PGE\textsubscript{2} EP\textsubscript{3} receptor downregulates COX-2 expression in the medullary thick ascending limb induced by hypertonic NaCl

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Hao S, Hernandez A, Quiroz-Munoz M, Cespedes C, Vio CP, Ferreri NR. PGE\textsubscript{2} EP\textsubscript{3} receptor downregulates COX-2 expression in the medullary thick ascending limb induced by hypertonic NaCl. \textit{Am J Physiol Renal Physiol} 307: F736–F746, 2014. First published July 30, 2014; doi:10.1152/ajprenal.00204.2014.—We tested the hypothesis that inhibition of EP\textsubscript{3} receptors augments cyclooxygenase (COX)-2 expression in the thick ascending limb (TAL) induced by hypertonic stimuli. COX-2 protein expression in the outer medulla increased approximately twofold in mice given free access to 1% NaCl in the drinking water for 3 days. The increase was associated with an approximate threefold elevation in COX-2 mRNA accumulation and an increase in PGE\textsubscript{2} production by isolated medullary (m)TAL tubules from 77.3 ± 8.4 to 165.7 ± 10.8 pg/mg protein. Moreover, administration of NS-398 abolished the increase in PGE\textsubscript{2} production induced by 1% NaCl. EP\textsubscript{3} receptor mRNA levels also increased approximately twofold in the outer medulla of mice that ingested 1% NaCl. The selective EP\textsubscript{3} receptor antagonist L-798106 increased COX-2 mRNA by twofold in mTAL tubules, and the elevation in COX-2 protein induced by 1% NaCl increased an additional 50% in mice given L-798106. COX-2 mRNA in primary mTAL cells increased twofold in response to media made hypertonic by the addition of NaCl (400 mosmol/kg H\textsubscript{2}O). L-798106 increased COX-2 mRNA twofold in isotonic media and fourfold in cells exposed to 400 mosmol/kg H\textsubscript{2}O. PGE\textsubscript{2} production by mTAL cells increased from 79.3 ± 4.6 to 286.7 ± 6.3 pg/mg protein after challenge with 400 mosmol/kg H\textsubscript{2}O and was inhibited in cells transiently transfected with a lentivirus short hairpin RNA construct targeting exon 5 of COX-2 to silence COX-2. Collectively, the data suggest that local hypertonicity in the mTAL is associated with increased COX-2 expression concomitant with elevated EP\textsubscript{3} receptor expression, which limits COX-2 activity in this segment of the nephron.

cyclooxygenase-2; prostaglandin E\textsubscript{2}; EP\textsubscript{3} receptors, thick ascending limb; hypertonic stress

Two isoforms of cyclooxygenase (COX), COX-1 and COX-2, are expressed in the mammalian kidney (16, 18, 19, 36, 42). The thick ascending limb (TAL) of Henle’s loop metabolizes arachidonic acid by the COX-2 pathway (10, 31, 35, 38) and is a nephron segment that can contribute to the development of salt-sensitive hypertension (11, 32). Expression of COX-2 but not COX-1 increases along the TAL in response to treatment with angiotensin-converting enzyme inhibitors, adenectomy, changes in salt intake, and diabetes (6, 16, 27, 35, 42). Studies regarding the regulation of COX-2 expression by inhibition of the renin-angiotensin system in neonates have demonstrated the localization of COX-2 to the medullary (m)TAL as well as the cortical TAL (24). Moreover, COX-2 expression increases axially along the TAL after adenectomy, is expressed in mTAL cells of juxtamedullary nephrons, and is associated with increased PGE\textsubscript{2} synthesis by mTAL tubules and primary cultures of mTAL cells (10, 31, 35).

