Nitric oxide stimulates cyclooxygenase-2 in cultured cTAL cells through a p38-dependent pathway

Hui-Fang Cheng, Ming-Zhi Zhang, and Raymond C. Harris

George M. O’Brien Kidney and Urologic Diseases Center and Division of Nephrology, Vanderbilt University School of Medicine and Nashville Veterans Affairs Hospital, Nashville, Tennessee

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Cheng, Hui-Fang, Ming-Zhi Zhang and Raymond C. Harris. Nitric oxide stimulates cyclooxygenase-2 in cultured cTAL cells through a p38-dependent pathway. Am J Physiol Renal Physiol 290: F1391–F1397, 2006.—To examine the interaction of nitric oxide (NO) and cyclooxygenase (COX-2) and the signaling pathway involved, primary cultured rabbit cortical thick ascending limb (cTAL) were used. In these cells, immunoactive COX-2 and vasodilatory prostaglandins were increased by a NO donor, S-nitros-N-acetylpenicillamine (SNAP; 2.5 ± 0.3-fold control, n = 6, P < 0.01). SNAP increased expression of phosphorylated p38 (pp38; 2.4 ± 0.3-fold control; n = 5; P < 0.01), which was inhibited by the p38 inhibitor SB-203580 (1.3 ± 0.1-fold control, n = 5, P < 0.01). SB-203580 inhibited SNAP-induced COX-2 expression [1.4 ± 0.2-fold control, n = 6, not significant (NS) vs. control] and levels of PGE2 significantly. In cTAL cells transfected with a luciferase reporter driven by the wild-type mouse COX-2 promoter, SNAP stimulated luciferase activity, which was reversed by SB-203580 (control vs. SNAP vs. SNAP + SB-203580: 1.4 ± 0.2-, 8.3 ± 1.4-, and 0.4 ± 0.1-fold control, respectively, n = 4, P < 0.01). Electrophoretic mobility shift assay indicated that SNAP stimulated nuclear factor (NF)-κB binding activity in cTAL that was also inhibited by the p38 inhibitor. SNAP was not able to stimulate a mutant COX-2 promoter construct that is not activated by NF-κB (0.9 ± 0.1, 1.2 ± 0.1, and 1.0 ± 0.2, respectively, n = 4, NS). Low chloride increased COX-2 expression (2.7 ± 0.4-fold control, n = 6, P < 0.01) and pp38 expression (2.8 ± 0.3-fold; n = 5, P < 0.01), which were reversed by the specific NO synthase (NOS) inhibitor 7-nitroindazole. Administration of a low-salt diet increased immunoreactive COX-2 and neuronal NOS (nNOS) in the macula densa and surrounding cTAL of kidneys of wild-type mice but did not significantly elevate COX-2 expression in nnos−/− mice. In summary, these studies indicate that, in cTAL, NO can increase COX-2 expression in cTAL and macula densa through p38-dependent signaling pathways via activation of NF-κB.

nitric oxide; cyclooxygenase-2; nuclear factor-κB; p38; transcriptional regulation

NITRIC OXIDE (NO) exhibits a broad spectrum of biological effects, including modulation of vascular tone, neurotransmission, hormone release, inflammation, and cell growth. In the kidney, NO is an important regulator of renal plasma flow, glomerular filtration rate, the renin-angiotensin system, and sodium excretion (4, 19). The three isoforms of nitric oxide synthase (NOS) have distinct localization patterns in the mammalian kidney. Type I NOS, the brain or neural isoform (nNOS), is highly expressed in the macula densa (3) and is thought to contribute to regulation of tubuloglomerular feedback and activation of renin production and release by the juxtaglomerular cells (41, 45). These effects may involve interactions between NO and other vasoconstrictors, such as ANG II and thromboxane (16, 37) and oxygen radicals and cyclooxygenase (COX)-2 (21).

In the kidney cortex, COX-2 is also localized to macula densa and adjacent cortical thick ascending limb (cTAL) (12, 20). Previous studies have shown that in vivo COX-2 expression in cTAL and macula densa increased in response to low salt (7, 20, 38, 40), and, in cultured cTAL and macula densa cells, COX-2 expression increased in response to decreased extracellular chloride (9, 47). Previous studies by our group indicated that administration of NO donors or cell-permeable cGMP analogs also stimulated COX-2 expression in cultured cTAL cells, and nNOS inhibitors decreased COX-2 expression in rats on a low-salt diet (11). The present studies examined whether common signal pathways are involved and whether these stimuli are necessarily integrated in regulating cortical COX-2 expression.

MATERIALS AND METHODS

Materials. The selective COX-2 inhibitor SC-58236, which exhibits its COX-2/COX-1 selectivity of 1,780-fold (32), was a gift from Searle Monsanto (St. Louis, MO). Rabbit anti-COX-2 antibody and PGE2, 6-keto-PGF1α, and thromboxane B2 (TXB2) EIA kits were from Cayman Chemical, rabbit anti-nNOS antibody was from Zymed Laboratories (San Francisco, CA), and monoclonal anti-pp38 and goat anti-β-actin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). The enhanced chemiluminescence (ECL) kit and ECL Hyperfilm were from Amersham (Arlington Heights, IL). The BCA protein assay reagent kit, immunopure ABC peroxidase staining kit, and biotin-labeled mouse anti-rabbit or rabbit anti-goat IgG (H + L) antibodies were from Pierce (Rockford, IL). 7-Nitroindazole (7-Ni) was from Calbiochem (La Jolla, CA). Other reagents were purchased from Sigma Chemical (St. Louis, MO).

