Increased dietary NaCl induces renal medullary PGE2 production and natriuresis via the EP2 receptor

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Divisions of 1Nephrology and 2Clinical Pharmacology, Department of Medicine, Vanderbilt University, and 3Veterans Affairs Medical Center, Nashville, Tennessee; 4DongFang Hospital, Fujian; 5Department of Cellular and Molecular Medicine, Kidney Research Centre, University of Ottawa, Ontario, Canada; and 6Huashan Hospital, Shanghai, China

Submitted 16 April 2008; accepted in final form 11 July 2008

Chen J, Zhao M, He W, Milne GL, Howard JR, Morrow J, Hébert RL, Breyer RM, Chen J, Hao C-M. Increased dietary NaCl induces renal medullary PGE2 production and natriuresis via the EP2 receptor. Am J Physiol Renal Physiol 295: F818–F825, 2008. First published July 16, 2008; doi:10.1152/ajprenal.90253.2008.—A high-NaCl diet induces renal medullary cyclooxygenase (COX)2 expression, and selective intramedullary infusion of a COX2 inhibitor increases blood pressure in rats on a high-salt diet. The present study characterized the specific prostanoid contributing to the antihypertensive effect of COX2. C57BL/6J mice placed on a high-NaCl diet exhibited increased medullary COX2 and microsomal prostaglandin E synthase 1 (mPGES1) expression as determined by immunoblot and real-time PCR. Cytosolic prostaglandin E synthase and prostacyclin synthase were not induced by the high-salt diet. Immunofluorescence showed mPGES1 in collecting ducts and interstitial cells. High salt increased renal medullary PGE2 as determined by gas chromatography-negative ion chemical ionization mass spectrometry. The effect of direct intramedullary PGE2 infusion was examined in anesthetized uninephrectomized mice. Intramedullary PGE2 infusion (10 ng/h) increased urine volume (from 3.3 ± 0.6 to 9.5 ± 1.6 μl/min) and urine sodium excretion (0.11 ± 0.02 to 0.32 ± 0.05 μeq/min). To determine which E-prostanoid (EP) receptor(s) mediated PGE2-dependent natriuresis, EP-selective prostanoids were infused. The EP2 agonist butaprost produced natriuresis (from 0.06 ± 0.02 to 0.32 ± 0.05 μeq/min). The natriuretic effect of intramedullary PGE2 or butaprost was abolished in EP2-deficient mice, which exhibited NaCl-dependent hypertension. In conclusion, a high-salt diet increases renal medullary PGE2 production and natriuresis via the EP2 receptor.

Inhibition of endogenous prostaglandin biosynthesis by cyclooxygenase (COX) inhibiting NSAIDs (including COX2-selective inhibitors) can result in de novo systemic hypertension or compromise the control of blood pressure in patients with preexisting salt-sensitive hypertension (7, 20, 38, 39). The kidney is an important target of prostanoid action (3, 14) and also critical for blood pressure regulation (5, 6, 13). Animal studies show that renal medullary COX2 expression increases following a high NaCl diet (40, 42) and that selective renal medullary infusion of a COX2 inhibitor results in hypertension in rats fed a high NaCl diet (41, 42). These studies are consistent with an important role of renal medullary COX2 in maintaining sodium homeostasis and blood pressure.

The COX enzyme represents the committed step for prostanoid biosynthesis. COX catalyzes the conversion of arachidonic acid to PGH2 (19, 32). PGH2 is then converted to five bioactive prostanoids via distinct prostanoid synthases, including PGE2, PGL2, PGF2α, PGD2, and TxB2 (21, 26, 34). Two isoforms of COX have been identified: COX1 and COX2. COX1 is constitutively expressed in most tissues examined and is thought to be involved in maintaining basic cellular function. In contrast, COX2 is induced by physiological and pathophysiological stressors and plays important roles in the cellular response to stress (19, 35). The prostanoids interact with a group of G protein-coupled receptors (27). Since prostanoids are rapidly metabolically degraded, their actions are limited to the immediate vicinity of their synthetic sites (2, 31). Therefore, the biological effects of COX-derived prostanoids depend on a distinct enzymatic machinery that couples COX to a specific prostanoid synthase, and the interaction of prostanoids with their respective receptors (14, 27, 32). The present studies (1) examine the effect of a high-salt diet on renal medullary prostanoid synthase expression and prostanoids synthesis and (2) characterize the receptor through which medullary prostanoids mediate sodium excretion.

