HYPERTONIC INDUCTION OF COX-2 IN COLLECTING DUCT CELLS BY REACTIVE OXYGEN SPECIES OF MITOCHONDRIAL ORIGIN

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Running title: ROSs mediate tonicity-stimulated COX-2 expression.

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Our previous studies have documented MAPK mediation of the hypertonicity-induced stimulation of COX-2 expression in cultured renal medullary epithelial cells. The present study extends this observation by examining the role of reactive oxygen species (ROSs). ROS levels, determined using dichlorodihydrofluorescence diacetate (DCFDA) and cytochrome c, were rapidly and significantly increased following exposure of mIMCD-K2 cells to media made hypertonic by adding NaCl. Hypertonic treatment (550 mosmol/kg) for 16 hours induced a 5.6-fold increase in COX-2 protein levels and comparable increases in PGE2 release, both of which were completely abolished by the NADPH oxidase inhibitor diphenyleneiodonium (25-50 µM). The general antioxidant N-acetyl-L-cysteine (6 mM), and the SOD mimetic tempo (2.0 mM) reduced COX-2 levels by 75.6% and 79.8%, respectively. Exposure of mIMCD-K2 cells to exogenous O2 generated by the xanthine/xanthine oxidase system mimicked the effect of hypertonicity on COX-2 expression and PGE2 release. The increases in phosphorylation of Erk1/2 and p38 were detected 20 minutes following the hypertonic treatment and were both prevented by NAC. The increases in ROSs in response to hypertonic treatment were completely blocked by either one of the mitochondrial inhibitors tested, such as rotenone, tenoyltrifluoroacetone, or carbonyl cyanide m-chlorophenylhydrazone, associated with remarkable inhibition of COX-2 expression. In contrast, the increases in ROSs were not significantly altered in IMCD cells deficient in either gp91phox or p47phox, nor were the increases in COX-2 expression. We conclude that ROSs derived from mitochondria, but not NADPH oxidase, mediate the hypertonicity-induced phosphorylation of MAPK, and the stimulation of COX-2 expression.

The abbreviations: ROSs, reactive oxygen species; COX-2, cyclooxygenase-2; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; IMCD, inner medullary collecting duct.

INTRODUCTION

In mammals, the renal medulla is one of the few tissues being constantly exposed to hypertonicity. Furthermore, urinary osmolality in rodents increases to levels greater than 3000 mosmol/kg following water deprivation. Survival of renal medullary cells in this hypertonic environment depends on accumulation of compatible organic osmolytes (1,2). This is accomplished by activation of a cluster of osmolyte-related genes, including aldose reductase and the transporters for betaine, taurine, and myo-inositol. Most of these genes are under the control of the tonicity-responsive transcription factor, TonEBP/NFAT5, which interacts with the tonicity enhancer element (TonE/ORE) (3,4).
Cyclooxygenase, also called prostaglandin H synthase, is the rate-limiting enzyme catalyzing the metabolism of arachidonic acid to prostaglandins (PGs). COX exists in two major isoforms, the inducible form, COX-2 and the constitutive form, COX-1 (5-8). COX-1 is expressed in a wide variety of tissues and its expression level does not appear to change significantly; it is implicated in regulation of house keeping functions such as platelet aggregation and cytoprotective function in the gastrointestinal tract. In contrast, COX-2 is much more restricted in its expression to certain cell types and its expression undergoes robust changes in response to growth factors and inflammatory stimuli. In addition to its role as an inducible generator of PGH in the inflammatory response, evidence is emerging to suggest that COX-2 also plays an important role in the regulation of physiological processes, including in the kidney. Within the kidney, COX-2 is abundantly expressed in the inner medulla and is further induced by water restriction (9,10) and chronic salt loading (11). In cultured renal medullary epithelial and interstitial cells (10,12), as well as in liver macrophages (13), hypertonicity exerts a direct stimulatory effect on COX-2 expression. COX-2 activity is required for osmolyte accumulation and adaptation to hypertonic stress (14).

