The PGE2-EP4 receptor is necessary for stimulation of the renin-angiotensin-aldosterone system in response to low dietary salt intake in vivo

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Pöschke A, Kern N, Maruyama T, Pavenstädt H, Narumiya S, Jensen BL, Nüsing RM. The PGE2-EP4 receptor is necessary for stimulation of the renin-angiotensin-aldosterone system in response to low dietary salt intake in vivo. Am J Physiol Renal Physiol 303: F1435–F1442, 2012. First published September 19, 2012; doi:10.1152/ajprenal.00512.2011.—Increased cyclooxygenase-2 (COX-2) expression and PGE2 synthesis have been shown to be prerequisites for renal renin release after Na+ deprivation. To answer the question of whether EP4 receptor type of PGE2 mediates renin regulation under a low-salt diet, we examined renin regulation in EP4+/−, EP4−/−, and in wild-type mice treated with EP4 receptor antagonist. After 2 wk of a low-salt diet (0.02% wt/wt NaCl), EP4−/− mice showed diminished Na+ excretion, unchanged K+ excretion, and reduced Ca2+ excretion. Diuresis and plasma electrolytes remained unchanged. EP4−/− exhibited a similar attenuation of Na+ excretion; however, diuresis and K+ excretion were enhanced, and plasma Na+ concentration was higher, whereas plasma K+ concentration was lower compared with control diet. There were no significant differences between EP4+/− and EP4−/− mice in blood pressure, creatinine clearance, and plasma antidiuretic hormone (ADH) concentration. Following salt restriction, plasma renin and aldosterone concentrations and kidney renin mRNA level rose significantly in EP4+/− but not in EP4−/− and in wild-type mice treated with EP4 antagonist ONO-AE3–208. In the latter two groups, the low-salt diet caused a significantly greater rise in PGE2 excretion. Furthermore, mRNA expression for COX-2 and PGE2 synthetic activity was significantly greater in EP4−/− than in EP4+/− mice. We conclude that low dietary salt intake induces expression of COX-2 followed by enhanced renal PGE2 synthesis, which stimulates the renin-angiotensin-aldosterone system by activation of EP4 receptor. Most likely, defects at the step of EP4 receptor block negative feedback mechanisms on the renal COX system, leading to persistently high PGE2 levels, diuresis, and K+ loss.

prostaglandins; prostanoid receptors; cyclooxygenase; renin-angiotensin-aldosterone system

ACTIVATION OF THE RENIN-ANGIOTENSIN -ALDOSTERONE-SYSTEM (RAAS) is an important physiological mechanism to counteract volume depletion, such as that due to limited amounts of NaCl intake or losses of blood or extracellular fluid volume. PGE2 represents a signaling molecule able to modulate RAAS. In general, prostaglandins are important renal modulators for renal salt- and water homeostasis and vascular tone and renal perfusion. Prostaglandins are synthesized by the cyclooxygenase (COX) pathway, and two isoforms of COX enzymes are known: COX-1 and COX-2. COX-1 represents a housekeeping enzyme expressed by virtually all types of tissue, whereas COX-2 is undetectable in most cells and tissues, and expression is induced by various stimuli, such as growth factors or cytokines (34). Both COX isoforms are expressed by the kidney, with a predominant occurrence in the renal inner medulla and lower concentrations in the outer medulla and renal cortex (8), but the specific roles of these isozymes in renal regulation have not been clarified in detail. Many studies in rodents and man indicate that COX-2 expression is an important requirement for renin stimulation (7, 10, 38) and show that PGE2 exerts key functions in the regulation of renal renin release (15, 33). Application of a low-salt diet (14, 38, 39) and loop diuretics (21) in mice and rats induces COX-2 expression in the MD, which leads to prostaglandin synthesis and contributes to renin secretion. That this mechanism holds also in humans has been shown in volunteers receiving furosemide or a low-salt diet (17), as well as in patients suffering from antenatal Bartter syndrome (18), clinical settings that are associated with elevated renal PGE2 formation and renin release. Consequently, renal PGE2 synthesis, and thereby renin secretion, can be blocked by nonspecific COX inhibitors, such as indomethacin, and also by specific COX-2 inhibitors in mice and man (30, 36).

