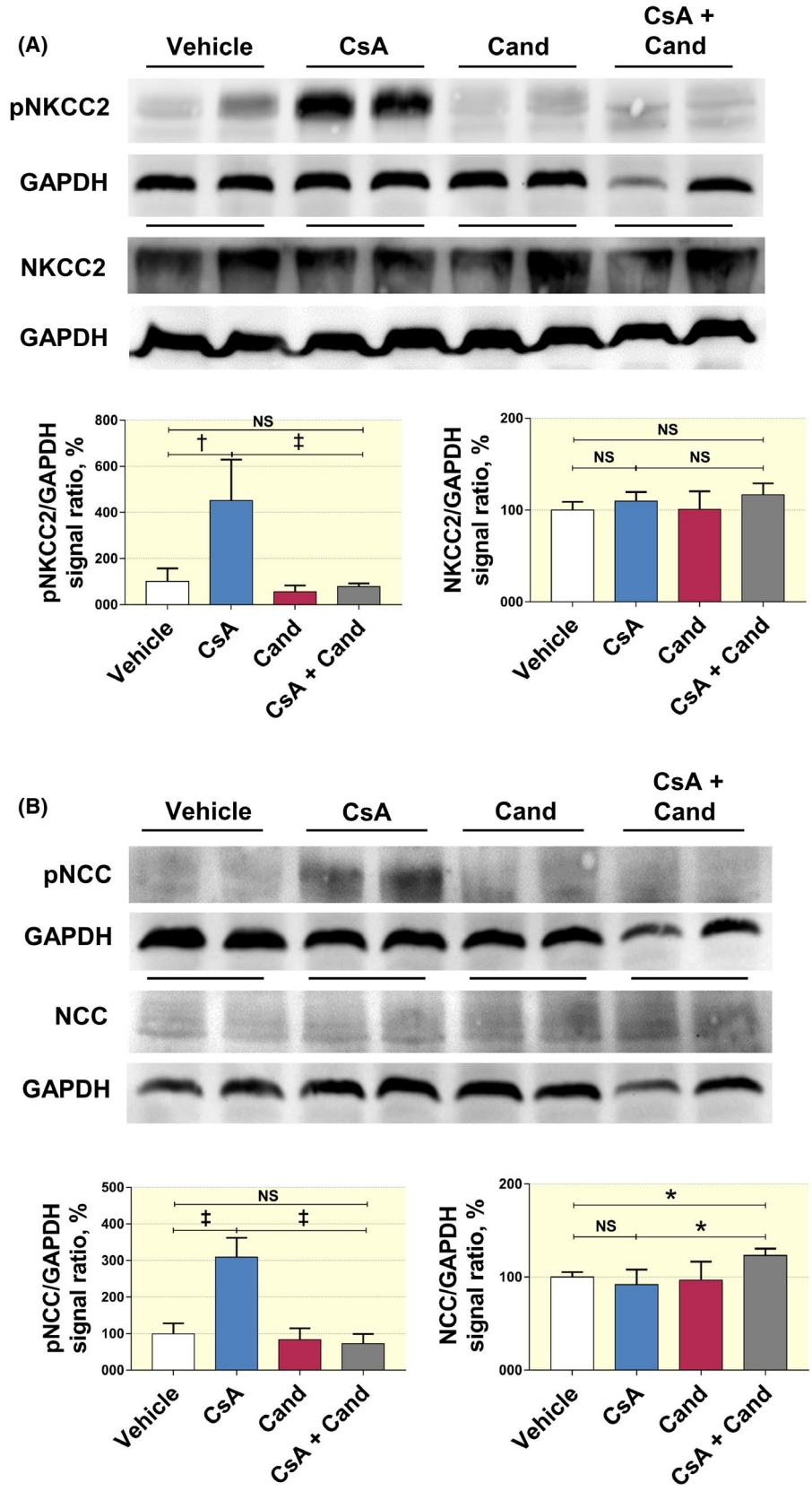


FIGURE 7 Effects of cyclosporine A (CsA), candesartan (Cand) or their combination on Na^+-K^+-$2Cl^-$ cotransporter (NKCC2) and Na^+-Cl^- cotransporter (NCC). A, B, Representative immunoblots of kidney lysates from rats treated with vehicle, CsA (25 mg/kg/d), Cand (5 mg/kg/d), or CsA + Cand for 3 d show 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; * $P < .05$, $^{\dagger}P < .01$, $^{\ddagger}P < .001$, 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

