MAPK Mediation of Hypertonicity-stimulated Cyclooxygenase-2 Expression in Renal Medullary Collecting Duct Cells*

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We have previously shown that hypertonicity stimulates cyclooxygenase-2 (COX-2) expression in cultured medullary epithelial cells. The aims of the present study were (i) to examine the role of cytoplasmic signaling through MAPK pathways in tonicity regulation of COX-2 expression in collecting duct cells and (ii) to assess the possible contribution of COX-2 to the survival of inner medullary collecting duct (IMCD) cells under hypertonic conditions. In mIMCD-K2 cells, a cell line derived from mouse IMCDs, hypertonicity induced a marked increase in COX-2 protein expression. The stimulation was reduced significantly by inhibition of MEK1 (PD-98059, 5–50 µM) and p38 (SB-203580, 5–100 µM) and was almost abolished by the combination of the two compounds. To study the role of JNK in tonicity-stimulated COX-2 expression, IMCD-3 cell lines stably transfected with dominant-negative mutants of three JNKs (JNK-1, -2, and -3) were used. Hypertonicity-stimulated COX-2 protein expression was significantly reduced in dominant-negative JNK-2-expressing cells and was unchanged in dominant-negative JNK-1- and JNK-3-expressing cells compared with controls. The reduction of COX-2 expression was associated with greatly reduced viability of dominant-negative JNK-2-expressing cells during hypertonicity treatment. 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) (2–8 µM), an inhibitor of Src kinases, reduced the tonicity-stimulated COX-2 expression in a dose-dependent manner, whereas PP3, an inactive analog of PP2, had no effect. Inhibition of COX-2 activity by NS-398 (30–90 µM) and SC-58236 (10–20 µM) significantly reduced viability of mIMCD-K2 cells subjected to prolonged hypertonic treatment. We conclude that 1) all three members of the MAPK family (ERK, JNK-2, and p38) as well as Src kinases are required for tonicity-stimulated COX-2 expression in mouse collecting duct cells and that 2) COX-2 may play a role in cell survival of medullary cells under hypertonic conditions.

A large body of experimental work has demonstrated that accumulation of compatible osmolytes such as sorbitol, inositol, taurine, glycerophosphorylcholine, and betaine is essential for survival of medullary cells under hypertonic conditions (1). Recent studies suggest that the osmotic responsiveness of cells is a complex process involving the participation of multiple mitogen-activated protein kinases (MAPKs).1 It has been shown in yeast that high osmolality glycerol (Hog1) kinase, the yeast homolog of p38 kinase, is activated by hypertonicity and is responsible for the induction of glycerol-3-phosphate dehydrogenase, the enzyme essential for production of glycerol, the major organic osmolyte in yeast (2, 3). Furthermore, a yeast Hog1 deletion mutant is lethal under hypertonic conditions, and the lethality is rescued by overexpression of mammalian wild-type p38 (4) or c-Jun NH2-terminal kinase (JNK) (5). The evidence supports a critical role of MAPKs in the regulation of osmolyte accumulation during osmotic stress in yeast. Several studies indicate that mammalian MAPKs may have a similar function in tonicity responses as their yeast homologs. Several groups have demonstrated that all three members of the MAPK family, p38, JNK, and the extracellular signal-regulated kinase (ERK), can be activated in mammalian cells by hypertonicity (6–9). In a study by Wojtaszek et al. (10), hypertonicity was shown to selectively activate JNK-2, but not JNK-1, in mouse medullary epithelial cells, and a dominant-negative mutant of JNK-2 reduced cell viability under hypertonic conditions independent of inositol accumulation.

