Nitric oxide stimulates COX-2 expression in cultured collecting duct cells through MAP kinases and superoxide but not cGMP

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IT IS WELL KNOWN that the renal medulla is enriched in nitric oxide (NO) synthetic capacity. Total nitric oxide synthase (NOS) enzymatic activity, determined by measurement of L-citrullin formation, is as much as 25 times greater in inner medulla than in cortex (22). Renal medullary interstitial NO concentration in anesthetized rats as determined by a microdialysis system coupled with an oxyhemoglobin-trapping technique is twice as much as in renal cortex (40). Immunoreactive eNOS, nNOS, and iNOS proteins are all predominantly present in renal inner medulla, and all three enzymes are induced by chronic salt loading (19). Using the renal medullary interstitial infusion technique, Mattson et al. (20, 21) selectively manipulated NO levels in the renal medulla and showed that acute infusion of Nω-nitro-L-arginine methyl ester (L-NAME) into rat renal medulla leads to a reduction of medullary blood flow (MBF) and urinary sodium excretion and that chronic medullary infusion of L-NAME over a period of 12 days significantly elevates arterial blood pressure. Thus renal medullary NO appears to participate in regulation of blood pressure, most likely through a local mechanism within the renal medulla. Similar to NO, prostaglandins (PGs) are also abundantly produced in renal medulla (3, 5). Constitutive expression of both COX-1 and COX-2 is detected at substantially higher levels in renal medulla than renal cortex (4, 9, 36, 37). Furthermore, Sprague-Dawley rats on a high-salt diet exhibit an over 10-fold increase in renal medullary COX-2 expression (37). Recent evidence suggests that renal medullary COX-2 plays an important role in stabilizing blood pressure during high-salt loading (38, 39), similar to renal medullary NOS. Given the general similarities between the effects of NO and PGs on renal sodium handling and blood pressure control, an interaction of the two systems is conceivable and might even be necessary for coordinating their antihypertensive actions in renal medulla. In support of this speculation, the induction of COX-2 expression in the rat renal medulla following chronic salt loading is inhibited by treatment with the nNOS inhibitor, 7-nitroindazole (7-NI), raising the possibility that renal medullary COX-2 expression might be under the control of NO (6). Therefore, we performed the present studies to determine whether NO exerts a direct stimulatory effect on COX-2 expression in a cell culture model of renal medullary origin and to investigate the intracellular signaling mechanisms underlying this interaction.

MATERIALS AND METHODS

Materials. Cell culture media and serum were from Life Technologies, PD-98059 was purchased from New England Biological Lab (Beverly, MA), and SB-203580 from Upstate Biotechnology (Lake Placid, NY). SNP was from Sigma. Murine COX-2 polyclonal antibody, PGE2 enzyme immunoassay kit, and S-nitroso-N-acetylpenicillamine (SNAP) were from Cayman (Ann Arbor, MI).

Cell culture. mLMCD-K2 is an established inner medullary collecting duct cell line provided by Dr. Bruce Stanton (11). The cells were routinely propagated in an Opti medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Western blotting for COX-2. mLMCD-K2 cells were lysed and subsequently sonicated in PBS containing 1% Triton X-100, 250 μM PMSF, 2 mM EDTA, and 5 mM DTT (pH 7.5). Protein concentration was determined by Coomassie reagent. Forty micrograms of protein from whole cell lysates were 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 TBS, followed by incubation for 1 h with rabbit antimurine polyclonal

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antiserum to COX-2 (Cayman, Ann Arbor, MI) at a dilution of 1:1,000. After being washed with TBS, blots were incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and visualized with ECL kits (Amersham).

**Real-time RT-PCR.** For real-time PCR, oligonucleotides were chosen by Primer Express 1.0 (PE Applied Biosystems) with probes positioned at an exon-intron junction (26). Sequences of oligonucleotides were COX-2 sense: 5'-CCCTGAAGCGGTACACATCA-3', antisense: 5'-TGTCATGAGGGTCTTCCAATT-3', and probe: 5'-(FAM)TG-CAGCCATTCTTCTCTCAGTTTCT-(TAMARA) (Accession#: BC052900). Real-time PCR amplification was performed using the TaqMan Universal PCR Master Mix and the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 repeats of 95°C for 0.15 min and 60°C for 1 min. Relative amounts of mRNA, normalized by β-actin, were calculated from threshold cycle numbers (CT; i.e., 2^-ΔΔCT), according to the manufacturer’s suggestions.