MATERIALS AND METHODS

Experimental animals. Male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME), EP2 receptor-deficient mice (C57BL/6J background) were generated at Vanderbilt University as previously described (24). The mice were maintained on standard rodent chow and allowed free access to water before the experiments. To examine the effect of a high-salt diet on renal medullary COX and prostanoid synthase expression, mice were fed with either a high-salt diet (8% NaCl, Research Diet) or a normal-salt diet (0.3% NaCl) for 3 days. At the end of the experiments, the mice were killed under anesthesia and the kidneys were harvested for immunoblotting, immunohistochemistry, and quantitative RT-PCR. All animal experiments were performed according to animal protocols approved by the Animal Care Use Committee at the Vanderbilt University School of Medicine.

Immunoblotting. After the mice were killed, the renal medulla was isolated, and protein was extracted. Protein concentration was determined using the bicinchoninic acid protein assay (Sigma, St. Louis, MO). Protein extract was loaded (30 μg/lane) on a 10% SDS-PAGE minigel and run at 120 V. Proteins were transferred to a polyvinylidene difluoride membrane and blocked with 5% nonfat dry milk in 1× Tris buffered saline containing 0.1% Tween 20. The membranes were probed with serum-free monoclonal antibody to COX1, COX2, PGE2, PGL2, PGF2α, PGD2, and TxB2 (21, 26, 34). The antibodies were detected by horseradish peroxidase-conjugated secondary antibody (Amersham, Buckinghamshire, UK) and chemiluminescence (Amersham). Densitometry of the blots was performed using the NIH Image program, and the data were expressed as the ratio of the band density relative to the sodium average density of each experimental group.
dene difluoride membrane at 100 V for 1 h on ice. The membrane was washed three times with TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20), incubated in blocking buffer (150 mM NaCl, 50 mM Tris, 0.05% Tween 20, and 5% Carnation nonfat dry milk, pH 7.5) for 1 h at room temperature, and then incubated with primary antibody in blocking buffer overnight at 4°C. The primary antibodies used for immunoblotting studies were anti-COX2 antibody (1:1,000), anti-COX1 antibody (1:1,000), anti-microsomal prostaglandin E synthase 1 (mPGES1; 1:1,000), anti-mPGES2 (1:1,000), anti-prostacyclin synthase (PGIS; 1:1,000), anti-cytoxic PGS1 (cPGES1; 1:1,000), and anti-thromboxane synthase (TxBS; 1:1,000). These primary antibodies were from Cayman Chemical (Ann Arbor, MI). After being washed three times, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000-1:20,000, Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature, followed by three washes. Antibody labeling was visualized by the addition of a chemiluminescence reagent (Renaissance, PerkinElmer Life Sciences), and the membrane was exposed to Kodak XAR-5 film.

Immunofluorescence staining. Kidney tissues were fixed in 4% paraformaldehyde and incubated in 30% sucrose overnight. Cryostat sections (5 μm) were blocked with 3% normal donkey serum for 20 min. The blocking buffer from the M.O.M. kit (Vector Laboratories, Burlingame, CA) was used with mouse monoclonal primary antibody. Sections were then incubated with primary antibody for 60 min at room temperature. After washing in PBS, the sections were incubated in Cy3-conjugated anti-IgG secondary antibody (Jackson ImmunoResearch Laboratories) for 30 min. Sections were viewed and imaged with a Zeiss Axioskop and spot-cam digital camera (Diagnostic Instruments) or confocal microscopy (Zeiss LSM510). The primary antibodies used for immunofluorescence studies were anti-COX2 antibody (1:1,000), anti-mPGES1 (1:100), anti-PGIS (1:50), and anti-cPGES1 (1:100) from Cayman Chemical and anti-aquaporin-2 (AQP2) antibody (1:1,000) from Alpha Diagnostic International (San Antonio, TX).

Quantitative RT-PCR. Total RNA was extracted from the renal medulla using TRIzol reagent (Invitrogen). Reverse transcription was performed using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using Applied Biosystems Taqman gene expression assay system. The PCR primers used were Mm00477214_m1 (mouse COX1), Mm00478374_m1 (mouse COX2), Mm00447271_m1 (mouse PGIS), Mm00452105_m1 (mouse mPGES1), and Mm00727367_s1 (mouse cPGES). Primers for eukaryotic 18S rRNA (4319413E) were used as an endogenous control. Gene expression values were calculated based on the comparative threshold cycle (Ct) method detailed in Applied Biosystem User Bulletin Number 2. Gene expression levels were normalized to the 18S rRNA and displayed as fold-induction of a high-salt diet relative to a normal-salt diet.