The major COX-2-derived prostanoid in the kidney is PGE\textsubscript{2}, which is synthesized by tubular epithelia and interstitial cells and plays an important role in the regulation of renal renin release (15, 21, 33) and salt balance (12). Interestingly, COX-2-derived PGE\textsubscript{2} synthesis is differentially regulated in the kidney in a cell type-dependent manner. Administration of a low-salt diet increased COX-2-derived PGE\textsubscript{2} levels in the renal cortex (16, 29, 41). In contrast, ingestion of a high-salt diet increased inner medullary COX-2 and PGE\textsubscript{2} synthesis (5, 42). These studies are consistent with the concept that medullary PG production is upregulated during volume expansion, thus promoting the excretion of NaCl and water. Despite these observations, the mechanisms that regulate COX-2 expression in the mTAL are not well understood.

The diverse biological actions of PGE\textsubscript{2} are mediated by four different G protein-coupled receptor subtypes, EP\textsubscript{1}–EP\textsubscript{4}, each activating distinct intracellular signaling pathways (5). We recently showed that a novel negative feedback mechanism in the mTAL, effected by PGE\textsubscript{2} acting via EP\textsubscript{3} receptors, attenuates COX-2 expression induced by COX-2 inhibitors (37). Expression of COX-2 in the TAL under basal conditions is less than that observed in the inner medulla (42). However, COX-2 expression is markedly increased along the mTAL by administration of the EP\textsubscript{3} antagonist L-798106 (37). We postulated that the suppression of COX-2 expression in the mTAL via EP\textsubscript{3} receptors limits the capacity of this nephron segment, which is responsible for the reabsorption of ~25% of filtered NaCl and Ca\textsuperscript{2+} and contributes to the generation of the osmotic gradient that drives vasopressin-dependent water reabsorption by the collecting duct, to produce COX-2-derived PGE\textsubscript{2} in response to diverse stimuli. In the present study, we tested the hypothesis that inhibition of EP\textsubscript{3} receptors augments COX-2 expression in the mTAL induced by hypertonic NaCl (HS).

METHODS

Chemicals and reagents. All chemicals were of the highest grade commercially available. Anti-COX-2 and anti-COX-1 antibody were purchased from Abcam (Cambridge, MA) and used at a 1:1,000 dilution for immunoblot analysis. Tissue culture media were obtained from Life Technologies (Grand Island, NY). Collagenase (type IA) was from Sigma (St. Louis, MO), and polyvinylidene difluoride membranes were obtained from Amersham (Arlington Heights, IL).

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The selective COX-2 inhibitor NS-398 and EP3 receptor antagonist L-798106 were purchased from Cayman Chemical (Ann Arbor, MI) or Santa Cruz Biotechnology (Santa Cruz, CA).

Animals. Male C57BL/6J mice (19–21 g, Jackson Laboratory) or male Sprague-Dawley rats (190–200 g, Charles River Laboratory) were maintained on standard diet and given tap water ad libitum. Experimental procedures were conducted in accordance with institutional and international guidelines for the welfare of animals (Animal Welfare Assurance nos. A3362-01 or A5848-01, Office of Laboratory Animal Welfare, Public Health Service, National Institutes of Health). Animals were administered the COX-2 inhibitor NS-398 (5 mg·kg⁻¹·day⁻¹) or the EP3 receptor antagonist L-798106 (100 µg·kg⁻¹·day⁻¹) subcutaneously for 6 days, starting 3 days before treatment with 1% NaCl in the drinking water for 3 days. Control groups consisted of animals given vehicle or treated with NS-398 or L-798106 alone for 6 days. At the end of the protocol, kidneys were removed and processed for immunohistochemistry, morphometric analysis, real-time RT-PCR, Western blot analysis, and PGE2 analysis.

