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# Everolimus treatment downregulates renocortical cyclooxygenase-2 expression in the rat kidney

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**1** Based on recent evidence that renal cyclooxygenase-2 (COX-2) gene expression is suppressed by immunosuppressive agents such as cyclosporin A (CsA), tacrolimus and dexamethasone, this study aimed to characterize the effect of the new immunosuppressant everolimus on COX-2 expression in the rat kidney.

**2** Oral application of everolimus ($3\,mg\,kg^{-1}\,day^{-1}$) to male Sprague–Dawley rats (175–200 g; $n=8$) for 7 days lowered COX-2 expression in the rat renal cortex and outer medulla, while COX-2 expression in the inner medulla as well as COX-1 expression remained unaltered. Furthermore, everolimus decreased renocortical prostaglandin (PG) $E_2$ concentration.

**3** Everolimus also attenuated the stimulation of renocortical COX-2 expression by furosemide ($12\,mg\,day^{-1}$ for 7 days; s.c. *via* osmotic minipumps), by low salt intake (0.02% NaCl, wt $wt^{-1}$) or by a combination of low salt intake with the $AT_1$-receptor antagonist valsartan ($30\,mg\,kg^{-1}\,day^{-1}$; oral). In line with these findings, everolimus decreased renocortical $PGE_2$ concentration during these treatment maneuvers.

**4** Everolimus moderately increased natriuresis and diuresis, while the urinary excretion of $PGE_2$, 6-keto $PGF_{1\alpha}$ and thromboxane $B_2$ was decreased.

**5** These findings suggest that everolimus inhibits basal and also stimulated expression of renocortical COX-2 and of tissue prostanoid formation. Since inhibition of renal prostanoid formation by everolimus was associated by an increased rather than decreased natriuresis and diuresis, it appears as if everolimus also inhibits tubular salt and water resorption.
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**Abbreviations:** ANG-II, angiotensin II; BW, body weight; COX, cyclooxygenase; CsA, cyclosporin A; GFR, glomerular filtration rate; mTOR, mammalian target of rapamycin; PG, prostaglandin; TALH, thick ascending limb of Henle; Tx, thromboxane

## Introduction

Prostaglandins (PGs) are formed by the action of cyclooxygenase (COX). Two isoforms of COX have been identified, COX-1 and COX-2. Within the kidney, COX-1 is localized in mesangial cells, arteriolar endothelial cells, pariethal epithelial cells of Bowman's capsule and cortical and medullary collecting ducts (Harris & Breyer, 2001; Krämer *et al*., 2004). COX-2 is also constitutively expressed in the kidney**,** where it is localized in glomeruli, in macula densa cells, cortical thick ascending limb of Henle (cTALH) cells and in medullary interstitial cells (Harris & Breyer, 2001; Krämer *et al*., 2004). The molecular signalling pathways triggering COX-2 expression in TALH and medullary interstitial cells are only poorly understood. It has been demonstrated that the expression of COX-2 in the TALH and in macula densa cells is physiologically regulated by the salt intake, the renal perfusion pressure and by angiotensin II (ANG-II) (Harris *et al*., 1994; Höcherl *et al*., 2001; Mann *et al*., 2001). Convincing evidence suggests that COX-derived prostanoids could be involved in the regulation of renin synthesis and secretion in the juxtaglomerular apparatus, as well as in tubular salt and water handling (Harris & Breyer, 2001; Krämer *et al*., 2004). Findings with selective COX-2 blockers suggest that inhibition of COX-2-derived prostanoids cause potassium and sodium retention, vasoconstriction of the afferent arterioles and reduction of glomerular filtration rate (GFR) (Harris & Breyer, 2001; Komers *et al*., 2001; Krämer *et al*., 2004); typical adverse effects that are also seen with the calcineurin inhibitor cyclosporin A (CsA) (Myers *et al*., 1984; Olyaei *et al*., 1999). The precise mechanism of CsA-induced nephrotoxicity is still unknown, but it is thought to involve an imbalance of a number of mediators including endothelins, thromboxane (Tx), prostaglandins, angiotensin II, nitric oxide and renal nerve activity (Ader & Rostaing, 1998; Darlametsos & Varonos, 2001).

Recent research has been conducted on developing other compounds to improve immunosuppression either as replacements for, or to work in synergy with, CsA. Everolimus (SDZ-RAD) is a derivative of rapamycin. Everolimus and

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rapamycin inhibit the proliferation of T cells by a different mechanism than CsA. CsA inhibits the $Ca^{2+}$ and calmodulin-dependent phosphatase calcineurin, leading to an inhibition of the activation of the transcription factor nuclear factor of activated T cells, which in turn blocks interleukin-2-mediated signal transduction pathways. In contrast, everolimus inhibits p70 S6 kinase activation (Nashan, 2001). The distinct differences in the mechanism of T-cell inhibition have also been suggested to result in a better renal side effect profile. However, high doses of rapamycin have also been shown to impair renal function in rats (DiJoseph *et al*., 1994). Therefore, we were interested in the effect of the new immunosuppressant everolimus on renal COX-isoform expression and renal function.

## Methods

### *Materials*

Everolimus (SDZ-RAD, a rapamycin derivative) and valsartan were kind gifts from Novartis AG (Basel, Switzerland). Furosemide was purchased from Sigma (Deisenhofen, Germany).

### *Animals*

Male Sprague–Dawley rats (175–200 g), obtained from Charles River (Sulzfeld, Germany), were housed in cages in a temperature- and light-controlled environment. The animals, which were maintained *ad libitum* with free access to tap water, were weighted and examined every day. Animals were divided into eight groups of eight rats each and were given vehicle or everolimus ($3\,mg\,kg^{-1}\,day^{-1}$) orally by a stomach tube and fed either a normal salt diet (0.6% NaCl wt $wt^{-1}$; Altromin, Lage, Germany), a low salt diet (0.02% NaCl wt $wt^{-1}$; Ssniff special diets, Soest, Germany), a combination of low salt diet with the $AT_1$-receptor antagonist valsartan ($30\,mg\,kg^{-1}\,day^{-1}$, orally by a stomach tube) or were chronically infused with the loop diuretic furosemide ($12\,mg\,day^{-1}$, subcutaneously *via* osmotic mini pumps; model 2ML1, ALZET Osmotic Pumps, Durect Corporation, Cupertino, CA, U.S.A.) for 1 week. Osmotic mini pumps were implanted subcutaneously under the skin of the neck during sevoflourane anaesthesia (3%, vol. $vol.^{-1}$). Furosemide-treated rats received in addition to tap water a solution containing 0.9% sodium chloride and 0.1% potassium chloride to compensate for salt and water loss.

The oral dose of everolimus used in this study has previously been shown to prevent kidney rejection after transplantation in rats (Schuurman *et al*., 1997).

Systolic blood pressure (SBP) and heart rate (HR) measurements were performed (tail cuff method) prior to treatment and every second day. For urinary measurements, rats were housed individually in metabolic cages during the last 2 days of treatment. At the end of the study period, the final doses were given 2 h prior to decapitation of rats during sevoflurane anaesthesia (3%, vol. $vol.^{-1}$). Blood was collected into tubes containing EDTA. The kidneys were quickly removed and were cut in longitudinal halves. Kidney zones were dissected with a scalpel blade under a stereomicroscope, 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 and German laws relating to the protection of animals and were approved by the local ethics committee.

### *Extraction of RNA*

Total RNA was extracted from renal cortex, outer medulla and inner medulla, basically according to the acid-guanidinium-phenol-chloroform protocol of Chomczynski & Sacchi (1987). RNA pellets were dissolved in diethylpyrocarbonate-treated water, the yield of RNA was quantified by spectroscopy at 260 nm, and aliquot samples were stored at −80°C until further processing.