PGE2 signals via four different types of receptor, EP1–EP4 (26), which are expressed in renal tissue (see Ref. 2 for review). The precise role of the different EP receptors in modulating renin in vivo still remains elusive. We recently observed, in isolated perfused kidneys of EP4-knockout mice that the stimulation of renin release by PGE2 was significantly lower compared with that in controls (33) and that in furosemide-treated EP4−/− mice, the increase in renin activity was lower compared with control mice (27). In support of these data on the regulation of renin, we observed EP4 mRNA expression in glomeruli and in renin-secreting juxtaglomerular (JG) granular (14) cells, as well as EP4 protein expression in preglomerular arterioles (5). These observations indicated that EP4 receptor is involved in PGE2-induced renin activity, at least following pharmacologically induced salt and water loss. Whether EP4 also plays a dominant role in more physiologically relevant states of variations in salt intake in vivo is unknown. In the present study, we investigated the role of the EP4 receptor in regulating renin release following administration of a chronic low-salt diet to mice with targeted deletion of EP4 and by the use of EP4-selective pharmacological tools.
Materials and Methods

Animals. All experiments were performed with female mice aged 8–12 wk. C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). EP4+/+ and EP4−/− mice were generated as previously described (11), and mice were weaned at the age of 21 days. Genotypes of the mice were determined by PCR analysis using specific oligonucleotides to the respective EP locus and the Neo cassette. RT-PCR using RNA isolated from EP4−/− kidneys revealed no cycle threshold (Ct) value significantly different from the water control. All knockout mice were backcrossed to C57BL/6 more than 10 times. Animals were kept under controlled conditions at an ambient temperature of 21 ± 1°C and a 12:12-h light-dark cycle. They had free access to water and standard chow diet containing 0.9% NaCl wt/wt (Altromin, Germany). In the low-salt studies, animals were fed a diet containing 0.02% wt/wt NaCl (Altromin, Germany). The State Agency Giessen approved all animal experiments, and the procedures followed were in accordance with institutional guidelines.

Analysis of experiments. After obtaining basal values, animals received a low-salt diet [0.02% NaCl wt/wt (Altromin, Germany)] for 14 days. The animals had access to water ad libitum. On experimental day 12, animals were placed in metabolic cages for 2 days to examine urine output with free access to water and a low-salt diet. Urine was collected for 24 h in a cooling device to prevent prostaglandins from decay. Body weight and drinking volume were determined daily. For plasma analysis of electrolyte levels, animals were anesthetized, and blood samples were taken by cardiac puncture. Plasma was separated and kept at −80°C until analysis. In urine samples, electrolyte concentrations were quantified by flame photometry (EFOX5053, Eppendorf), as reported previously (27). In experiments using EP4 receptor antagonist ONO AE3–208, the drug was given in the drinking water (1 mg/ml) for 14 days.

Measurement of plasma renin concentration. For determination of plasma renin concentration (PRC), the ANG I produced was measured by ultrasensitive assay (27). Five serial dilutions from the same plasma sample were assayed in duplicate for all samples. Linearity over three serial dilutions was required for a value to be accepted. Renin concentrations were expressed in Goldblatt units compared with renin standards from the National Institute for Biological Standards and Control (Hertfordshire, UK).

Determination of mean arterial blood pressure in anesthetized mice and systolic blood pressure in conscious mice. Mice were anesthetized with pentobarbital sodium (50 μg/kg ip) and placed on a heated table at 37°C to maintain body temperature. Cannulas were placed into the trachea to facilitate breathing and into the carotid artery to measure systemic mean arterial pressure (Cardiomax-III; Columbus Instruments; Columbus, OH), as described by Nüssing et al. (27). Systolic blood pressure was determined in conscious mice using a noninvasive computerized automated tail-cuff system (BP-2000; Visitech Systems, Apex, NC). The animals were trained by placing them in restrainers on a heated platform daily at 1:00 PM for three consecutive days to get them accustomed to the procedure, followed by additional measurements of systolic blood pressure and heart rate on two consecutive days. They underwent five preliminary cycles and a 10-cycle measurement, which each have a minimum of 6 out of 10 successful measurements. Mean values obtained on days 4 and 5 were used for comparisons. Following recording of baseline measurements under a control diet, animals obtained a low-salt diet for 14 days, and blood pressure was recorded as before.