Cyclooxygenase (COX) plays a key role in regulation of prostaglandin synthesis and is probably rate-limiting under some circumstances. Two isoforms of cyclooxygenase have been identified by molecular approaches, a constitutive form (COX-1) and an inducible form (COX-2). The two forms share similar enzymatic properties, but differ markedly with respect to cellular expression pattern and regulation. In general, COX-1 is expressed constitutively in a wide variety of tissues and is considered to have “housekeeping” functions. COX-2 is more restricted in its expression and can be dramatically induced in inflammatory cells by cytokines as well as by mitogenic factors (11). Inducibility is also a characteristic of COX-2 expression in a variety of organ systems, including the kidney. Previous studies from our laboratory have shown that chronic salt loading (12) or water deprivation (13) induced COX-2, but not COX-1, expression in the renal medulla in vivo and that hypertonicity induced COX-2, but not COX-1, expression in collecting duct cell lines in vitro. The present study examined the

1 The abbreviations used are: MAPKs, mitogen-activated protein kinases; JNK, c-Jun NH2-terminal kinase; ERK, extracellular signal-regulated kinase; COX, cyclooxygenase; IMCD, inner medullary collecting duct; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PGE2, prostaglandin E2; MEK, MAPK/ERK kinase.
signaling pathway responsible for the tonicity-stimulated COX-2 expression in inner medullary collecting duct (IMCD) cell lines. We demonstrate that inhibition of any one of the three MAPKs p38, ERK, and JNK-2 by SB-203580, PD-58059, and a dominant-negative mutant, respectively, significantly suppressed hypertonicity-stimulated COX-2 expression in cultured medullary epithelial cells. Blockade of JNK-1 or JNK-3 was without effect, however. Inhibition of the Src kinase family by PP2 also suppressed hypertonicity-stimulated COX-2 expression. We further show that COX-2 inhibitors induced a marked reduction of cell viability of medullary epithelial cells under hypertonic conditions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture media and serum were from Life Technologies, Inc. PD-98059 was obtained from New England Biolabs Inc. (Beverly, MA), and SB-203580 from Upstate Biotechnology, Inc. (Lake Placid, NY). Tyrophostin-23 and -51 and MTT tetrazolium salt were from Sigma. Anti-murine COX-2 polyclonal antibody and NS-398 were obtained from Cayman Chemical Co., Inc. (Ann Arbor MI). SC-58236 was a generous gift from Monsanto.

**Cell Culture**—mIMCD-K2 is an established inner medullary collecting duct cell line that was provided by Dr. Bruce Stanton (14). The cells were routinely propagated in Opti-MEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. JNK dominant-negative mutant cells were generated from the mouse IMCD-3 cell line provided by Dr. Steve Gullans (10). IMCD-3 cells were propagated in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 nutrient mixture supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Generation of Stable JNK-1, -2, and -3 Transfectants**—Generation of the three stable JNK dominant-negative cell lines has been described previously (10). Experiments were performed in two independent clones of each stable cell line.

**Western Blotting**—Cells were lysed and sonicated in 30 mM Tris-Cl, pH 7.5, and 100 μM phenylmethylsulfonyl fluoride. Protein concentrations were determined by Coomasie reagent. 100 μg of protein from the whole cell lysate were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The blots were blocked overnight with 5% nonfat dry milk in Tris-buffered saline, followed by incubation for 1 h with rabbit anti-murine COX-2 polyclonal antiserum at a dilution of 1:1000. After washing with Tris-buffered saline, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and visualized with ECL kits (Amersham Pharmacia Biotech).

**MTT Assay**—The assay was performed as described previously (15). Briefly, mIMCD-K2 cells grown in six-well plates were washed with phosphate-buffered saline and incubated at 37 °C with 1 ml of MTT (1 mg/ml) for 2 h. After replacing the MTT solution with 1 ml of 1-propa- nolethanol, cells were gently shaken at room temperature for 10 min. Absorpti- ance at 540 and 690 nm was read in 20-μl samples in a spectrophotometer, and results are expressed as the difference between specific absorbance at 540 nm and background absorbance at 690 nm.

**PGE2 Enzyme Immunoassay**—PGE2 in the culture medium was measured with an enzyme immunoassay kit (Cayman Chemical Co., Inc.). The assay was performed according to the manufacturer’s instructions. Briefly, 25 or 50 μl of the medium, along with a serial dilution of PGE2 standard samples, were mixed with appropriate amounts 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 containing substrate for acetylcholinesterase were added. The enzyme reaction was carried out on a slow shaker at room temperature for 1 h. The plates were read at 415 nm, and the results were analyzed by DeltaSOF2 II software.