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

Cytoplasmic $\beta$-actin, COX-1 and COX-2 mRNA were determined by specific RNase protection assays as described previously (Jensen & Kurtz, 1997). In brief, after linearization and phenol/chloroform purification, the plasmids yielded radiolabelled antisense cRNA transcripts by incubation with SP6 polymerase (Promega) and $\alpha$-$^{32}$P-GTP (Amersham-Pharmacia) according to the Promega riboprobe *in vitro* transcription protocol. cRNA probes ($5 \times 10^5$ c.p.m.) were hybridized with 100 $\mu$g of total RNA (renal cortex and outer medulla, COX-1 and COX-2), 10 $\mu$g of total RNA (inner medulla, COX-1 and COX-2), 1 $\mu$g of total RNA ($\beta$-actin) and 20 $\mu$g of tRNA (negative control) at 60°C overnight and then digested with RNase A/T1 (room temperature/30 min) and proteinase K (37°C/30 min). After phenol/chloroform extraction and ethanol precipitation, protected fragments were separated on an 8% polyacrylamide gel. The gel was dried for 2 h, and bands were quantified in a Phosphoimager (Instant Imager 2024, Packard). Autoradiography was performed at −80°C for 1–3 days. The abundance of COX-1 and COX-2 is presented relative to the level of $\beta$-actin mRNA as a standard.

### *Determination of prostanoids*

Urinary prostanoid excretion ($TxB_2$, 6-keto $PGF_{1\alpha}$ and $PGE_2$) was assayed by using monoclonal EIA kits (Cayman Chemical, Ann Arbor, U.S.A.). Tissue levels of $PGE_2$ were assayed as described previously (Höcherl *et al*., 2002c, d).

### *Determination of urinary sodium and potassium*

Urinary sodium and potassium levels were determined by flame-photometry as described previously (Höcherl *et al*., 2004).

### *Determination of creatinine and urea*

Plasma and urinary concentrations of creatinine were measured with the Jaffe reaction as described previously (Höcherl *et al*., 2004). Plasma and urinary concentrations of urea were determined by a commercially available kinetic UV test (Ecoline® 25, Diagnostica Merck KGaA, Darmstadt, Germany).

*Immunoblotting for COX-1 and COX-2 protein*

COX-2 and COX-1 immunoblotting were performed as described previously (Höcherl *et al*., 2002c; 2004). In total, 100 $\mu$g protein of the renal cortex or the outer medulla or 20 $\mu$g protein of the inner medulla were loaded per lane, separated by a 10% Tris-HCl gel and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked overnight with 5% nonfat dry milk in Tris-buffered saline at 4°C and incubated for 2 h 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-labelled secondary antibody (goat anti-rabbit IgG; 1 : 500; Santa Cruz). Detection was achieved by enhanced chemiluminescence (Amersham). The band intensities (COX-2 72 kDa; COX-1 70 kDa) were quantified densitometrically.

*COX-2 immunoreactivity*

COX-2 immunoreactivity was performed as described previously (Mann *et al*., 2001). In brief, sections were layered with the primary antibody (dilution 1 : 500; M19; Santa Cruz Biotechnology, Ann Arbor, U.S.A.) and incubated at 4°C overnight. After addition of the secondary antibody (dilution 1 : 500; biotin-conjugated, rabbit anti-goat IgG), the sections were incubated with streptavidin D horseradish peroxidase complex (Vectastain DAB kit; Vector Laboratory) and exposed to 0.1% diaminobenzidine tetrahydrochloride and 0.02% $H_2O_2$ as the source of peroxidase substrate. Each slide was counterstained with hematoxylin-eosin. As a negative control, we used the same dilutions of preimmune goat serum (for the primary antibody) or normal rabbit IgG (for the second antibody).

Quantification of COX-2 immunoreactivity was performed by counting the glomeruli with an adjacent juxtaglomerular apparatus staining positive for COX-2. The identity of the tissue used for counting was coded, and counting was performed by persons who were not involved in animal handling or tissue fixation in order to assure nonbias. In each kidney (left and right), 250–300 glomeruli were counted, and the number of positive glomeruli was expressed as a percentage of the total number of glomeruli counted in the section. These percentage values were used for statistical analysis.

*Statistical analysis*

Level of significance was calculated by one-way ANOVA followed by Student's *t*-test. A *P*-value $<0.05$ was considered significant.

## Results

*Effect of everolimus on SBP, HR, hematocrit, daily body weight (BW) gain, food intake, water intake and plasma creatinine and urea levels*

SBP during normal salt intake was $131\pm5$ mmHg. Low salt intake and furosemide treatment did not change SBP, but the combination of low salt diet with valsartan clearly decreased SBP to $91\pm4$ mmHg ($P<0.05$). Additional treatment with everolimus had no effect on SBP compared with the respective control groups (Table 1).

HR during normal salt intake was $389\pm7$ beats per minute (b.p.m.) and did not change during low salt intake. The combination of low salt intake with valsartan and furosemide treatment increased HR to $423\pm8$ ($P<0.05$) and $428\pm9$ b.p.m. ($P<0.05$), respectively. Everolimus had no effect on HR compared with the respective controls (Table 1).

Hematocrit during normal salt intake was $38\pm1$% and was not affected by low salt intake, furosemide treatment or the combination of low salt intake with valsartan. Everolimus increased hematocrit during normal salt intake, low salt diet, furosemide or the combination of low salt intake with valsartan to $41\pm1$ ($P<0.05$), $42\pm1$ ($P<0.05$), $42\pm1$ ($P<0.05$) and $43\pm1$% ($P<0.05$), respectively (Table 1).

Daily BW gain during normal salt intake was $7.3\pm0.2$ g day$^{-1}$ and was not affected by low salt intake or furosemide treatment. Daily BW gain was reduced in rats receiving the combination of low salt diet with valsartan to $5.0\pm0.2$ g day$^{-1}$ ($P<0.05$). Rats treated with everolimus showed a reduced weight gain in all treatment groups (Table 1). Daily BW gain in everolimus-treated rats was decreased during low salt intake ($P<0.05$), the combination of low salt intake with valsartan ($P<0.05$) and during furosemide treatment ($P<0.05$). No signs of diarrhea occurred in everolimus-treated rats.

Daily food intake during normal salt intake was $22.5\pm1.0$ g day$^{-1}$ and was not affected by low salt intake, the

**Table 1** Effect of everolimus on SBP, HR, hematocrit (Hct), plasma creatinine levels, plasma urea levels, daily food intake, daily water intake and daily BW gain