Prostaglandin measurement. The prostaglandin profile in the renal medulla before and after a high-salt diet was determined using gas chromatography/negative ion chemical ionization mass spectrometric assays, as described previously (15). The amount of each prostaglandin was normalized to the total amount of protein in the renal medulla. Data are presented as fold-induction of prostaglandin after a high-salt diet relative to a normal-salt diet.

Effect of renal medullary prostaglandin infusion on urinary sodium excretion. One week before the experiment, the right kidney of the mice increased significantly as assessed by immunoblotting studies, as described previously (15). Densitometric analysis showed that mPGES1 expression increased by 170% (P < 0.05), mPGES2, cPGES, and PGIS were detected in the renal medulla, but their expression levels did not change significantly after a high-salt diet (Fig. 1). Although TxBS protein was detected in the renal cortex, no TxBS was detected by immunoblotting in the renal medulla of mice on either a normal-salt diet or a high-salt diet (Fig. 1).

Quantitative real-time PCR showed that renal medullary mPGES1 mRNA expression levels significantly increased following a high-salt diet compared with a normal-salt diet (1.7-fold, P < 0.05, Fig. 2), consistent with its protein expression. In contrast, a high-salt diet did not alter cPGES and PGIS mRNA expression (Fig. 2). A high-salt diet also increased renal medullary COX2 mRNA expression (Fig. 2).

Immunofluorescence staining demonstrated that mPGES1 was mainly expressed in the collecting ducts and the cells in the interstitium (Fig. 3). cPGES was primarily expressed in the collecting ducts. PGIS was mainly detected in the interstitium (Fig. 3).

A high-salt diet increases renal medullary PGE2 biosynthesis. As shown in Fig. 4, PGE2 was the major prostaglandin present in the renal medulla of mice on a normal-salt diet (0.3% NaCl).
Low levels of PGF2α, PGI2 metabolite 6-keto PGF1α, and PGD2 were also detected. TxB2 was not detected. Following a high-salt diet, renal medullary PGE2 levels increased by 190% (181 ± 83 vs. 345 ± 48 ng/g, P < 0.05). A high-salt diet also increased medullary PGF2α and 6-keto PGF1α, but the difference did not reach statistical significance. PGD2 levels were unchanged after a high-salt diet (Fig. 4).

Intramedullary infusion of PGE2 increases urinary sodium excretion. To determine whether increased renal medulla PGE2 had any effect on urinary sodium excretion, we infused PGE2 directly into renal medulla in anesthetized mice and examined urinary sodium excretion. Direct intramedullary infusion of PGE2 (10 ng·20 µl⁻¹·h⁻¹), but not of vehicle, significantly increased urine volume (UV; PGE2, from 3.3 ± 0.6 to 9.5 ± 1.6 µl/min vs. vehicle, from 2.2 ± 0.4 to 1.5 ± 0.8, n = 7, P < 0.01) and UNaV (PGE2 from 0.11 ± 0.02 to 0.32 ± 0.05 vs. vehicle 0.07 ± 0.01 to 0.05 ± 0.03 µeq/min, n = 7, P < 0.01) (Fig. 5). The same dose of PGE2 administered intravenously did not change UV or UNaV (Fig. 5), suggesting that the intramedullary PGE2-induced natriuresis was not due to systemic effects due to “spillover” of PGE2 into the systemic circulation. Infusion of PGE2 into the renal cortex (10 ng·20 µl⁻¹·h⁻¹) did not increase UV (from 0.8 ± 0.18 at 0 h to 0.6 ± 0.18 µl/min at 2 h after PGE2 infusion, n = 4) and UNaV (from 0.05 ± 0.014 at 0 h to 0.04 ± 0.016 µeq/min at 2 h after PGE2 infusion, n = 4). Renal medullary PGE2 infusion did not significantly increase urine potassium excretion (from 0.16 ± 0.03 to 0.25 ± 0.03 µeq/min at 2 h after PGE2 infusion, n = 7, P > 0.05). Intramedullary infusion of PGE2 did not change systemic blood pressure (Fig. 5). These studies suggest a selective effect of renal medullary PGE2 on sodium excretion.

EP2 receptor mediates renal medullary PGE2-induced natriuresis. PGE2 exerts its biological function by interacting with one of four E-prostanoid receptors, e.g., EP1–4. To determine which receptor mediates medullary PGE2-induced natriuresis, agonists for each receptor were administered directly into the renal medulla. Intramedullary infusion of sulprostone (10 ng/h), which activates the EP1 and the EP3 receptors, slightly
increased UV and UNaV (Fig. 6). However, compared with vehicle, the difference did not reach statistical significance. PGE$_1$-OH (10 ng/h), a ligand for the EP4 receptor, also did not alter UV or UNaV. In contrast, intramedullary infusion of the EP2 agonist butaprost (10 ng/h) significantly increased UV by 280% ($n=9$, $P<0.01$) and UNaV by 533% ($n=9$, $P<0.01$). Intravenous administration of the same amount of butaprost did not alter UNaV (Fig. 6). No change in blood pressure was observed following either intramedullary or intravenous infusion of the same amount of butaprost (Fig. 6).