Previous studies from this laboratory have identified multiple members of the mitogen-activated protein kinase (MAPK) family, namely Erk1/2, p38, and JNK, as factors mediating toxicity-induced induction of COX-2 in cultured renal medullary epithelial cells (12). In these experiments, COX-2 induction by hypertonicity was partially inhibited by blockade of each of these MAPKs, and was completely blocked by simultaneous blockade of Erk1/2 and p38. The hypertonic induction of COX-2 expression in IMCD3 cells requires transactivation of the epidermal growth factor receptor (EGFR) tyrosine kinase (15).

In cultured renal medullary interstitial cells, the COX-2 induction is dependent on NFkB (10).

The present study was performed to further define the transduction pathway responsible for osmotic regulation of COX-2 expression. Oxygen species (ROSs) which are highly reactive by carrying one or more unpaired electrons in the outer orbits, are typically generated as a by-product of oxygen metabolism. Large amount of ROSs produced in phagocytes serve to kill invading microorganisms, while small amounts of ROSs produced in non-phagocytic tissues participate in signaling pathways. Increased formation of ROSs occurs when cells are receiving stress signaling. Since some of the best known targets of ROSs include MAPK as well as NFkB (16), we examined whether ROSs participate in the mediation of the hypertonic induction of COX-2 expression via MAPK in cultured renal medullary epithelial cells.

**EXPERIMENTAL PROCEDURES**

**Animals.** The gp91phox-null mutant mice were a gift from Mary C. Dinauer (University of Indiana) (17), and the p47phox-null mutant mice were provided by Steve Holland (National Institutes of Health)(18).

**Materials.** Cell culture media and serum were from Life Technologies, Inc. PD-98059 and SB-203580 were from New England Biological Lab, Upstate, respectively. Cytochrome c, SOD, genistein, tempo, xanthine, xanthine oxidase, rotenone (ROT), carbonyl cyanide m-chlorophenylhydrazone (CCCP), thenoyltrifluoroacetone (TTFA), and indomethacin were from Sigma. Murine COX-2 polyclonal antibody and PGE2 enzyme immunoassay kit were from Cayman. Polyclonal antibodies against phosphorylated p-44/42 and phosphorylated p38 were from Cell Signaling Technology.

**Cell Culture.** mIMCD-K2 is an established inner medullary collecting duct cell line provided by Dr. Bruce Stanton (14). The cells were routinely propagated in an Opti medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100
µg/ml streptomycin. Primary cultures of kidney IM cells were generated with modifications of previously described protocols (19). In brief, mice were anesthetized by ketamine/xylazine, and kidneys were quickly removed under sterile conditions. The renal inner medulla was dissected, minced, and digested for 45 min in 10 ml medium (DMEM:Ham’s F12, 1:1 vol/vol) containing 0.2% collagenase and 0.2% hyaluronidase (w/v) at 37°C with a stirrer. After incubation, 20 ml of distilled water was added to lyse cells other than collecting duct cells by osmotic shock (100 mosmol/kg). Cells were then centrifuged at 150 g for 10 min, the supernatant was discarded and the pellet was resuspended in the modified DM medium (DMEM:Ham’s F12, 1:1 vol/vol; 50 nM hydrocortisone, 5 pM 3,3,5-triiodo-L-thyronine, 1 nM sodium selenate, 10 ng/ml epidermal growth factor, 5 mg/l transferrin, 2 mM L-glutamine, 100 U/ml penicillin G, 100 U/ml streptomycin sulfate and 10% FBS (vol/vol). Cells were kept in a 6-well plate for at least 10 days until confluence. At least 48 hours before experiments, medium incubating cells was replaced with a medium that contained no drugs and hormones.

DCFDA fluorescence measurement of ROSs. The fluorogenic substrate 2’,7’-dichlorofluorescein diacetate (DCFDA) is a cell-permeable dye that is oxidized to highly fluorescent 2’,7’-dichlorofluorescein (DCF) by H₂O₂ and can therefore be used to monitor intracellular generation of ROSs. For measurement of ROSs, cells were grown onto glass cover slides. When the cells reached confluence, they were washed twice with PBS and incubated for 30 min with 50 µM DCFH-DA diluted in Opti-MEM with 10% FCS. Then hypertonic medium was added. At the end of the incubation period, the cells were again washed twice with PBS, and imaged by confocal laser microscopy. To quantify ROS levels, cells were seeded to 96-well plate, and were treated as above-mentioned. Relative fluorescence was measured by a fluorescence plate reader (FLUOstar OPTIMA) at excitation and emission wavelengths of 485 and 528 nm, respectively, three times at 90-s intervals. Relative fluorescence units (RFU) was expressed as fold increase over untreated cells.