| | *SBP* (mmHg) | *HR* (bpm) | *BW gain* (g day$^{-1}$) | *Hct* (%) | *Plasma creatinine* (mg dl$^{-1}$) | *Plasma urea* (mg dl$^{-1}$) | *Food intake* (g d$^{-1}$) | *Water intake* (ml day$^{-1}$ kg$^{-1}$ BW) |
|---|---|---|---|---|---|---|---|---|
| Normal salt | $131\pm5$ | $389\pm7$ | $7.3\pm0.2$ | $38\pm1$ | $0.82\pm0.04$ | $23\pm2$ | $22.5\pm1.0$ | $163\pm4$ |
| Normal salt + everolimus | $134\pm5$ | $400\pm9$ | $4.2\pm0.4$* | $41\pm1$* | $1.05\pm0.04$* | $29\pm3$ | $18.3\pm0.4$* | $196\pm6$* |
| Low salt | $127\pm4$ | $407\pm8$ | $6.3\pm0.6$ | $37\pm1$ | $0.81\pm0.03$ | $22\pm1$ | $22.6\pm0.5$ | $138\pm4^{\dagger}$ |
| Low salt + everolimus | $130\pm4$ | $403\pm11$ | $2.2\pm0.7$*$^{\#}$ | $42\pm1$* | $1.10\pm0.05$* | $44\pm2$*$^{\#}$ | $19.5\pm1.0$* | $314\pm22$*$^{\#}$ |
| Low salt + valsartan | $91\pm4^{\dagger\ddagger}$ | $423\pm8^{\dagger\ddagger}$ | $5.0\pm0.2^{\dagger}$ | $37\pm1$ | $0.98\pm0.03^{\dagger}$ | $30\pm3$ | $21.9\pm0.7$ | $132\pm3^{\dagger}$ |
| Low salt + valsartan + everolimus | $96\pm5^{\#+}$ | $422\pm10$ | $2.4\pm0.6$*$^{\#}$ | $43\pm1$* | $1.49\pm0.10$*$^{\#+}$ | $83\pm6$*$^{\#+}$ | $17.2\pm1.4$* | $171\pm3$*$^{\#+}$ |
| Furosemide | $124\pm5$ | $428\pm9^{\dagger}$ | $6.4\pm0.6$ | $38\pm1$ | $0.87\pm0.02$ | $25\pm1$ | $22.2\pm1.2$ | $720\pm21^{\dagger}$ |
| Furosemide + everolimus | $131\pm4$ | $433\pm11^{\#}$ | $2.3\pm0.6$*$^{\#}$ | $42\pm1$* | $0.97\pm0.04$ | $32\pm2$ | $17.3\pm0.6$* | $936\pm53$*$^{\#}$ |

Results are represented as mean$\pm$s.e.m. of eight rats per group. *$P<0.05$ compared to the respective control. $^{\dagger}P<0.05$ vs normal salt diet. $^{\ddagger}P<0.05$ vs low salt diet. $^{\#}P<0.05$ vs normal salt intake and everolimus, $^{+}P<0.05$ vs low salt intake and everolimus.

combination of low salt diet with valsartan or furosemide treatment. Everolimus reduced daily food intake during normal salt intake, low salt intake, low salt diet in combination with valsartan or furosemide treatment to $18.3 \pm 0.4$ ($P<0.05$), $19.5 \pm 1.0$ ($P<0.05$), $17.2 \pm 1.4$ ($P<0.05$) and $17.3 \pm 0.6$ g day$^{-1}$ ($P<0.05$), respectively (Table 1).

Daily water intake during normal salt intake was $163 \pm 4$ ml day$^{-1}$ kg$^{-1}$ BW. Daily water intake was decreased by low salt intake and by low salt diet in combination with valsartan to $138 \pm 4$ ($P<0.05$) and $132 \pm 3$ ml day$^{-1}$ kg$^{-1}$ BW ($P<0.05$), respectively. Furosemide treatment increased daily water intake to $720 \pm 21$ ml day$^{-1}$ kg$^{-1}$ BW ($P<0.05$). Additional treatment with everolimus increased daily water intake during normal salt intake, low salt intake, low salt diet in combination with valsartan and furosemide treatment to $196 \pm 6$ ($P<0.05$), $314 \pm 22$ ($P<0.05$), $171 \pm 3$ ($P<0.05$) and $936 \pm 53$ ml day$^{-1}$ kg$^{-1}$ BW ($P<0.05$), respectively (Table 1). Daily water intake in everolimus-treated rats was increased during furosemide treatment ($P<0.05$) and by low salt intake ($P<0.05$). Additional treatment with valsartan attenuated the increase in fluid intake by low salt intake and everolimus ($P<0.05$).

Plasma creatinine concentration during normal salt intake was 0.82 mg dl$^{-1}$ and did not alter during low salt intake or chronic furosemide infusion. The combination of low salt diet with valsartan increased plasma creatinine concentration to $0.98 \pm 0.03$ mg dl$^{-1}$ ($P<0.05$). Additional treatment with everolimus increased plasma creatinine levels during normal salt intake, low salt intake, low salt diet in combination with valsartan or furosemide treatment to $1.05 \pm 0.04$ ($P<0.05$), $1.10 \pm 0.05$ ($P<0.05$), $1.49 \pm 0.10$ ($P<0.05$) and $0.97 \pm 0.04$ mg dl$^{-1}$ ($P<0.05$), respectively (Table 1). Plasma creatinine concentration in everolimus-treated rats increased during the combination of low salt with valsartan ($P<0.05$).

Plasma urea concentration during normal salt intake was $23 \pm 2$ mg dl$^{-1}$ and was not altered by low salt diet, by the combination of low salt diet with valsartan or by furosemide treatment. Everolimus did not alter plasma urea concentration during normal salt intake and furosemide treatment. Additional treatment with everolimus increased plasma urea concentration during low salt intake and low salt diet in combination with valsartan to $44 \pm 2$ ($P<0.05$) and $83 \pm 6$ mg dl$^{-1}$ ($P<0.05$), respectively (Table 1).

### *Effect of everolimus on COX isoform expression during normal salt intake*

COX-1 (Figure 1a) and COX-2 (Figure 1b) mRNA levels ((c.p.m. c.p.m.$^{-1}$) $10^3$) clearly increased from the rat renal cortex to the inner medulla in control rats. COX-1 mRNA abundance during normal salt intake was not influenced by everolimus in the renal cortex, outer and inner medulla (Figure 1a). In contrast, the levels of COX-2 mRNA of animals receiving normal salt intake and everolimus were reduced in the renal cortex from $2.0 \pm 0.1$ to $1.3 \pm 0.1$ ($P<0.05$) and in the outer medulla from $3.4 \pm 0.3$ to $2.1 \pm 0.3$ ($P<0.05$). In the inner medulla, COX-2 mRNA levels were not altered by everolimus treatment (Figure 1b). The decrease of COX-2 mRNA abundance was paralleled by a decrease of COX-2 immunoreactive protein levels (expressed as fold of control). In animals receiving everolimus, COX-2 protein in the renal cortex decreased from $1.0 \pm 0.2$ to $0.4 \pm 0.2$ ($P<0.05$) and in the outer medulla from $1.5 \pm 0.3$ to $0.7 \pm 0.2$ ($P<0.05$). COX-2 protein level in the inner medulla was not altered by