Determination of urinary PGE2. Urinary PGE2 concentration was determined by PGE2 competitive BiotreK enzyme immunoassay system (PGE2-EIA-kit) from Amersham Biosciences (Buckinghamshire, UK). Urine excretion rates were calculated on the basis of daily excretion.

Determination of vasopressin and aldosterone in plasma. Plasma levels of vasopressin and aldosterone were measured by specific enzyme immunoassays (Arg8-vasopressin EIA kit) from R&D Systems (Wiesbaden-Nordenstadt, Germany) and aldosterone EIA kit from Enzo Life Sciences (Lörrach, Germany), according to the instructions of the suppliers.

Determination of plasma and urinary creatinine levels and electrolytes. Plasma and urinary creatinine levels were determined using a creatinine kit from Randox Laboratories (Ardmore, UK), according to the instructions of the supplier. Electrolytes were determined by flame photometry, as described previously (27).

Immunoblotting. To obtain whole tissue extracts, kidneys were homogenized in 50 mM Tris-HCl, 1% Triton X-100, pH 7.4, supplemented with protease inhibitor cocktail (Merck, Darmstadt, Germany) using a Potter S homogenizer and sonification. Following a 30-min centrifugation at 18,000 g and 4°C, the supernatant was removed. Protein concentrations were determined by the bicinchoninic acid method, according to the manufacturer’s protocol (Pierce, Rockford, IL) using BSA as the protein standard. Samples of 50-μg protein were mixed with 4× Laemmli sample buffer (0.2 mM Tris-HCl, 20% SDS, 40% glycerol, 0.04% bromophenol blue, pH 6.8, 20% β-mercaptoethanol) and heated for 10 min at 95°C. The samples were separated on 15% polyacrylamide gels and transferred to nitrocellulose membranes (Protran, Whatmann, Dassel, Germany). The membranes were blocked with 5% nonfat dry milk in PBS and incubated with the primary antibodies. The following antibodies were used: anti-COX-1 and anti-COX-2 (Cayman Chemical, Ann Arbor, MI) diluted 1:150 in 5% nonfat dry milk in PBS, containing 0.1% Tween 20 and left overnight at 4°C. After washing 3 times, membranes were incubated with secondary horseradish peroxidase-labeled antibody, diluted 1:5,000 in 5% nonfat dry milk in PBS, containing 0.1% Tween 20 for 1 h at room temperature. An antibody against ERK-2 (Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect for equal loading, as previously described (1). Immunodetected protein bands were visualized with the ECL detection system, according to the instructions of the supplier (GE Healthcare, Freiburg, Germany). Densitometric analysis was performed using ImageJ software (National Institutes of Health).

Quantitative real-time PCR. Kidneys from mice were dissected and stored at −80°C until analysis. Total RNA was isolated using RNeasy kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. The RNA concentration was quantified by A260 using a NanoDrop-2000 spectrophotometer (Peglab Biotechnologie, Erlangen, Germany). 0.5 μg RNA was reverse transcribed using superscript II RNase and random hexamers (Invitrogen, Karlsruhe, Germany), and 1/40 volumes of the resulting cDNA samples were used as templates for real-time PCR usingSYBR Green supermix reaction procedure with the 7500 Fast System (Applied Biosystems, Darmstadt, Germany). All reactions were run in triplicate to minimize experimental error. The following primer pairs were used: for COX-1, GTG GCT ATT TTC ATC AGC TC and CAG TGC CTC AAC CCC ATA GT; for COX-2, GCA GTT GTT CCA GAC CAA AGG CAA and AAG AGG ATG CCA GTG ATA GA; for renin, ATG AAG GGG GTG TCT GTG GGG AND ATG TCG GGG AGG GTG ACC TG; and for β-actin, TTC ATC ATG AAG GTG TCT GAG CAA TTA GTC TTA GCT TCA T. PCR was initiated at 95°C for 15 min followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C for annealing and extension. As an internal control, we used 18S-rRNA (32). The expression of mRNA was assessed relative to that of β-actin, and the relative quantitative level of samples was determined by the standard 2−ΔΔCT method and expressed as change (fold) relative to expression levels under control condition.

