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Inhibition of COX-2 counteracts the effects of diuretics in rats

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Inhibition of COX-2 counteracts the effects of diuretics in rat.

Background. It is well established that the diuretic- and renin-stimulated effects of loop diuretics can be attenuated by nonselective cyclooxygenase inhibitors. Since it is yet unclear which of the isoforms of cyclooxygenases, COX-1 and COX-2, is relevant in this context, our study aimed to determine the effects of selective COX-2 inhibition on the renal effects of the loop diuretic furosemide, as well as the diuretic hydrochlorothiazide, which acts on the distal tubule.

Method. Male Sprague-Dawley rats were treated with furosemide (12 mg/day subcutaneously by osmotic pump) or hydrochlorothiazide (30 mg/kg body weight/day orally by gavage). In addition, parallel groups received rofecoxib (1 to 10 mg/kg body weight/day) for selective inhibition of COX-2. Controls were treated with vehicle.

Results. Induction of COX-2 mRNA expression due to furosemide was paralleled by increased renal excretion of prostanoids. Also, hydrochlorothiazide led to a rise in prostanoid excretion. Rofecoxib blunted the diuretic-induced increase in prostanoid excretion, thus confirming an effective blockade of COX-2. Moreover, the COX-2 inhibitor rofecoxib dose-dependently attenuated diuresis and saluresis, as well as the stimulation of the renin system induced by furosemide. Furthermore, rofecoxib completely reversed diuresis and saluresis and prevented the increase of plasma renin activity induced by hydrochlorothiazide.

Conclusions. These findings suggest that COX-2–derived prostanoids are of major relevance in modulating the renal effects of diuretics. COX-2 inhibitors might be valuable drugs to treat salt and water wasting during Bartter and Gitelman diseases.

It is well established that prostaglandins can exert major influence on renal salt and water handling as well as on the activity of the renin system in the kidney [1, 2]. As a consequence, inhibition of prostanoid formation reduces renal salt and water excretion and attenuates the activity of the renin system [3, 4]. This effect becomes more evident in states of diuresis and saluresis induced by diuretics or in renal salt and water wasting disorders such as the Bartter disease [5, 6]. A variety of species, including humans and rat, express both cyclooxygenase isoforms, namely COX-1 and COX-2, in the kidney [7, 8]. COX-1 is mainly localized in the inner medulla [9], whereas COX-2 is found in the thick ascending limb of Henle including the macula densa region as well as in collecting ducts [9, 10]. Which isoform of cyclooxygenases is of major relevance for the generation of prostaglandins involved in renal salt and water excretion is yet unknown. As to the stimulation of the renin system by loop diuretics, there is first evidence for COX-2– rather than COX-1–derived prostanoids that mediate the stimulation of renin secretion [11]. Such an involvement of COX-2–derived prostanoids is in good accordance with reports that macula densa cells, which are closely neighbored to the renin-producing juxtaglomerular cells, express COX-2 [12] and that selective COX-2 inhibitors inhibit the stimulation of the renin system by different maneuvers [13–15]. Moreover, the expression of COX-2 in the thick ascending limb of Henle, including the macula densa region, is up-regulated by loop diuretics [16] and in Bartter disease [17]. These findings prompted us to investigate a possible mediator function of COX-2–derived prostanoids for renal salt and water excretion in states of diuresis resembling genetic renal disorders such as Bartter and Gitelman diseases. Bartter disease results from insufficient salt reabsorption in the thick ascending limb of Henle, which can be caused by defects of the apical Na, K, 2Cl-cotransporter NKCC-2, the apical potassium channel ROMK, or the basolateral chloride channel ClC-Kb [18]. We aimed to mimic the Bartter disease in the rat by chronic infusion of the loop diuretic furosemide, which blocks NKCC-2 activity in the thick ascending limb of Henle. Gitelman disease results from an insufficient salt reabsorption in the convoluted part of the distal tubule by a defect of the apical NaCl cotransporter (NCC) [18]. Gitelman disease was mimicked by chronic administration of hydrochlorothiazide, which specifically blocks

Key words: salt and water wasting, Bartter disease, Gitelman disease, loop diuretics, furosemide, hydrochlorothiazide, renin system, prostaglandins, saluresis, urinary excretion.

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NCC in the kidney [19]. To allow compensation of salt and water loss, the animals had free access to salt water and free water. To assess the possible renal effects of COX-2–derived prostanoids in these animal models, the rats were treated with the selective COX-2 inhibitor rofecoxib [20]. We found that the COX-2 inhibitor markedly attenuated salt and water excretion induced by both diuretics. In parallel, the COX-2 inhibitor attenuated the well-known stimulation of renin secretion by the diuretics. If these findings obtained in rats can be transferred to humans, then inhibition of COX-2 activity could be a viable therapy for alleviating the symptoms of renal salt wasting diseases.