**Statistical Analysis**—Values represent means ± S.D. Statistical analysis was performed by Student’s t test, with p < 0.05 being considered statistically significant.

**RESULTS**

**Effect of MAPK Inhibitors and NS-398 on Hypertonicity-stimulated PGE2 Release from Cultured IMCD-K2 Cells**—Confluent mIMCD-K2 cells were pretreated with SB-203580, PD-98059, or NS-398 for 30 min, followed by hypertonic treatment for 16 h. PGE2 release was determined by enzyme immunoassay. As we have shown previously (13), hypertonic treatment induced a 50-fold increase in PGE2 release from mIMCD-K2 cells. The stimulation was significantly suppressed by all three compounds (Fig. 1). Among these inhibitors, SB-203580 exhibited the most potent effect, and it also abolished basal PGE2 release.

**Effect of p38 and MEK1 Inhibitors on Hypertonicity-stimulated COX-2 Expression**—Consistent with our previous observations (13), hypertonicity induced a marked increase in COX-2 protein expression in mIMCD-K2 cells. As shown in Figs. 2 and 3, this stimulation was partially suppressed by both SB-203580 and PD-98059, commonly used inhibitors of p38 kinase and MEK1 (16, 17). Combined treatment with the two inhibitors almost completely abolished the stimulatory effect of hypertonicity (Fig. 3C). It has been reported that SB-203580 can inhibit cyclooxygenase activity (18). To rule out the possibility that the effect of SB-203580 might be a secondary consequence of COX inhibition, we examined the effect of SC-58236, a COX-2-specific inhibitor, on COX-2 expression in mIMCD-K2 cells. In contrast to the inhibitory effect of SB-203580 on COX-2 expression, SC-58236 stimulated COX-2 expression (Fig. 2D).

**Effect of Overexpression of Dominant-negative Mutants of JNK Isoforms on Hypertonicity-stimulated COX-2 Expression**—To study the role of JNK in hypertonicity-stimulated COX-2 expression, we used IMCD-3 cell lines stably transfected with dominant-negative mutants of JNK-1, -2, or -3 (10). Densitometric values of COX-2 protein from two independent clones of each cell line were averaged. Hypertonicity significantly induced COX-2 expression in neomycin control and JNK-1 and JNK-3 dominant-negative cell lines. In contrast, stimulation was significantly blunted in the two clones of the JNK-2 dominant-negative cell line (Fig. 4). COX-2 protein in JNK-2 dominant-negative cells in the basal state was also reduced compared with neomycin control and JNK-1 dominant-negative cell lines. The reduction of COX-2 expression was associated with greatly reduced viability of cells expressing dominant-negative JNK-2 during the hypertonic treatment as determined by MTT assay (Fig. 5).

**Effect of Tyrosine Kinase Inhibitors on Hypertonicity-stimulated COX-2 Expression**—Tyrophostin-23 and -51, specific inhibitors of epidermal growth factor receptor tyrosine kinase, had no obvious effect on toxicity-stimulated COX-2 expression (Fig. 6C). In contrast, PP2, an inhibitor of non-receptor Src tyrosine kinases (2–8 μM), significantly reduced toxicity-stimulated COX-2 expression in a dose-dependent manner, whereas...
PP3, an inactive analog of PP2, had no effect (Fig. 6A).

