COX2 Activity Promotes Organic Osmolyte Accumulation and Adaptation of Renal Medullary Interstitial Cells to Hypertonic Stress*

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The mechanism by which COX2 inhibition decreases renal cell survival is poorly understood. In the present study we examined the effect of COX2 activity on organic osmolyte accumulation in renal medulla and in cultured mouse renal medullary interstitial cells (MMICs) and its role in facilitating cell survival. Hypertonicity increased accumulation of the organic osmolytes inositol, sorbitol, and betaine in cultured mouse medulla in medullary interstitial cells. Pretreatment of MMICs with a COX2-specific inhibitor (SC58236, 10 μmol/liter) dramatically reduced osmolyte accumulation (by 79 ± 9, 57 ± 12, and 96 ± 10% for inositol, sorbitol, and betaine respectively, p < 0.05). Similarly, 24 h of dehydration increased inner medullary inositol, sorbitol, and betaine concentrations in vivo by 85 ± 10, 197 ± 28, and 190 ± 24 pmol/μg of protein, respectively, but this increase was also blunted (by 100 ± 5, 66 ± 15, and 81 ± 9% for inositol, sorbitol, and betaine, respectively, p < 0.05) by pretreatment with an oral COX2 inhibitor. Dehydrated COX2−/− mice also exhibited an impressive defect in sorbitol accumulation (88 ± 9% less than wild type, p < 0.05) after dehydration. COX2 inhibition (COX2 inhibitor-treated or COX2−/− MMICs) dramatically reduced the expression of organic osmolyte uptake mechanisms including betaine (BGT1) and sodium-myo-inositol transporter and aldose reductase mRNA expression under hypertonic conditions. Importantly, preincubation of COX2 inhibitor-treated MMICs with organic osmolytes restored their ability to survive hypertonic stress. In conclusion, osmolyte accumulation in the kidney inner medulla is dependent on COX2 activity, and providing exogenous osmolytes reverses COX2-induced cell death. These findings may have implications for the pathogenesis of analgesic nephropathy.

The maintenance of intracellular solute concentrations and cell volume is a prerequisite for optimal cell function. Changes in extracellular tonicity typically result in corresponding changes in intracellular solute concentration that allow cells to regain their normal size (1). In renomedullary cells, osmotic equilibrium with elevated extracellular NaCl concentrations is achieved primarily by intracellular accumulation of small organic solutes (“organic osmolytes”) (2, 3). In contrast to inorganic electrolytes, organic osmolytes do not severely impair cell function even at high concentrations and are thus considered “compatible osmolytes” (3). Reduced accumulation of compatible osmolytes may cause decreased cell viability in the renal medulla (4) as well as inhibition of growth and, ultimately, cell death (5).

Prostanoids comprise a diverse family of biologically active lipids derived from the enzymatic metabolism of arachidonic acid by prostaglandin G2/H2 synthase (cyclo-oxygenase (COX)).1 Although prostanoid synthesis occurs in all cells and tissues, the kidney is a particularly rich source for prostanoids. Cyclo-oxygenase is a key enzyme regulating the formation of prostaglandins from arachidonic acid, and two isoforms of COX have been identified, a constitutively expressed isoform (COX1) and an inducible isoform (COX2) (6–8). These two proteins are encoded by different genes, which are ~60% homologous and are both inhibited by non-selective nonsteroidal anti-inflammatory drugs (9). In contrast to these similarities, the expression patterns of COX1 and COX2 genes are quite different. COX1 is constitutively expressed in several tissues and is thought to participate in housekeeping functions, including the maintenance of gastric epithelial integrity. COX2 expression can be dramatically induced in inflammatory cells by cytokines as well as by mitogenic factors (10). COX2 is expressed in several tissues, including those of the kidney, lung, and brain (11–14). In the kidney, COX2 is expressed in the cortical thick ascending limb and MICs, whereas COX1 mRNA appears to be mainly expressed in the collecting duct (12, 15). Recent studies have documented toxicity-dependent regulation of COX2, but not COX1, expression in MICs (16) in vivo and in vitro (17). These studies suggest that COX2, a major target for nonsteroidal anti-inflammatory drug action, might serve an osmoprotective function in MICs, a site of early injury in analgesic nephropathy (18). This study explored the possible relationship between COX2 activity and organic osmolyte-dependent adaptation of medullary interstitial cells to hyperosmotic stress.