Immunohistochemistry and morphometric analysis. Kidney samples were fixed by immersion in Bouin’s solution for 24 h at room temperature and then embedded in paraffin wax. Immunostaining was performed using an indirect immunoperoxidase technique as previously described to localize COX-2 in rat kidneys (26, 35). Tissue sections (5 µm thickness) were incubated overnight at 22°C with a primary antiserum raised against COX-2 (catalog no. sc-1747, Santa Cruz Biotechnology). The secondary antibody and corresponding peroxidase-anti-peroxidase complex (MP Biomedicals, Aurora, OH) were applied for 30 min each at 22°C. The immunoperoxidase reaction was visualized after incubation of sections in 0.1% (wt/vol) diaminobenzidine and 0.03% H₂O₂. Omission of the primary antibody or replacing it with preimmune serum served as controls. Tissue samples from all groups were coded and studied independently by two observers in a blinded coded fashion; sections selected at random were used for each animal, and 4 fields/section were studied. Images were examined and acquired using a Nikon Eclipse E600 microscope and Nikon DS-Ri1 digital camera, respectively (35). The COX-2-stained area in each image was quantified utilizing computer-assisted image-analysis software (Simple PCI, Hamamatsu). Values corresponding to the total area of immunostained cells were averaged and expressed as mean absolute values per square micron expressed as fold changes compared with control, as previously described (26).

Localization of EP3 receptors. PGE2 EP3 receptors were localized in renal tissue from normal and 1% NaCl-treated animals using specific anti-EP3 receptor antibody (catalog no. 101760, Cayman Chemicals) with the immunoperoxidase method (26, 35, 37).

Isolation of mTAL tubules and cells. mTAL tubules and cells (90–95% purity) were isolated from mice as previously described (14). Briefly, kidneys were perfused with sterile 0.9% saline via retrograde perfusion of the aorta. The outer medulla (OM) was excised, minced with a sterile blade, and incubated for 10 min at 37°C in 0.01% collagenase solution gassed with 95% O₂. The suspension was sedimented on ice and mixed with HBSS containing 2% BSA (Life Technologies), and the supernatant containing the crude suspension of tubules was collected. The collagenase digestion step was repeated three times, and the remaining undigested tissue and combined supernatants were centrifuged for 10 min, resuspended in HBSS, and filtered through a 53-µm nylon mesh membrane (Fisher Scientific, Springfield, NJ). The filtered solution was discarded, and tubules retained on the mesh were resuspended in HBSS and centrifuged at 500 rpm for 10 min; pelleted tubules were used in experiments or to establish primary cultures of mouse mTAL cells. Cells were grown on membrane inserts in six-well plates using renal epithelial cell basal medium (Cambrex Bio Science, Walkersville, MD). After 6–7 days, monolayers of cells were 70–80% confluent. Cells were quiesced for 24 h in RPMI medium containing 0.5% FBS, L-glutamine (2 mM), 100 U/ml streptomycin-penicillin, MEM nonessential amino acids, MEM sodium pyruvate, and β-mercaptoethanol before their use (39).

Plasmid constructs and virus preparation. All constructs were generated using standard cloning procedures and verified by restriction enzyme analysis and DNA sequencing. The inhibitory construct for COX-2 was designed using a short hairpin (sh)RNA-expressing construct targeting exon 5 of murine COX-2 (U6-C2-ex5) under control of the murine U6 small nuclear RNA promoter as previously described (14); scrambled U6-shRNA (U6) was used as a negative control of the murine U6 small nuclear RNA promoter as previously described (14); scrambled U6-shRNA (U6) was used as a negative control of the murine U6 small nuclear RNA promoter as previously described (14).
control. Subcloning of U6-C2-ex5 into a pLKO.1 vector and cotransfection of human embryonic kidney (HEK)-293-T cells with pLKO.1 was performed to generate lentivirus encoding U6-C2-ex5. psPAX2 and pMD2.G plasmids were used for the preparation of lentivirus (Addgene MIT, Cambridge, MA).

**Gene transfection.** HEK-293-T cells were grown in 20-cm² flasks to generate lentivirus. Briefly, the packaging cells were seeded at a density of $7 \times 10^5$ cells/flask in 5 ml media (DMEM-10% FBS-no antibiotics) 24 h before transfection and grown at 37°C and 5% CO₂. DNA for transfection was prepared by mixing 1.0 μg psPAX2 and 0.1 μg pMD2.G.