To further determine the role of the EP2 receptor in mediating renal medullary PGE$_2$-induced natriuresis, the effect of PGE$_2$ and the EP2 receptor agonist butaprost on UNaV was examined in EP2 receptor knockout mice. As shown in Fig. 7, genetic deletion of the EP2 receptor abolished the natriuretic effect of PGE$_2$ and butaprost, supporting that EP2 played a critical role in mediating PGE$_2$-induced natriuresis.

**Discussion**

It has been demonstrated that a high-salt diet increases renal medullary COX2 expression (40–42). Blockade of renal medullary COX2 results in sodium-dependent hypertension in uninephrectomized rats (41, 42). The present studies demonstrate that 1) PGE$_2$ is the major prostanooid induced by high-salt diet; 2) increased mPGES1 in the renal medulla likely contributes to increased PGE$_2$ synthesis following a high-salt diet; 3) renal medullary PGE$_2$ promotes renal sodium excretion via the EP2 receptor.

COX mediates the initial committed step in prostanoid synthesis by converting arachidonic acid to PGH$_2$. The synthesis of bioactive prostanoids requires a sequential enzymatic
machinery that couples COX with a distinct prostanoid synthase, converting PGH2 to the bioactive product (2, 14, 32). Distinct cellular localization of the two isoforms of COX in the kidney has been well documented (9, 16, 17, 30). COX1 is predominantly expressed in the collecting ducts, while COX2 is mainly expressed in the renal medullary interstitial cells and the cortical thick ascending limb/macular densa. Recent studies demonstrate that a high-salt diet induces COX2 expression, and this induction is predominantly localized in renal medullary interstitial cells (9, 41). In contrast, COX1, which is primarily expressed in collecting ducts, is not altered by a high-salt diet (40). In the present study, we examined the expression and cellular localization of prostanoid synthases in the renal medulla of mice on a normal-salt or a high-salt diet. The present studies show that mPGES1, mPGES2, cPGES, and PGIS were detected in the renal medulla by both immunoblotting and quantitative RT-PCR. A high-salt diet increased mPGES1 protein and mRNA expression but did not change the expression of mPGES2, cPGES, or PGIS. Immunofluorescence staining showed that the mPGES1 was expressed in both collecting ducts and renal medullary interstitial cells, suggesting that mPGES1 is coupled to COX2 in renal medullary interstitial cells and to COX1 in the collecting ducts. Consistent with increased medullary mPGES1 expression, renal medullary PGE2 biosynthesis was also significantly increased after a high-salt diet. Interestingly, in the present study gas chromatography/negative ion chemical ionization mass spectrometry did not detect an increase in renal medulla PGF2α synthesis after a high-salt diet, consistent with a recent study (23) but in contrast to other published studies (36, 37). The reason of this discrepancy is not known, but differences in the detection
Urine was collected every 30 min before and during PGE$_2$ or butaprost infusion. UV and UNaV were determined. Values are means ± SE.

Methods (gas chromatography/negative ion chemical ionization mass spectrometric vs. ELISA) or species differences could contribute. Based on the distribution of COX and PGES in the kidney, it is suggested that the high-salt diet induced-PGE$_2$ synthesis in the renal medulla may come from renal medullary interstitial cells via a COX2/mPGES1 pathway, and collecting ducts via a COX1/mPGES1 pathway. Ye et al. (41) have shown that inhibition of either COX2 or COX1 in the renal medulla resulted in increased blood pressure after a high-salt diet, supporting that both COX2-derived PGE$_2$ from renal medullary interstitial cells and COX1-derived PGE$_2$ from collecting ducts are involved in the maintenance of normal blood pressure after a high-salt diet.

The renal medulla has been suggested to play a critical role in the maintenance of blood pressure by modulating sodium balance (1). To examine whether increased renal medullary PGE$_2$ levels promote sodium excretion, we infused PGE$_2$ directly into the renal medulla via an intramedullary catheter and examined its effect on sodium excretion. The result shows that intramedullary infusion of PGE$_2$ significantly increased UNaV, consistent with a natriuretic effect of PGE$_2$ in the renal medulla. The same dose of PGE$_2$ administered intravenously failed to produce this natriuretic effect, consistent with a local effect of PGE$_2$ rather than a systemic effect due to leakage into the circulation. In the present study, low-dose intravenous PGE$_2$ did not decrease systemic blood pressure, excluding the possibility that systemic hypotension counteracted the natriuretic effect of PGE$_2$.