1 The abbreviations used are: COX, cyclo-oxygenase; MIC, medullary interstitial cells; MMIC, mouse medullary interstitial cells; SMIT, sodium-myo-inositol transporter; DME, Dulbecco’s modified Eagle’s medium; HPLC, high pressure liquid chromatography; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; WT, wild type; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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Mouse medullary interstitial cells were prepared as described previously (19). C57BL mice, obtained from The Jackson Laboratory (Bar Harbor, ME), were sacrificed, both kidneys were rapidly removed under sterile conditions, and the inner medullary regions were excised, minced, and suspended in Dulbecco’s minimum essential medium (DMEM, Invitrogen) containing 10% fetal calf serum. The suspension was then injected intracutaneously under sterile conditions at two or three different locations of the ventral abdominal wall of an isogenic recipient mouse. After 3–4 days the recipient animal was sacrificed, and the firm yellow nodules were removed aseptically. The nodules were minced under sterile conditions, and cells were then trypsinized in 0.05% trypsin-EDTA at 37 °C for 15 min, washed, and pelleted at 300 × g. The pellet was resuspended in DMEM containing 10% fetal calf serum and 50 μg/ml each penicillin and streptomycin (Sigma). Cells were plated in 75-ml flasks and grown until confluent. Mouse medullary interstitial cells (MMICs) were studied after three or four passages were plated in 75-ml flasks and grown until confluent. Mouse medullary interstitial cells were grown to confluency in 96-well plates and either adapted to increasing medium osmolarity (430 mosmol/liter) with medium containing 1.0 mmol/liter of the individual osmolyte (inositol, sorbitol, or betaine), before the addition of newborn mouse interstitial cells (MMICs) were kept at 600 mosmol/liter and then incubated for 60 min with 2% agarose gel showing a product length of 545 bp. The cDNA probe for sodium myo-inositol transporter (SMIT) was a generous gift from Dr. Moo Kwon, Johns Hopkins University, Baltimore, MD. A glycerol-dehyde-3-phosphate dehydrogenase probe was amplified by PCR as described previously (20). All probes were 32P-labeled using the Prime-it RII random primer labeling kit (Stratagene, La Jolla, CA). Percent viability was calculated as (OD of drug-treated sample/OD of control sample) × 100.

**COX2 Activity Promotes Organic Osmolyte Accumulation**

**MATERIALS AND METHODS**

**Cell Culture and Animals**

Mouse medullary interstitial cells were grown to confluency (26) using an isocratic HPLC system as described previously (23). Briefly, cells were grown to confluence and then adapted to hypertonic conditions in 100 mosmol/liter increments (exposure to each increment for 24 h, final osmolality of 600 mosmol/liter, produced with NaCl, in DMEM, and 2% fetal calf serum). MMICs were kept at 600 mosmol/liter for 24 h before harvest. In some experiments SC58236 (16), a COX2-specific inhibitor (Monsanto Searle, St. Louis, MO), was added to all incubation media at a concentration of 10 μmol/liter. At the end of the incubation period cells were harvested, washed with hypotonic medium (600 mosmol/liter), and pelleted using 5 min of centrifugation at 200 x g. Osmolytes were quantitated in the neutralized perchloric acid extracts of the pellets as described previously (24).

