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Cyclosporine A attenuates the natriuretic action of loop diuretics by inhibition of renal COX-2 expression

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Cyclosporine A attenuates the natriuretic action of loop diuretics by inhibition of renal COX-2 expression.

Background. It is known that inhibition of cyclooxygenase (COX) impairs the renal actions of loop diuretics. Recently, we found that cyclosporine A (CsA) inhibits renal COX-2 expression. Therefore, we examined the interferences of CsA with the renal actions of loop diuretics.

Method. We investigated the renal effects of furosemide administration (12 mg/day subcutaneously) in male Sprague-Dawley rats receiving in addition vehicle, CsA (15 mg/kg × day), rofecoxib (10 mg/kg × day), or a combination of both.

Results. CsA, rofecoxib, and their combination lowered the furosemide-induced increase of prostaglandin E_2 (PGE_2) and of 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) excretion by 55% and by 70%. They also lowered furosemide stimulated renal excretion of sodium and water by about 65% and 60%. Basal as well as furosemide-induced stimulation of plasma renin activity (PRA) and of renal renin mRNA was further enhanced by CsA. In contrast, rofecoxib attenuated the furosemide-induced rise of PRA and of renin mRNA, both in the absence and in the presence of CsA. In addition, the increase in plasma 6-keto $PGF_{1\alpha}$ levels by furosemide was further enhanced by CsA and was attenuated by rofecoxib.

Conclusion. Taken together, our data suggest that CsA acts as an antinatriuretic, likely by the inhibition of COX-2–mediated renal prostanoid formation. Since the furosemide-induced stimulation of the renin system is not attenuated by CsA but by COX-2 inhibition, we speculate that extrarenal COX-2–derived prostanoids may be involved in the stimulation of the renin system by CsA and by loop diuretics.

Loop diuretics such as furosemide exert diverse renal effects, which in part involve prostanoids [1]. It is known that natriuresis caused by impaired salt transport in the loop of Henle as well as the concomitant stimulation of the renin system by furosemide are attenuated or even abrogated by inhibition of cyclooxygenase (COX) in humans and laboratory animals [2, 3]. More recent results suggest that prostanoids, which apparently favor natriuresis and diuresis and which concomitantly stimulate renin synthesis and secretion, are mainly produced by the cyclooxygenase-2 (COX-2) isoform [4]. It has been shown in this context that selective COX-2 inhibitors attenuate natriuresis and the stimulation of the renin system in patients suffering from the Bartter syndrome as well as in men and rats treated with the loop diuretic furosemide [5–8]. COX-2 is expressed in the normal kidney and its expression in the loop of Henle, including the macula densa region, is further enhanced in Bartter patients as well as in rats treated with furosemide [4–9]. The close anatomic relation between COX-2 expressing macula densa cells and renin-producing juxtaglomerular cells has led to the concept that COX-2–derived prostanoids from the macula densa may be importantly involved in the regulation of renin secretion and renin synthesis [10]. Whether such a linkage really exists is still a matter of debate, since findings have previously been reported supporting [10, 11] or not supporting [12–15] such a role.

We have previously observed that treatment with calcineurin inhibitors, such as cyclosporine A (CsA) and tacrolimus strongly attenuates the expression of COX-2 in the rat kidney and reduces renal tissue prostanoid formation [16]. In view of the prostanoid dependency of the renal effects of loop diuretics [5, 6], it appeared of interest to us to find out if and how the renal effects of loop diuretics are altered in the presence of the calcineurin inhibitor CsA.

METHODS

In vivo experiments

Male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) weighing 150 to 175 g were used for this study. Rats received standard rat chow (0.6% NaCl wt/wt) (Trouw Nutrition, Burgheim, Germany) and had free access to tap water and a solution containing 0.9% sodium chloride and 0.1% potassium chloride. Body weight, water intake, food intake, and systolic blood pressure (tail

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cuff method) were monitored daily. For urinary measurements, rats were housed individually in metabolic cages during the last 2 days of treatment. Rats were divided into eight groups, consisting of eight rats each and treated as follows: group 1 were controls; group 2 were given CsA (15 mg/kg × day for 5 days) orally via a stomach tube; group 3 were given rofecoxib (10 mg/kg × day for 5 days) orally via a stomach tube; group 4 were given CsA and rofecoxib for 5 days; group 5 were given furosemide (12 mg/day) chronically infused subcutaneously via osmotic minipump (model 2ML1) (Alzet Osmotic Pumps, Durect Corporation, Cupertino, CA, USA) for 5 days; group 6 were given furosemide and CsA; group 7 were given furosemide and rofecoxib; and group 8 were given furosemide, CsA, and rofecoxib.

Samples

Rats were sacrificed by decapitation during anesthesia with sevoflurane. Blood was collected into ethylenediaminetetraacetic acid (EDTA) tubes. The kidneys were quickly removed and were cut in longitudinal halves. Cortex and outer and inner medulla were dissected with a scalpel blade under a stereomicroscope, frozen in liquid nitrogen, and stored at −80°C until extraction of total RNA.

Ribonuclease protection assays for β-actin, COX-2, COX-1, and renin mRNA

Cytoplasmic β-actin, COX-1, COX-2, and renin mRNA were determined by specific RNase protection assays as described previously [17].

Determination of plasma renin activity (PRA)

PRA was determined using a commercially available radioimmunoassay (RIA) kit (Sorin Biomedica, Düsseldorf, Germany).

Determination of prostanoids

Plasma 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PFG_{1\alpha}$) levels and daily urinary prostanoid excretion [thromboxane B_2(TxB_2), 6-keto $PGF_{1\alpha}$, and prostaglandin E_2 (PGE_2)] were assayed by using monoclonal enzyme immunoassay (EIA) kits (Cayman Chemical, Ann Arbor, MI, USA). Tissue levels of prostanoids were assayed as described previously [18].

Determination of urinary sodium and potassium

Urinary sodium and potassium concentrations were determined by flame photometry using a low temperature, single-channel emission flame photometer (PFP7) (Jenway, Dunmow, Essex, UK). Excretion rates for sodium and potassium were calculated.