METHODS

In vivo experiments

For all animal experiments, male Sprague-Dawley rats weighing 150 to 175 g were used. They received standard rat chow (0.5% NaCl wt/wt; Trouw Nutrition, Burgheim, Germany) with free access to tap water and a solution containing 0.9% sodium chloride and 0.1% potassium chloride. They were housed individually in metabolic cages with a 12-hour light/dark cycle. Urine collections were performed for 24 hours during the last two days.

Control animals ($N = 6$) were treated with vehicle or rofecoxib (10 mg/kg body weight/day in 2 single doses by gavage). For the experiments with furosemide and hydrochlorothiazide, the control groups ($N = 3$ to 4) were treated with vehicle, one group with the diuretic and vehicle, and one group with the diuretic and rofecoxib ($N = 10$). Therefore, furosemide was chronically infused subcutaneously for seven days via an osmotic pump with a dose of 12 mg/day (model 2ML1, ALZET Osmotic Pumps, Durect Corporation, Cupertino, CA, USA). The osmotic pumps were placed subcutaneously under general anesthesia with methohexital 50 mg/kg body wt (Lilly, Bad Homburg, Germany). Controls received saline-filled osmotic pumps. Hydrochlorothiazide (Novartis, Wehr, Germany) was dissolved in propylenglycol (Bufa Pharmaceutical Products, Uttgeest, Holland); for COX-2 inhibition, the commercially available oral suspension of rofecoxib was used (MSD, Haar, Germany). All drugs except furosemide were administered for five days with a total daily dose of 30 mg/kg body weight for hydrochlorothiazide and 10 mg/kg body weight for rofecoxib. They were applied orally by gavage twice per day.

Furthermore, for the dose response experiments, furosemide-treated rats received a daily dose of either vehicle or rofecocxib at 1, 3, or 10 mg/kg body weight ($N = 3$).

The animals were killed by decapitation, and blood was collected from the carotid arteries in ethylenediaminetetraacetic acid (EDTA) pretreated tubes. Kidneys were removed rapidly for dissection of the cortices, which were frozen in liquid nitrogen and stored at −80°C until extraction of total RNA.

All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Extraction of RNA

Total cortical RNA was extracted according to the acid-guanidinium-phenol-chloroform protocol described in detail by Chomczynski and Sacchi [21].

RNA pellets were dissolved in diethylpyrocarbonate-treated water. The yield of RNA was quantified by spectroscopy at 260 nm. Samples were aliquoted and stored at −80°C until further processing.

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

Renin, COX-2 and β-actin mRNA levels were measured by RNase protection assay basically as described previously [5, 22, 23]. In brief, after linearization and purification with phenol/chloroform, the plasmids yielded radiolabeled antisense cRNA transcripts by incubation with SP6 polymerase (Promega, Mannheim, Germany) and α-32 P-GTP (Amersham Pharmacia Biotech, Freiburg, Germany) according to the Promega riboprobe in vitro transcription protocol; 40 μg total RNA and 40 μg t-RNA (negative control) were hybridized with 500,000 cpm of the cRNA probes at 60°C overnight. Thereafter, RNase A/T1 (RT/30 min) and proteinase K (37°C for 30 min) were used for digestion. After phenol/chloroform extraction and ethanol precipitation, protected fragments were separated on an 8% polyacrylamide gel. The gel was dried for two hours. Bands were quantitated in a Phosphoimager (Instant Imager 2024; Packard, Meriden, CT, USA). Autoradiography was performed at −80°C for one day.

Determination of plasma renin activity

Plasma renin activity (PRA) was determined by a commercially available radioimmunoassay (DiaSorin, Düsseldorf, Germany).

Urinary electrolytes and osmolality

Urinary sodium and potassium concentrations were measured 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.

Urinary osmolality was measured by depression of the freezing point using a semi-micro osmometer (Knauer, Berlin, Germany).