**Fig. 2.** HS intake increases COX-2 protein expression in the OM. A–D: Western blot analysis of COX-2 (A and B) and COX-1 (C and D) protein expression in the OM from mice given tap water or 1% NaCl in drinking water. Data in B are presented as means ± SE of three independent experiments; n = 3 (P < 0.05). E and F: renal sections from the cortex and OM were immunostained for COX-2. G: morphometric analysis of COX-2-immunostained cells after rats were given 1% NaCl or tap water for 7 days.
µg pMD2.G with 1.5 µg pLKO.1 or psiLV plasmids in each flask. A mixture of 150 µl OptiMEM and 6 µl FUGENE (Roche, Mannheim, Germany) was then added to the DNA, and this mixture was incubated for 15 min before addition to the packaging cells. Cells were incubated for 12 h, and media were changed to remove the remaining transfection reagent. Lentiviral supernatants were collected 48 h after transfection; the supernatants were pooled and frozen at −80°C for long-term storage.

Primary cultures of murine mTAL cells were cultured to 70–80% confluence in six-well plates with membrane inserts (BD Biosciences, San Jose, CA) as indicated (8), the medium was removed, and cells were placed in 1 ml of serum-free OPTI-MEM containing different plasmid DNA constructs and 10 µl Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions, for 4 h at 37°C and 5% CO2. After the transfection period, 1 ml of renal epithelial cell growth medium containing 20% FBS was added, and cells were incubated overnight at 37°C and 5% CO2. The medium was then removed, and cells were cultured for an additional 12–48 h in renal epithelial cell growth medium containing 10% FBS. Transfection efficiency was evaluated by flow cytometry as previously described (13).

Isolation of total RNA and amplification of cDNA fragments. Total RNA was isolated using TRIzol reagent (Ambion, Carlsbad, CA). Chloroform (0.2 ml) was added at room temperature for 2–3 min followed by centrifugation at 4°C at 12,000 rpm for 15 min. Isopropanol (3 vol) was added to the recovered supernatant, and the mixture was incubated at room temperature for 10 min and then centrifuged at 4°C at 12,000 rpm for 15 min. The pellet was washed in 1 ml of 75% ethanol, mixed gently, and centrifuged for 5 min at 7,500 rpm at 4°C; the supernatant was removed, and the pellet was dried for 5–10 min. Total RNA was treated with deoxyribonuclease I for 30 min, and a 3-µg aliquot was used for cDNA synthesis using the Superscript Preamplification system (Life Technologies) in a 20-µl reaction mixture containing Superscript II reverse transcriptase (200 U/µl) and random hexamers (50 ng/µl). The reaction was incubated at room temperature for 10 min to allow extension of the primers by reverse

Fig. 3. HS intake increases PGE2 content and EP3 receptor mRNA abundance. A and B: PGE2 content in freshly isolated murine mTAL tubules (A) and the rat OM (B). C: the effect of the COX-2 inhibitor NS-398 on the PGE2 content increase in response to administration of 1% NaCl in the drinking water was determined in freshly isolated murine mTAL tubules.
A 0.5-μg aliquot of total RNA was converted to cDNA using random primers and PowerScript RT (Clontech, Mountain View, CA). cDNA from each RNA sample was placed in a 20 μl RT-PCR mixture using a FastStart DNA Master SYBR Green I kit (Roche) supplemented with 3 mM MgCl₂ and Platinum Taq polymerase (Invitrogen). Quantitative real-time PCR was used to determine the accumulation of mRNA using the following specific primer pairs: murine COX-2, forward 5'-GAGAAGGAAATGCTGCAGA-3' and reverse 5'-GATATTGAGGAGAACAGATGGG-3'; COX-1, forward 5'-GACACATGGATACTGGCTCT-3' and reverse 5'-GCCGAAGGCTGAACATCTG-3'; mouse EP₃ receptor, forward 5'-GCAAGACACAGATGGAAAG-3' and reverse 5'-GGGAAACAGGTACTGCAATG-3'; and rat EP₃ receptor, forward 5'-GAGACCGTATCCAGCTTAT-3' and reverse 5'-GGGATTAGGAAGGAATTGC-3'. Input cDNAs were normalized using β-actin, and the efficiency of primer pair amplification was determined using a standard curve generated using previously described protocols (28, 30). The 2⁻ΔΔCt method (where Ct is threshold cycle) was used to evaluate changes in COX-2, COX-1, and EP₃ receptor mRNA accumulation (25).