Determination of furosemide levels

Furosemide was determined by reversed-phase high-performance liquid chromatography (HPLC) adapting a published method for the determination of ketorolac [19], which in turn was used as internal standard. In brief, serum or urine was mixed with $ZnSO_4$/methanol as described [19]. Renal tissue was homogenized (Ultra-Turrax, IKA, Freiburg, Germany) in 5 volumes (weight/volume) of 5% aqueous $ZnSO_4$/methanol (20:80; vol/vol). Following centrifugation, 10 to 20 μL of the supernatant was injected into the HPLC system. Chromatographic separation was performed using a reversed-phase Luna 5 C18 column (internal diameter 150 × 4.6 mm) (Phenomenex, Aschaffenburg, Germany) with acetonitrile/water (40:60, vol/vol, pH 2.8, with 85% H_3PO_4) as eluent and photometric detection at 235 nm. At a flow rate of 1 mL/min (column temperature 30°C) furosemide eluted after 5 minutes, and ketorolac (internal standard) after 6 minutes.

Immunoblotting for β-actin, COX-1, and COX-2 protein

One hundred micrograms of total renocortical protein were loaded per lane, separated by an 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Membranes were blocked overnight at 4°C and incubated for 2 hours at room temperature with the primary antibody (COX-2 murine polyclonal AB, 1:500) (Cayman Chemicals) (COX-1 murine polyclonal AB 1:500) (Cayman Chemicals) and a horseradish peroxidase (HPR)-labeled secondary antibody (goat antirabbit IgG; 1:500) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Detection was achieved by enhanced chemiluminescence (ECL) (Amersham, Braunschweig, Germany). The band intensities were quantified densitometrically. For the detection of β-actin protein (mouse monoclonal AB, 1:2000) (Sigma Chemical Co., St. Louis, MO, USA) 20 μg of protein were analyzed.

Creatinine clearance

Plasma and urinary concentrations of creatinine were measured with the Jaffe reaction. Clearances were calculated.

Statistical analysis

Data are presented as mean ± SEM. Levels of significance were calculated by analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons. $P < 0.05$ was considered statistically significant.

Table 1. Systolic blood pressure, heart rate, daily body weight gain, hematocrit, and creatinine clearance in the different groups

	Systolic blood pressure *mm Hg*	Heart rate *bpm*	Daily body weight gain *g/day*	Hematocrit %	Creatinine clearance *mL/min*
Control	122 ± 5	388 ± 12	8.2 ± 0.8	38.1 ± 2.0	0.60 ± 0.09
Cyclosporine A	141 ± 5[a]	432 ± 13[a]	6.5 ± 0.7[a]	39.5 ± 1.5	0.52 ± 0.09
Rofecoxib	139 ± 4[a]	395 ± 10	8.0 ± 0.6	37.8 ± 2.5	0.52 ± 0.07
Cyclosporine A + rofecoxib	145 ± 6[a]	435 ± 11[a]	6.3 ± 0.5[a]	39.0 ± 1.9	0.53 ± 0.08
Furosemide	117 ± 4	428 ± 13[a]	4.5 ± 0.4[a]	39.1 ± 1.5	0.52 ± 0.09
Furosemide + cyclosporine A	129 ± 5	462 ± 16[b]	1.8 ± 0.3[b]	42.0 ± 2.5	0.42 ± 0.09
Furosemide + rofecoxib	127 ± 5	422 ± 13	4.8 ± 0.4	37.1 ± 2.1	0.43 ± 0.07
Furosemide + cyclosporine A + rofecoxib	145 ± 7[b]	465 ± 14[b]	2.0 ± 0.2[b]	37.8 ± 2.2	0.43 ± 0.08

Data are mean ± SEM; $N = 8$.
[a] $P < 0.05$ compared with control; [b] $P < 0.05$ compared with furosemide-treated rats.

RESULTS

Influence of furosemide, CsA, and rofecoxib treatment on blood pressure, heart rate, daily body weight gain, hematocrit, and creatinine clearance

Systolic blood pressure of control rats was 122 ± 5 mm Hg. Treatment with CsA, rofecoxib, or the combination of CsA with rofecoxib increased systolic blood pressure to 141 ± 5, 139 ± 4, or 145 ± 6 mm Hg, respectively. Systolic blood pressure of furosemide-treated animals was 117 ± 4 mm Hg. Additional treatment with CsA or rofecoxib increased blood pressure to 129 ± 5 or 127 ± 5 mm Hg, respectively. The combination of furosemide with CsA and rofecoxib increased systolic blood pressure to 145 ± 7 mm Hg (Table 1). Heart rate of control rats was 388 ± 12 bpm. Rofecoxib treatment did not alter heart rate. CsA or the combination of CsA with rofecoxib increased heart rate to 432 ± 13 or 435 ± 11 bpm, respectively. Heart rate of furosemide-treated animals was 428 ± 13 bpm and increased during co-administration of CsA or CsA in combination with rofecoxib to 462 ± 16 or 465 ± 14 bpm, respectively (Table 1). Daily body weight gain was decreased to about 80% of control values during treatment with CsA or during treatment with the combination of CsA and rofecoxib. Rofecoxib had no influence on the body weight gain. Furosemide treatment decreased daily body weight gain to about 55% of control values. Additional treatment with rofecoxib did not change body weight gain. Additional treatment with CsA or the combination of CsA with rofecoxib further decreased daily body weight gain to about 40% of furosemide-treated rats (Table 1). The hematocrit was not altered by any treatment maneuver (Table 1). Creatinine clearance was not altered by CsA, rofecoxib, furosemide, or the combination of CsA with rofecoxib. Furthermore, creatinine clearances of furosemide-treated rats were not altered by any additional co-treatment (Table 1).