**Experimental Protocols**

**In Vitro**—The accumulation of organic osmolytes in cultured MMICs was quantitated using an isocratic HPLC system as described previously (23). Briefly, cells were grown to confluence and then adapted to hypertonic conditions in 100 mosmol/liter increments (exposure to each increment for 24 h, final osmolality of 600 mosmol/liter, produced with NaCl, in DMEM, and 2% fetal calf serum). MMICs were kept at 600 mosmol/liter for 24 h before harvest. In some experiments SC58236 (16), a COX2-specific inhibitor (Monsanto Searle, St. Louis, MO), was added to all incubation media at a concentration of 10 μmol/liter. At the end of the incubation period cells were harvested, washed with hypotonic medium (600 mosmol/liter), and pelleted using 5 min of centrifugation at 200 x g. Osmolytes were quantitated in the neutralized perchloric acid extracts of the pellets as described previously (24).

**In Vivo**—To examine the effect of COX2 inhibition on mouse renal medullary organic osmolyte content, C57BL mice were treated with (n = 6) or without (n = 6) the COX2-specific inhibitor SC58236. COX2 inhibitor was diluted to a final concentration of 0.6 mg/liter in H2O with 0.1% Tween and 0.2% polyethylene glycol 200 as solvents and given to mice via drinking water 3–4 days before a 24-h dehydration (25). Inner medullary osmolyte concentrations were quantitated in kidneys of C57BL WT (+ SC58236) and COX2 /− mice after a 24-h dehydration. Neutralized perchloric acid extracts from renal inner medullary tissue sections were prepared as described previously in detail (26), and cellular organic osmolyte concentrations were quantitated by an isocratic HPLC system. Intracellular osmolyte concentrations in both in vivo and in vitro studies were normalized to tissue protein concentrations in each of the samples. Protein concentrations were determined using the method described by Bradford (27).

**myo-[3H]Inositol Uptake Study**

Mouse medullary interstitial cells were grown to confluency in 96-well plates (Costar 3598, Corning Inc., Corning, NY), stepwise adapted to a final medium osmolality of 600 mosmol/liter using NaCl, and then incubated for 60 min with different concentrations of unlabelled and [3H]-labelled inositol (1000–125 μmol/liter unlabelled inositol mixed with 1 nmol of [3H]inositol). [3H]Inositol was purchased from ICN, Boston, MA, with a specific activity of 10–20 Ci/mmol/liter. Isotonic controls were kept at 295 mosmol/liter and then incubated for 60 min with identical inositol solutions as in hypertonic experiments. Selected hypotonic and isotonic groups were incubated with 10 μmol/liter SC58236 before inositol incubation. Cell count and viability studies were performed before and immediately after SC58236 incubation. At the end of the inositol incubation, cells were washed twice with ice-cold isotonic and hypertonic DMEM, respectively, and lysed in 1% SDS. [3H]Inositol counts were measured using a Beckman scintillation counter.

**DNA Fragmentation Assay**

For detection of apoptotic DNA cleavage, DNA fragmentation assay was performed using Wizard genomic DNA purification kit (Promega,
Madison, WI). MMICs were either adapted to a final osmolarity of 600 mosmol/liter with and without 20 μmol/liter SC58236 or preincubated for 24 h with mildly elevated hyperosmolarity (430 mosmol/liter) with medium containing 0.5 or 1.0 mmol/liter of an osmolyte mixture (inositol, sorbitol, and betaine together), before the addition of COX2 inhibitor and raising of the osmolarity of the medium to 600 mosmol/liter. DNA fragmentation was visualized by electrophoresis on a 1% agarose gel containing ethidium bromide.

**Statistical Analysis**

Results are presented as mean ± S.D. of 4–6 replicates. Differences were considered statistically significant if p < 0.05, using analysis of variance and Bonferroni t tests.