Assay for PGE2 synthetic activity. For determination of PGE2 synthesis, kidneys were weighed and rapidly homogenized in 2 vol (wt/vol) of cold tissue-buffer (50 mM Tris pH 7.4, 1 mM phenol, 1× protease inhibitor cocktail set; Merck Chemicals, Darmstadt, Germany) with an ultratruars followed by ultrasonification (5–10× for 2–4 s). Homogenates were centrifuged for 10 min at 3,000 g, and protein concentration of the supernatants was determined. Samples of 100 μg protein were prewarmed at 37°C for 1 min, and reactions were recorded by PGE2 competitive BiotreK enzyme immunoassay system (PGE2-EIA-kit) from Amersham Biosciences (Buckinghamshire, UK). Urine excretion rates were calculated on the basis of daily excretion.
Calculations. A Prism 5.0 software (GraphPad, San Diego, CA) was used for all statistical analysis. Normality was determined by the D’Agostino-Pearson test. Data are presented as means ± SE (n = 12–14). c, control; ls, low salt, WT, wild type. *Significant difference (P < 0.05) between the c and ls groups.

Table 1. Blood electrolytes in wild-type and EP4 receptor knockout mice under basal conditions and after a low-salt diet for 14 days

<table>
<thead>
<tr>
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<th>Na⁺, mmol/l</th>
<th>K⁺, mmol/l</th>
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<tr>
<td></td>
<td>c</td>
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<td>WT</td>
<td>147.3</td>
<td>147.3</td>
<td>5.23</td>
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<td></td>
<td>± 0.3</td>
<td>± 0.6</td>
<td>± 0.29</td>
</tr>
<tr>
<td>EP4⁻/⁻</td>
<td>147.1</td>
<td>150.6*</td>
<td>5.10</td>
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<td></td>
<td>± 0.6</td>
<td>± 0.9</td>
<td>± 0.16</td>
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Values are expressed as means ± SE (n = 12–14). c, control; ls, low salt, WT, wild type. *Significant difference (P < 0.05) between the c and ls groups.

RESULTS

EP4⁺/⁺ control mice and EP4⁻/⁻ mice were fed for 14 days with a low-salt diet containing 0.02% NaCl. Analysis of blood electrolytes (Table 1) revealed that in control mice, Na⁺, K⁺, and Ca²⁺ concentrations remained unchanged under a low-salt diet compared with a normal diet. In EP4⁻/⁻ mice, low-salt conditions caused a slight, but significant, increase in plasma Na⁺ and a significant decrease in K⁺ concentration, whereas blood Ca²⁺ remained unchanged. Following salt restriction, diuresis was not affected in control mice. However, EP4⁻/⁻ mice exhibited a significant higher output of urine volume (Fig. 1A). With regard to excretion rates of the electrolytes, Na⁺ declined by 85% (Fig. 1B), K⁺ remained unchanged (Fig. 1C) and Ca²⁺ decreased by ~40% in control mice on a low-salt intake. In EP4⁻/⁻ mice, we observed that daily Na⁺ excretion was also dramatically diminished by about 86% under a low-salt diet (Fig. 1B). However, potassium excretion was significantly higher compared with that under control conditions (Fig. 1C). The excretion rate of calcium did not differ before or after salt restriction (Fig. 1D).

Calculation of creatinine clearance, using plasma and urine creatinine levels, revealed no differences in either control mice or EP4⁻/⁻ mice under our experimental conditions (Fig. 2A). In EP4⁺/⁺ and in EP4⁻/⁻ mice, there was a tendency that plasma concentrations of antidiuretic hormone were enhanced following a low-salt diet, but this did not reach statistical significance (Fig. 2B). Mean arterial pressure in anesthetized mice was not significantly different between EP4⁺/⁺, EP4⁻/⁻, and wild-type mice treated with EP4 antagonist ONO AE3–208 (Fig. 2C). Furthermore, systolic blood pressure as measured.

![Graph](AJP-Renal Physiol • doi:10.1152/ajprenal.00512.2011 • www.ajprenal.org)

Fig. 1. Urine volume (A), sodium excretion (B), potassium excretion (C), and calcium excretion (D) in EP4⁺/⁺ and EP4⁻/⁻ mice under normal diet (c) and low salt diet (ls) conditions. Following 14 days on a control diet or under salt restriction, animals were placed in metabolic cages and 24-h urine collection was performed. Data are expressed as means ± SE. *Significant difference, P < 0.05 between the indicated groups (n = 12).
sured by the tail-cuff method in conscious EP4+/+ and EP4−/− mice showed also no difference, neither under the control diet nor under a low-salt diet (Fig. 2D). Interestingly, compared with EP4+/+, heart rate of EP4−/− mice was significantly lower under a control diet, but not under a low-salt diet (Fig. 2E).