Plasma levels, tissue levels, and daily urinary excretion of furosemide

Plasma level of furosemide was 4.93 ± 0.65 μg/mL for furosemide-treated rats and did not change during additional treatment with CsA (4.78 ± 0.57 μg/mL), rofecoxib (4.48 ± 0.48 μg/mL), or the combination of CsA with rofecoxib (5.13 ± 0.54 μg/mL). The tissue concentration of furosemide in the renal cortex of furosemide-treated rats was 7.22 ± 1.46 μg/g wet weight for furosemide-treated rats and did not change during additional treatment with CsA (9.14 ± 1.55 μg/g wet weight), rofecoxib (7.07 ± 1.08 μg/g wet weight), or the combination of CsA with rofecoxib (9.20 ± 1.21 μg/g wet weight). The daily urinary excretion of furosemide was 5.79 ± 0.64 mg for furosemide-treated rats and did not change during additional treatment with CsA (4.96 ± 1.32 mg), rofecoxib (5.87 ± 0.60 mg), or the combination of CsA with rofecoxib (6.57 ± 0.54 mg).

Influence of furosemide, CsA, and rofecoxib treatment on COX-1 and COX-2 mRNA abundance in different kidney zones

COX-1 mRNA was detected by RNase protection assay in renal cortex and outer and inner medulla, with the highest expression in the inner medulla. In the renal cortex, COX-1 mRNA was not altered by any treatment maneuver (Fig. 1A). Furosemide treatment decreased COX-1 mRNA abundance in the outer and inner medulla to about 50% of control levels (Fig. 1B and C). CsA, rofecoxib, or the combination of CsA with rofecoxib did not influence COX-1 mRNA abundance at basal levels nor during additional treatment with furosemide (Fig. 1B and C).

COX-2 mRNA expression also displayed a gradient from the cortex to the inner medulla, where the highest expression of COX-2 mRNA was found. Rofecoxib had no effect on COX-2 mRNA expression in any kidney zone (Fig. 2). CsA and the combination of CsA with rofecoxib clearly suppressed basal COX-2 mRNA abundance in the renal cortex and outer and inner medulla to about 25%, 45%, and 25% of basal values, respectively (Fig. 2). Furosemide treatment increased COX-2 mRNA abundance in the renal cortex about twofold and suppressed COX-2 mRNA abundance in the inner medulla to about

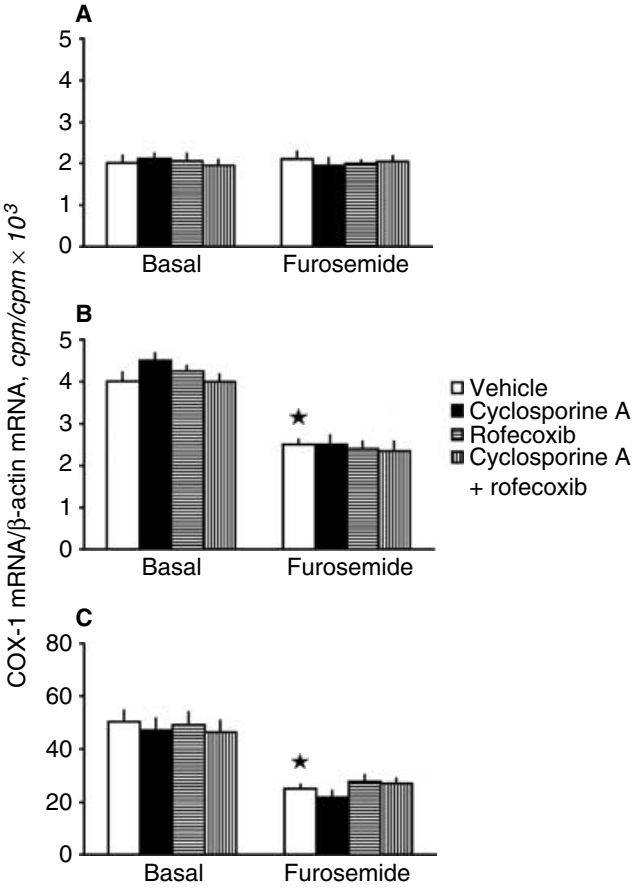

Fig. 1. Effect of furosemide, cyclosporine A (CsA), rofecoxib, or their combinations on cyclooxygenase 1 (COX-1) mRNA abundance in the rat renal cortex (*A*) and outer (*B*) and inner medulla (*C*). Furosemide decreased COX-1 mRNA in the outer and inner medulla (mean ± SEM; $N = 8$). $^{*}P < 0.05$ compared with vehicle-treated control.

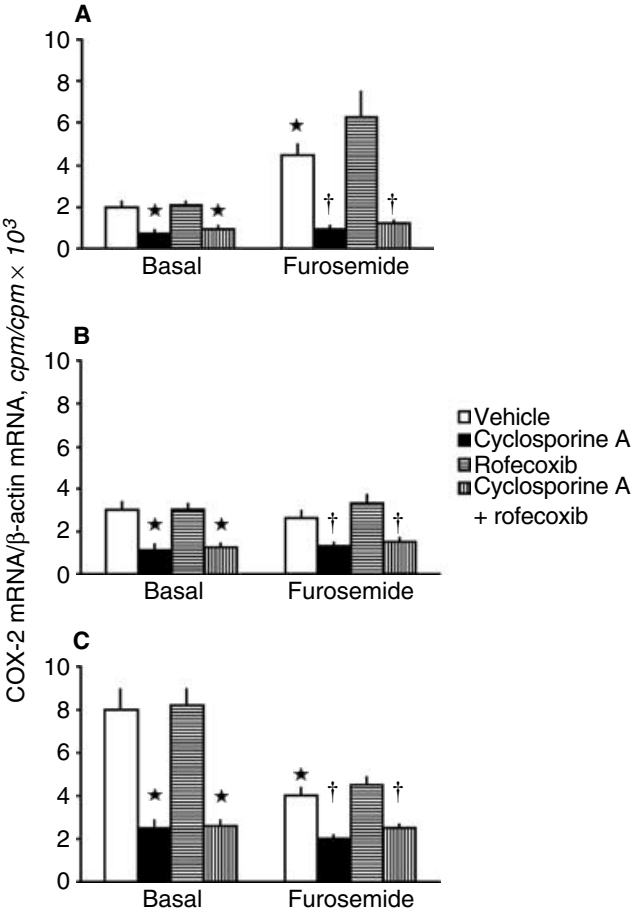

Fig. 2. Effect of furosemide, cyclosporine A (CsA), rofecoxib, or their combinations on cyclooxygenase 2 (COX-2) mRNA abundance in the rat renal cortex (*A*) and outer (*B*) and inner medulla (*C*). CsA decreased basal as well as furosemide-stimulated renocortical COX-2 mRNA abundance (A) and decreased COX-2 mRNA in the outer medulla of control or furosemide treated rats (B). CsA further decreased COX-2 mRNA abundance in the rat inner medulla of vehicle and furosemide treated rats (C) (mean ± SEM; $N = 8$). $^{*}P < 0.05$ compared with vehicle-treated control. $^{\dagger}P < 0.05$ compared with furosemide-treated rats.