**RESULTS**

Accumulation of Organic Osmolytes by Medullary Interstitial Cells Is COX2-dependent—Primary cultures of MMICs were more than 99% pure for interstitial cells with the presence of characteristic abundant cytoplasmatic lipid droplets (Fig. 1). MMICs accumulated the organic osmolytes inositol, sorbitol, and betaine in response to hyperosmotic conditions. Compared with isotonic conditions (295 mosmol/liter), the intracellular accumulation of inositol, sorbitol, and betaine in MMICs increased by 234 ± 19, 74 ± 11, and 134 ± 26 pmol/μg of protein, respectively, after culture in 600 mosmol/liter. In contrast, when MMICs were cultured in the presence of a COX2-specific inhibitor (SC58236, 10 μmol/liter) (Table I), accumulation of inositol, sorbitol, and betaine after hypertonicity was dramatically reduced by 79 ± 9% for inositol, 57 ± 12% for sorbitol, and 96 ± 10% for betaine (Fig. 2).

To assess in vivo osmolyte accumulation, we measured inner medullary osmolyte concentrations in dehydrated C57BL WT, COX2−/−, and SC58236-treated WT mice versus animals provided with water ad libitum. Inner medullary concentrations of inositol, sorbitol, and betaine increased by 85 ± 10, 197 ± 28, and 190 ± 24 pmol/μg of protein, respectively, in WT animals subjected to a 24-h dehydration (Fig. 3). This increase was markedly diminished by a COX2 inhibitor (SC58236, 600 μg/liter administered in the drinking water × 4 days), with complete inhibition (100 ± 5%) of inositol, 66 ± 15% reduction for sorbitol, and a 81 ± 9% reduction for betaine accumulation. Similarly dehydrated COX2−/− mice showed an even more dramatic reduction in medullary sorbitol accumulation (173 ± 30 pmol/μg of protein; 88 ± 9%) as compared with SC58236-treated WT mice (Fig. 3). Also inositol and betaine concentrations were further reduced by 235 ± 35 and 267 ± 15 pmol/μg of protein, respectively, in COX2−/− animals.

**Effect of COX2-specific Inhibitor on Osmolyte Gene mRNA Expression**—To explore the underlying mechanisms of the above observations, we investigated the effect of SC58236 on betaine (BGT1) and SMIT transporter mRNA levels in MMICs. Messenger RNA levels for both the betaine transporter BGT1 and SMIT transporter were diminished by 44 ± 11 and 23 ± 7%, respectively, after 48 h at 600 mosmol/liter with...
COX2 inhibitor (Fig. 4). Furthermore, cultured MMICs from COX2/−/− mice also exhibited dramatically reduced medullary sorbitol accumulation and aldose reductase mRNA levels (27 ± 3% at 400 and 63 ± 3% at 500 mosmol/liter medium tonicity, respectively, p < 0.05, see Fig. 5). Interestingly, under isotonic conditions cultured COX2/−/− MMICs showed similar aldose reductase messenger RNA levels as compared with MMICs from WT mice.

To exclude the possibility that SC58236 directly blocked osmotic osmolyte transporters, we investigated the effect of SC58236 on [3H]inositol uptake in hyperosmotically treated MMICs, compared with isotonic controls. Hypertonicity (600 mosmol/liter) produced a 4-fold increase in [3H]inositol uptake as compared with isotonic controls (7778 ± 1400 versus 1288 ± 558 cpm/100 μg of protein, respectively) that was independent of SC58236 treatment (10 μM, 2 h) (data not shown). Furthermore, preincubation with SC58236 for 2 h did not significantly affect cell count or viability under isotonic or hypertonic conditions.

Medullary Interstitial Cell Survival under Hypertonic Stress

Is Osmolyte-dependent—To evaluate the role of organic osmolytes in determining the increased MMIC fragility after hyperosmotic stress, we facilitated cellular osmolyte uptake by adding betaine, inositol, and sorbitol to the extracellular medium. When adapted to 600 mosmol/liter culture media by stepwise increases in media tonicity, MMICs tolerated hyperosmotic conditions well without a significant decrease in viability compared with isotonic controls (Fig. 6). Even in these gradually adapted MMICs, COX2 inhibition (SC58236, 10 μmol/liter) significantly reduced cell viability (30 ± 14%, p < 0.05). Importantly, preincubation with the organic osmolytes betaine and sorbitol dramatically increased MMIC survival in COX2-inhibited cells (65 ± 12% improvement in betaine-treated and 27 ± 6% in sorbitol-treated MMICs, p < 0.05) compared with SC-treated cells without osmolytes. Inositol did not significantly increase cell viability in SC58236-treated MMICs (Fig. 6).