Measurement of urinary excreted prostaglandins

By gas chromatography and mass spectroscopy, the concentrations of the renally excreted prostanoids, namely

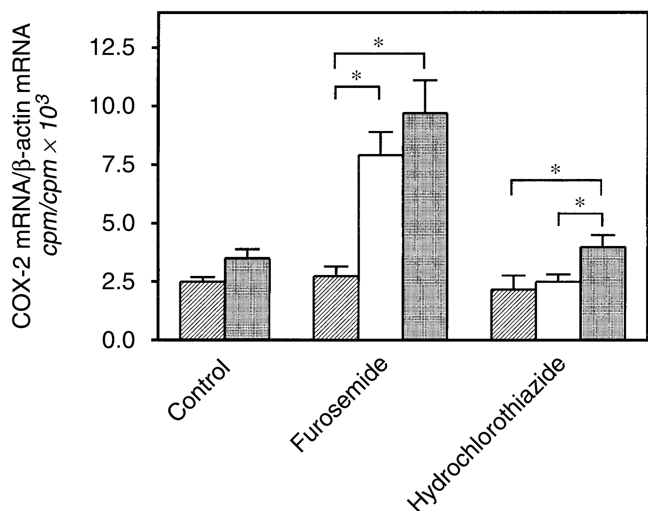

Fig. 1. COX-2 mRNA after seven days of treatment with furosemide or hydrochlorothiazide (5 days). Parallel groups were additionally treated with vehicle or rofecoxib (10 mg/kg body weight/day) for 5 days. Symbols are: (■) control (□) diuretic; (▨) additional rofecoxib. Data are means ± SEM, and the asterisk marks significant differences with $P < 0.05$.

of the PGE_2 group (PGE_2, PGE-M), the $PGF_{1\alpha}$ group ($6kPGF_{1\alpha}$, $d6kPGF_{1\alpha}$), and the thromboxane group (TXB_2, $2,3dTXB_2$, $11dhTXB_2$), were measured as described in detail previously [24].

Statistical analysis

Differences between the groups were analyzed by analysis of variance with multiple comparisons. *P* values less than 0.05 were considered significant.

RESULTS

Effect of diuretics and rofecoxib on renal COX-2 expression and prostaglandin excretion

The loop diuretic furosemide led to a significant increase of renocortical COX-2 mRNA expression (Fig. 1). In parallel with the induction of COX-2, renal excretion of prostaglandins was stimulated by furosemide two- to fivefold compared with controls (Fig. 2).

Inhibition of COX-2 with rofecoxib led to a rather enhanced stimulation of COX-2 mRNA expression during treatment with furosemide (Fig.1). In contrast, the furosemide-induced stimulation of prostanoid excretion was completely reversed by rofecoxib, thus confirming the effective inhibition of COX-2 (Fig. 2). To decrease the furosemide-induced prostanoid excretion significantly, rofecoxib doses higher than 3 mg/kg body weight were necessary (Fig. 3)

Hydrochlorothiazide, a blocker of NaCl cotransport in the convoluted part of the distal tubule, had no effect on basal COX-2 mRNA expression (Fig. 1). However, there was an increase in renal prostanoid excretion of the $PGF_{1\alpha}$ and TXB_2 groups (Fig. 2).