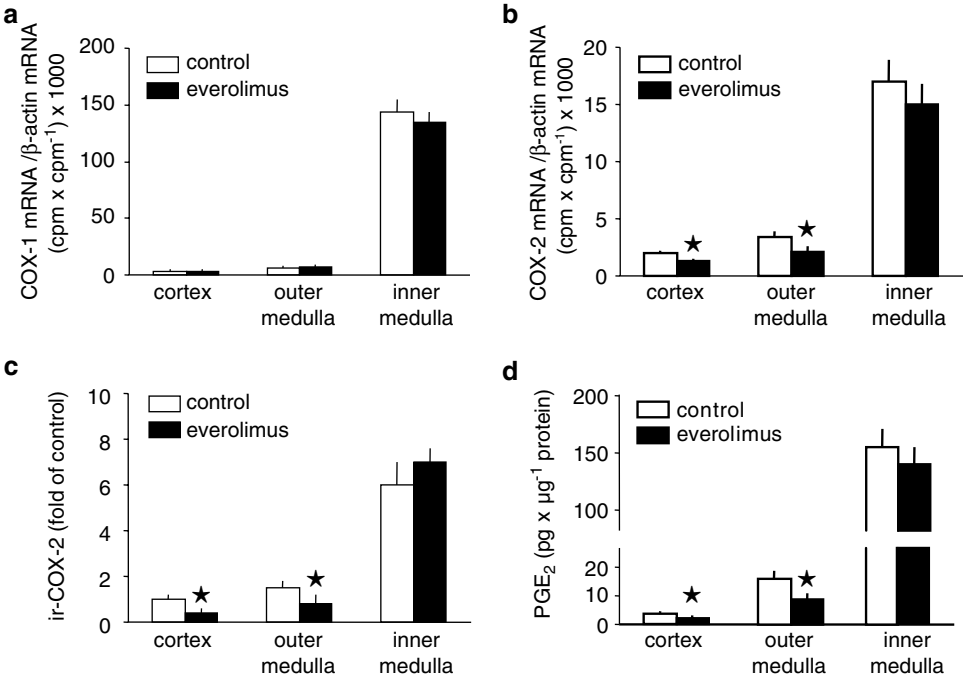


**Figure 1** Effect of everolimus (3 mg kg$^{-1}$ day$^{-1}$) on cyclooxygenase (COX)-1 mRNA abundance (a), COX-2 mRNA abundance (b), COX-2 protein (c) expression and prostaglandin $E_2$ ($PGE_2$) tissue concentration (d) in the rat renal cortex, outer and inner medulla during normal salt intake (0.6% NaCl, wt wt$^{-1}$). Data represent the mean ± s.e.m. of $n=8$ rats. *$P<0.05$ vs respective control.

everolimus (Figure 1c). Similar to the data obtained for COX-2 expression, renocortical tissue $PGE_2$ level ($pg\,\mu g^{-1}$ protein) was decreased in animals receiving everolimus from $3.5\pm0.2$ to $2.1\pm0.2$ ($P<0.05$) (Figure 1d). Further, tissue level of $PGE_2$ in the outer medulla decreased from $16.2\pm2.6$ to $9.0\pm2.5$ ($P<0.05$). Everolimus did not alter prostanoid concentration in the inner medulla (Figure 1d).

### *Effect of everolimus on renocortical COX-2 expression during low salt intake, a combination of low salt intake with the $AT_1$ receptor antagonist valsartan or chronic furosemide infusion*

In subsequent studies, we examined the effect of everolimus on typical stimulations of renocortical COX-2 expression. Renocortical COX-1 expression was not influenced by a low salt diet, by a combination of low salt diet with valsartan or by chronic furosemide infusion for 1 week. Additional treatment with everolimus did not affect renocortical COX-1 expression during these treatment manoeuvres (Figure 2a). Renocortical COX-1 protein was not altered by any treatment maneuvres (data not shown). In contrast, low salt diet, the combination of low salt diet with valsartan and furosemide infusion increased renocortical COX-2 mRNA ((c.p.m. c.p.m.$^{-1}$) $10^3$) levels from $2.0\pm0.2$ to $3.4\pm0.2$ ($P<0.05$), $15\pm2$ ($P<0.05$) and $4.0\pm0.3$ ($P<0.05$), respectively (Figure 2b). Everolimus attenuated the stimulations of renocortical COX-2 mRNA abundance by low salt intake, a combination of low salt intake with valsartan and furosemide to $1.8\pm0.2$ ($P<0.05$), $5.0\pm0.8$ ($P<0.05$) and $2.2\pm0.3$ ($P<0.05$), respectively (Figure 2b). Furosemide and the combination of low salt intake with valsartan increased COX-2 mRNA in everolimus-treated rats (Figure 2b). The alterations in renocortical COX-2 mRNA were paralleled by changes in COX-2 immunoreactive protein (fold of control). Renocortical COX-2 immunoreactive protein increased during low salt intake, the combination of low salt intake with valsartan and furosemide treatment to $1.5\pm0.2$ ($P<0.05$), $4.0\pm0.4$ ($P<0.05$) and $2.0\pm0.3$ ($P<0.05$), respectively (Figure 2c). Additional treatment with everolimus during low salt intake, the combination of low salt intake with valsartan and furosemide treatment lowered renocortical COX-2 protein levels to $0.9\pm0.1$ ($P<0.05$), $2.5\pm0.4$ ($P<0.05$) and $1.1\pm0.2$ ($P<0.05$), respectively (Figure 2c). Furosemide and the combination of low salt intake with valsartan increased COX-2 protein levels in everolimus-treated rats (Figure 2c). The data on COX-2 expression were paralleled by respective changes of COX-2 immunoreactivity in the TALH/macula densa structures (Figure 3). The percentage of glomeruli with adjacent COX-2 immunoreactivity during normal salt intake was $6\pm1\%$ and increased during low salt intake, low salt diet in combination with valsartan and chronic furosemide infusion to $14\pm2\%$ ($P<0.05$), $53\pm16\%$ ($P<0.05$) and $19\pm2\%$ ($P<0.05$), respectively (Figure 3). Furosemide and the combination of low salt intake with valsartan increased COX-2 immunoreactivity in everolimus-treated rats (Figure 3). Additional treatment with everolimus during normal salt intake, low salt diet, low salt diet in combination with valsartan and chronic furosemide infusion decreased the percentage of glomeruli with adjacent COX-2 immunoreactivity to $3\pm1\%$ ($P<0.05$), $6\pm2\%$ ($P<0.05$), $28\pm4\%$ ($P<0.05$) and $9\pm2\%$ ($P<0.05$), respectively (Figure 3). Similarly, renocortical tissue $PGE_2$ ($pg\,\mu g^{-1}$ protein) concentration

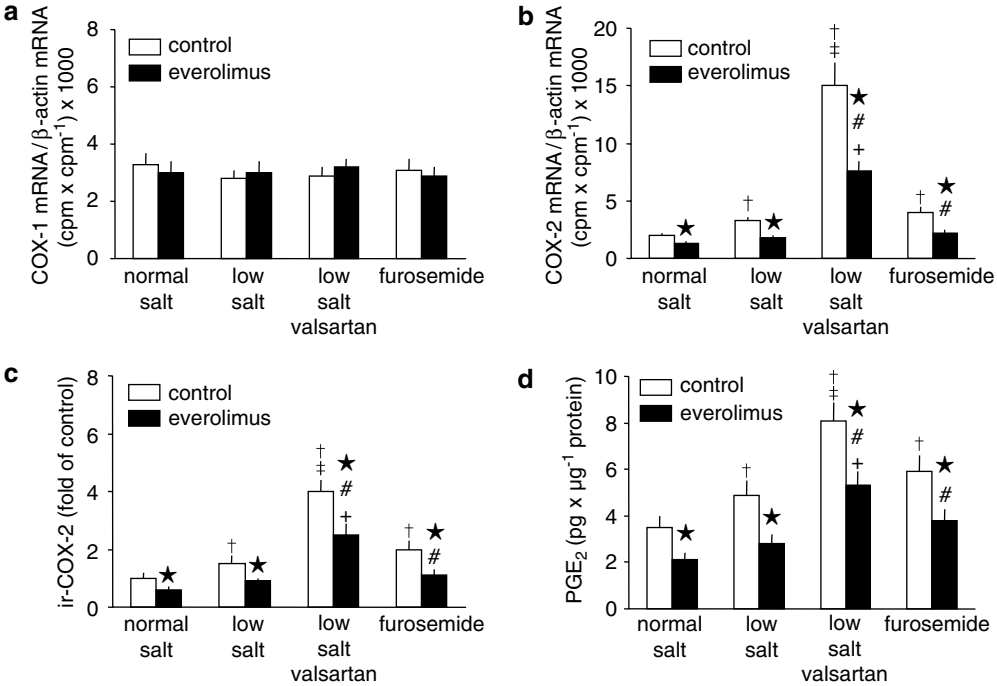


**Figure 2** Effect of everolimus ($3\,mg\,kg^{-1}\,day^{-1}$) on cyclooxygenase (COX)-1 mRNA abundance (a), COX-2 mRNA abundance (b), COX-2 protein expression (c) and prostaglandin (PG) $E_2$ concentration (d) in the renal cortex during normal salt intake (0.6% NaCl, wt wt$^{-1}$), low salt intake (0.02% NaCl, wt wt$^{-1}$), low salt intake combined with valsartan ($30\,mg\,kg^{-1}\,day^{-1}$) treatment and furosemide ($12\,mg\,day^{-1}$, s.c.) infusion. Data represent the mean$\pm$s.e.m. of $n=8$ rats. *$P<0.05$ vs respective control. $^{\dagger}P<0.05$ vs normal salt intake. $^{\ddagger}P<0.05$ vs low salt intake. $^{\#}P<0.05$ vs normal salt intake and everolimus, $^{+}P<0.05$ vs low salt intake and everolimus.