In a subsequent set of experiments, we studied the role of the EP4 receptor in activating renin and aldosterone, two components of the RAAS. In EP4+/+ mice, salt restriction caused a significant rise in plasma renin concentration (Fig. 3A). In striking contrast, no significant difference was observed in EP4−/− mice and also not in C57BL6 wild-type mice treated with EP4 antagonist ONO AE3–208 (Fig. 3A). Analysis of renin mRNA by real-time PCR methodology revealed an increase in renin mRNA expression following a low-salt diet only in EP4+/+ mice (Fig. 3B). Neither in EP4−/− nor in wild-type mice treated with EP4 antagonist was a significant change in renin mRNA expression observed. With regard to plasma aldosterone concentrations, we observed a significant rise following a low-salt diet in EP4+/+ mice but not in mice deficient in EP4 receptor function, achieved either by knockout or by pharmacological blockade (Fig. 3C).

We determined 6-keto-PGF1α and TXB2, the more stable hydrolyzation products of PGI2 and TXB2, respectively, and PGE2 in urine samples by enzyme immunoassay to calculate daily prostanoid excretion under normal and low-salt diet conditions. In EP4+/+ and EP4−/− mice, a low-salt diet for 14 days caused a significant increase in the urinary PGE2 excretion rate, but the increases were significantly different between both groups. In EP4+/+ mice, salt restriction caused an increase of PGE2 excretion of 220% compared with the normal diet, whereas the urinary PGE2 excretion in EP4−/− mice was enhanced by about 650%, a significant augmentation compared with
crease in PGE2 excretion, we analyzed renal expression of EP4 with that in EP4\(^{+/+}\) mice. In both mouse lines, a tendency toward an increase in COX-1 mRNA expression was observed, which did not achieve statistical significance. In contrast, COX-2 mRNA expression was significantly induced by a low-salt diet in EP4\(^{+/+}\) and EP4\(^{-/-}\) mice, and the induction of COX-2 mRNA expression was also significantly higher in EP4\(^{-/-}\) mice than in control mice (Fig. 5C). To investigate whether changes in COX-2 expression were paralleled by changes in renal PGE2 synthetic capacity, we measured PGE2 formed by kidney homogenates from EP4\(^{+/+}\) and EP4\(^{-/-}\) mice on a control and a low-salt diet following the addition of exogenous arachidonic acid. Under a low-salt diet, significantly elevated PGE2-synthesizing activities were observed in both groups (Fig. 5D), whereas PGE2 synthesis was significantly higher in the EP4\(^{-/-}\) group.

**DISCUSSION**

The purpose of our study was to determine the relevance of PGE2 receptor subtype EP4 for the regulation of the RAAS by salt restriction in vivo. Our findings demonstrate that salt restriction involves COX-2 induction, enhanced PGE2 release, and requirement for the PGE2 receptor type EP4 for RAAS activation. Abolition of the signaling system through the EP4 receptor not only attenuates RAAS activation but also blocks negative feedback mechanisms on the COX system, leading to overstimulated COX-2 expression and PGE2 synthesis, thereby contributing to further electrolyte alterations, such as raised urine and potassium output.

In general, loss of NaCl and water and, hence, extracellular fluid volume activates the RAAS to counteract volume depletion. The juxtaglomerular apparatus senses the luminal NaCl concentration in the area of the macula densa (9), involving the Na-K-2Cl cotransporter, NKCC2. A decrease in luminal salt concentration and in plasma volume stimulates renin release. The activation of the RAAS leads to NaCl and water retention and maintenance of blood pressure. The role of COX isoenzymes in this type of macula densa-dependent RAAS regulation is not clear. On the one hand, in rats, abolition of COX-2 expression and PGE2 synthesis is not clear. On the one hand, in rats, abolition of overstimulated COX-2 expression and PGE2 synthesis, thereby contributing to raised urine and potassium output.