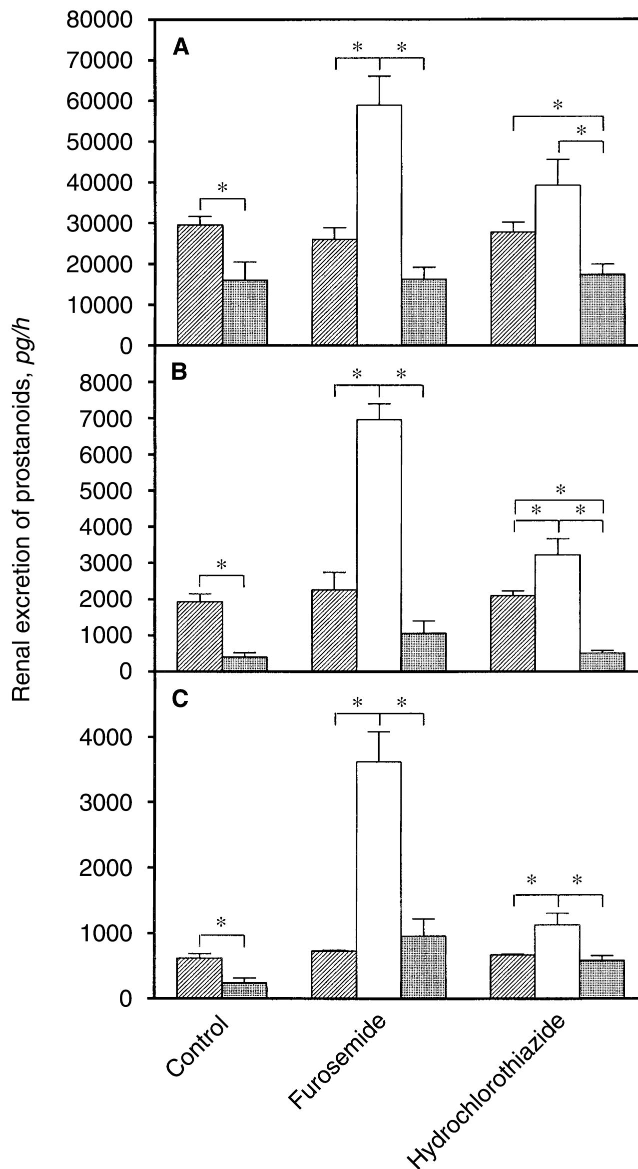

Fig. 2. Urinary prostaglandin excretion after seven days of treatment with furosemide or hydrochlorothiazide (5 days). (*A*) PGE_2 + metabolite; (*B*) $PGF_{1\alpha}$ + metabolites; (*C*) TXB_2 + metabolites. Parallel groups were additionally treated with vehicle or rofecoxib (10 mg/kg body weight/day) for 5 days. Symbols are: (■) control (□) diuretic; (▨) additional rofecoxib. Data are means ± SEM, and the asterisk marks significant differences with $P < 0.05$.

Additional rofecoxib administration during hydrochlorothiazide treatment caused an increase in COX-2 mRNA expression (Fig. 1) and blunted the stimulation of urinary prostanoid excretion. Prostanoid excretion was suppressed partially below control levels (Fig. 2).

Effect of rofecoxib on diuretic-induced salt and water excretion

Furosemide infusion increased urine production approximately fivefold, sodium excretion about 3.5-fold,

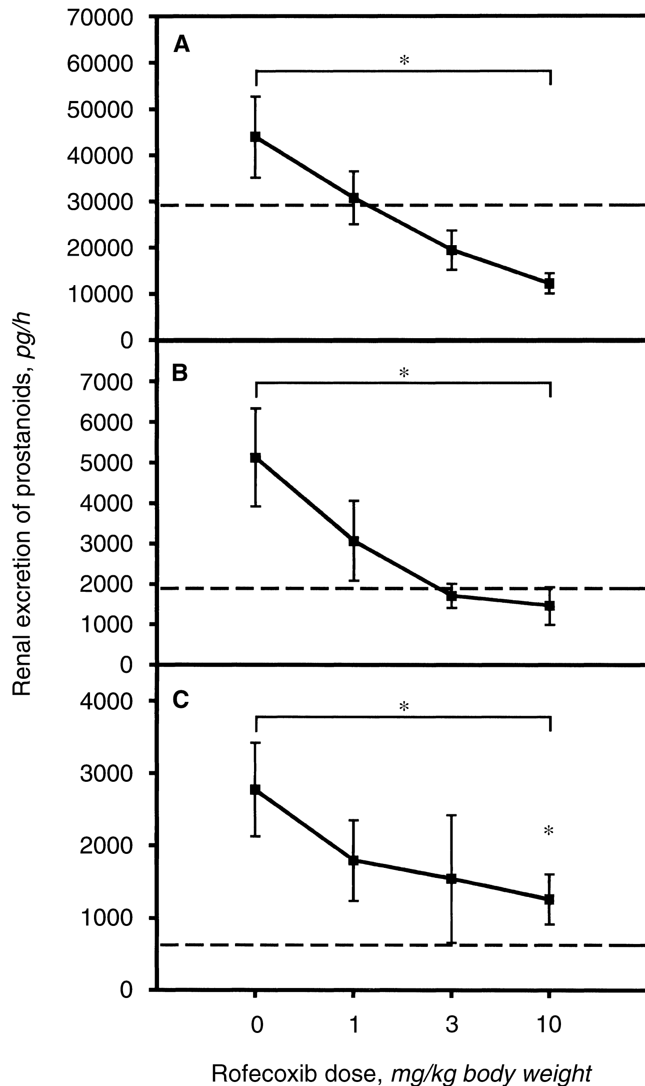

Fig. 3. Urinary prostaglandin excretion after seven days of furosemide infusion and additional treatment with vehicle or rofecoxib (1 to 10 mg/kg body weight/day) for five days. (*A*) PGE_2 + metabolites; (*B*) $PGF_{1\alpha}$ + metabolites; (*C*) TXB_2 + metabolites. Dotted lines represent values of vehicle-treated controls; data are means ± SEM, and the asterisk marks significant difference compared to control with $P < 0.05$.