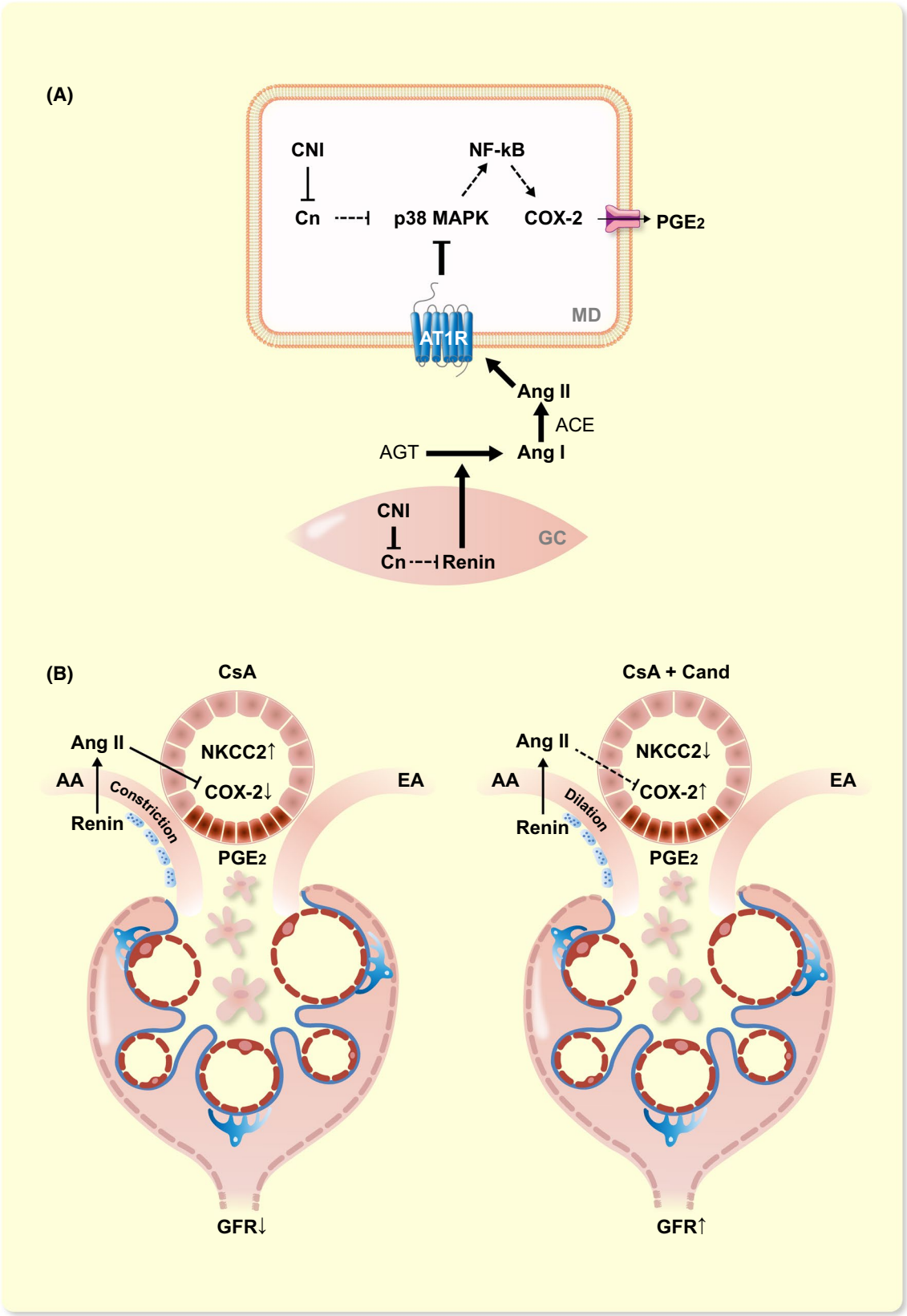


FIGURE 8 Schematic summary of cell-autonomous and systemic effects of calcineurin inhibitors (CNI) affecting expression of the juxtaglomerular cyclooxygenase 2 (COX-2). A, Calcineurin (Cn) inhibits p38 MAPK and NF-kB pathway to suppress COX-2 expression in macula densa (MD) cells. In contrast, CNI increases 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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