Cytochrome c reduction based superoxide assay. The extracellular superoxide production by hypertonicity treated cells seeded in 24-well plates was determined from the SOD-inhibitable reduction of cytochrome c (20). Confluent cells were treated with isotonic or hypertonic medium (550 mosmol/kg by adding NaCl) in the presence or absence of 50 µM genistein. 160 µM cytochrome c was added to all wells and 100 U/ml SOD was added to a second well for each sample. Absorbance was measured in a plate reader at 550 nm. Superoxide production was calculated from the difference in the absorbance in the absence and presence of SOD.

Western blotting for COX-2. mIMCD-K2 cells were lysed and subsequently sonicated in PBS containing 1% Tx-100, 250 µM PMSF, 2 mM EDTA, and 5 mM DTT (pH 7.5). Protein concentration was determined by Coomassie reagent. 40 µg of protein from whole cell lysates was denatured in boiling water for 10 min, separated by SDS-PAGE and transferred onto nitrocellulose membranes. 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 at a dilution of 1:1000. After washing with TBS, blots were incubated with a goat anti-rabbit HRP-conjugated secondary antibody and visualized with ECL kits (Amersham).

Phosphorylation of MAP kinases. mIMCD-K2 cells grown in a 6-well plate were lysed by sonication for 10 seconds in 300 µl of 1 x Laemmli sample buffer containing 10 mM Tris, 1.4% SDS, and 40 mM DTT (pH 6.8). The protein samples were heated at 60 °C for 15 min and electrophoresis was performed as described above. The blots were blocked in 5% non-fat dry milk for 1 h, and incubated overnight at 4 °C with the primary antibodies.
against phospho-Erk1/2 and phospho-p38 at a dilution of 1:1000. The secondary antibody and ECL reaction were the same as described above.

COX-2 promoter activity assay. A 2.7 kb of 5′ flanking region of the mouse COX-2 gene was amplified by PCR and subcloned into the pGL3-Basic vector (Promega) upstream of the luciferase reporter gene. Transfection of mIMCD-K2 cells was performed using FUGENE6 according to the instruction from the manufacturer (Roche). Luciferase activity was determined using a luminescence/fluorescence plate reader (FLUOstar OPTIMA).

PGE₂ enzyme immunoassay. PGE₂ in the culture media was measured with an enzyme immunoassay kit. The assay was performed according to the manufacturer's instruction. 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 hours. After the wells were emptied and rinsed with wash buffer, 200 µl of Ellman's reagent.

Statistical analysis. Values shown represent means ± SE. Statistical analysis was performed by ANOVA and Bonferroni tests with a p value of less than 0.05 being considered statistically significant.

RESULTS

Effect of hypertonicity on ROSs.

ROS levels in mIMCD-K2 cells exposed to isotonic or hypertonic medium were determined using dichlorodihydrofluorescence diacetate (DCFDA) and cytochrome c. Osmolality in the medium was raised by adding NaCl. Exposure of the cells to hypertonicity markedly increased ROS levels as determined by the two independent methods (Figs. 1 and 2). The stimulation of ROSs was time dependent, being detected at 2 min, gradually increasing with time, and peaking at 30 min following hypertonic treatment (Fig. 1 lower panel). The increases in ROS levels were prevented by 50 µM genistein (Figs. 1 and 2).

The source of increased ROSs in response to hypertonicity

Two potential sources of the ROSs, e.g. mitochondria and NADPH oxidase, were examined. A 30-min exposure of mIMCD-K2 cells to hypertonic medium induced an over 2-fold increase in DCF fluorescence that was completely abolished by 5 µM rotenone, an inhibitor of complex I, 10 µM tenoyltrifluoroacetone (TTFA), an inhibitor of mitochondrial electron transport chain complex II, or 0.5 µM carbonyl cyanide m-chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation (Fig. 3A). To examine NADPH oxidase as the source of the ROSs, DCF fluorescence was determined in cells derived from the renal inner medulla from gp91phox -/- and p47phox -/- and wild type control mice. The majority of cells exhibited epithelial cell-like morphology (data not shown) and were considered to be IMCD cells although contamination of other cell types such as interstitial cells was inevitable. Hypertonic treatment induced an approximately 2-fold increase in DCF fluorescence in primary cultures of both wild type and in mIMCD-K2 cells (Fig. 3B). However, the increases in DCF fluorescence were not significantly altered in gp91phox -/- or p47phox -/- cells as compared to wild type controls (WT 1.03 ± 0.04 vs. 2.06 ± 0.12, P<0.05; gp91phox -/- 1.14 ± 0.07 vs 1.81 ± 0.07, P<0.05; P47 -/- 1.21 ± 0.07 vs. 1.87 ± 0.04, P<0.05, N = 8 in each group).

