Regulation of cyclooxygenase-2 expression in renal medulla by tonicity in vivo and in vitro

TIANXIN YANG,1 JURGEN B. SCHNERMANN,2 AND JOSEPHINE P. BRIGGS1,2,3

Departments of 1Internal Medicine and 2Physiology, University of Michigan, Ann Arbor, Michigan 48104; and 3National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland 20892

Yang, Tianxin, Jurgen B. Schnermann, and Josephine P. Briggs. Regulation of cyclooxygenase-2 expression in renal medulla by tonicity in vivo and in vitro. Am. J. Physiol. 277 (Renal Physiol. 46): F1–F9, 1999.—Renal medullary prostaglandins are believed to exert an important functional role in antagonizing vasopressin effects in dehydration. Studies were undertaken to determine the effect of hyperosmolality on cyclooxygenase (COX) isoform expression in the renal medulla. COX-1 and COX-2 mRNA and protein levels were determined by RT-PCR or Western blotting in Sprague-Dawley rats on varying water intakes, in Brattleboro rats and in Long-Evans controls. Over a wide range of urinary tonicity, COX-2 expression correlated closely with urine osmolality levels (R = 0.872). COX-1 levels did not vary. Immunolocalization showed that the stimulation of COX-2 expression by dehydration occurred predominantly in the collecting duct. Hypertonicity caused by addition of NaCl produced a dose- and time-dependent stimulation of COX-2 expression in mMCD-K2 cells as well as in MDCK cells. COX-1 was unaffected. In the same cell lines, mannotol, sucrose, and raffinose also had a stimulatory effect. The toxicity-stimulated COX-2 expression in mMCD-K2 cells was almost completely blocked by a tyrosine kinase inhibitor, genistein at 100 µM. In MDCK cells transfected with a 2.7-kb COX-2 promoter and lacZ reporter construct, NacI induced a twofold increase in β-galactosidase activity. Using mRNA from COX-2 cells, hypertonic NaCl (600 mosmol/kg H2O for 24 h) induced a 33-fold increase in PGE2 release determined by enzyme immunoassay, an effect completely blocked by 3 µM indomethacin or the COX-2-specific blocker N-(2-cyclohexy-4-nitrophenyl)methanesulphonamide (NS-398). We conclude that in inner medulla, COX-1 but not COX-1 is upregulated by hyperosmolality.

prostaglandin H synthase; inner medulla; hyperosmolality; prostaglandins; sodium chloride; promoter

There is strong evidence to suggest that prostaglandins (PGs) participate in the regulation of urinary water excretion. Infusion of PGs of the E series have been shown to cause water diuresis in both human and animals (2) and to inhibit water absorption in collecting ducts (24, 36). PGs appear to act as functional antagonists of the hydromotonic effect of vasopressin (AVP). AVP stimulates water transport in collecting ducts. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
cell lysate were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The blots were blocked overnight with 5% nonfat dry milk in Tris-buffered saline (TBS), followed by incubation for 1 h with rabbit anti-murine polyclonal antiserum to COX-2 (Cayman) at a dilution of 1:500. After washing with TBS, blots were incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and visualized with enhanced chemiluminescence cassettes (ECL, Amersham). For immunoblotting of COX-1, the blot was stripped in 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-Cl (pH 6.7), and incubated at 50°C for 30 min. Immunodetection was performed as described except that a murine monoclonal antibody to COX-1 (Cayman) was used for the primary antibody incubation.