50% of control values (Fig. 2). Additional treatment with CsA or the combination of CsA with rofecoxib decreased furosemide-altered COX-2 levels in the renal cortex and outer and inner medulla to about 15%, 45%, and 45% of the respective furosemide levels (Fig. 2).

Influence of furosemide, CsA, and rofecoxib treatment on renocortical COX-2 and COX-1 protein expression

Basal renocortical COX-2 protein expression was clearly decreased by CsA or the combination of CsA with rofecoxib to about 40% of control values. Rofecoxib alone had no influence on COX-2 protein levels. Furosemide increased renocortical COX-2 protein levels about twofold. The increase was not attenuated by rofecoxib, but was decreased to about 20% by additional treatment with CsA and to about 25% by additional treatment with the combination of CsA with rofecoxib (Fig. 3A). Renocortical COX-1 (Fig. 3B) and β-actin protein were not altered by any treatment maneuver.

Influence of furosemide, CsA, and rofecoxib treatment on renocortical prostanoid levels

The basal formation of renocortical PGE_2 decreased to about 65% of control values by treatment with CsA, rofecoxib, or the combination of CsA with rofecoxib. Furosemide treatment increased renocortical PGE_2 concentration 1.4-fold and this increase was attenuated by co-administration of CsA, rofecoxib, or the combination of CsA with rofecoxib (Fig. 4A). The basal formation of renocortical 6-keto $PGF_{1\alpha}$ decreased to about 70% of control values by treatment with CsA, rofecoxib, or the combination of CsA with rofecoxib. Furosemide increased renocortical 6-keto $PGF_{1\alpha}$ concentration 1.4-fold and this increase was attenuated by co-administration of CsA, rofecoxib, or a combination of both (Fig. 4B). Renocortical TxB_2 formation was not altered by any treatment maneuver (Fig. 4C).

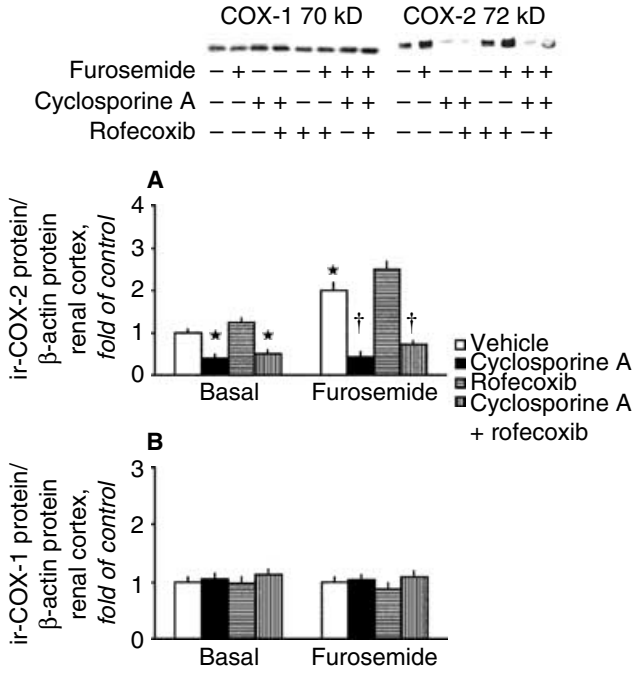

Fig. 3. Renocortical cyclooxygenase 2 (COX-2) (*A*) and COX-1 (*B*) protein levels in rats treated with either vehicle or furosemide and in addition with cyclosporine A (CsA), rofecoxib, or the combination of rofecoxib with CsA. The insert shows representative blots for COX-1 and COX-2 protein (−, untreated; +, treated). The furosemide induced increase in COX-2 protein expression was attenuated by CsA given alone or in combination with rofecoxib, whereas rofecoxib had no influence (A). COX-1 protein abundance was not influenced by any treatment maneuver (B) (mean ± SEM; $N = 8$). $^{*}P < 0.05$ compared with vehicle-treated control; $^{\dagger}P < 0.05$ compared with furosemide-treated rats.