**Western blot analysis.** Cells and tissue were solubilized with Triton X-100 lysis buffer after protease inhibitors (Roche) were added for COX-2 or COX-1. Protein samples were heated in boiling water with loading buffer, and concentrations were determined with a Bio-Rad protein assay kit (Hercules, CA). Equal amounts of protein were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. After being blocked, membranes were probed at 4°C overnight with appropriate primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Pittsburgh, PA). Membranes were washed, and proteins were detected by ECL (Amersham).

**Measurement of PGE₂.** PGE₂ content in murine mTAL tubules and rat OM tissues was determined by liquid chromatography-tandem mass spectroscopy (LC-MS/MS; Life Technologies) using a modified method according to the previously published assay (40). Briefly, mTAL tubules or frozen OM tissue were homogenized and extracted for prostaglandins and quantified by LC-MS/MS. Analysis of cellular PGE₂ levels was also determined using an enzyme immunoassay kit (catalog no. RP222210, GE Healthcare) according to the manufacturer’s instructions. Data were normalized by protein amount, which was determined by a Bradford protein assay (Bio-Rad).

**Statistics.** All data are presented as means ± SE. Statistical analyses were performed using one-way ANOVA followed by a Tukey’s multiple-comparisons test and an unpaired t-test using the appropriate GraphPad Prism software (GraphPad Software, San Diego, CA). Differences with P values of <0.05 were considered statistically significant.

**HS intake increases COX-2 mRNA abundance in vivo.** The relative abundance of mRNA levels for COX-1 and COX-2 in the OM from mice given tap water or 1% NaCl in the drinking water for 3 days was determined using quantitative RT-PCR. Evaluation of the relative levels indicated that mice given 1% NaCl exhibited about a threefold increase in COX-2 mRNA accumulation compared with mice given tap water (Fig. 1A). In contrast, COX-1 mRNA accumulation did not change in response to an increase in NaCl intake (Fig. 1B). Similarly, COX-2 but not COX-1 mRNA accumulation increased in freshly isolated mTAL tubules from mice given 1% NaCl for 3 days (Fig. 1, C and D). Collectively, these data indicate that HS selectively induces COX-2 mRNA accumulation in the OM TAL and suggest that other cell types in this region of the kidney do not contribute importantly to this effect. In control experiments, total RNA was amplified before cDNA synthesis to exclude possible contamination with genomic DNA, and DNA sequences for each of the PCR fragments were identical to those previously published for the corresponding COX isoforms (not shown).

**HS intake increases COX-2 protein expression in vivo.** Western blot analysis showed that COX-2 protein expression in the OM was approximately twofold higher in mice given 1% NaCl for 3 days compared with mice given tap water (Fig. 2, A and B). In contrast, COX-1 protein expression did not change in response to an increase in NaCl intake (Fig. 2, C and D). Similarly, immunohistochemical data revealed that the baseline level of COX-2 expression, which is typically limited to a small percentage of TAL cells, was increased after rats consumed 1% NaCl in the drinking water (Fig. 2, E and F). Morphometric analysis of these data revealed an approximate twofold increase in COX-2 expression in TAL cells (Fig. 2G). Collectively, these data indicate that COX-2 is selectively induced after HS intake in the OM TAL.