Osmolytes Decrease MMIC Apoptosis and Caspase Activity after Hypertonic Stress—To explore the mechanism of osmoprotection we evaluated apoptotic MMICs using an in situ TUNEL assay. Under isotonic conditions only 1.9 ± 0.1% of MMICs were apoptotic. Stepwise adaptation to 600 mosmol/liter medium tonicity produced a 6-fold increase in TUNEL-positive cell numbers (12 ± 1.0%, respectively). However, in MMICs treated with the COX2-specific inhibitor (SC58236, 10 μM) a 25-fold increase in fluorescence-positive apoptotic cells was observed at 600 mosmol/liter culture conditions (50 ± 2.7%, p < 0.05) compared with isotonic controls (Fig. 7A). Preincubation with a mixture of organic osmolytes (sorbitol, betaine, and inositol, all 1 mmol/liter) dramatically decreased apoptosis by 77 ± 18% in SC58236-treated MMICs cultured at 600 mosmol/liter.

As shown in Fig. 7B, caspase-3 activity is enhanced in an osmolarity-dependent fashion in cultured MMICs. Caspase-3 activity increased 4-fold when medium tonicity was increased to 600 mosmol/liter, and preincubation with both betaine and sorbitol (1 mmol/liter) significantly reduced caspase activity (by 41 ± 10 and 59 ± 12%, respectively, p < 0.05) in MMICs cultured at 600 mosmol/liter. This protective effect was maintained in MMICs treated with the COX2-specific inhibitor (SC58236, 10 μM) and subjected to hypertonic stress as shown in Fig. 8. A and B. Fig. 8A shows nuclear fluorescein positivity in MMICs cultured at either isotonic (295 mosmol/liter) or hypertonic (500 mosmol/liter) medium conditions. Treatment
with SC58236 (10 μM) without osmolytes significantly increased the number of apoptotic cells (TDT) and decreased overall cell numbers (PI). This observation was dramatically ameliorated by preincubation with a mixture of organic osmolytes (inositol, sorbitol, and betaine, 1 mmol/liter each). Fig. 8B depicts apoptotic DNA laddering under hyperosmotic stress (lane B, 600 mosmol/liter), which is enhanced by the addition of SC58236 and reversed by increasing concentrations of organic osmolytes.

**DISCUSSION**

**Effect of Organic Osmolytes on COX2 Inhibitor-mediated Decrease in MMIC Viability**—Previous studies (30) found that the COX2-specific inhibitor SC58236 reduces COX2 activity and COX2 products in renal medullary interstitial cells. The present study shows that inhibition of COX2 activity is associated with decreased hypertonicity-induced osmolyte accumulation in MMICs (Fig. 2). COX2 inhibition most dramatically inhibited betaine accumulation (96 ± 10%), whereas inositol and sorbitol accumulation were less affected by treatment with SC58236. Decreased accumulation of intracellular osmolyte concentrations was also associated with diminished mRNA levels of genes coding for osmolyte transporters and aldose reductase (Figs. 5 and 6). The reductions in betaine and myo-inositol transporter mRNA levels seen with the COX2 inhibitor were proportional to the reduction of cellular concentrations of these compounds. In fact, BGT1 showed a nearly 2-fold greater reduction in mRNA levels compared with SMIT (Fig. 4). SC58236 did not have any direct effect on [3H]inositol uptake in MMIC, suggesting that COX2 activity exerts its effect on osmolyte transporter genes possibly at the transcriptional level, rather than on the cytoplasmic membrane osmolyte transporters. The mechanism of the particular sensitivity of BGT1 to COX2 inhibition might therefore be because of transcriptional regulation, but this remains to be elucidated.