**Effect of COX-2 Inhibitors on Viability of mIMCD-K2 Cells**—To test a potential function of COX-2 as a cytoprotective agent during an osmotic challenge, we examined the effect of COX-2 inhibitors on cell viability of mIMCD-K2 cells exposed to hypertonic medium. mIMCD-K2 cells were grown for 7 days in hypertonic or isotonic medium in the presence or absence of the COX-2-specific inhibitors NS-398 and SC-58236. To achieve maximal osmotic stress, the initial medium osmolarity of 600 mosmol/liter was increased over 3 days to 750 mosmol/liter by adding NaCl and was maintained at this level until day 7. Cell viability was assessed by morphological analysis (Fig. 7) and MTT assay (Fig. 8). Under hypertonic conditions, both NS-398 (30–90 μM) and SC-58236 (10–20 μM) significantly reduced cell viability in a time- and dose-dependent manner. These effects became apparent at 3 days and were maximal at 7 days of drug treatment. At this time, NS-398 (90 μM) and SC-58236 (20 μM) reduced the optical density from 0.085 ± 0.027 to 0.059 ± 0.020 (n = 4, p < 0.05) and 0.035 ± 0.008 (n = 4, p < 0.01), respectively. In contrast, neither drug had any obvious effect on cell viability under isotonic conditions.

**DISCUSSION**

The study demonstrates that inhibition of any one of the three MAPKs p38, ERK, and JNK-2 significantly suppresses hypertonicity-stimulated COX-2 expression and that the simultaneous inhibition of the p38 and ERK pathways almost completely prevents COX-2 induction. In addition, the selective non-receptor tyrosine kinase inhibitor PP2 suppresses the stimulation of COX-2 by hypertonicity, whereas inhibition of epidermal growth factor receptor tyrosine kinase has no effect.
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These findings indicate that activation of multiple MAPKs as well as non-receptor tyrosine kinases participates in the cytoplasmic signaling pathways that mediate the effect of hypertonicity on COX-2 expression in medullary epithelial cells. Finally, our data show that COX-2 inhibitors cause a marked reduction of cell viability of medullary epithelial cells under hypertonic, but not isotonic, conditions, supporting the notion of a cytoprotective function of COX-2 in the cellular response to hypertonicity.

An impressive body of work has established that cells adapt to environmental hypertonicity by inducing a cluster of osmolyte-associated genes, including aldose reductase and the transporters for betaine, taurine, and myo-inositol. The expressed proteins are responsible for the synthesis or uptake of compatible organic osmolytes (1, 19). The complexity of the intracellular mechanisms mediating the response to environmental osmotic changes is revealed by recent observations that hypertonicity activates multiple protein kinases, including all three MAPKs p38, JNK, and ERK, as well as tyrosine kinases, including epidermal growth factor receptor tyrosine kinase (20), and that activation of these kinases is associated with the induction of immediate early genes, including Egr-1 and c-fos (21, 22). It is well established that the p38 homolog Hog1 regulates the production of the major yeast osmolyte glycerol, a prerequisite for yeast growth under hypertonic conditions. Conflicting observations exist regarding the association of MAPKs with osmolyte accumulation in mammals (23, 24). There is increasing evidence that the function of MAPKs in the osmotic response in mammalian cells is not limited to regulating the accumulation of compatible osmolytes. In support of this notion is the finding that p38 and ERK play a role in hypotonicity-stimulated HSP-70 expression (16). In addition, a study by Wojtaszek et al. (10) has demonstrated that a dominant-negative mutant of JNK-2 reduced cell viability without affecting inositol uptake. It appears that MAPKs are involved in the osmotic response in mammalian cells through more diverse mechanisms than in yeast and that the activation of multiple MAPK pathways may affect both osmolyte-dependent and -independent mechanisms. The major contribution of the present study is the demonstration that COX-2 is a target of MAPKs in mammalian cells during osmotic perturbations and that it exerts a cytoprotective function during hypertonic stress. The MAPK/COX-2 relationship appears to be a novel pathway in the overall osmotic response of mammalian cells that is probably dissociated from osmolyte accumulation.