Role of ROSs in mediation of hypertonicity stimulation of COX-2 expression.

We examined the effects of antioxidants on the stimulation of COX-2 expression by hypertonicity in cultured mIMCD-K2 cells. To interfere with different steps in O₂⁻ metabolism, four classes of antioxidants, mitochondrial inhibitors (ROT, CCCP, and TTFA), the NADPH oxidase inhibitor DPI, the synthetic SOD mimetic tempo, and the
general antioxidant NAC, were used. When mIMCD-K2 cells were exposed to media made hypertonic (550 mosmol/kg) by the addition of NaCl for 16 hours, an over 5-fold increase in COX-2 protein expression was noted that was remarkably inhibited or abolished by 5 µM ROT, 10 µM TTF-A, 0.5 µM CCCP, or 25-50 µM DPI. NAC (6 mM) or tempo (2.0 mM) reduced COX-2 levels by 75.6% and 79.8%, respectively (Fig. 4). The inhibition of COX-2 expression was paralleled by complete blockade of hypertonicity-stimulated PGE2 release (Fig. 5).

To examine the role of NADPH oxidase in mediation of the hypertonicity-dependent stimulation of COX-2 expression, cells from the renal inner medulla from gp91phox -/- and p47phox -/- mice and wild type controls were exposed to hypertonicity for 16 h, and COX-2 expression was examined by immunoblotting. The magnitude of COX-2 stimulation in response to hypertonic treatment was comparable between gp91phox -/- and p47phox -/- cells and their wild type controls (Fig. 6).

To examine the effect of antioxidants on the hypertonicity-induced COX-2 transcripational activity, mIMCD-K2 cells were transiently transfected with a reporter construct containing the luciferase gene driven by a 2.7-kb COX-2 mouse promoter. Hypertonic treatment for 16 hours markedly induced COX-2 promoter activity that was abolished by 25 µM DPI and significantly inhibited by 3 mM NAC (Fig. 7). NAC and tempo were not toxic to the cells in the doses and durations used while modest toxicity was noticed with 50 µM but not 25 µM DPI.

We examined whether oxidative stress generated by the xanthine/xanthine oxidase system had a direct effect on COX-2 expression. mIMCD-K2 cells were treated for 16 hours with vehicle, xanthine alone, or xanthine plus xanthine oxidase. COX-2 expression was not affected by xanthine alone, but markedly stimulated by xanthine plus xanthine oxidase (Fig. 8A). The expression of COX-2 correlated well with PGE2 levels (Fig. 8B).

COX-2 expression can be regulated in a feed forward process by its own prostaglandins and there is a possibility that the effects observed with the antioxidants might be due to effects on COX activity. To rule out this possibility, we examined the effects of non-selective and selective non-steroidal anti-inflammatory agents on COX-2 expression. As shown in Fig. 9, neither indomethacin nor SC-58635 caused significant changes of hypertonicity-induced COX-2 expression. Thus, it is unlikely that in our model system COX-2 expression is regulated in a feed forward process by its own products.

Role of ROSs in mediation of the activation of Erk1/2 and p38 by hypertonicity.

Based on the observation that MAPK mediates the hypertonicity-induced stimulation of COX-2 expression, we hypothesized that ROSs may act via MAPK. To test this hypothesis, we determined the effects of antioxidant treatment on the activation of MAPK by hypertonicity. Phosphorylation of Erk1/2 and p38 was determined by immunoblotting using phosphorylation-specific antibodies. Exposure of mIMCD-K2 cells to hypertonicity for 20 minutes markedly increased the abundance of the phosphorylated forms of both Erk1/2 (p-44/42) and p38. The activation of both Erk ½ and p38 by hypertonicity was prevented by NAC (Fig. 10).