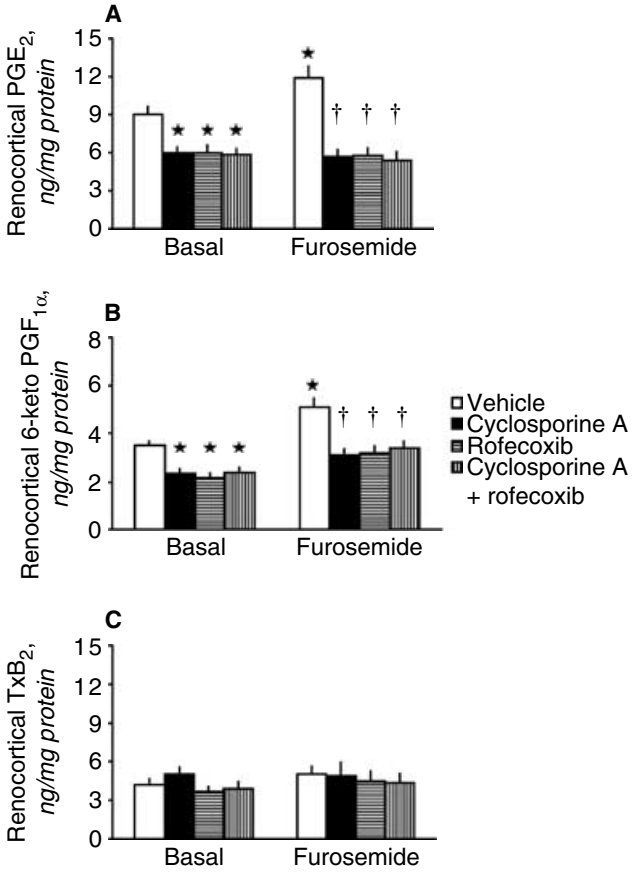

Fig. 4. Renocortical prostaglandin E_2 (PGE_2) (*A*), 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) (*B*), and thromboxane B_2 (TxB_2) (*C*) levels in rats treated with either cyclosporine A (CsA), rofecoxib, furosemide, or the combination of CsA with rofecoxib. CsA, rofecoxib, and the combination of CsA with rofecoxib decreased basal as well as furosemide stimulated tissue levels of PGE_2 (A) and 6-keto $PGF_{1\alpha}$ (B). Tissue levels of TxB_2 were not altered by any treatment maneuver (C) (mean ± SEM; $N = 8$). $^{*}P < 0.05$ compared with vehicle-treated control; $^{\dagger}P < 0.05$ compared with furosemide-treated rats.

Influence of furosemide, CsA, and rofecoxib treatment on renal prostanoid excretion

Daily urinary excretion of PGE_2 decreased to about 60% of control values by CsA and by rofecoxib treatment. The combination of rofecoxib with CsA did not further decrease PGE_2 excretion. Furosemide treatment increased PGE_2 excretion about 2.5-fold. Co-treatment with CsA or rofecoxib attenuated the furosemide-induced excretion of PGE_2 to about 45% of furosemide levels. The combination of CsA with rofecoxib did not further decrease PGE_2 excretion (Fig. 5A).

Daily urinary excretion of 6-keto $PGF_{1\alpha}$, a metabolite of prostacyclin, decreased to about 70% of control values by treatment with CsA or rofecoxib. The combination of rofecoxib with CsA did not further decrease 6-keto $PGF_{1\alpha}$ excretion. Furosemide treatment increased 6-keto $PGF_{1\alpha}$ excretion about fourfold and additional treatment with CsA or rofecoxib attenuated the furosemide-induced excretion of 6-keto $PGF_{1\alpha}$. The combination of CsA with rofecoxib did not further decrease 6-keto $PGF_{1\alpha}$ excretion (Fig. 5B).

Daily urinary excretion of TxB_2, a metabolite of TxA_2, increased 2.5-fold over basal levels by CsA treatment, and decreased to about 70% of basal levels by rofecoxib treatment. Rofecoxib also attenuated the CsA-induced rise in TxB_2 excretion. Furosemide treatment increased TxB_2 excretion about twofold and additional treatment with CsA further increased TxB_2 excretion. Rofecoxib treatment attenuated these increases to about 50% of the levels found in furosemide-treated rats (Fig. 5C).

Influence of furosemide, CsA, and rofecoxib treatment on water, sodium, and potassium excretion

Daily urine excretion of control rats was 107 ± 12 mL/day × kg body weight. CsA and the combination of CsA with rofecoxib, but not rofecoxib alone, decreased daily urine excretion to about 75% of control values. Furosemide treatment increased daily urine excretion about 7.5-fold. The increase was clearly attenuated by co-administration of CsA, rofecoxib, or the combination

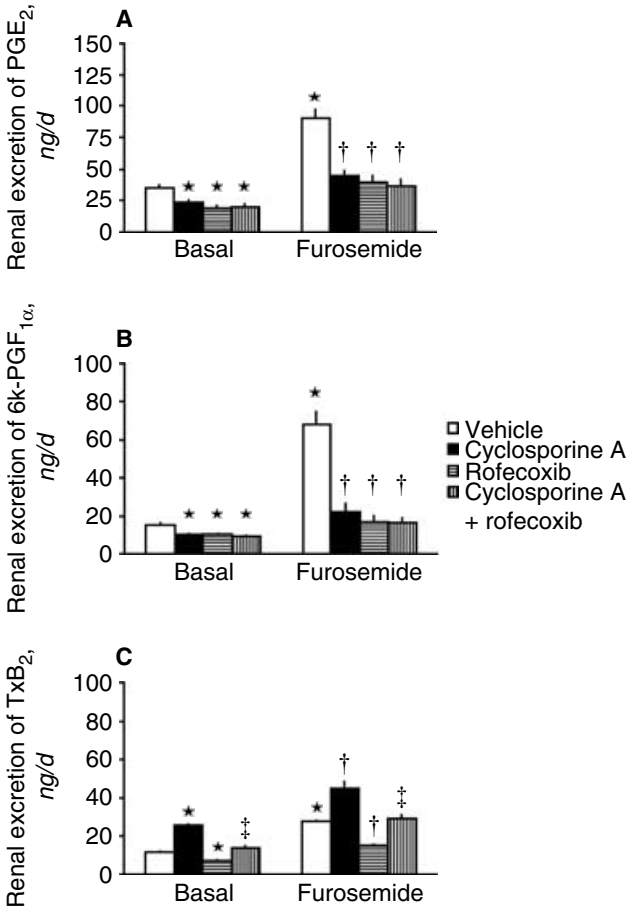

Fig. 5. Urinary prostaglandin E_2 (PGE_2) (*A*), 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) (*B*), and thromboxane B_2 (TxB_2) (*C*) excretion in rats treated with either cyclosporine A (CsA), rofecoxib, furosemide, or the combination of CsA with rofecoxib. CsA and rofecoxib decreased basal as well as furosemide stimulated daily urinary excretion of PGE_2 (A) and 6-keto $PGF_{1\alpha}$ (B). CsA increased TxB_2 excretion, what was attenuated by additional rofecoxib treatment (C). Rofecoxib decreased basal as well as furosemide-induced TxB_2 excretion (mean ± SEM; $N = 8$). $^*P < 0.05$ compared with vehicle-treated control; $^\dagger P < 0.05$ compared with furosemide-treated rats; $\ddagger P < 0.05$ compared with CsA.