**HS intake stimulates the COX-2/PGE₂/EP₃ receptor signaling pathway in vivo.** We (10) have previously demonstrated that mTAL cells in primary culture express COX-2 protein and synthesize PGE₂ in response to PMA and TNF. However, the effect of in vivo HS intake, which increases medullary toxicity (17), on PGE₂ production by mTAL tubules has not yet been determined. Indeed, PGE₂ content in mTAL tubules from mice treated with 1% NaCl in the drinking water for 3 days in-
increased more than twofold compared with basal PGE2 production (Fig. 3A), which was similar to the levels of medullary PGE2 increased by ingestion of a high-salt diet (5). Similar results were obtained when rats were treated with 1% NaCl in drinking water, indicating that these responses are not restricted to mice (Fig. 3B). The increase in PGE2 content in mTAL tubules from mice treated with 1% NaCl was completely inhibited by administration of the selective COX-2 inhibitor NS-398, indicating that PGE2 production in the mTAL is COX-2 derived under these conditions (Fig. 3C). Interestingly, there was a concomitant twofold increase in EP3 receptor mRNA accumulation in mouse mTAL tubules and rat OMs in response to HS (Fig. 4, A and B). An increase in EP3 receptor expression localized to the apical membrane of the TAL segment was also observed 3 days after administration of 1% NaCl in the drinking water and was sustained up to 7 days (Fig. 5, A–C). Collectively, these data provide the anatomic basis for the regulation of COX-2, not COX-1, in the TAL by EP3 receptors and suggest that hypertonic stress activates a COX-2/PGE2/EP3 receptor signaling pathway in the renal outer medulla.

Inhibition of EP3 receptors increases COX-2 mRNA abundance and protein expression in vivo. We recently showed that activation of EP3 receptors during normal Na+ intake is part of a negative feedback mechanism that attenuates COX-2 expression in the TAL. However, it is not known to what extent this feedback inhibition regulates COX-2 in response to diverse stimuli. In the present experiments, the relative abundance of COX-2 mRNA levels in TAL tubules was determined in mice given tap water or 1% NaCl in drinking water for 3 days after administration of a selective EP3 receptor antagonist [L-798106 (100 μg·kg⁻¹·day⁻¹)]. COX-2 mRNA accumulation was increased in mice that received L-798106 compared with mice given tap water (Fig. 6). Moreover, the increase in COX-2 mRNA induced by HS intake was significantly increased in mice that were also treated with L-798106 (Fig. 6). Similarly, L-798106 increased basal levels of COX-2 protein.
expression in the OM and further increased expression levels of COX-2 induced by HS intake (Fig. 7, A and B). Taken together, the data demonstrate that antagonism of EP3 receptors increases both basal levels of COX-2 and those induced by HS intake, thus supporting the notion that activation of EP3 receptors limits the extent of COX-2 expression in vivo.

**Inhibition of EP3 receptors increases COX-2 mRNA in vitro.** We determined if the regulation of COX-2 by EP3 receptors could also be observed in the absence of integrated in vivo responses. Thus, primary cultures of mTAL cells were quiesced overnight and then challenged with a hypertonic stimulus [NaCl (400 mosmol/kg H2O)] for 9 h in the absence or presence of L-798106 (100 nM). Indeed, COX-2 mRNA accumulation in mTAL cells increased approximately twofold in response to media made hypertonic by the addition of NaCl compared with cells incubated in isotonic media (Fig. 8). Moreover, the increase in COX-2 mRNA induced by the addition of NaCl was potentiated in mTAL cells pretreated with L-798106. It is also interesting to note that L-798106 increased basal COX-2 mRNA accumulation (Fig. 8). These data suggest that inhibition of EP3 receptors mitigates its suppressive influence and enables a net increase in COX-2 mRNA accumulation in mTAL cells. Moreover, the tonic suppressive effect of EP3 receptor activation was evident in vitro as well as in vivo.

**Knockdown of COX-2 in mTAL cells.** A lentivirus-based shRNA vector targeting a region in exon 5 of the COX-2 gene (Fig. 9A) and subjected to a BLAST search to ensure specificity was used to silence COX-2 in mTAL cells. The psiLV-U6 vector that specifically targets COX-2 was designed as previously described (14). The COX-2 shRNA construct (U6-C2-ex5) specifically knocked down COX-2 but not COX-1 mRNA in mTAL cells, whereas empty vector (U6) had no effect on either isoform (Fig. 9B). Pretreatment of mTAL cells with the lentivirus construct psiLV-U6-C2-ex5 reduced basal mRNA accumulation and was the same as the increase in COX-2 mRNA observed in cells treated with PMA (1 μM; Fig. 9C). Neither basal nor stimulated COX-2 mRNA levels were affected in mTAL cells infected with the negative control shRNA lentivirus U6 (Fig. 9C). Collectively, these data show that lentivirus-based shRNA targeting successfully silences COX-2 in mTAL cells.