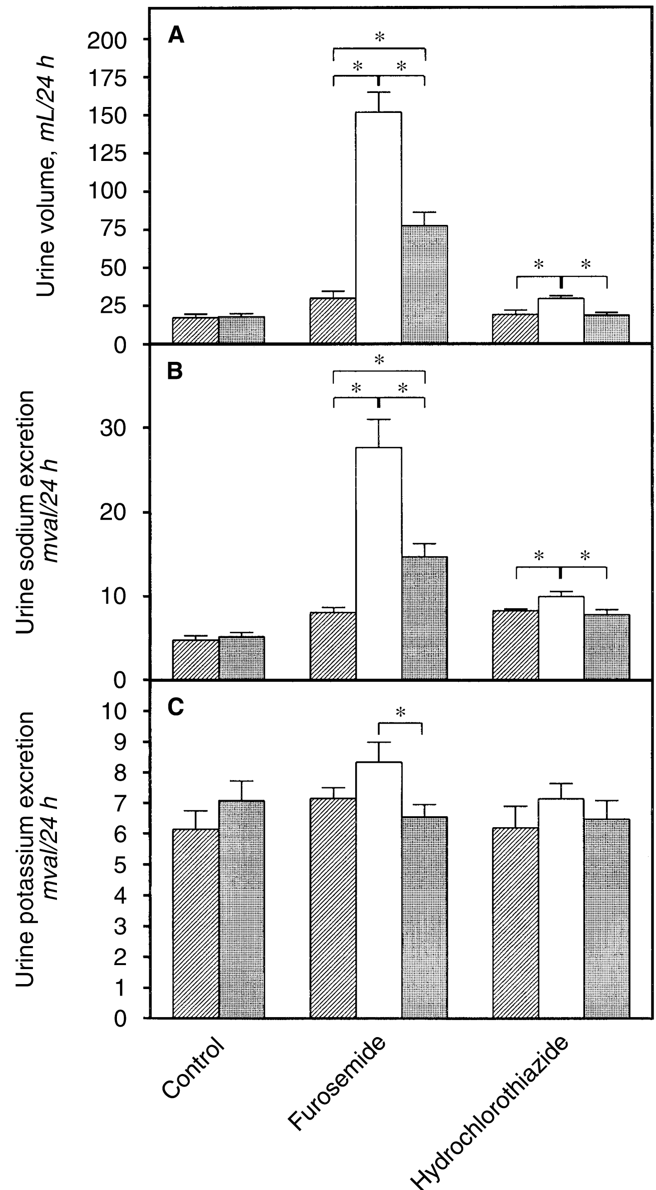

Fig. 4. Urine volume (*A*), urine sodium excretion (*B*), and urine potassium excretion (*C*) after seven days of treatment with furosemide or hydrochlorothiazide (5 days). Parallel groups were additionally treated with vehicle or rofecoxib (10 mg/kg body weight/day) for five days. Symbols are: (▥) control (□) diuretic; (▨) additional rofecoxib. Data are means ± SEM, and the asterisk marks significant differences with $P < 0.05$.

and potassium excretion by 15% (Fig. 4). When the rats were treated with furosemide in combination with the COX-2 blocker rofecoxib (10 mg/kg body weight/day), the urine, sodium, and potassium outputs were significantly reduced when compared with furosemide alone. Relative to untreated controls, urine output increased 2.5-fold and sodium excretion increased twofold, whereas potassium excretion remained normal with the combination of furosemide and rofecoxib (Fig. 4). Furosemide treatment also led to a marked fall of urine osmolality (Fig. 5). This effect of furosemide was not changed by rofecoxib. In further considering the dose dependency for rofecoxib on the diuretic effect of furosemide (Fig. 6), the half-maximal effect on salt and water retention occurred with a dose between 1 to 3 mg/kg per day of rofecoxib.

Hydrochlorothiazide increased urine and sodium output by about 50% and 20%, respectively, and did not change potassium excretion (Fig. 4). This increase of urine and sodium output was absent if the rats received hydrochlorothiazide in combination with rofecoxib (Fig. 4). Hydrochlorothiazide moderately lowered urine osmolality, an effect that was reversed by rofecoxib (Fig. 5).

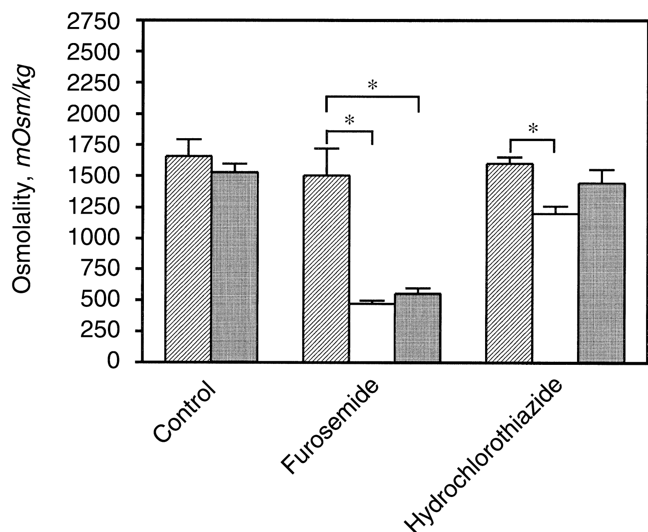

Fig. 5. Urine osmolality after seven days of treatment with furosemide or hydrochlorothiazide (5 days). Parallel groups were additionally treated with vehicle or rofecoxib (10 mg/kg body weight/day) for five days. Symbols are: (▩) control (□) diuretic; (▨) additional rofecoxib. Data are means ± SEM, and the asterisk marks significant differences with $P < 0.05$.