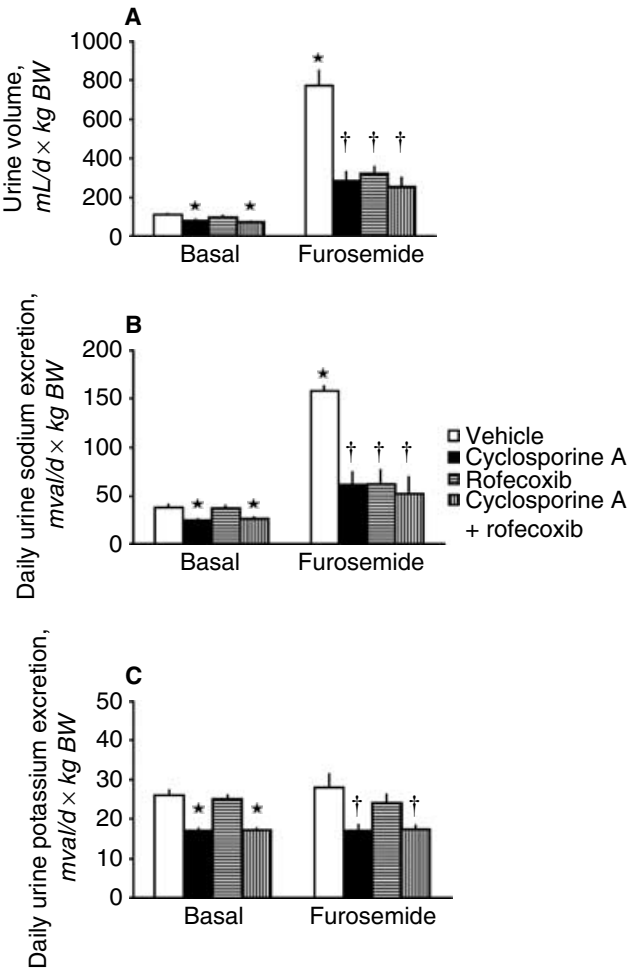

Fig. 6. Urinary volume (*A*) and daily urinary sodium (*B*) and potassium excretion (*C*) in rats treated with either vehicle and furosemide and in addition with cyclosporine A (CsA), rofecoxib, or the combination of rofecoxib with CsA. Furosemide increased urinary volume (A) and daily urinary sodium excretion (B). These increases were attenuated by co-administration of rofecoxib, CsA, or a combination of rofecoxib with CsA. Urinary potassium excretion was decreased by CsA treatment (C) (mean ± SEM; $N = 8$). $^*P < 0.05$ compared with vehicle-treated control; $^\dagger P < 0.05$ compared with furosemide-treated rats.

of CsA with rofecoxib to about 40% of furosemide levels (Fig. 6A).

Daily sodium excretion of control rats was 38 ± 4 mval/day × kg body weight and was not altered by rofecoxib treatment. CsA alone or in combination with rofecoxib decreased basal sodium excretion to about 70% of control values. Furosemide treatment increased daily sodium excretion about 4.5-fold. This increase was clearly attenuated by CsA, rofecoxib, or the combination of both to about 35% of furosemide levels (Fig. 6B).

Daily potassium excretion of control rats was 26 ± 3 mval/day × kg body weight and was not altered by rofecoxib or furosemide. CsA, whether given alone or in combination with rofecoxib, decreased potassium excretion (Fig. 6C) to about 65% of the respectative control values.

Influence of furosemide, CsA, and rofecoxib treatment on PRA and renal renin mRNA abundance

PRA was 7.5 ± 0.9 ng angiotensin I (Ang I)/hour × mL in control rats. Treatment with CsA increased PRA 1.8-fold and this increase was attenuated by additional rofecoxib treatment. Rofecoxib alone did not alter PRA. Furosemide increased PRA 6.6-fold and co-administration of CsA further increased PRA. The furosemide-induced rise in PRA as well as the increase by additional CsA treatment was attenuated by co-administration of rofecoxib to 30 ± 5 ng Ang I/hour × mL (Fig. 7A).

Similar results were obtained with regard to renocortical renin mRNA abundance. CsA increased renin mRNA abundance about twofold over control values

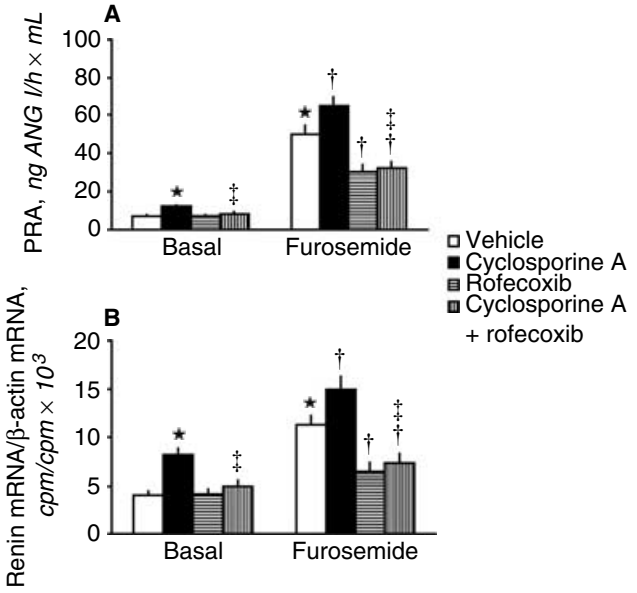

Fig. 7. Plasma renin activity (PRA) (*A*) and renocortical renin mRNA abundance (*B*) in rats treated with either vehicle or furosemide and in addition with cyclosporine A (CsA), rofecoxib, or the combination of rofecoxib with CsA. The furosemide induced raise in PRA was attenuated by rofecoxib or the combination of rofecoxib with CsA. CsA further increased PRA (A). The furosemide-induced increase in renocortical renin mRNA abundance was attenuated by rofecoxib or the combination of rofecoxib with CsA. CsA further increased renocortical renin mRNA abundance (B) (mean ± SEM; $N = 8$). $^{*}P < 0.05$ compared with vehicle-treated control; $^{\dagger}P < 0.05$ compared with furosemide-treated rats; $\ddagger P < 0.05$ compared with CsA.