**The PGE2 production increase is dependent on COX-2.** The COX-2 shRNA construct was used to determine if PGE2 production induced in response to high NaCl concentration was dependent on COX-2. Primary cultures of mTAL cells were transduced with lentivirus packaged with the shRNA vector targeting COX-2 (EGFP-C2-ex5) to directly evaluate the role of COX-2 on PGE2 production. Cells were quiesced overnight and then challenged with media made hypertonic by the addition of NaCl to infected cells and cells infected with control lentivirus vector (EGFP-U6; Fig. 10). Neither basal nor stimulated COX-2 mRNA levels were affected in mTAL cells infected with the negative control shRNA lentivirus U6 (Fig. 9C). Collectively, these data show that lentivirus-based shRNA targeting successfully silences COX-2 in mTAL cells.
Fig. 9. Validation of mTAL cell knockdown using a COX-2 isoform targeting vector. A: design and validation of COX-2 isoform specificity in the generation of the EGFP-C2-ex5 lentivirus construct for COX-2 short hairpin (sh)RNA according to cDNA sequences. B: cDNA fragments for COX isoforms were generated by RT-PCR and evaluated by separation on a 1% agarose gel stained with ethidium bromide. C: specific knockdown of COX-2 mRNA in mTAL cells after the EGFP-C2-ex5 lentivirus construct was transfected as described in METHODS. Accumulation of COX-2 mRNA in mTAL cells was evaluated by quantitative real-time RT-PCR. The results showed that shRNA knockdown of COX-2 effectively inhibited COX-2 mRNA levels induced by PMA in mTAL cells (n = 3, P < 0.05). Empty vector (U6) had no effect on COX-2 mRNA. M, marker; − RT and + RT, without and with reverse transcriptase, respectively.
PGE2 levels were markedly diminished in mTAL cells infected with the EGFP-C2-ex5 vector (Fig. 10). These data are consistent with those showing that PGE2 production is COX-2 dependent in mTAL tubules isolated from mice given 1% NaCl and indicate that mTAL cells also produce PGE2 in response to HS intake via a mechanism dependent on COX-2.

**DISCUSSION**

In the present study, we demonstrated that EP3 receptors in the TAL contribute to the regulation of COX-2 expression and enzymatic activity in this segment of the nephron. Moreover, COX-2 itself provides the signaling prostanoid (PGE2), via activation of EP3 receptors, to limit PGE2 production by the mTAL. The anatomic basis for this negative feedback loop is supported by data showing the localization and induction by hypertonic saline of EP3 receptors in the mTAL. We showed that COX-2 mRNA levels in the OM increased about threefold when mice or rats were given 1% NaCl in the drinking water. In contrast, COX-1 mRNA levels did not change. Western blot analysis of mTAL tubules and immunohistochemical analysis of renal sections confirmed that COX-2 was induced and activated along the TAL in response to HS intake. This notion is consistent with the reduction in COX-2-derived PGE2 synthesis observed in mTAL tubules isolated from mice treated with NS-398 and after knockdown of COX-2 using the EGFP-C2-ex5 lentivirus construct in primary cultures of mTAL cells. The concomitant increase in PGE2 production and EP3 receptor mRNA by mTAL tubules isolated from mice given 1% NaCl suggest that the selective increase in COX-2 mRNA accumulation and protein expression are part of a linked regulatory system that adjusts the extent of PGE2 synthesis in response to hypertonic stress.