PGE$_2$ exerts its function by interacting with four G protein-coupled receptors, EP1, EP2, EP3, and EP4. The intrarenal expression and localization of EP receptors in the kidney have been characterized, primarily at mRNA levels (4, 22). In the renal medulla, EP1 mRNA expression is detected in the medullary collecting ducts, with EP3 predominantly expressed in the thick ascending limb and outer medullary collecting duct (4). EP2 and EP4 mRNA has been reported in vas recta by RT-PCR of the dissected vessels (22). To examine which PGE$_2$ receptor contributed to the natriuretic effect of PGE$_2$ in the renal medulla, we infused a specific ligand for each EP receptor into the medulla and examined sodium excretion. Our results show that the EP2 agonist butaprost produced natriuresis when administered via the intramedullary catheter. The diuretic and natriuretic effects of intramedullary infused PGE$_2$ and butaprost were abolished in mice deficient in the EP2 receptor, further supporting the role of the EP2 receptor in mediating natriuresis in the renal medulla. Importantly, EP2 receptor deletion has been demonstrated to cause salt-sensitive hypertension (24). These studies support a critical role of the renal medullary EP2 receptor in modulating salt balance and the blood pressure. In the present study, intramedullary infusion of sulprostone (the EP1/EP3 ligand) slightly increased UV and UNaV, but the change was not statistically significant. Activation of the EP1 and EP3 receptors has been reported to inhibit sodium reabsorption and inhibit AVP-induced water absorption in isolated, microperfused cortical collecting ducts (11, 18, 33). However, an in vivo study demonstrated that deletion of the EP1 gene failed to cause significant sodium retention or hypertension following a high-salt diet (12). Deletion of the EP3 receptor also did not alter concentrating ability in response to vasopressin (8). The reason for this inconsistency between in vivo and in vitro studies is not clear. The local effect of EP1 and EP3 on sodium absorption remains to be further investigated.

The mechanism by which the EP2 receptor mediates the natriuretic effect of PGE$_2$ is incompletely defined. PGE$_2$ has been suggested to exert its natriuretic and diuretic effects by directly dilating the medullary vasculature (vasa recta) or/and inhibiting epithelial salt absorption in the thick ascending limb and collecting duct (30). The vasa recta blood flow plays a critical role in pressure natriuresis and is a major determinant for systemic blood pressure (1, 29). Vasa recta blood flow is controlled by a balance between vasoconstrictors such as angiotensin II and vasodilators (1, 28). COX2-derived prostanooid has been reported to play an important role in counteracting the vasoconstrictor effect of angiotensin II (30). EP2 mRNA is detected in rat and rabbit renal medulla by nuclease protection assay (10, 22). RT-
PCR of microdissected rat nephron segments and vessels shows that EP2 mRNA is expressed in the vasa recta (22). These studies suggest that EP2 activation may cause dilation of the vasa rectae, increasing renal medullary blood flow and contributing to PGE2-induced natriuresis (22). EP2 mRNA is also detected in the descending thin limb and medulla. Renal medullary PGE2 promotes sodium excretion via the direct effect of EP2 on tubular sodium reabsorption, its vasodilator effect on vasa recta, or both remains to be further explored. Taken together, the present studies have demonstrated that a high-salt diet increases microsomal prostaglandin E synthase-1 expression and PGE2 biosynthesis in the renal medulla. Renal medullary PGE2 promotes sodium excretion via the EP2 receptor. These studies provide further insights into the involvement of EP2 knockout mice with regard to the development of NaCl-sensitive hypertension. The studies suggest that the COX2/PGES1/PGE2/EP2 pathway in the vasa rectae, increasing renal medullary blood flow and sodium excretion, or both may play an important role in modulating sodium excretion and maintaining body sodium balance and systemic blood pressure.

ACKNOWLEDGMENTS

Immunoﬂuorescence experiments were performed in part through the use of the VUMC Cell Imaging Shared Resource.

GRANTS

These studies were supported by National Institutes of Health Grants DK-071876 to C.-M. Hao, DK-48831 and ES-13125 to J. Morrow, GM-15431 to R. Breyer and J. Morrow, and DK-37097 to R. Breyer. J. Chen is an awardee of National Natural Science Foundation (30400211).

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