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Although the importance of COX-2 expression and PGE2 formation for renin release has been emphasized, the PGE2-
dependent signaling pathway, has not been clarified in vivo. Four types of PGE2 receptors, EP1 to EP4, are expressed within the kidney (3, 14, 16, 24), and coupling to distinct renal functions has been suggested. Experiments using furosemide to block NaCl reabsorption by NKCC2 have shown that PGE2 mainly signals via the EP4 receptor to activate the RAAS in mice (4, 27). In the present study, we show that enhanced plasma renin and aldosterone concentrations following salt restriction were also dependent on EP4 receptor function. In EP4−/− mice, a low-salt diet for 14 days did not stimulate plasma renin activity, renin mRNA expression, or plasma aldosterone concentration. Strong support for the role of the EP4 receptor in salt-dependent renin regulation came from our experiments using the selective EP4 receptor antagonist, ONO-AE3–208, in wild-type mice. Similar to targeted gene disruption, pharmacological blockade leads to suppression of renin

Fig. 4. Urinary PGE2 (A), 6-keto-PGF1α (B), and TXB2 (C) excretion by EP4+/+ mice and EP4−/− mice under normal diet (c) and low-salt diet (ls) conditions. Following 14 days of salt restriction, animals were placed in metabolic cages, and 24-h urine collection was performed. Prostanoid concentrations in urine samples were determined by ELAs. Data are expressed as means ± SE; n = 10–12. *P < 0.05 compared with control. #P < 0.05 compared with EP4+/+ low-salt group.

Fig. 5. Expression of COX-1 and COX-2 protein (A and B) and mRNA (C) by EP4+/+ mice and EP4−/− mice under normal diet (c) and low-salt diet (ls) conditions, and PGE2 synthetic activity (D). Following 14 days of a control diet or salt restriction, animals were killed and kidneys were removed. COX mRNA expression was analyzed by real-time PCR and COX protein expression by Western blot analysis. Relative expression to ERK-2 is presented for at least three independent experiments. For determination of PGE2 synthetic activity, kidney homogenates were prepared and incubated with arachidonic acid. Formed PGE2 was determined by LS-MS/MS. Data are expressed as means ± SE; n = 4. *P < 0.05 compared with control; #P < 0.05 compared with EP4+/+ low-salt group.
activity, renin expression, and aldosterone level. Several hints support our observation on the role of EP4 receptor as the type of PGE2 receptor responsible for coupling alterations in salt intake to renin secretion. First, the EP4 receptor is coupled to intracellular cAMP elevation, which is known to be an essential prerequisite for renin secretion (19), and in isolated juxtaglomerular apparatus cells, a selective EP4 agonist led to a rapid cAMP formation and renin secretion (5). Second, renin secretion rates stimulated by PGE2 are strongly attenuated in isolated, perfused kidneys from EP4−/− mice (33). Third, the EP4 receptor is expressed in renin-secreting juxtaglomerular granular cells (5), and EP4 transcripts were increased twofold by salt deprivation (14). Fourth, activation of renin activity by furosemide also depends on the EP4 receptor (4, 27). Although we cannot exclude a role of the EP2 receptor type, which is also coupled to intracellular cAMP elevation, a predominant role of this receptor is unlikely, as EP2 was found to be expressed in the kidney medulla. Furthermore, EP2 receptor deficiency exerts no significant effect on furosemide-induced renin activity (4, 27). Regarding the juxtaglomerular expression of EP4 receptor, a direct effect of PGE2 on JG cells via EP4 to secrete renin is assumed; however, our data do not exclude the possibility that other EP4-expressing cell types are involved.

Aldosterone secretion is enhanced by ANG II, ACTH, and increased plasma potassium concentration (20). Because plasma ANG II likely mirrors suppressed renin concentration in EP4−/− and plasma K+ concentration is also lower following salt restriction, both of these factors probably contribute to the observed lower plasma aldosterone concentration in EP4-deleted mice compared with wild-type mice. In addition, a recent report indicated that EP4 receptor directly enhances aldosterone secretion from zona glomerulosa cells (25). Therefore, blockade of the EP4 receptor could also contribute to lower aldosterone plasma concentrations in EP4−/− mice. Na+ excretion was not different in EP4−/− mice, and blood pressure was not altered by a low-salt diet. Therefore, other mechanisms, e.g., sympathetic nervous system, medullary blood flow, or vasopressin (23), as recently recognized, independent of RAAS must be operating in the nephron to enhance salt reabsorption and to maintain salt balance and blood pressure in EP4−/− mice. Further studies are necessary to address the identification of such a compensatory mechanism and whether this may be operable only in mice, which are known for their great ability to reabsorb salt under low-salt conditions or also in other species, such as in rat or man.