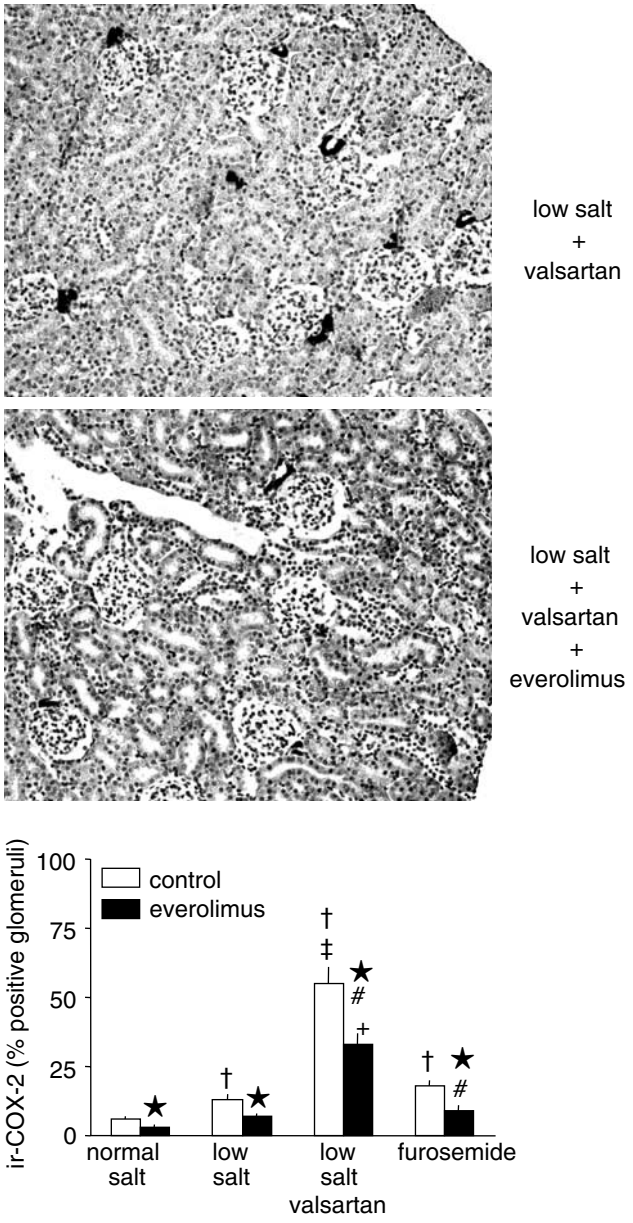


**Figure 3** Localization of renocortical ir-COX-2 expression in low salt (0.02% NaCl, wt wt$^{-1}$) and valsartan (30 mg kg$^{-1}$ day$^{-1}$) (upper inset) or low salt in combination with valsartan and everolimus (3 mg kg$^{-1}$ day$^{-1}$) (lower inset) treated rats. Effect of everolimus on COX-2 immunoreactivity related to macula densa structures during normal salt intake (0.6% NaCl, wt wt$^{-1}$), low salt intake (0.02% NaCl, wt wt$^{-1}$), low salt intake combined with valsartan (30 mg kg$^{-1}$ day$^{-1}$) treatment and furosemide (12 mg day$^{-1}$, s.c.) infusion. Data represent the mean ± s.e.m. of $n=8$ rats. *$P<0.05$, vs respective control. $^{\dagger}P<0.05$ vs normal salt intake. $^{\ddagger}P<0.05$ vs low salt intake. $^{\#}P<0.05$ vs normal salt intake and everolimus, $^{+}P<0.05$ vs low salt intake and everolimus.

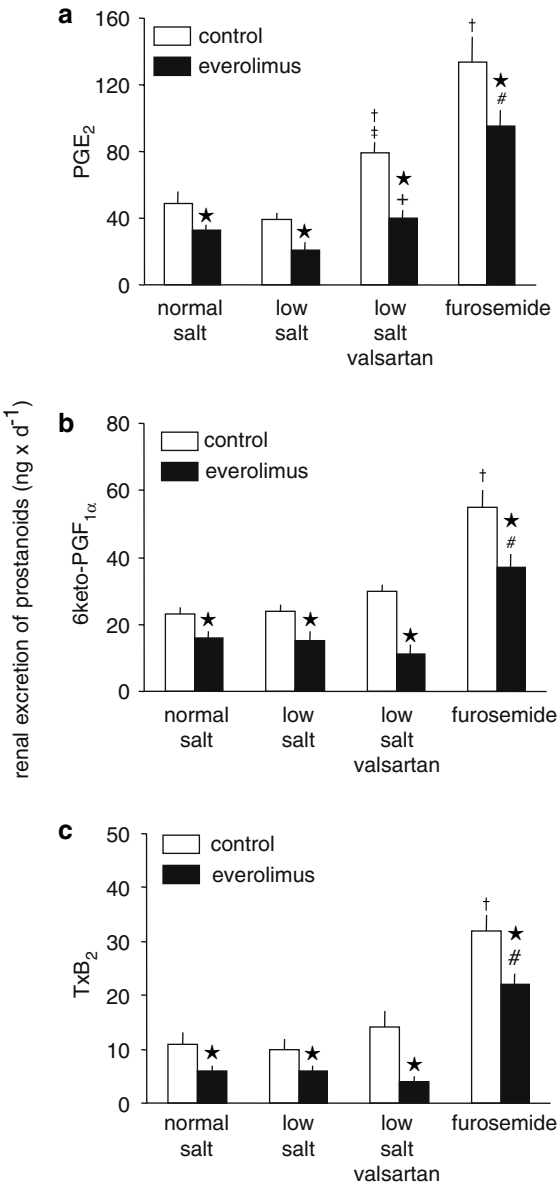


**Figure 4** Effect of everolimus (3 mg kg$^{-1}$ day$^{-1}$) on daily urinary $PGE_2$ (a), 6-keto $PGF_{1\alpha}$ (b) and $TxB_2$ (c) excretion in rats during normal salt intake (0.6% NaCl, wt wt$^{-1}$), low salt intake (0.02% NaCl, wt wt$^{-1}$), low salt intake combined with valsartan (30 mg kg$^{-1}$ day$^{-1}$) treatment and furosemide (12 mg day$^{-1}$, s.c.) infusion. Data represent the mean ± s.e.m. of $n=8$ rats. *$P<0.05$ vs respective control. $^{\dagger}P<0.05$ vs normal salt intake. $^{\ddagger}P<0.05$, vs low salt intake. $^{\#}P<0.05$ vs normal salt intake and everolimus, $^{+}P<0.05$ vs low salt intake and everolimus.

increased during low salt intake, the combination of low salt diet with valsartan and furosemide infusion to 4.9 ± 0.4 ($P<0.05$), 8.1 ± 0.6 ($P<0.05$) and 5.8 ± 0.3 ($P<0.05$), respectively (Figure 2d). Additional treatment with everolimus during low salt intake, the combination of low salt diet with valsartan and furosemide infusion lowered renocortical $PGE_2$ levels to 2.6 ± 0.3 ($P<0.05$), 4.6 ± 0.4 ($P<0.05$), and 3.2 ± 0.3 ($P<0.05$), respectively (Figure 2d). Furosemide and the combination of low salt intake with valsartan increased renocortical $PGE_2$ levels in everolimus-treated rats (Figure 2d).