DISCUSSION

The present study attempts to extend our previous observation that MAPK mediates the stimulation of COX-2 expression by hypertonicity in cultured renal medullary epithelial cells. In the present study, we identified ROSs as upstream mediators of the activation of MAPK in the response to hypertonic stress. We found that exposure of mIMCD-K2 cells to hypertonic stress induced rapid release of ROSs that were derived from mitochondria, but not NADPH.
oxidase. The notion of a causal relationship between ROS generation and COX-2 induction is based on the observation that treatment of mIMCD-K2 cells with exogenous $O_2$ generated by the xanthine/xanthine oxidase system mimicked the hypertonicity effect on COX-2 expression and PGE$_2$ release. To interfere with intracellular production of ROSs, we employed structurally unrelated antioxidants, the NADPH oxidase inhibitor DPI, the superoxide scavenger tempo, and the general antioxidant NAC. The induction of COX-2 expression was effectively blocked by all three classes of antioxidants. Commensurate with the protein expression data, antioxidants also effectively blocked PGE$_2$ release. Furthermore, the hypertonicity-induced phosphorylation of Erk1/2 and p38 was completely blocked by antioxidant treatment. These observations reveal the existence of a novel ROSs/MAPK/COX-2 pathway in the osmotic response of collecting duct cells.

The present study determined ROS levels in mIMCD-K2 cells exposed to hypertonic NaCl treatment, using two independent methods involving the use of cytochrome c and dichlorodihydrofluorescence diacetate. Both methods were able to detect increases in ROS levels as early as 2 minutes following exposure to a hypertonic environment, indicating that oxidative stress is an early event in the osmosensing pathway. This finding agreed with the study of Zhang et al. who observed a similar phenomenon in cultured IMCD3 cells (21).

It is of great significance to identify the source of increased ROSs in response to hypertonic treatment. NADPH oxidase is best characterized as a major superoxide generating enzyme in phagocytes. It is a multicomponent enzyme comprised of five subunits: p40$^{\text{phox}}$, p47$^{\text{phox}}$, p67$^{\text{phox}}$, p22$^{\text{phox}}$, and gp91$^{\text{phox}}$ (22). In resting cells, the membrane portion of enzyme, also known as cytochrome b558 consisting of gp91$^{\text{phox}}$ and p22$^{\text{phox}}$, is dissociated from the cytosolic portion that consists of p40$^{\text{phox}}$, p47$^{\text{phox}}$, and p67$^{\text{phox}}$. When the cells are exposed to stimuli, p47$^{\text{phox}}$ is usually heavily phosphorylated, leading to translocation of the whole cytosolic complex to the membrane where the enzyme is reassembled into an active oxidase. Among the five subunits, gp91$^{\text{phox}}$ and p47$^{\text{phox}}$ are of particular significance as the former is the catalytic subunit and the latter is regulated by phosphorylation. Among the five subunits of NADPH oxidase detected in the kidney, only gp91$^{\text{phox}}$ and p47$^{\text{phox}}$ were regulated by sodium intake (23). However, neither the ROS formation nor the COX-2 stimulation in response to hypertonic treatment were measurably affected in gp91$^{\text{phox}}$ or p47$^{\text{phox}}$ deficient cells. These data virtually exclude gp91$^{\text{phox}}$ or p47$^{\text{phox}}$ as a source of hypertonicity-induced increases in ROS production.

We subsequently examined the possibility that mitochondria might be a source of the ROSs. Indeed, the hypertonicity-induced increases in ROSs were abolished by various mitochondrial inhibitors, including rotenone, an inhibitor of complex I, tenoyltrifluoroacetone (TTFA), an inhibitor of mitochondrial electron transport chain complex II, and carbonyl cyanide m-chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation. Furthermore, all of these inhibitors consistently blocked hypertonicity-induced COX-2 expression. Taken together, these observations suggest that mitochondria, but not NADPH oxidase, contribute to the hypertonicity-induced ROS production and COX-2 expression.