RT-PCR. RT-PCR was performed as previously described (41). Briefly, total RNA was isolated from the frozen tissues using TRI-reagent, and CDNA was synthesized by H-MMLV reverse transcriptase (Superscript; BRL, Gaithersburg, MD). Primers for COX-2 were derived from published sequences (18). Primers were chosen in areas of minimal cross-homology between COX-1 and COX-2, high species conservation, and positioned to span at least two intron-exon junctions to distinguish cDNA from genomic DNA. The sequence of the oligonucleotide primers and their location for COX-2 is as follows: sense COX-2, 5'-AAG GAA GGG ACA CCC TTT CAC AT-3' (bp 1229–1248); antisense COX-2, 5'-GAA GGG ACA CCC TTT CAC AT-3' (bp 1794–1813). PCR reaction was performed in the presence of 1.5 [µl/50 µl [32P]dCTP (Amersham), and incubated at 50°C for 30 min. A serial dilution of β-galactosidase was used as standard assay according to the manufacturer's instruction. PGE2 enzyme immunoassay. PGE2 in the media was measured by an enzyme immunoassay kit (Cayman). The assay was performed according to the manufacturer's instructions. Briefly, 25 or 50 µl of the medium, along with the serial diluted PGE2 standard samples, was mixed with appropriate amount of acetylcholinesterase-labeled tracer and PGE2 antiserum and incubated at room temperature for 18 h. After the wells were emptied and rinsed with wash buffer, 200 µl of Ellman's reagent that contained substrate for acetylcholinesterase was added. The enzyme reaction was carried out in a slow shaker at room temperature for 1 h. The plate was read at 415 nm and the result was analyzed by DeltaSOFT II software.

Statistical analysis. Data are expressed as means ± SD. All statistical comparisons were made with a paired Student's t-test with P < 0.05 being considered significant.

RESULTS

Tissue distribution of COX-1 and COX-2 expression. The expression of COX isofoms in various tissues in Sprague-Dawley rats was determined by Western blotting (Fig. 1). Each isofom exhibited a unique distribution pattern. COX-2 protein was expressed at highest levels in brain, to a lesser extent in renal inner medulla and renal cortex, and it was essentially undetectable in all other tissues tested. COX-1 protein was widely expressed in almost all organ systems except brain and heart, with highest levels of expression being found in renal inner medulla.

Effect of dehydration on gene expression of the two COX isofoms in renal inner medulla of Sprague-Dawley rats. Sprague-Dawley rats were dehydrated for 3 days, and protein and mRNA expression of COX isofoms in renal inner medulla was examined by Western blotting and RT-PCR with serial dilutions of cDNA, respectively. Densitometric analysis shows that dehydration induced a 3.4-fold increase in COX-2 protein (66.2 ± 1.7 vs. 19.9 ± 3.8, n = 6, P < 0.01) (Fig. 2A) and a 3.0-fold increase in COX-2 mRNA levels in the inner medulla (Fig. 2B), whereas the expression of COX-1 in this kidney region was not significantly altered (Fig. 2A). Immunohistochemistry studies showed stimulation of COX-2 expression in dehydration predominantly in collecting ducts (Fig. 3).

Regulation of COX-2 protein expression in inner medulla of Brattleboro and Long-Evans rats by dehydration. Dehydration for 36 h significantly stimulated COX-2 protein expression in the renal inner medulla of both Brattleboro and Long-Evans rats, but the expression level in Brattleboro rats in either control or dehydrated state was reduced compared with that of Long-Evans rats (Fig. 4A). Band intensity of the COX-2 protein was plotted against urine osmolality levels, indicating a dose correlation (R = 0.873).

Regulation of gene expression of the two COX isofoms in cultured kidney cells. The effect of hyperosmolality on COX isoform gene expression was evaluated in cultured MDCK and mIMCD-K2 cells. In MDCK cells, raising medium osmolality by the addition of NaCl from 300 to 600 mosmol/kgH2O caused a dose-dep-
dent increase in COX-2 protein expression (Fig. 5A). NaCl-stimulated COX-2 protein expression was detectable at 2 h, increased over incubation time, and peaked at 24 h (Fig. 5B). The stimulatory effect of osmolality was not inhibited by furosemide, amiloride, or the chloride channel blocker 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB, 100 µM, each for 12 h) (data not shown). Effect of different solutes including NaCl, mannitol, raffinose, and sucrose at the similar osmolality levels (600 mosmol/kgH2O) was compared. All solutes stimulated COX-2 expression, but the stimulation appeared to be dependent on specific solutes (Fig. 5C). COX-1 regulation in MDCK cells could not be assayed because of antibody species specificity, so additional experiments were performed in mIMCD-K2 cells. In mIMCD-K2 cells, NaCl as well as urea stimulated COX-2 protein expression, whereas COX-1 expression by mIMCD-K2 was not affected by changes in osmolality (Fig. 6).