### *Effect of everolimus on daily urinary prostanoid excretion*

Daily urinary excretion of $PGE_2$ (ng day$^{-1}$), 6-keto $PGF_{1\alpha}$ (ng day$^{-1}$) (a stable metabolite of $PGI_2$) and $TxB_2$ (ng day$^{-1}$) (a stable metabolite of $TxA_2$) was not altered by low salt intake (Figure 4a–c). The combination of low salt diet and valsartan increased daily urinary $PGE_2$ excretion from 49 ± 7 to 79 ± 7 ($P<0.05$) (Figure 4a), but had no effect on the daily urinary

excretion of 6-keto $PGF_{1\alpha}$ and $TxB_2$ (Figure 4b and c). Chronic furosemide infusion increased daily urinary excretion of $PGE_2$, 6-keto $PGF_{1\alpha}$ and $TxB_2$ to $134\pm15$ ($P<0.05$), $55\pm5$ ($P<0.05$) and $32\pm3$ ($P<0.05$) (Figure 4a–c). Additional treatment with everolimus during normal salt intake decreased the daily urinary excretion of $PGE_2$, 6-keto $PGF_{1\alpha}$ and $TxB_2$ to $33\pm3$ ($P<0.05$), $16\pm2$ ($P<0.05$), and $6\pm1$ ($P<0.05$), respectively (Figure 4a–c). Additional treatment with everolimus during low salt intake decreased the daily urinary excretion of $PGE_2$, 6-keto $PGF_{1\alpha}$ and $TxB_2$ to $21\pm4$ ($P<0.05$), $15\pm3$ ($P<0.05$), and $6\pm1$ ($P<0.05$), respectively (Figure 4a–c). Additional treatment with everolimus during low salt intake and valsartan treatment decreased the daily urinary excretion of $PGE_2$, 6-keto $PGF_{1\alpha}$ and $TxB_2$ to $40\pm5$ ($P<0.05$), $11\pm3$ ($P<0.05$), and $4\pm1$ ($P<0.05$), respectively (Figure 4a–c). Additional treatment with everolimus during furosemide infusion decreased the daily urinary excretion of $PGE_2$, 6-keto $PGF_{1\alpha}$ and $TxB_2$ to $95\pm5$ ($P<0.05$), $37\pm4$ ($P<0.05$), and $22\pm2$ ($P<0.05$), respectively (Figure 4a–c). With regard to everolimus-treated rats, furosemide and additional treatment with valsartan during low salt intake increased daily urinary excretion of $PGE_2$ ($P<0.05$) (Figure 4a). Furosemide increased daily urinary excretion of 6-keto $PGF_{1\alpha}$ and $TxB_2$ ($P<0.05$) in everolimus-treated rats (Figure 4b and c).

### *Effect of everolimus on urine output and daily urinary sodium and potassium excretion*

Daily urine production, normalized to the BW ($ml\,day^{-1}\,kg^{-1}$ BW), was $71\pm6$ in rats fed a normal salt diet, $58\pm6$ in rats fed a low salt diet ($P<0.05$) and $76\pm11$ in rats during the combination of low salt diet with valsartan. Urine output clearly increased in furosemide-infused rats to $723\pm38$ ($P<0.05$). Additional treatment with everolimus increased daily urine production during normal salt intake, low salt intake, the combination of low salt diet with valsartan and furosemide infusion to $111\pm8$ ($P<0.05$), $165\pm25$ ($P<0.05$), $101\pm9$ ($P<0.05$) and $972\pm102$ ($P<0.05$), respectively (Figure 5a). Daily urine excretion in everolimus-treated rats was increased during furosemide intake and low salt intake ($P<0.05$). Additional treatment with valsartan during the combination of low salt intake and everolimus attenuated the increase in daily urine excretion ($P<0.05$) (Figure 5a).

Daily urinary sodium excretion, normalized to the BW ($mmol\,day^{-1}\,kg^{-1}$ BW), was $37\pm4$ in rats fed a normal salt diet. Low salt diet for 1 week clearly decreased urinary sodium excretion to $1.5\pm1$ ($P<0.05$). Urinary sodium excretion during the combination of low salt diet with valsartan decreased to $6.9\pm2$ ($P<0.05$). Chronic furosemide infusion increased daily urinary sodium excretion to $286\pm25$ ($P<0.05$) (Figure 5b). Additional treatment with everolimus increased urinary sodium excretion during normal salt intake, low salt intake and during chronic furosemide infusion to $51\pm4$ ($P<0.05$), $7.5\pm2$ ($P<0.05$) and $382\pm26$ ($P<0.05$), respectively (Figure 5b). Daily urinary sodium excretion in everolimus-treated rats was decreased during low salt intake and the combination of low salt intake with valsartan ($P<0.05$). Daily urinary sodium excretion in everolimus-treated rats was increased during furosemide treatment ($P<0.05$) (Figure 5b).

Everolimus had no effect on daily urinary potassium excretion ($mmol\,day^{-1}\,kg^{-1}$ BW) (Figure 5c). Potassium excretion was decreased in rats fed a low salt diet and by the combination of low salt diet with valsartan to $12\pm2$ ($P<0.05$) and $14\pm2$ ($P<0.05$). Chronic furosemide infusion increased daily urinary potassium excretion to $30\pm4$ ($P<0.05$) (Figure 5c).