The HS-dependent increase in COX-2 expression in the OM most likely reflects an increase in the TAL, since similar increases were observed when mTAL tubules were isolated from the OM. A previous study (23) has shown that hypertonicity induced COX-2 expression and PGE2 synthesis in the medullary region of the kidney, which could be part of an adaptive mechanism that modulates the action of vasopressin. The data in the present study are consistent with such a model and provide another level of regulation by limiting the extent of COX-2-derived PGE2 synthesis, via EP3 receptor activation, thereby limiting the extent of water and NaCl excretion (7, 9, 22). It is well established that COX-2 expression along the TAL segment and PGE2 production by mTAL cells can be induced by diverse stimuli (1, 2, 10, 35, 38). Indeed, PGE2 inhibits the expression and activity of bumetanide/furosemide-sensitive Na+\(^+\)-K\(^+\)-Cl\(^-\) cotransporter 2 (9, 22), which contributes importantly to NaCl reabsorption by the TAL. As hypertonicity stimulates COX-2 expression and activity in the mTAL, the negative feedback mechanism involving EP3 receptors likely subserves an adaptive mechanism that remains to be fully defined.

The negative feedback regulation of de novo COX-2 expression along TAL via PGE2 activation of EP3 receptors was unmasked by pretreatment with a selective EP3 receptor antagonist (L-798106), which increased basal COX-2 and potentiated COX-2-derived PGE2 synthesis induced by hypertonic stress. We (37) have also previously found that administration of the EP3 receptor agonist sulprostone decreased the number of cells expressing COX-2. Upregulation of EP3 receptors in response to HS intake is consistent with data showing that administration of a high-salt diet (4% NaCl) to rats increased EP3 receptor mRNA levels (20) and may be part of a mechanism that regulates the COX-2/PGE2 signaling pathway. Although urinary PGE2 levels reflect renal production of this prostanoid, the cell type and mechanism responsible for the COX-2-dependent increase in urinary PGE2, especially the negative feedback effect of EP3 receptors in response to ingestion of high salt, remains to be determined. The EP3 receptor is expressed in the TAL and collecting ducts (3, 4), and a functional role of EP3 receptors to the COX-2/PGE2 signaling pathway in proximal interlobular arteries of the kidney has also been suggested (34). This may constitute that another benefit of limiting PGE2 synthesis via a feedback mechanism, since vasoconstriction via vascular EP3 receptors would tend to offset the natriuretic effects of this prostanoid. Accordingly, the functional effects of PGE2 to the regulation of salt and water homeostasis may be balanced by several cell type-specific fine-tuning mechanisms.

COX-2 is constitutively expressed and tightly regulated along the TAL, but the mechanisms that regulate its expression are not fully understood. The present study provides a mechanism that explains why COX-2 expression induced by HS is muted in the OM compared with that observed in the inner medulla. We hypothesize that COX-2 is under tonic suppression by a negative feedback mechanism involving EP3 receptors, as COX-2-dependent PGE2 synthesis by the mTAL after exposure to HS was potentiated when EP3 receptors were inhibited. As EP3 receptor-dependent feedback inhibition in
the TAL was also observed in response to treatment with COX-2-selective inhibitors (37), the present study suggests that PGE2 signaling via EP3 receptors in the TAL may be part of a mechanism that regulates mTAL COX-2 expression and function in response to diverse stimuli. Since PGE2 inhibits Na+-K+-Cl− cotransporter 2 activity (22), this mechanism may be teleologically important to prevent excessive loss of NaCl and/or the regulation of urinary concentrating mechanisms due to the prodigious ability of the TAL to reabsorb NaCl. Further studies are required to explore the complex interactions between EP3 receptor signaling pathways in the mTAL that contribute to the maintenance of Na+ and water homeostasis.

REFERENCES


DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


EXTRA TEXT

16. The TAL was also observed in response to treatment with COX-2-selective inhibitors, the present study suggests that PGE2 signaling via EP3 receptors in the TAL may be part of a mechanism that regulates mTAL COX-2 expression and function in response to diverse stimuli. Since PGE2 inhibits Na+-K+-Cl− cotransporter 2 activity, this mechanism may be teleologically important to prevent excessive loss of NaCl and/or the regulation of urinary concentrating mechanisms due to the prodigious ability of the TAL to reabsorb NaCl. Further studies are required to explore the complex interactions between EP3 receptor signaling pathways in the mTAL that contribute to the maintenance of Na+ and water homeostasis.