We examined the effect of 1-desamino-8-0-arginine vasopressin (DDAVP) as well as forskolin on COX-2 expression in cultured MDCK cells and renal medullary interstitial cells (RMIC). Treatment with DDAVP at 10 µM or and with forskolin at 100 µM had no significant effect on the expression of COX-2 protein in either MDCK cells or RMIC (data not shown).

Effect of hypertonic NaCl on COX-2 promoter activity in transfected MDCK cells. MDCK cells were transiently transfected with a construct containing a 2.7-kb sequence of the rat COX-2 promoter coupled to a nuclear localization signal and lacZ or with a promoterless construct served as control. β-Galactosidase activity was analyzed by X-gal staining or by a quantitative assay system using ONPG as a substrate. Cells transfected with the promoter construct showed a certain degree of lacZ activity under basal condition, consistent with the constitutive expression of COX-2 in these cells. Treatment with hypertonic NaCl (600 mosmol/kgH2O) for 12 h significantly increased the number of blue staining cells as well as the intensity of the blue staining in the promoter construct transfected cells (Fig. 7). As shown in high power, the blue staining was localized to nuclei. The quantitative assay showed that NaCl treatment induced an approximately twofold increase in the β-galactosidase activity in the promoter construct transfected cells (Fig. 8). In contrast, in cells transfected with the promoterless construct, there was little β-galactosidase activity under basal condition, and NaCl treatment did not change the activity (Figs. 7 and 8). Two independent assays, X-gal staining and the ONPG quantitative assay, performed in separate experiments, both consistently showed similar results, suggesting little influence from variation of transfection efficiency. In addition, we obtained similar data from the passaged cells derived from the same transfected cells (data not shown). For these reasons, no control for transfection efficiency experiment was performed.

Effect of COX inhibitors on NaCl-stimulated PGE2 release from cultured mIMCD-K2 cells. Confluent mIMCD-K2 cells were pretreated with COX inhibitors or the ethanol as vehicle control for 2 h and then subjected to NaCl stimulation (600 mosmol/kgH2O) in the presence of 5 µM of arachidonic acid. PGE2 release was determined by enzyme immunoassay. NaCl treatment for 24 h induced a 33-fold increase in PGE2 release from mIMCD-K2 cells, and this effect was completely blocked by N-(2-cyclohexyl-4-nitrophenyl)methanesulfonamide (NS-398) and indomethacin (30 µM each) (Fig. 9).

Effect of genistein on osmotic stimulation of COX-2 expression in mIMCD-K2 cells. To determine whether tyrosine kinase activation participates in osmotic stimulation of COX-2 by medullary epithelial cells, the effect of genistein on hypertonic NaCl-induced COX-2 protein expression in mIMCD-K2 cells was examined. The confluent mIMCD-K2 cells were pretreated with DMSO or genistein at various concentrations for 2 h and then
treated with isotonic or hypertonic medium (600 mosmol/kgH₂O by NaCl) for 20 h. COX-2 protein expression was determined by Western blotting analysis. NaCl treatment induced a fivefold increase in COX-2 protein expression level and this effect was markedly inhibited by genistein treatment in a dose-dependent manner (Fig. 10).