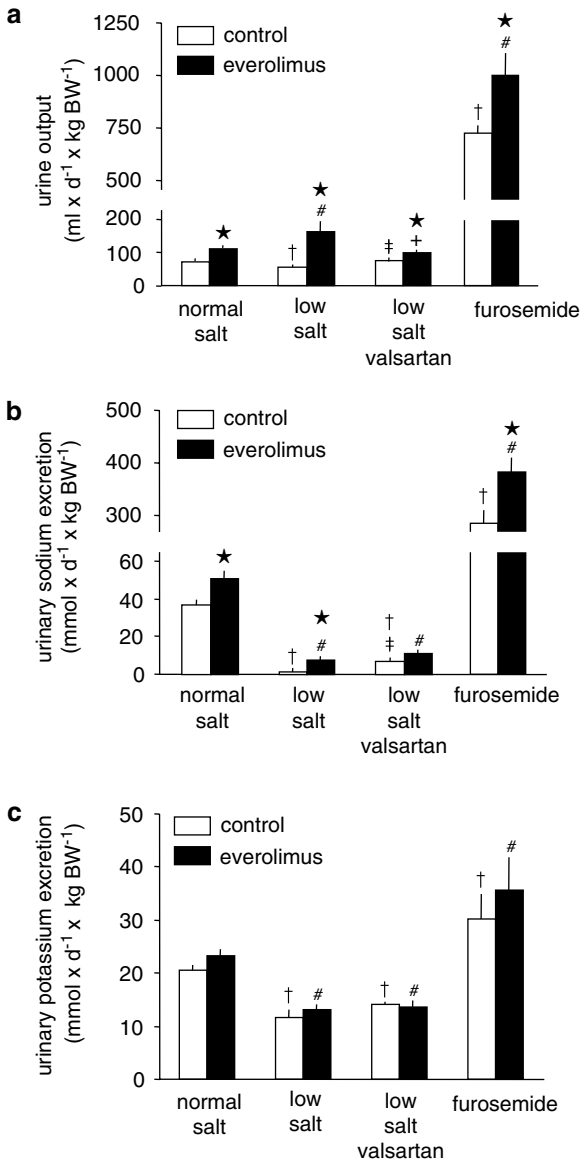


**Figure 5** Effect of everolimus ($3\,mg\,kg^{-1}\,day^{-1}$) on daily urine excretion (a), daily urinary sodium (b) and potassium excretion (c) in rats during normal salt intake (0.6% NaCl, $wt\,wt^{-1}$), low salt intake (0.02% NaCl, $wt\,wt^{-1}$), low salt intake combined with valsartan ($30\,mg\,kg^{-1}\,day^{-1}$) treatment and furosemide ($12\,mg\,day^{-1}$, s.c.) infusion. Data represent the mean±s.e.m. of $n=8$ rats. $^{*}P<0.05$, vs respective control. $^{\dagger}P<0.05$ vs normal salt intake. $^{\ddagger}P<0.05$ vs low salt intake. $^{\#}P<0.05$ vs normal salt intake and everolimus, $^{+}P<0.05$ vs low salt intake and everolimus.

### *Effect of everolimus on creatinine and urea clearance*

Creatinine clearance ($ml\,min^{-1}\,100\,g\,BW^{-1}$) during normal salt intake was $0.97\pm0.07$ and decreased during low salt intake and the combination of low salt intake with valsartan to $0.75\pm0.07$ ($P<0.05$) and $0.58\pm0.06$ ($P<0.05$), respectively.

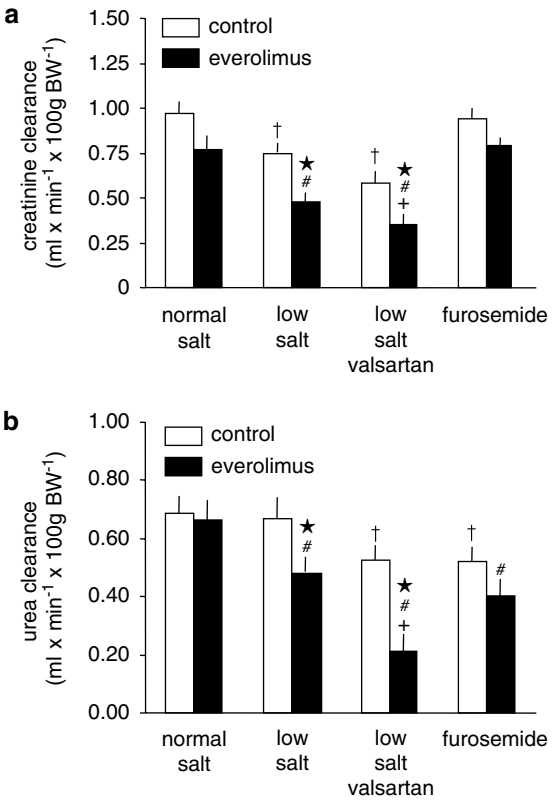


**Figure 6** Effect of everolimus ($3\,mg\,kg^{-1}\,day^{-1}$) on creatinine clearance (a) and urea clearance (b) in rats during normal salt intake (0.6% NaCl, wt $wt^{-1}$), low salt intake (0.02% NaCl, wt $wt^{-1}$), low salt intake combined with valsartan ($30\,mg\,kg^{-1}\,day^{-1}$) treatment and furosemide ($12\,mg\,day^{-1}$, s.c.) infusion. Data represent the mean ± s.e.m. of $n=8$ rats. *$P<0.05$ vs respective control. $^{\dagger}P<0.05$ vs normal salt intake. $^{\ddagger}P<0.05$ vs low salt intake. $^{\#}P<0.05$ vs normal salt intake and everolimus, $^{+}P<0.05$ vs low salt intake and everolimus.

Furosemide treatment did not alter creatinine clearance (Figure 6a). Additional treatment with everolimus had no effect on creatinine clearance during normal salt intake. Additional treatment with everolimus during low salt intake and the combination of low salt intake with valsartan decreased creatinine clearance to $0.48\pm0.05$ ($P<0.05$) and $0.35\pm0.05$ ($P<0.05$), respectively (Figure 6a). Everolimus did not alter creatinine clearance during furosemide infusion. Creatinine clearance was decreased by low salt intake in everolimus-treated rats ($P<0.05$) and additional treatment with valsartan further decreased creatinine clearance ($P<0.05$) (Figure 6a).

Urea clearance ($ml\,min^{-1}$ $100\,g$ $BW^{-1}$) during normal salt intake was $0.69\pm0.04$ and was not altered by low salt intake. Urea clearance decreased during the combination of low salt diet with valsartan and during chronic furosemide treatment to $0.53\pm0.04$ ($P<0.05$) and $0.52\pm0.04$ ($P<0.05$), respectively (Figure 6b). Additional treatment with everolimus decreased urea clearance during low salt intake and low salt intake in combination with valsartan to $0.49\pm0.04$ ($P<0.05$) and $0.21\pm0.06$ ($P<0.05$), respectively (Figure 6b). Urea clearance was not affected by additional treatment with everolimus during normal salt intake or during furosemide infusion (Figure 6b). Urea clearance was decreased by furosemide treatment and by low salt intake in everolimus-treated rats ($P<0.05$). Additional treatment with valsartan during low salt intake and everolimus treatment further decreased urea clearance ($P<0.05$) (Figure 6b).

## Discussion

The purpose of our study was to investigate the effect of everolimus, a derivative of rapamycin, on renal cyclooxygenase-2 (COX-2) expression and renal function, since rapamycin has been shown to impair renal function in rats (DiJoseph *et al*., 1994) and since we found that inhibition of renal COX-2 expression by other immunosuppressant like CsA could be involved in the renal adverse effects of these drugs (Höcherl *et al*., 2002a; 2004). Therefore, we studied the effect of everolimus on basal renal COX-2 expression and during various conditions that lead to an increase in renocortical COX-2 expression.

In accordance with previous results obtained for rapamycin, we found that everolimus did not affect SBP (Andoh *et al*., 1996; Shihab *et al*., 2004) and HR (Gardiner *et al*., 2004). Rapamycin increased the hematocrit during all experimental maneuvres, which might be related to an increased salt and water excretion. In addition, we found that everolimus did not attenuate the fall in blood pressure induced by valsartan treatment. Furthermore, rats that have been treated with everolimus gained less weight (Andoh *et al*., 1996; Podder *et al*., 2001). The mTOR pathway has been reported to be involved in food consumption. It has been proposed that leucin activates mTOR, which in turn stimulates leptin production and secretion. An increase in circulating leptin may cause satiety and inhibit food intake (Roh *et al*., 2003). Inhibition of mTOR may, therefore, increase food intake. However, we found that everolimus-treated rats had a reduced food intake. Furthermore, it has been reported that during a normal diet, BW gain was decreased in S6 Kinase 1 (S6K1)-deficient mice compared to wild-type mice, although food intake was normal (Um *et al*., 2004). Therefore, the reduced weight gain by everolimus treatment in our study may be related to the lower food intake and to a direct effect of everolimus on the mTOR/S6K1 pathway.