In vivo the effect of COX2 inhibition either with SC58236 or COX2 gene disruption was associated with a more pronounced impairment of osmolyte accumulation than in cultured MMICs (Fig. 3). Nevertheless, as seen in cultured interstitial cells, medullary concentrations of betaine and inositol also showed the greatest sensitivity to COX2 inhibition, with inositol levels in COX2 inhibitor-treated or COX2−/− dehydrated mice being below even those of control animals (Fig. 3). Because aldose...
reductase mRNA levels diminish after increased toxicity in cultured COX2−/− MMICs, the existence of a down-regulatory factor that becomes fully active in the absence of COX2 could possibly explain these observations. Elucidation of these regulatory mechanisms are under current investigation.

When MMICs were slowly adapted to medium osmolarities of 600 mosmol/liter (100 mosmol/liter increments/24 h), cell survival was equal to isotonic controls (Fig. 6). However, MMICs were quite sensitive to abrupt increases in medium tonicity of more than 200 mosmol/liter/24 h, which were poorly tolerated (data not shown). This time-dependent adaptation is consistent with insufficient time to transcribe and translate genes encoding for osmolyte transporters and enzymes. We therefore tested the effect of COX2 inhibitors on cell survival only in adapted MMICs. SC58236 reduced MMC survival by 30% at 600 mosmol/liter, even when adequate time to adapt was provided. Preincubation with 1 mmol/liter betaine or sorbitol rescued MMICs and returned survival rates back to normal levels. Betaine showed the greatest potential to prevent MMC death, similar to its protective capacity in Madin-Darby canine kidney cells exposed to hypertonic stress (31).

Apoptosis was a major mechanism contributing to cell demise in MMICs exposed to hyperosmotic stress, as shown by TUNEL stain and DNA laddering (Fig. 8, A and B). In contrast, provision of exogenous organic osmolytes largely prevented apoptosis in COX2 inhibitor-treated MMICs after hypertonic stress. Similarly, in cultured cells toxicity-dependent activation of caspase-3 activity was reduced by betaine and sorbitol but not inositol (Fig. 7B). These findings suggest that the inability to adequately accumulate organic osmolytes significantly contributes to COX2 inhibitor-mediated action to decrease MMC survival after hypertonic stress. Furthermore, the results of our studies argue against a direct toxic effect induced by the COX2-specific inhibitor per se.

COX2-dependent Osmolyte Accumulation in MMC and Mouse Inner Medulla—The accumulation of organic osmolytes in response to exposure of hypertonic medium has been described previously for rat kidney papillary interstitial cells (20). Similar to this study, our results in mouse medullary interstitial cells show that betaine and inositol are the predominant osmolytes accumulated at 600 mosmol/liter medium tonicity, with a lower increase in sorbitol. Burger-Kentischer et al. (20) also detected low levels of glycerophosphorylcholine accumulation, an observation that we could not confirm. This might be because of lesser amounts of cell protein in our experiments, leaving glycerophosphorylcholine concentrations below detection levels or a particular species difference. Interestingly, the present studies showed a different pattern of osmolyte accumulation in the in vivo setting in which sorbitol accumulation predominated as compared with the in vitro setting in which betaine and inositol predominated. Because inner medullary whole tissue sections contain many additional cell types (i.e. collecting duct cells), this discrepancy could be because of a larger relative increase in sorbitol accumulation in papillary-collecting duct cells as compared with interstitial cells, as shown previously (20).

In conclusion, the present studies demonstrate that accumulation of organic osmolytes in kidney medullary cells exposed to hypertonic stress is dependent on COX2 activity. The diminished ability of MMCs to tolerate hypertonic stress after COX2 inhibition can be reversed by providing exogenous organic osmolytes to these cells. To our knowledge this is the first study to link organic osmolyte accumulation to COX2 activity, which provides a possible molecular mechanism contributing to medullary interstitial cell injury in patients with analgesic nephropathy.

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