DISCUSSION

There is substantial evidence in support of a role of PGs in the regulation of collecting duct NaCl and water absorption (3, 5, 7). In view of the general notion that PGs regulate cellular functions in a paracrine fashion, it is likely that transport modulation along collecting ducts is the result of formation of PGs in the renal medulla. This conclusion is consistent with the high levels of COX activity found in the medullary region of the kidney, both in collecting duct cells and in RMICs (21, 23, 43). Since two isoforms of COX have been identified and since both isoforms are expressed in the inner medulla of the kidney, it is not clear whether COX-1, COX-2, or both may be responsible for the modulation of collecting duct NaCl and water absorption by PGs.

COX-1, the constitutive form of COX, is generally considered to have housekeeping functions that in the kidney include the regulation of H₂O and Na⁺ transport (14). It is consistent with this notion that COX-1 protein was found to be expressed in a wide variety of tissues. Nevertheless, clear differences in relative COX-1 expression were observed between tissues tested in the present study with highest levels in renal inner medulla and lowest expression in brain and heart. More importantly, COX-1 protein expression in renal medulla was not altered by dehydration in vivo or by hyperosmolality in vitro. This finding is not consistent
with the study by Cowley et al. (10), in which hyperosmolality was shown to increase COX-1 mRNA expression by Northern blotting analysis. The discrepancy is likely due to difference in techniques used in the two studies. In the study of Cowley et al. (10), Northern blotting analysis was used as the only technique to distinguish the two COX isoforms based on the size of their bands.

Fig. 4. Regulation of COX-2 protein expression in inner medulla of Brattleboro and Long-Evans rats by dehydration for 36 h. A: COX-2 Western blotting on inner medulla from control and dehydrated animals and the correspondent urine osmolality. B: plot of COX-2 band intensity and urine osmolality.

Fig. 5. Effect of extracellular solutes on COX-2 expression in MDCK cells by Western blotting analysis. A: cells were treated with hypertonic NaCl at various osmolalities made by addition of NaCl to isotonic medium for 12 h. B: cells were treated with 600 mosmol/kg H2O made by addition of NaCl to isotonic medium for the indicated period of time. C: comparison of effect of various solutes including mannitol, raffinose, sucrose, and NaCl at same osmolality (600 mosmol/kg H2O).

Fig. 6. Effect of hyperosmolality on protein expression of the two COX isoforms in mMCD-K2 cells. A: effect of hypertonic NaCl on COX-2 protein expression. B: blot from A was stripped and reprobed with COX-1 antibody. C: effect of urea (200 mM urea added to isotonic medium) on COX-2 protein expression.

Fig. 7. COX-2 promoter activity shown by X-gal staining. MDCK cells were transiently transfected with COX-2 promoter and promoterless constructs for 3 days and then treated with hypertonic NaCl (600 mosmol/kg H2O) for 12 h. Cells were fixed by 0.5% glutaraldehyde for 15 min, washed with PBS, and incubated with 0.2% X-gal.
the signals (4.2 kb for COX-2 and 3.2 kb for COX-1). We used two independent techniques, RT-PCR using isoform-specific primers, and Western blotting using the isoform-specific antibodies (Cayman) with an established specificity to distinguish the two isoforms. The specificity of the detection system with these two techniques will be advantageous over Northern blotting. Earlier studies from our laboratory have reported that pretreatment of rats with diets containing varying amounts of NaCl also did not alter levels of COX-1 expression in the kidney (43). Widespread tissue expression under baseline conditions and nonresponsiveness to variations in salt intake and to dehydration are consistent with the characterization of COX-1 as a constitutive gene product that does not appear to undergo rapid and intense regulation of expression.

COX-2 was initially identified as a cytokine-inducible enzyme implicated in inflammatory responses (14, 25). However, previous evidence as well as the present results suggest a more complex physiological role of this enzyme. Commensurate with the pronounced cytokine inducibility is a much more restricted expression pattern under baseline conditions. In the present studies, COX-2 protein was only observed in the brain and in the kidney, mostly in the inner medulla and to a lesser extent in the renal cortex, and was barely

Fig. 8. Quantitative analysis of COX-2 promoter activity. A: standard curve with various amounts of β-galactosidase. B: effect of hypertonic NaCl on activity of β-galactosidase (mU/mg protein) from MDCK cells transfected with COX-2 promoter construct and blank vector.