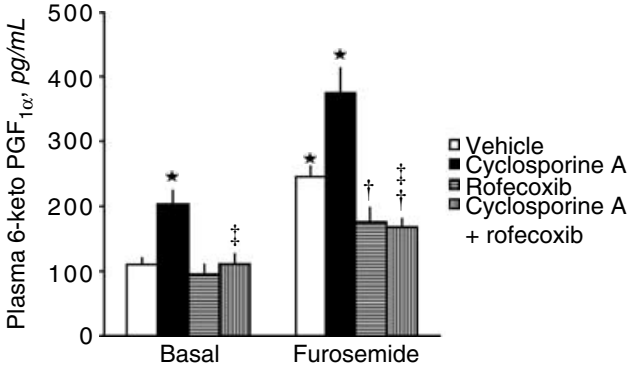

Fig. 8. Plasma concentrations of 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) in rats treated with either cyclosporine A (CsA), rofecoxib, furosemide, or the combination of CsA with rofecoxib. Furosemide increased plasma levels of 6-keto $PGF_{1\alpha}$. CsA further increased 6-keto $PGF_{1\alpha}$ levels. Rofecoxib attenuated the furosemide-stimulated rise in plasma 6-keto $PGF_{1\alpha}$ levels and attenuated the CsA-stimulated increase in plasma levels of 6-keto $PGF_{1\alpha}$ (mean ± SEM; $N = 8$). $^{*}P < 0.05$ compared with vehicle-treated control; $^{\dagger}P < 0.05$ compared with furosemide-treated rats; $\ddagger P < 0.05$ compared with CsA.

and additional rofecoxib treatment attenuated this increase. Rofecoxib alone had no effect on basal renocortical renin mRNA abundance. Furosemide treatment increased renin mRNA about 2.2-fold compared to basal levels. Additional treatment with CsA further increased renin mRNA abundance, which was attenuated by additional rofecoxib treatment. Furthermore, the furosemide-induced increase in renin mRNA abundance was attenuated by rofecoxib (Fig. 7B).

Influence of furosemide, CsA, and rofecoxib treatment on 6-keto $PGF_{1\alpha}$ plasma levels

CsA increased 6-keto $PGF_{1\alpha}$ plasma levels about twofold (Fig. 8). Rofecoxib had no influence on basal levels, but clearly prevented the CsA-induced rise in 6-keto $PGF_{1\alpha}$ plasma levels (Fig. 8). Furosemide increased 6-keto $PGF_{1\alpha}$ plasma levels about 2.2-fold. Coadministration of CsA further increased 6-keto $PGF_{1\alpha}$ plasma levels. Additionally, treatment with rofecoxib decreased furosemide and CsA-induced 6-keto $PGF_{1\alpha}$ plasma levels (Fig. 8).

DISCUSSION

On the basis of observations that the renal actions of loop diuretics are sensitive toward inhibition of COX-2 [5, 6] and because inhibitors of calcineurin phosphatase, like CsA, decrease renal COX-2 expression [16], our study aimed to characterize the interference of CsA with the renal actions of loop diuretics, especially with regard to diuresis and natriuresis.

The data obtained in this study clearly demonstrate that the calcineurin inhibitor attenuates the well-established increase in diuresis and natriuresis induced by the loop diuretic furosemide. Rats that have been treated with CsA or furosemide had less weight gain, confirming previous observations [20, 4]. The reduced weight gain of CsA-treated rats may be related to a moderate drug-induced diarrhea, because there was no observation of anorexia as estimated by the food intake, which was in the range of control rats. The furosemide-induced decrease in body weight may be related to a decreased food intake during the first 2 days after implantation of the osmotic minipumps. However, the food intake returned to control levels from day 3 on. It should be noted that we did not obtain evidence that the rats were volume depleted after prolonged treatment with furosemide.

In accordance to previous observations, CsA treatment increased systolic blood pressure [21]. Our data suggest that the increment in arterial pressure induced by CsA may be in part secondary to the increment in TxA_2, as reflected by increased daily urinary excretion of TxB_2, the stable metabolite of TxA_2, but also secondary to an increase in Ang II since we found that CsA increased the activity of the renin system. We further observed an increased heart rate in CsA-treated rats, suggesting an activation of the sympathetic nervous system, as reported by others [22, 23].

In line with previous reports, we found that CsA inhibits renal COX-2 expression [16], that furosemide

increases renocortical COX-2 expression [4, 6], that furosemide down-regulates COX-2 and COX-1 expression in the rat medulla [24], and that rofecoxib has no effect on renal COX-1 and COX-2 expression up to 7 days of treatment [13]. The opposite regulation of the COX isoforms by furosemide in the renal cortex and inner medulla has been previously described. Thus, it has been suggested that inhibition of the salt transport in the loop of Henle causes a selective stimulation of COX-2 expression in the macula densa [4], whereas down-regulation of COX-2 and COX-1 expression in the inner medulla of the rat kidney is likely related to a breakdown of the papillary osmotic gradient [24]. Although CsA attenuates COX-2 expression in the rat renal cortex and the medulla, the precise mechanism involved in the opposite regulation could not be answered from our findings and needs further investigation.

We now found that CsA also attenuates furosemide-stimulated COX-2 expression in the rat kidney cortex. Further, CsA decreased renocortical PGE_2 and 6-keto $PGF_{1\alpha}$ formation as well as daily urinary excretion of PGE_2 and 6-keto $PGF_{1\alpha}$ in furosemide-treated rats like the COX-2 blocker rofecoxib did. Although it is suggested that the dose of rofecoxib used in our study is selective for COX-2 in the rat [25, 26], rofecoxib may also inhibit the COX-1 isoform, since there was a decrease in the urinary excretion of TxB_2. In addition, we found that a combination of CsA with the COX-2 blocker rofecoxib has no additive effect on the formation and excretion of PGE_2 and 6-keto $PGF_{1\alpha}$.