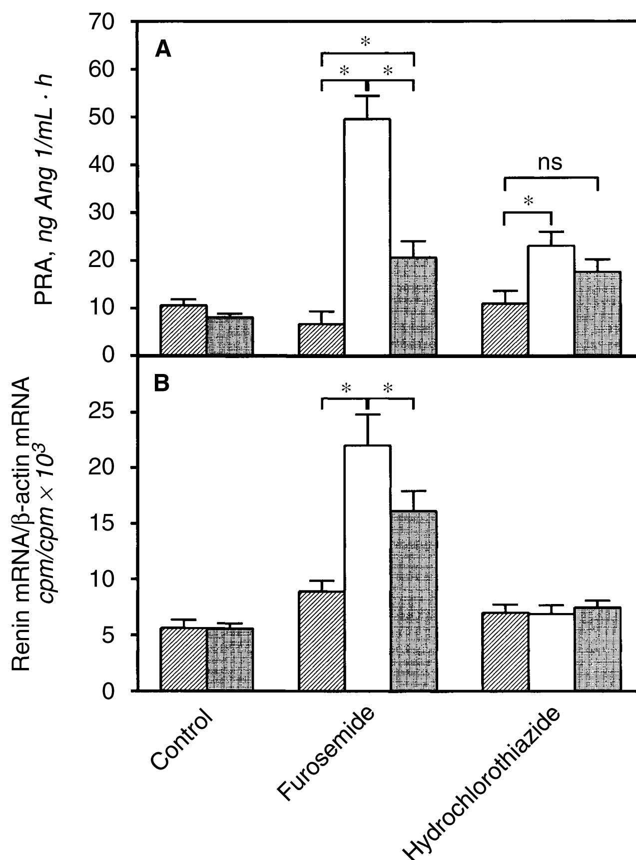

Fig. 7. Plasma renin activity (PRA) (*A*) and renin mRNA (*B*) after seven days of treatment with furosemide or hydrochlorothiazide (5 days). Parallel groups were additionally treated with vehicle or rofecoxib (10 mg/kg body weight/day) for five days. Symbols are: (▩) control (□) diuretic; (▨) additional rofecoxib. Data are means ± SEM, and the asterisk marks significant differences with $P < 0.05$; ns is not significant.

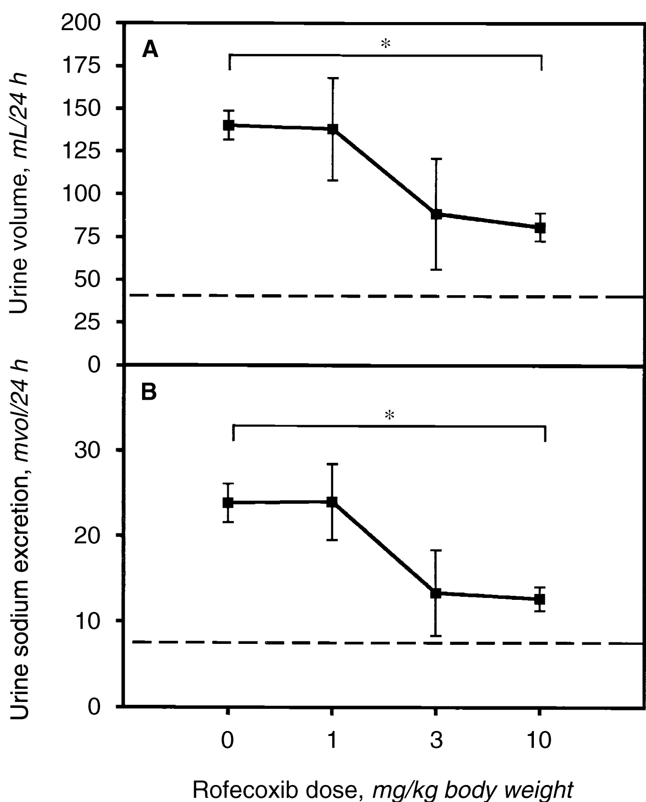

Fig. 6. Urine volume (*A*) and urine sodium excretion (*B*) after seven days of furosemide infusion and additional treatment with vehicle or rofecoxib (1 to 10 mg/kg body weight/day) for five days. Dotted lines represent values of vehicle-treated controls. Data are means ± SEM, and the asterisk marks significant difference compared to control with $P < 0.05$.