Fig. 9. Effect of COX inhibitors on prostaglandin (PG) release from cultured mIMCD-K2 cells. Confluent mIMCD-K2 cells were treated with isotonic or hypertonic medium (600 mosmol/kg H2O) in presence of ethanol, indomethacin, or NS-398, for 24 h. PGE2 release from the medium was determined by enzyme immunoassay.

Fig. 10. Effect of genistein on NaCl-induced COX-2 protein expression in cultured mIMCD-K2 cells. Confluent mIMCD-K2 cells were pretreated with DMSO or genistein at indicated concentration for 2 h and then treated with isotonic or hypertonic medium (600 mosmol/kg H2O by NaCl) for 20 h. A: Western blotting of COX-2. B: densitometry analysis of band intensity. * P < 0.05 vs. DMSO.
detectable in other tissues. Whereas the application of methods with relatively low sensitivity such as in situ hybridization or immunohistochemistry yielded results in agreement with our conclusion that COX-2 is constitutively expressed in only a limited number of tissues, analysis of mRNA levels by RT-PCR showed expression in virtually all tissues examined (34).

One of main results in this study is the observation that COX-2 expression in renal inner medulla, at both protein and mRNA levels, was markedly stimulated by dehydration. This phenomenon was observed in different strains of rats and was found to be related to the time of dehydration within the time span from 36 h to 3 days. The mechanism initiating COX-2 stimulation in vivo is unclear, but the medullary site specificity of COX-2 upregulation would seem to be most compatible with a dehydration-dependent alteration that is restricted to the renal medulla such as the change in tissue osmolality. The potential for hyperosmolality to stimulate COX-2 expression was demonstrated in two renal epithelial cell lines, MDCK and mIMCD-K2 cells, where raising medium NaCl concentration to hyperosmotic levels was found to increase COX-2 protein abundance. Consistent with an earlier study by Cowley et al. (10) stimulation of COX-2 expression in vitro occurred in a dose- and time-dependent manner.

We also demonstrated that hypertonic NaCl induced a marked stimulation in PGE2 release from mIMCD-K2 cells, which is consistent with the observations by majority of but not all of the previous studies (11, 12, 37, 38). We also found that the hyperosmolality-stimulated PGE2 release was completely blocked by a COX-2-specific inhibitor, NS-398, and a nonselective COX inhibitor, indomethacin, with almost equal potency, strongly suggesting that COX-2 but not COX-1 is responsible for the hyperosmolality-stimulated PGE2 release in renal medullary cells.

The precise mechanism of the stimulation of COX-2 expression by hyperosmolality is currently unclear. The possibility was considered that the effect of increased NaCl concentration may be mediated by stimulation of Na uptake through specific NaCl transport proteins. Although various transport mechanisms have been implicated in NaCl-mediated PG production (27, 28) and in NaCl-mediated JNK activation (41), the presence of furosemide, amiloride, or the Cl- channel blocker NPPB did not alter the response of COX-2 expression. Since various organic solutes exhibited a stimulatory effect on COX-2 expression in MDCK cells, it may seem more likely that the NaCl-mediated COX-2 induction is due to the osmotic effect of NaCl. Considering the facts that tyrosine kinase is involved in the signaling mechanism for both cytokine- and mitogen-stimulated COX-2 expression in pancreatic islet and mesangial cells, respectively (8, 9), and that hyperosmolality activates tyrosine kinase in various cell culture systems (35, 39), we examined the possibility that tyrosine kinase activation is involved in hyperosmolality-stimulated COX-2 expression in medullary epithelial cells. The present study demonstrates that tyrosine kinase inhibition by genistein almost completely blocks hyperosmolality-induced COX-2 expression in mIMCD-K2 cells, strongly suggesting that tyrosine kinase activation is required for the osmotic stimulation of COX-2 in medullary epithelial cells.