Interestingly, under a low-salt diet, urinary PGE2 excretion rate was significantly higher in EP4-deficient mice than in control mice. We speculate that because of genetic or pharmacological abolition of EP4 function, negative feedback mechanisms are disrupted leading to overexpressed renal COX-2 and PGE2 synthesis. As we did not observe a significant difference in COX-2 expression under normal salt diet in EP4+/+ and EP4−/− mice, we assume that higher PGE2 levels are necessary to trigger the negative feedback mechanism. We can only speculate on the mechanisms involved, but the most likely candidates are ANG II and aldosterone. On the one hand, an indirect negative feedback control of the renin system by ANG II is possible. ACE inhibition and AT1-receptor blockade lead to a rise in renin and COX-2 expression in rats (37) and chronic infusion of ANG II attenuated cortical COX-2 expression (41). Moreover, in the cultured macula densa cell line, MMDD1, ANG II inhibited COX-2 expression (41). On the other hand, mineralocorticoids have been shown to be the dominant adrenal steroids regulating expression of COX-2 in the renal cortex. In mature rats, adrenalectomy causes an increase in COX-2 expression in the cortical thick ascending limb and most dramatic in macula densa (40). Administration of the aldosterone analog, DOCA, to adrenalectomized rats suppressed COX-2 expression to undetectable levels (40). Further studies are necessary to clarify the negative feedback mechanisms of the renin system.

In control mice, a low-salt diet caused activation of the RAAS with an elevation in renin activity, renin mRNA expression, and increased plasma aldosterone concentration. Consequently, Na+ excretion was reduced by ~90%, whereas K+ excretion was unaffected, and the plasma concentrations of electrolytes were constant. In salt-restricted EP4−/− mice, Na+ excretion was diminished to a similar extent as in EP4+/+ mice, but diuresis was enhanced, plasma Na+ and daily K+ excretion increased, and plasma K+ concentration was reduced. Alterations in glomerular filtrate rate (GFR) or in ADH concentrations appear not to be responsible for this difference, although, high PGE2 levels most likely contributed to the small, but insignificant, increase in GFR in EP4−/−, as well as in EP4+/−. In both mouse groups, salt restriction caused minimal, but nonsignificant, increases in plasma ADH concentration. The reason for the increased diuresis in salt-restricted EP4−/− mice could have been increased activation of AVP-antagonizing EP1/EP3 receptors in the collecting duct system due to strongly elevated PGE2 levels or, alternatively, to lack of an EP4 receptor-mediated effect in promoting water reabsorption in the collecting ducts. Very recently, it has been shown that EP4 agonist CAY10580 is able to increase trafficking of AQP2 in the collecting duct cell line MDCK, independent of vasopressin (28). Thus, it appears possible that EP4 receptor deficiency under certain circumstances favors water loss, and the small increase in GFR as observed in our study, may have fostered a rise in diuresis. Interestingly, potassium excretion significantly increased in EP4−/− mice due to the low-salt diet, which was reflected in a lowered plasma K+ concentration. In control mice, no diet-dependent differences were observed. Most likely, a rise in distal flow provokes flow-mediated K+ secretion, a well-known phenomenon, in which large-conductance voltage-activated Ca2+ (BK) channels are involved (29). A direct modulatory role on BK channels in the action of PGE2 is unlikely, as cyclooxygenase inhibitors do not blunt arachidonic acid-induced activation of BK channels in the collecting duct system (35).

In summary, we conclude that the PGE2 receptor type EP4 is necessary for stimulation of the RAAS in response to a low-salt condition in vivo, a condition that has been the predominant evolutionary challenge.

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GRANTS

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ROLE OF EP4 RECEPTOR IN RENIN RELEASE

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: A.P., N.K., and B.L.J. performed experiments; A.P., H.P., and B.L.J. analyzed data; A.P., T.M., H.P., S.N., and R.M.N. interpreted results of experiments; B.L.J. and R.M.N., edited and revised manuscript; R.M.N. conception and design of research; R.M.N. prepared figures; R.M.N. drafted manuscript; R.M.N. approved final version of manuscript.

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