Effect of rofecoxib on diuretics induced stimulation of renin

Furosemide infusion also led to a sixfold increase in plasma renin activity and to a 2.5-fold increase in renal renin mRNA levels (Fig. 7). Rofecoxib significantly attenuated this stimulation of renin secretion and renin gene expression, but did not reduce these parameters to basal control levels. The effect of rofecoxib to attenuate the furosemide-induced stimulation of the renin system required doses greater than 3 mg/kg/day (Fig. 8).

Hydrochlorothiazide treatment increased plasma renin activity twofold without changing renal renin mRNA levels. Rofecoxib prevented the increase of PRA by hydrochlorothiazide (Fig. 7).

DISCUSSION

Since it is well known that inhibition of prostanoid formation attenuates the salt and water loss that occurs during treatment with loop diuretics or in the case of

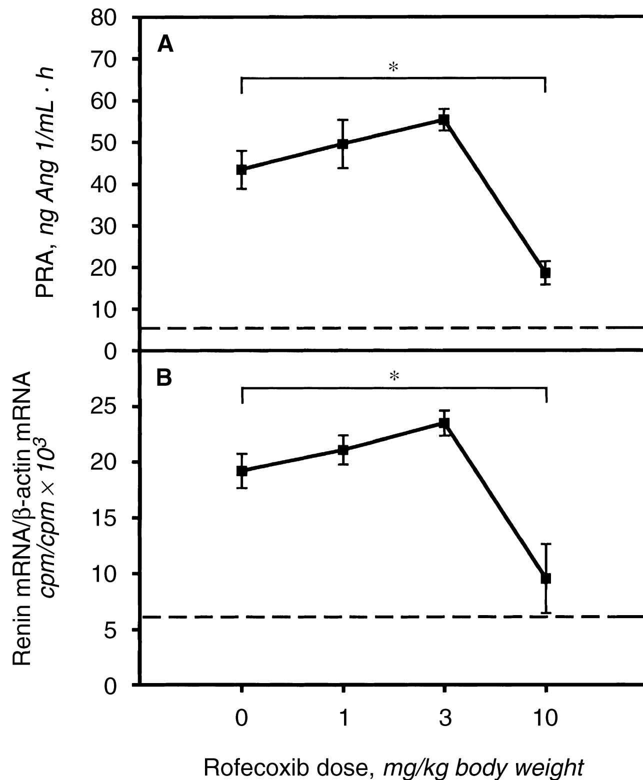

Fig. 8. PRA (*A*) and renin mRNA (*B*) after seven days of furosemide infusion and additional treatment with vehicle or rofecoxib (1 to 10 mg/kg body weight/day) for five days. Dotted lines represent values of vehicle-treated controls. Data are means ± SEM, and the asterisk marks significant difference compared with control with $P < 0.05$.

Bartter disease, this study aimed to determine the role of cyclooxygenase-2 in this context. This question was interesting because the kidneys of different species, including humans and rats, express both the COX-1 and COX-2 isoforms [7, 8]. The role of COX-2–derived prostanoids has gained much interest during the last years, since COX-2 activity appears to be required for normal renal development [25] and for the stimulation of the renin system under a variety of conditions [13–15]. COX-2 is evidently expressed in the cortical thick ascending limb of Henle (TALH), including the macula densa region and in the collecting duct system [9, 10]. The expression of COX-2 in the TALH and the macula densa, moreover, is subject to regulation in the way that it is up-regulated during a low-salt diet, renal hypoperfusion, and treatment with angiotensin II antagonists, and down-regulated in states of salt overload [9, 10, 14, 15, 22, 26]. It has been suggested that the chloride concentration in the TALH cells is an important regulator of COX-2 expression [27]. In line with this hypothesis, we previously reported that inhibition of salt transport in the TALH by the loop diuretic furosemide up-regulates COX-2 expression in the TALH and in the macula densa [16], thus providing a possible explanation for the enhancement of renal prostaglandin output and stimulation of renin secretion by furosemide.

Our data now show that the highly selective COX-2 inhibitor, rofecoxib, in fact markedly attenuates the stimulation of the renin system by furosemide, suggesting that the up-regulation of COX-2 in the macula densa and consequent up-regulation of COX-2–derived prostaglandins mediate the stimulation of renin secretion and of renin synthesis induced by loop diuretics. A similar conclusion was reached previously using the less selective COX-2 inhibitor, meloxicam, in humans [11].