**PGE2 enzyme immunoassay.** PGE2 in the culture media were measured with an enzyme immunoassay kit (Cayman). 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 acetycholinesterase-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 at room temperature for 1 h on a slow shaker. Plates were read at 415 nm.

**cGMP assay.** mIMCD-K2 cells grown in six-well plates were pretreated with 100 μM 3-isobutyl-1-methylxanthine for 30 min and then treated for 30 min with SNP or SNAP at appropriate concentrations. After treatment, medium was removed and the cells were washed with PBS. Immediately after being washed, 0.3 ml of 0.1 M HCl were added. After 20-min incubation, the cells were scraped and transferred into a centrifuge tube and spun for 10 min at 1,000 g to pellet the cell debris. The supernatant was neutralized with 20 μl of 0.5 M Tris, acetylated, and used for determination of cGMP by enzyme immunoassay (Cayman Chemicals).

**Statistical analysis.** Values shown represent means ± SE. Statistical analysis was performed by ANOVA and Bonferroni tests with a P value <0.05 being considered statistically significant.

**RESULTS**

**Stimulation of COX-2 expression by NO donors.** Figure 1A shows the dose-response relationship between SNP and COX-2 protein expression. Confluent mIMCD-K2 cells were treated with SNP at various doses for 16 h (A, dose response) or at 100 μM for various durations (B, time course). COX-2 protein expression was determined by immunoblotting. Shown are representatives of 3 independent experiments.

**Role of cGMP.** There is abundant evidence to show that guanylyl cyclase activation and cGMP production constitute the major signaling pathway mediating NO actions. Indeed, cGMP has been shown to mediate the COX-2 induction in response to NO donors in a primary culture of cortical thick ascending limb cells (6). Therefore, we examined whether cGMP mediates NO stimulation of COX-2 expression in cultured mIMCD-K2 cells. Contrary to our expectation, blockade of the cGMP pathway with either the soluble guanylyl cyclase inhibitor methylene blue (MB; Fig. 5A) or the protein kinase G (PKG) inhibitor KT-5823 (Fig. 5B) had no effect on SNP-

![Fig. 1. Effect of the NO donor Na nitroprusside (SNP) on cyclooxygenase (COX)-2 protein expression. Confluent mIMCD-K2 cells were treated with SNP at various doses for 16 h (A, dose response) or at 100 μM for various durations (B, time course). COX-2 protein expression was determined by immunoblotting. Shown are representatives of 3 independent experiments.](image1.png)

**Fig. 1.** Effect of SNP on COX-2 mRNA expression. Confluent mIMCD-K2 cells were treated with 100 μM SNP for the indicated period of time. COX-2 mRNA was determined by real-time RT-PCR and normalized by β-actin. Values are means ± SE. *P < 0.05, #P < 0.01; n = 4 in each group.

![Fig. 2. Effect of SNP on COX-2 mRNA expression.](image2.png)

**Fig. 2.** Effect of SNP on COX-2 mRNA expression. Confluent mIMCD-K2 cells were treated with 100 μM SNP for the indicated period of time. COX-2 mRNA was determined by real-time RT-PCR and normalized by β-actin. Values are means ± SE. *P < 0.05, #P < 0.01; n = 4 in each group.
induced COX-2 expression. Furthermore, treatment with the cGMP agonist 8-bromo-cGMP was without an effect on COX-2 expression (Fig. 5A). To rule out the possibility that this phenomenon might be the result of deficiency of the cGMP system in mIMCD-K2 cells, we measured cGMP content in these cells following treatments with the two NO donors. As shown in Fig. 5C, treatments for 30 min with SNP and SNAP both were able to elevate intracellular cGMP levels in mIMCD-K2 cells, suggesting an intact cGMP system in these cells.

Role of MAP kinases. NO has previously been shown in other cell types to activate ERK and p38 (10, 30). Therefore, we examined the role of MAP kinases in NO stimulation of COX-2 expression in cultured mIMCD-K2 cells. As shown in Fig. 6, the SNP-induced COX-2 expression was significantly reduced by either the ERK1/2 inhibitor PD-98059 or the p38 inhibitor SB-203580 and was completely blocked by the combined treatment with the two compounds.