We further provide evidence for involvement of tyrosine kinases in the ROS production by showing that the hypertonicity-related induction of ROSs was completely blocked by genistein, a non-selective tyrosine kinase inhibitor. The data were validated by two independent methods of determining ROS levels, the DCF fluorescence and cytochrome c methods. In line with the finding, a number of tyrosine kinases, including receptor tyrosine kinase,
EGF receptor tyrosine kinase (24), as well as non-receptor tyrosine kinases such as Src (25), FAK (26) and Fyn (27) are activated by osmotic stress. Both non-selective inhibition of tyrosine kinase with genistein, and selective inhibition of EGF receptor tyrosine kinase with AG-1478 and PD-153035 significantly blocked the tonicity-dependent induction of COX-2 expression (9,15). It is possible that tyrosine kinases may represent an upstream component in the hypertonicity-initiated cascade leading to oxidative stress. Future studies need to determine the identity of the kinases and the nature of their relationship with mitochondria.

A major contribution of this study is the identification of the ROSs/MAPK/COX-2 pathway in the osmotic response. A significant role of this pathway is suggested by previous evidence implicating MAPK and COX-2 in the osmotic response. There is an impressive body of work supporting the cytoprotective role of MAPK, especially of p38, against osmotic stress. In yeast, deletion of HOG1, a yeast homolog of p38, is lethal under hypertonic conditions, and the lethality is rescued by overexpression of wild type mammalian p38 (28) or c-Jun NH2-terminal kinase (JNK) (29). HOG1 is directly responsible for the induction of glycerol-3-phosphate dehydrogenase, the enzyme essential for production of glycerol, the major organic osmolyte in yeast (30,31). In mammalian cells, hypertonicity activates multiple MAP kinases including Erk1/2, JNK, and p38 in IMCD cells (32). Dominant negative inactivation of JNK2 sensitizes renal inner medullary collecting duct cells to hypertonicity-induced cell death (33).

There is also increasing evidence supporting a cytoprotective role of COX-2 in the osmotic response. Our previous study shows that inhibition of COX-2 increased cell death in mMCD-K2 cells in hypertonic, but not in isotonic conditions (12). A similar phenomenon has been observed in cultured RMIC cells in which COX-2 activity is required for osmolyte accumulation (14). In rodents, expression of renal medullary COX-2 is variably found in either renal medullary interstitial cells (RMIC) (34,35) or in the collecting duct (CD) (36,37). COX-2 inhibition induced apoptosis in RMIC of water deprived rabbits (10), but in the CD of water deprived rats (38). In a recent study of 53 normal human nephrectomy specimens, renal medullary COX-2 was constitutively expressed in vasa recta endothelial cells and in the CD, but not in RMIC (39). It is unclear whether the osmosensing pathway identified by the present study will apply to RMIC or/and the CD, or other cell types in the renal medulla in vivo.

Whether osmolyte-related genes are directly under the control of MAPK is still unclear due to conflicting reports. Pharmacological inhibition of p38 in monocytes and renal epithelial cells blocked the induction of mRNAs for betaine and myo-inositol transporters, and for aldose reductase, as well as ORE-driven reporter gene expression (40-42). Ko et al. showed that inhibition of p38 by either a pharmacological approach or dominant negative mutation of p38α (27) significantly blocked ORE reporter activity. Inconsistent with these observations, however, is the finding showing that dominant negative mutation of MKK3, an upstream activator of p38, did not affect the hypertonicity induced ORE activity, nor did mutation of JNK (43). Furthermore, hypertonicity-induced phosphorylation of TonEBP was not affected by the p38 inhibitor SB-203580. It seems possible that the ROSs/MAPK/COX-2 pathway might be distinct from the conventional TonEBP-mediated pathway in the osmotic response.

In summary, the present study describes a novel hypertonicity-activated pathway in which hypertonicity induces a rapid release of ROSs from mitochondria that activate both ErK½ and p38, leading to induction of COX-2 expression. The ROSs/MAPK/COX-2 pathway is expected to play a distinct role in the osmotic response.
ACKNOWLEDGMENTS