With regard to renal cyclooxygenases expression, we found that basal COX-2 and COX-1 expression as well as tissue prostaglandin $E_2$ ($PGE_2$) concentration in the rat renal cortex increased towards the inner medulla (Jensen & Kurtz, 1997). Administration of everolimus did not affect COX-1 mRNA abundance in the rat kidney, but decreased cortical COX-2 expression as well as renocortical tissue $PGE_2$ concentration. In accordance with previous reports, also in this study a low salt diet (Harris *et al*., 1994), the combination of low salt diet with an $AT_1$-receptor antagonist (Höcherl *et al*., 2001) and chronic furosemide (Kammerl *et al*., 2001a) treatment led to increases of renocortical COX-2 expression and renocortical $PGE_2$ formation. We found that the stimulation of COX-2 by these manoeuvres was sensitive towards additional treatment with everolimus, in the way that everolimus diminished these increases. In addition, we found that everolimus decreased macula densa cell-related COX-2 expression.

In accordance with previous observations, we found that a low salt diet did not increase urinary excretion of prostanoids, although renocortical COX-2 expression was increased (Kammerl *et al*., 2001b). Since the renal medulla is the major source

of renal prostanoid formation (Dunn & Hood, 1977) and since a low salt intake decreases medullary COX-2 expression (Jensen & Kurtz, 1997), urinary prostanoid excretion during low salt intake may well reflect the decreased formation of medullary prostanoids, compensating for the increased formation of prostanoids in the renal cortex. Everolimus decreased the urinary excretion of prostanoids during normal and low salt intake. Furthermore, the increases of urinary prostanoid excretion induced by low salt intake in combination with ANG-II inhibition and by furosemide treatment were also attenuated by everolimus treatment (Kammerl *et al*., 2001a, b).

Everolimus also attenuated the urinary excretion of $TxB_2$. In line with this, the COX-2 inhibitor rofecoxib, used at a dose selective for COX-2 (Gretzer *et al*., 2001; Höcherl *et al*., 2002b), has also been shown to decrease urinary excretion of $TxB_2$ (Kammerl *et al*., 2001a, b). However, it has been suggested that the urinary excretion of metabolites of $TxA_2$ is closely related to platelet COX-1 activity (Seyberth *et al*., 1991). Since we did not find that everolimus attenuated renal COX-1 expression, the decrease in urinary $TxB_2$ excretion could be due to a decrease in glomerular filtration rate. Another explanation would be that renal Tx production may partly depend on renal COX-2 activity. It should be noted that COX-2 and Tx synthase are both expressed within the glomerulus (Vitzthum *et al*., 2002), which might be therefore capable of generating Tx *via* COX-2 (Klein *et al*., 2001). Compared to our previous findings on renal COX-2 expression obtained by CsA treatment (Höcherl *et al*., 2002a; 2004), the inhibitory effect of everolimus was rather moderate, suggesting a different mode of action.

Everolimus and rapamycin are well-known inhibitors of the mammalian target of rapamycin (mTOR), a Ser/Thr protein kinase, which is involved in several steps in the control of mRNA translation (Gingras *et al*., 2001). A well-described downstream effector of mTOR is the ribosomal unit S6 kinase (S6K). The activation of S6K is stimulated by mitogens, and mTOR is required to trigger this mitogen response (Brown *et al*., 1995). In addition, the phosphatidylinositol 3-kinase (PI3 K) pathway is required for the mitogenic activation of S6K (Sekulic *et al*., 2000). Rapamycin has been found to inhibit lipopolysaccharide-induced COX-2 expression in murine macrophages (Attur *et al*., 2000) and recently, it has been reported that the PI3K-mTor-p70 S6K1 signalling pathway is of importance for the regulation of COX-2 gene expression *in vitro* (Lim *et al*., 2003). Moreover, activation of mitogen-activated protein kinases (MAPk), like p38 and p44/42, has been demonstrated in the upregulation of COX-2 expression by low chloride in a mouse macula densa cell line (Yang *et al*., 2000) and in cultured cells of rabbit cTALH (Cheng *et al*., 2000), and it has been shown *in vitro* that rapamycin inhibits the activation of p38 MAPk (Oh *et al*., 2001). Our *in vivo* data strongly support the proposed pathway linking mTOR and COX-2 expression in the rat renal cortex.

COX-2-derived prostanoids are of importance for renal blood flow and salt and water excretion (Krämer *et al*., 2004). It has been suggested, that inhibition of COX-2-derived prostanoids leads to sodium and water retention (Komers *et al*., 2001). Conversely, we found that everolimus treatment led to an increased diuresis and natriuresis especially during low salt intake, although renal excretion of prostanoids was attenuated. Such an increase in diuresis during rapamycin treatment has already been observed by other investigators (DiJoseph *et al*., 1994, Andoh *et al*., 1996; Podder *et al*., 2001). Regarding renal function, the PI3 K pathway has been suggested to be involved in sodium homeostasis. Angiotensin II (ANG-II) is known to cause sodium retention through stimulation of the $Na^+/H^+$ exchanger 3 (NHE3) activity in renal proximal tubule, mainly *via* activation of ANG-II type 1-receptors. Recently, it has been reported that the PI3 K pathway, which may be attenuated by everolimus, mediates ANG-II-stimulated NHE3 activity (Du Cheyron *et al*., 2003). Since low salt intake activates the renin system (Höcherl *et al*., 2002d) and subsequently leads to an increased synthesis of ANG-II, the observed effects of rapamycin on natriuresis, which were strongest during low salt intake, might be linked to an inhibition of the PI3K-mTor-p70 S6K1 signalling pathway by low salt diet in the rat renal cortex.

Rapamycin treatment at supratherapeutic doses, as used in our study for everolimus, did not cause renal toxicity in rats receiving a normal diet (Ninova *et al*., 2004). Evaluation of the effects of rapamycin in different rat models using salt-depleted rats (Podder *et al*., 2001) or spontaneously hypertensive rats (DiJoseph *et al*., 1994) revealed functional as well as mild to moderate morphological alterations, which where different to those observed by CsA treatment (DiJoseph *et al*., 1994), but which may also lead to an impaired renal function. Administration of mTOR inhibitors at higher doses in rat models prone to spontaneous kidney lesions or with induced renal lesions may, therefore, result in an exacerbation of renal function, as seen in our study during low salt intake. This effect may be related to the antiproliferative potential of mTOR inhibitors (Lieberthal *et al*., 2001). In our study, everolimus treatment increased plasma creatinine and urea levels and decreased glomerular filtration rate during low salt intake, as it has been found for rapamycin treatment (DiJoseph *et al*., 1994; Podder *et al*., 2001), suggesting that renal function is impaired during everolimus treatment. Since COX-2 inhibition decreases glomerular filtration rate when sodium intake is low, but does not influence glomerular filtration rate when sodium intake is normal (Rodriguez *et al*., 2000), the effect of everolimus on creatinine clearance and plasma creatinine concentration may be only in part due to COX-2 inhibition. Since we used supratherapeutic doses of everolimus, the impact of everolimus on renal function might be of minor relevance for the therapeutic use of the drug.

In accordance with previous observations, the combination of low salt diet with $AT_1$-receptor antagonism increased plasma concentrations of urea and creatinine, probably due to the low blood pressure and changes in intrarenal hemodynamics (Griffiths *et al*., 2001; Kammerl *et al*., 2002; Richer-Giudicelli *et al*., 2004).

In conclusion, our findings suggest that rapamycin attenuates basal and also stimulates expression of renocortical COX-2 and of tissue prostanoid formation. Despite the decrease of COX-2 expression, everolimus is associated with increased natriuresis and diuresis.

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