We further found that CsA treatment reduces natriuresis and diuresis confirming previous reports [20, 21]. The antinatriuretic effect of CsA has long since been known, but the precise mechanisms causing the decrease in natriuresis have not been really understood. It has been suggested in this context that the decrease in sodium excretion may be secondary to a CsA induced fall in glomerular filtration rate (GFR) [20]. To investigate such a possible influence of CsA or rofecoxib on GFR, we measured creatinine clearance. Confirming previous studies, we found that neither CsA [27] nor COX-2 inhibition [28] negatively influenced GFR, suggesting that a fall in GFR is of minor relevance for the decrease in natriuresis in our study.

It is well known, that prostanoids, especially PGE_2, increase natriuresis and diuresis [29–32]. At the cellular level, PGE_2 directly inhibits the reabsorption of NaCl in the medullary thick ascending limb and in the collecting duct [29–31]. This action of PGE_2 may explain why cyclooxygenase inhibitors cause sodium and water retention, an effect that predominantly occurs in the thick ascending limb of Henle [33]. In the thick ascending limb of Henle the expression of the Na-K-2 Cl co-transporter can be modulated by renal prostanoids in the way that PGE_2 decreases its expression and leads therefore to an enhanced diuresis and sodium excretion [34]. In addition, the vasopressin-induced resorption of water in the collecting duct is reduced by PGE_2 [29]. With regard to the data obtained in our study, it seems likely that inhibition of renal PGE_2 formation and urinary excretion by CsA attenuates the furosemide-induced natriuresis and diuresis. This conclusion would be further supported by the finding that the COX-2 blocker rofecoxib has similar effects on renal PGE_2 formation and on natriuresis and diuresis in furosemide-treated rats. Furthermore, a combination of CsA with rofecoxib had no additive effects on PGE_2 formation, natriuresis, and diuresis.

Taken into consideration that the renin system and the sympathetic nervous system is activated by CsA, the antinatriuretic and antidiuretic effect of CsA may also be in part secondary to the activation of the renin system and the sympathetic nervous system and therefore an additive effect may not be observed. Further, an additive effect may not be observed because of the differences in blood pressure in the treatment groups. However, additional treatment with the angiotensin-converting enzyme (ACE) inhibitor enalapril or the angiotensin II type 1 (AT_1) receptor antagonist valsartan did not ameliorate CsA-induced antinatriuresis in spontaneously hypertensive rats, but attenuated CsA-induced hypertension [35].

Although, we cannot exclude further tubular effects of CsA, which may influence natriuresis and diuresis in our study, the results obtained demonstrate that there is an association between furosemide induced natriuresis with COX-2–derived prostanoids.

Aside from the effects on salt and water reabsorption, PGE_2 and in particular prostacyclin (PGI_2) also stimulate renin synthesis and secretion [36, 37]. It has been found that inhibition of COX activity reduces renin synthesis and secretion [38]. Since macula densa cells can express COX-2, it has been hypothesized that COX-2–derived prostanoids might be the mediators of the macula densa control of renin secretion and renin synthesis [10, 11]. Loop diuretics block salt transport, increase the expression of COX-2 in the macula densa region, and at the same time stimulate the activity of the renin system in a COX-2–dependent fashion [4–6]. Therefore, it is an obvious conclusion that the stimulatory effect of loop diuretics is mediated by the macula densa. However, our data show that CsA markedly attenuates the stimulation of COX-2 expression and of renal prostanoid excretion by furosemide but does not prevent the stimulation of the renin system by furosemide, while a COX-2 blocker inhibits the stimulation of the renin system by furosemide in presence of CsA. It appears, therefore, as if COX-2–derived prostanoids but not macula densa–derived prostanoids or even renal prostanoids in general are required for the effect of loop diuretics on the renin system.

We are aware that a marked decrease of COX expression in the medulla, which is the main site of renal prostanoid synthesis, is at first glance difficult to reconcile with the enhancement of renal prostanoid excretion by furosemide, but there is evidence that loop diuretics increase PGI_2 formation in extrarenal tissues leading to increased plasma PGI_2 formation, for example [39–41]. Thus, it has been shown that furosemide stimulates prostacyclin levels in human endothelial cells, suggesting that the vasculature could be a predominant site of PGI_2 synthesis [41]. Therefore, we determined plasma concentration of PGI_2 as indicated by 6-keto $PGF_{1\alpha}$ levels. Control levels of 6-keto $PGF_{1\alpha}$, which were in the range of a previous report of us [42], were increased by treatment with CsA, furosemide, and much stronger by the combination of CsA with furosemide. We further found that additional treatment with the selective COX-2 blocker rofecoxib attenuated these increases, suggesting that COX-2 is responsible for increased PGI_2 plasma levels and, since PGI_2 is known to be a potent stimulator of the renin system [36, 37], increases in plasma PGI_2 levels may be involved in the stimulation of the renin system during CsA and furosemide treatment. We speculate, therefore, that furosemide-induced stimulation of the renin systems may partially depend on extrarenal prostanoid formation. However, the question arises, where the increased formation of PGI_2 during CsA treatment comes from. This question could not be answered from our study, but it has been shown in vitro that rat aortic endothelial cells increase the synthesis of prostacyclin in response to CsA [43], suggesting that CsA increases the formation of PGI_2 in endothelial cells. However, it is generally believed that PGI_2 is an autacoid that is quickly metabolized to 6-keto $PGF_{1\alpha}$ before reaching to the kidney and given the case that a small portion of not metabolized PGI_2 reaches the kidney, as it has been shown for intravenous administration of PGI_2 [44, 45], the increment of PGI_2 may increase not only renin [44, 45] release but also urinary sodium and water excretion [44], a possibility that would favor our findings for furosemide treatment, but not for CsA, suggesting that there are additional tubular effects involved in CsA-associated antinatriuresis and antidiuresis. Further, the small increment of prostacyclin in our study may be sufficient to activate the prostacyclin receptor [46].

Although differences between the treatment groups in the levels of blood pressure, renin, and probably sympathetic activity may complicate a comparison, our data suggest that CsA attenuates furosemide-induced natriuresis, likely by the inhibition of COX-2–mediated renal prostanoid formation. Since the furosemide-induced stimulation of the renin system is not attenuated by CsA but by COX-2 inhibitors, we speculate that extrarenal COX-2–derived prostanoids may be involved in the stimulation of the renin system by loop diuretics.

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