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FIGURE LEGENDS

Fig. 1. Detection of hypertonicity-stimulated induction of ROSs by 2′,7′-dichlorofluorescein (DCF) fluorescence. Upper panel: ROS production in response to osmotic stress; confluent mIMCD-K2 cells in chamber slides were exposed to isotonic or hypertonic (600 mosmol/kg by adding NaCl) medium in the presence of DCF. A: isotonic, 2 min; B: isotonic, 5 min; C: hypertonic, 2 min; D: hypertonic, for 5 min. Middle panel: time course of ROS induction following the hypertonic treatment. Confluent mIMCD-K2 cells in 24-well plates were exposed to isotonic (300 mosmol/kg) or hypertonic (600 mosmol/kg by adding NaCl) medium in the presence of DCF. At the indicated time points, fluorescence was quantified using FLUOstar OPTIMA. #P<0.01, * P<0.05 compared with the corresponding isotonic group. Lower panel: effects of genistein. Cells were treated with isotonic or hypertonic (600 mosmol/kg by NaCl) medium in presence or absence of 50 µM genistein. Results are expressed as fold increases over untreated cells.

Fig. 2. Detection of hypertonicity induction of ROSs by the cytochrome c method. Confluent mIMCD-K2 cells in 24-well plates were treated with isotonic or hypertonic medium (550 mosmol/kg by adding NaCl) in the presence of NaCl in the presence or absence of 50 µM genistein. 160 µM cytochrome c was added to all wells and 100 U/ml SOD was added to a second well for each sample. Absorbance was measured in a plate reader at 550 nm. Shown are SOD-inhibitable absorbencies.

Fig. 3. The contribution of mitochondria or NADPH oxidase to the hypertonicity-induced generation of ROSs. A) effects of the mitochondrial inhibitors ROT, CCCP, and TTFA on ROS production in mIMCD-K2 cells. Confluent mIMCD-K2 cells in 96-well plates were treated for 30 min with isotonic or hypertonic medium (550 mosmol/kg by adding NaCl) in the presence or absence of 5 µM ROT, 10 µM TTFA, or 0.5 µM CCCP, and then subjected to the ROS assay. #, P<0.01 compared with the isotonic group. B), ROS generation in response to osmotic stress in IMCD cells derived from gp91phox -/- and p47 phox -/- mice and from wild type control mice. * P<0.01 compared with the corresponding isotonic group; Ú, P>0.05 compared with the hypertonicity treated wild type group.

Fig. 4. Effects of antioxidants on hypertonicity-stimulated COX-2 expression in mIMCD-K2 Cells. Confluent mIMCD-K2 cells were exposed to isotonic or hypertonic medium for 16 hours in the presence or absence of ROT, CCCP, and TTFA (A), DPI (B), NAC (C), or tempo (D). Hypertonic medium (550 mosmol/kg) was made by adding NaCl to isotonic medium. COX-2 protein expression was determined by immunoblotting. Shown are representative results from 2-3 separate experiments.

Fig. 5. Effects of antioxidants on hypertonicity-stimulated PGE2 release in mIMCD-K2 Cells. Confluent mIMCD-K2 cells were exposed to isotonic or hypertonic medium for 16 hours in the presence or absence of DPI or NAC. Medium PGE2 concentration was determined by EIA.

Fig. 6. COX-2 expression in primary cultures of control and NADPH oxidase deficient IMCD cells in response to hypertonicity. IMCD cells from gp91phox -/- and p47 phox -/- mice were isolated using standard methods. Confluent cells were treated for 16 hours with isotonic or hypertonic medium. Hypertonic medium (550 mosmol/kg) was made by adding NaCl. COX-2 expression is determined by
immunoblotting. Shown are representative results from 3 separate experiments.

Fig. 7. Effect of antioxidants on hypertonicity-induced COX-2 promoter activity. mIMCD-K2 cells were transiently transfected with the mouse COX-2 promoter-luciferase construct. Forty eight hours later, the cells were treated for 16 hours with hypertonic medium (550 mosmol/kg by adding NaCl) in the presence or absence of 25 µM DPI or 3 mM NAC. Luciferase activity was assayed and expressed as RLU/ mg protein.

Fig. 8. Effect of xanthine/xanthine oxidase on COX-2 expression. Confluent mIMCD-K2 cells were exposed to vehicle, xanthine (160 µM) alone or in combination with xanthine oxidase (0.6 U/ml) for 16 hours. A), immunoblotting analysis of COX-2 protein expression, and B), EIA for PGE2 concentration.