We further showed that hypertonicity induced COX-2 promoter activity in MDCK cells transiently transfected with a 2.7-kb flanking sequence of rat COX-2 promoter (Figs. 6 and 7), suggesting that sequences necessary for tonicity-stimulated COX-2 expression are included in this flanking region. Analysis of the COX-2 promoter sequence revealed an element (failed to identify the complete osmotic response element AGGAAATA-CAC) that with an exception of 1-bp mismatch is identical to the osmotic responsive element in the promoter region of aldose reductase gene (19). Whether this element confers osmotic responsiveness to COX-2 gene expression remains to be determined.

Speculations about the significance of hyperosmolality-induced stimulation of COX-2 expression can be made on the basis of the known function of COX products in the kidney. PGs and AVP exert opposing effects in regulating duct water permeability and water transport. AVP stimulates water transport in collecting duct primarily through V2/Gs-mediated activation of adenyl cyclase and cAMP-dependent activation of protein kinase A. In contrast, PGs inhibit water transport, an effect that appears to be mediated through activation of protein kinase C (5). The operation of these antagonistic actions of the two hormonal systems in vivo is supported by the observation that COX inhibition by nonsteroidal antiinflammatory drugs enhances the antidiuretic effect of AVP (1, 17). Since AVP and hyperosmolality stimulate PG production from renal medulla (4, 11, 12), one may postulate that PGs serve as local feedback regulators of the antidiuretic action of AVP. It is possible that the hyperosmolality-stimulated COX-2 expression may be involved in this feedback loop. COX-2-derived products may also be important in protecting the medullary blood flow that tends to be decreased by volume depletion.

COX-2 may also serve as a survival gene in medullary epithelial cells in response to osmotic stress. Osmotic adaptation of medullary epithelial cells requires the induction of a group of genes involved in osmolyte transport or synthesis (20, 29, 42). In addition, recent studies have shown that hyperosmolality also stimulates the expression of nonsymolyte-associated genes, most of which represent members of the immediate early response gene family including Egr-1 and c-fos (6). It is conceivable that COX-2 may be added to the list of nonsymolyte-associated genes induced by hyperosmolality. COX-2 shares some key features with immediate early genes. For example, its expression is inducible by serum and growth factors, suggesting that COX-2 may play a role in cell growth. The function of COX-2 as survival gene is particularly supported by the observation that overexpression of COX-2 is protective against apoptosis in intestinal epithelial cells (40). In comparison with other members of the immediate early gene family, COX-2 is relatively unique to the kidney so that COX-2 may play a specific role in the survival of
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In this regard, genetic deficiency of COX-2 in vivo causes abnormalities in postnatal kidney function (32), and inhibition of COX-2 with NS-398 in vitro causes cell death in cultured medullary interstitial cells (22). A well-noted phenomenon in clinical observation is that phenacetin and other COX inhibitory drugs cause necrosis of the renal papilla (13, 16). This evidence taken together suggests that COX-2 may have a unique role for the survival of medullary cells in response to osmotic stress. Studies utilizing COX-2-specific inhibitors and COX-2-deficient medullary cell lines may provide further insight into this possibility.

In summary, stimulation of renal medullary COX-2 expression by dehydration was demonstrated in different strains of rats with various time course, at both protein and message levels. In vitro experiments demonstrated that hypertonic NaCl significantly stimulated COX-2 expression in MDCK and mIMCD-K2 cells. Further, hypertonic NaCl increased COX-2 promoter transcriptional activity in MDCK cells. These data suggest that the dehydration-stimulated medullary COX-2 expression is likely due to the effect of increased extracellular solutes on COX-2 expression in medullary epithelial cells. In contrast to COX-2, renal medullary COX-1 was not regulated by either dehydration in vivo or hypertonic NaCl in vitro. The present studies contribute to clarify the distinct role of the two COX isomers in regulation of water balance.

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