In parallel, the COX-2 blocker markedly attenuated diuresis and saluresis by furosemide, suggesting that inhibition of COX-2–derived prostaglandins mainly accounts for the salt- and water-sparing effects of nonselective COX inhibitors during treatment with loop diuretics or during Bartter disease [5, 6, 11]. This finding is in accordance with a preliminary report that the COX-2 inhibitor nimesulide attenuates salt and water loss in Bartter patients (abstract; Nüsing et al, *Eur J Clin Pharmacol* 55:A10, 1999).

Since both inhibition of saluresis/diuresis and of the renin system depend on the dose of rofecoxib, these parameters could serve as possible markers for adjusting the therapeutic dose in patients with Bartter disease.

The well-known fall of urine osmolality induced by inhibition of salt transport in the TALH was not influenced by COX-2 inhibition. This is in accordance with an action of prostaglandins in the distal nephron, and indicates that COX-2–derived prostanoids are not involved in the breakdown of the corticomedullary osmolality gradient induced by loop diuretics [28]. Considering the enhancement of COX-2 expression and renal prostaglandin production by impaired salt transport in the TALH, it is tempting to speculate that the diuretic and natriuretic actions of furosemide comprise two effects, namely, a direct COX-2–independent inhibition of salt transport at the level of TALH and an increased formation of COX-2–derived prostaglandins leading to inhibition of salt transport at the level of the collecting duct, which is consistent with the expression of COX-2 in the cortical thick ascending limb and the macula densa [9].

Our findings also show that the diuretic and saluretic effects of hydrochlorothiazide, which inhibits the NCC in the distal tubule [18], are completely reversed by COX-2 inhibition. Compared with furosemide, however, hydrochlorothiazide leads to a weaker enhancement of renal prostaglandin excretion. Furthermore, no stimulation of cortical COX-2 expression by hydrochlorothiazide was found. We assume that inhibition of NCC does not stimulate the formation of renal COX-2–derived prostanoids. The antidiuretic and antisaluretic effects of rofecoxib during hydrochlorothiazide treatment may result from either preventing the normal action of COX-2–derived prostanoids or from a reversed stimulation of systemic

COX-2–dependent prostanoid formation or flow-dependent prostanoid excretion [29]. Thus, rofecoxib could reverse the effect of hydrochlorothiazide by increasing salt and water reabsorption in tubular cells. Obvious target cells are as outlined before: the collecting duct cells, which themselves express COX-2 and, therefore, may modulate their ability for salt and water reabsorption through prostaglandins acting in an autocrine or paracrine fashion. In addition, inhibition of COX-2–derived prostanoids might increase salt reabsorption in the TALH, resulting in a decreased salt and water load of the distal nephron [30].

In any case, it appears from our findings that an enhanced renal COX-2 expression and subsequent enhanced prostanoid formation are not prerequisites for COX-2 inhibitors to attenuate diuresis and saluresis. A similar conclusion also holds for the stimulation of the renin system in states of renal salt and water wasting. The stimulation of the renin system by furosemide, which increases COX-2 expression in the macula densa, is attenuated by the COX-2 inhibitor. Furthermore, stimulation of the renin system by hydrochlorothiazide, which does not change COX-2 expression, was prevented by rofecoxib. A possible explanation for this phenomenon is that COX-2 activity—but not COX-2 protein expression—in the macula densa is stimulated during treatment with hydrochlorothiazide. A more provocative explanation could be that the effects of the COX-2 inhibitor on the renin system are more related to the salt and water balance of the organism, which possibly is influenced by systemic COX-2–dependent prostanoids, rather than a direct effect on macula densa cells. This explanation is suggested by the parallel changes of salt and water excretion and plasma renin activity induced by the COX-2 blocker. Although we did not find a significant change of the extracellular volume during treatment with furosemide [16], it is likely that a salt/volume sensor is highly activated during treatment with the diuretic, since the rats drank exactly as much of free water and of salt water as they excreted through the kidney. It is thought that this volume sensor regulates salt and water uptake as well as the activity of the renin system [31]. With this view in mind, we cannot exclude that the effects of the COX-2 inhibitor on the renin system could be secondary to the salt- and water-sparing effects.

Regardless of the detailed mechanisms by which COX-2 blockers inhibit salt and water secretion and renin secretion in states of renal salt and water wasting, our data suggest that COX-2 blockers might be useful drugs for treating patients with Bartter disease and Gitelman disease, if the latter suffer from a significant loss of body volume. A preliminary study with a single Bartter patient in fact supports this conclusion (unpublished observations).

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