Fig. 9. Effects of non-selective and selective COX inhibitors on COX-2 expression. Confluent mIMCD-K2 cells were treated for 16 hours with hypertonic medium (550 mosmol/kg by adding NaCl) in the presence or absence of 10 µM indomethacin or 10 µM SC-58635. Shown are representative results from 3 separate experiments.

Fig. 10. Effect of antioxidants on the hypertonicity activation of p-44/42 and p38. Confluent mIMCD-K2 cells were exposed to isotonic or hypertonic medium for 20 minutes in the presence or absence of NAC at 6 mM. Prior to the hypertonic treatment, cells were pretreated by vehicle or NAC for 24 hours. Phosphorylation of p-44/42 and p38 was determined by immunoblotting using phosphorylation-specific antibodies. Shown are representative results from 3 separate experiments.

REFERENCES

Fig. 1

**DCF fluorescence (fold change over untreated cells)**

- Isotonic
- Hypertonic

* n = 8 in each group

**Time (min)**

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**DCF Fluorescence**

- Isotonic
- Hypertonic
- Hypertonic + Genistein

* n = 5 in each group
Fig. 2

Isotonic  Hypertonic  Hypertonic + Genistein

SOD inhibitable cytochrome c reduction
(delta OD550 nm)

+, p < 0.05 vs Isotonic
#, p < 0.05 vs. Hypertonic
n = 8 in each group

Treatment

n = 8 in each group

* p < 0.05 vs Isotonic
# p < 0.05 vs. Hypertonic
**A**

- DCF Fluorescence
- Isotonic
- Hypertonic
- Hypertonic + CCCP
- Hypertonic + ROT
- Hypertonic + TTFA
- n=8 in each group

**B**

- DCF Fluorescence
- WT
- gp91phox -/-
- p47phox -/-
- n=8 in each group
- Isotonic
- Hypertonic

*Fig. 3*
Fig. 4

A. Isotonic Hypertonic

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>CCCP</th>
<th>ROT</th>
<th>TTFA</th>
</tr>
</thead>
</table>

B. Isotonic Hypertonic

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>DPI (25 µM)</th>
<th>DPI (50 µM)</th>
</tr>
</thead>
</table>

C. Isotonic Hypertonic

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>NAC (6 mM)</th>
</tr>
</thead>
</table>

D. Isotonic Hypertonic

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Tempo (2 mM)</th>
</tr>
</thead>
</table>
**Fig. 5**

- Isotonic Vehicle DPI (25 µM)
- NAC (3 mM)
- NAC (6 mM)

* , p < 0.05 v.s. Hypertonic Vehicle

n = 3 in each group
Fig. 6

**A**

<table>
<thead>
<tr>
<th>gp91\text{phox} +/+</th>
<th>gp91\text{phox} -/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotonic</td>
<td>Hypertonic</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>p47\text{phox} +/+</th>
<th>p47\text{phox} -/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotonic</td>
<td>Hypertonic</td>
</tr>
</tbody>
</table>
Luciferase activity (RLU/mg protein)

Fig. 7

*, p<0.001 vs. Hypertonic Vehicle
#, p<0.001 vs. Isotonic
n = 6 in each group
Fig. 8

A

Vehicle Xanthine Xanthine/Xanthine Oxidase

B

p < 0.05 vs. Vehicle or Xanthine alone
n = 3 in each group
Fig. 9

<table>
<thead>
<tr>
<th>Isotonic</th>
<th>Hypertonic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>Indomethacin</td>
</tr>
</tbody>
</table>
Fig. 10

A

Hypertonic (550 mosmol/kg by NaCl)

<table>
<thead>
<tr>
<th>Isotonic</th>
<th>Vehicle</th>
<th>NAC (6 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>

phospho-p44/42

B

Hypertonic (550 mosmol/kg by NaCl)

<table>
<thead>
<tr>
<th>Isotonic</th>
<th>Vehicle</th>
<th>NAC (6 mM)</th>
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</thead>
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</table>

phospho-p38
Hypertonic induction of COX-2 in collecting duct cells by reactive oxygen species of mitochondrial origin

Tianxin Yang, Aihua Zhang, Matthew Honeggar, Donald E. Kohan, Diane Mizel, Karl Sanders, John R. Hoidal, Josephine P. Briggs and Jurgen B. Schnermann

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