Role of superoxide. There is evidence that peroxynitrite produced by the reaction of NO with superoxide can stimulate COX-2 expression (23) and activate MAP kinase (27). Therefore, we examined the possibility that NO stimulation of COX-2 expression may include the participation of superoxide. As shown in Fig. 7, tempol treatment effectively blocked the COX-2 induction by SNP.

**DISCUSSION**

The present study was undertaken to examine the role and mechanism of NO in regulation of COX-2 expression in cultured renal medullary epithelial cells. Our results indicate that the NO donor SNP has a stimulatory effect on COX-2 mRNA and protein expression, paralleled by stimulation of PGE2 release. Although cGMP is the major signaling pathway of NO, blockade of the cGMP pathway by the guanylyl cyclase inhibitor MB or the PKG inhibitor KT-5823 did not affect SNP-induced COX-2 expression, and activation of the cGMP pathway using 8-bromo-cGMP was also without an effect. In contrast, the COX-2 induction by SNP was effectively blocked by the MAP kinase inhibitors PD-98059 and SB-230580, as well as superoxide scavenger tempol, suggesting involvement of ERK 1/2 and p38, as well as superoxide.

It is well known that within the kidney the renal medulla has the greatest capacity of NO and PG synthesis. This is in agreement with the fact that COX-2 and all three isoforms of NOS are predominantly expressed in renal medulla. Furthermore, COX-2 and NOS are stimulated in parallel by a high-salt diet, and both pathways play a similar role in stabilizing blood
had a measurable effect on COX-2 expression. The cGMP system in mIMCD-K2 cells appeared intact as intracellular cGMP levels increased following treatment with NO donors. These findings strongly argue against an involvement of cGMP in NO-induced signaling in cultured mIMCD-K2 cells. In apparent contradiction to our observations are results from a previous study documenting cGMP mediation of NO stimulation of COX-2 expression in cultured cortical thick ascending limb cells (6). The difference in the requirement of cGMP for the NO signaling in cortical thick ascending limb cells and medullary collecting duct cells may indicate a cell type-specific phenomenon. Indeed, there are several fundamental differences between renal cortical and medullary cells. For example, in the former, a parallel induction of COX-2 and nNOS results from a low-salt diet (2, 9, 25, 32, 33), while in medullary cells the same result is achieved by a high-salt diet (19, 37).

NO has previously been shown in other cell types to activate ERK and p38 (10, 30). Therefore, we examined the role of MAP kinases in NO stimulation of COX-2 expression in cultured mIMCD-K2 cells. We found that the COX-2 stimulation by NO was significantly reduced by the use of either PD-98059 or SB-203580 and was completely abolished by combination of the two compounds. This finding is compatible with our previous studies documenting a significant role of MAP kinases in mediating COX-2 induction in response to hypertonicity (34) and low chloride (35). Taken together, this evidence strongly suggests that MAP kinase activation is a common terminal pathway of COX-2 induction, at least in renal cells.

NO and O$_2^-$ can react to produce peroxynitrite (ONOO$^-$), which is a key oxidant and nitrating molecule (1, 15, 24). The amount of ONOO$^-$ depends on the competition for O$_2^-$ between superoxide dismutase (SOD) and NO. Because the NO/O$_2^+$ reaction occurs at a rate of $2 \times 10^{10}$ M$^{-1}$s$^{-1}$ (16), it is thought that NO effectively competes with SOD for scavenging O$_2^+$. Emerging evidence suggests that ONOO$^-$ functions as an active signaling molecule likely owing to its nitrating proper-
ties (28). Our studies show that NO stimulation of COX-2 expression was remarkably blocked by the superoxide scavenger tempol, indicating a requirement of O$_2^-$ as a reactive oxygen species (28). This work was supported by National Institutes of Health Grants RO-1-HL-079453, RO-1-DK-066592, and RO-1-DK-064981 and by intramural grants.

In summary, our results show that the NO donor SIN-1 stimulates COX-2 expression in cultured renal collecting cells and that the signaling pathway involves MAP kinases and superoxide, but not cGMP. This study contributes to a better understanding of the interaction between NO and PGs in the renal medulla.

REFERENCES