Using pharmacological blockade of p38 and ERK pathways and dominant-negative mutants of various forms of JNK (JNK-1, -2, and -3), we have demonstrated that interference with each of the three MAPK pathways significantly suppresses hypertonicity-stimulated COX-2 expression and that combined inhibition of p38 and ERK virtually completely abolishes the stimulation. This is consistent with the finding that MAPK inhibitors suppress hypertonicity-stimulated PGE2 release. The inhibition of COX activity by SB-203580 is particularly potent in that it abolishes even basal PGE2 release. This is likely due to an inhibitory effect of SB-203580 on COX-2 expression and COX activity (18). Our findings suggest that all three MAPK pathways are required for the induction of COX-2 by hypertonicity and that the different MAPK pathways act in an additive fashion. Simultaneous activation of more than one MAPK pathway is also required for the stimulation of COX-2 expression by both cytokines (17) and ceramide (25). Taken together, these findings suggest that cooperative action of multiple MAPKs mediates the activation of COX-2 in response to various external stimuli. The detailed mechanism for MAPK-mediated activation of COX-2 expression is not clear. It is possible that the three MAPKs p38, ERK, and JNK-2 phosphor- ylate different transcription factors that form a complex and that bind to osmolality-responsive elements in the COX-2 promoter region. The specificity of the signaling pathway for a given stimulus is likely determined by differences in upstream mediators. However, the existence of various isoforms of MAPKs could also contribute to the specificity of the signaling. In support of this notion are the findings that hypertonicity selectively activates JNK-2, but not the other isoforms of JNK, and that hypertonicity-stimulated COX-2 is reduced only in JNK-2-expressing cells, but not in dominant-negative JNK-1- and JNK-3-expressing cells.

It has been noted that tyrosine kinases are involved in the signaling mechanism for both cytokine- and mitogen-stimu- lated COX-2 expression in pancreatic islet and mesangial cells.
Confluent mIMCD-K2 cells were treated with isotonic or hypertonic medium in the presence or absence of 90 μM NS-398 (10–20 μM) or SC-58236 for 7 days. To achieve maximal osmotic stress, the initial medium osmolarity of 600 mosmol/liter was increased over 3 days to 750 mosmol/liter by adding NaCl and was maintained at this level until day 7. Shown are mIMCD-K2 cells grown at the indicated days following hypertonic treatment in the presence of 20 μM SC-58236 or 90 μM NS-398. Cells treated with an equivalent amount of solvent (Me2SO) served as controls (at day 7).

Our present results show that the COX-2 inhibitors NS-398 and SC-58236 reduced cell viability of medullary epithelial cells during exposure to hypertonicity, but had no obvious effect under isotonic conditions, supporting the cytoprotective function of COX-2 in medullary cells. This finding is consistent with a number of previous observations on the growth-promoting and cytoprotective function of COX-2. For example, it has been shown that inhibition of COX-2 induces cell death in cultured medullary interstitial cells and colon cancer cells (31, 32) and that overexpression of COX-2 is protective against apoptosis in intestinal endothelial cells (33). The mechanism of the cytoprotective action of COX-2 is not clear. Recent studies have demonstrated that 15-deoxy-Δ12,14-prostaglandin J2 is a natural ligand for the γ-isofrom of the peroxisomal proliferator-activated receptor, an observation that, together with the presence of COX-2 in the nuclear envelope, establishes the existence of a nuclear pathway for the action of prostaglandins (34, 35). Studies from our (36) and other (37) laboratories have demonstrated predominant expression of peroxisomal proliferator-activated receptor-γ in the renal medulla, where COX-2 is abundantly expressed. These findings raise the possibility that COX-2 products may exert their cytoprotective role through nuclear receptors.

In summary, our data show that inhibition of one of the three MAPKs p38, ERK, and JNK-2 significantly suppresses hypertonicity-stimulated COX-2 expression in cultured medullary epithelial cells. Selective inhibition of the Src kinase family by PP2 also suppresses the hypertonicity-stimulated COX-2 expression. Furthermore, COX-2 inhibitors induce a marked reduction of cell viability of medullary epithelial cells under hypertonic conditions. We conclude that hypertonicity stimulates COX-2 expression through activation of multiple MAPKs as well as Src kinases and that the induction of COX-2 may exert a specific cytoprotective function in the overall osmotic response of medullary epithelial cells.

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