Primary culture of rabbit cTAL cells. cTAL cells were isolated from homogenates of rabbit renal cortex by immunodissection with anti-Tamm Horsfall (TH) antibody, as previously described (1, 11, 13). Briefly, the renal cortex was dissected, minced, and digested with 0.1% collagenase. After being blocked with 10% BSA, the sieved homogenates were incubated with goat anti-human TH (1:500) and rabbit anti-COX-2 antibody (1:50) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG (1:1000). Attached cells resistant to washing were dislodged and grown to confluence in DMEM-F-12 with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 95% air-5% CO2.

Transfection and luciferase reporter assay. Primary cultured cTLAH cells at 50–60% confluence were transiently transfected with lipofectamine reagent (GIBCO-BRL) according to instructions.
Briefly, after changing to serum-free culture medium, a DNA-lipofectamine mixture (3 μg DNA and 10 μl lipofectamine reagent in 150 μl DMEM-F-12) was added to the culture medium and allowed to react with cells at 37°C, 5% CO2. A pcMV-β-gal plasmid (1 μg) encoding β-galactosidase regulated by the CMV promoter was co-transfected with the pGAL constructs as a control for transfection efficiency. After transfection with lipofectamine for 6–8 h, DMEM-F-12 containing 20% FBS was added back to the cell culture. The mouse COX-2–S185 luciferase reporter construct was a generous gift from Dr. K. Kamamoto (46), and we have previously reported that it mediates similar activity as the full-length promoter (9). As previously described (9), the nuclear factor (NF)-κB mutant represents a point mutation from GA to CC at the NF-κB binding site at -410 in the COX-2–S185 luciferase reporter construct. When cells grew to confluence (48 h later), they were made quiescent with serum-free medium for 16 h and then changed to the indicated condition for 6 h. The cells were extracted as previously described (49). Briefly, cells were homogenized with a Dounce homogenizer in buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10% glycerol, and 0.1% leupeptin with [32P]ATP by T4 polynucleotide kinase. Nuclear protein (5 μg) was added to the reaction mixture, incubated for 30 min at 25°C, and resolved on 6% nondenatured polyacrylamide gels.

**Immunohistochemistry.** Under deep anesthesia with Nembulator (70 mg/kg ip), mice were exsanguinated with 50 ml/100 g heparinized saline (0.9% NaCl, 2 U/ml heparin, 0.02% sodium nitrite) through a transcardial aortic cannula and fixed with glutaraldehyde-peroxide acid saline (GPAS) for COX-2 staining as previously described. GPAS contains final concentrations of 2.5% glutaraldehyde, 0.011 M sodium metaperiodate, 0.04 M sodium phosphate, 1% acetic acid, and 0.1 M NaCl and provides excellent preservation of tissue structure and antigenicity. Antigen was retrieved in 0.01 M citrate buffer, pH 6.0, by microwave for 2 min.

COX-2 immunoreactivity was localized with COX-2 antiserum diluted to 2.5 ng/ml; nNOS immunoreactivity was with anti-nNOS antibody (5 μg/ml). The first antibody was localized using Vectastain ABC-Elite (Vector, Burlingame, CA) with diaminobenzidine as the chromogen, followed by a light counterstain with toluidine blue. The fixed kidneys were dehydrated through a graded series of ethanol, embedded in paraffin, sectioned at 4 μm thickness, and mounted on glass slides.

**Statistical analysis.** All values are presented as means ± SE. ANOVA and Bonferroni t-tests were used for statistical analysis, and differences were considered significant when P < 0.05.

**RESULTS**

The NO donor, S-nitros-N-acetylpenicillamine, stimulated COX-2 expression and prostaglandin production in cTAL cells. Cultured cTAL cells that were incubated with S-nitros-N-acetylpenicillamine (SNAP, 10⁻⁴ M) for 16 h increased expression of immunoreactive COX-2 (2.5 ± 0.3-fold control, n = 6, P < 0.01; Fig. 1). After addition of external arachidonic acid (10⁻⁵ M), SNAP increased PGE₂ (from 39.3 ± 11.3 to 200.5 ± 36.8 ng/mg protein, n = 4, P < 0.05; Fig. 2A) and 6-keto-PGF₁α (from 20.9 ± 3.0 to 100.4 ± 31.6 ng/mg protein, n = 4, P < 0.05; Fig. 2B) production but did not change TxB₂ [0.9 ± 0.2 vs. 0.9 ± 0.3 ng/mg protein, n = 4, not significant (NS); Fig. 2C].

The p38 mitogen-activated protein kinase pathway regulated NO-mediated increases in COX-2 expression. To explore the effect of p38 in signaling of COX-2 by NO, a specific p38 inhibitor, SB-203580 (10⁻⁵ M), was added to the medium of cTAL cells during coincubation with SNAP. Increases in COX-2 expression were significantly blunted (from 2.5 ± 0.3- to 1.4 ± 0.2-fold control, n = 6, NS vs. control; Fig. 1). The increase in PGE₂ production was also prevented by SB-203580 (to 37.4 ± 13.5 ng/mg protein, n = 4, NS vs. control; Fig. 2, A and B). Expression of the active form of p38, phosphorylated p38 (pp38), was increased in cTAL cells by SNAP (2.4 ± 0.3-fold control; n = 5; P < 0.01; Fig. 3).

NO-induced transcriptional regulation of COX-2 was mediated by p38-dependent NF-κB activation. Our previous studies indicated that decreased extracellular chloride induced COX-2 upregulation in cTAL at the transcriptional level via NF-κB (9). To investigate if the regulation of COX-2 by NO was also mediated by NF-κB, cultured cTAL cells were transiently transfected with a luciferase reporter construct driven by the proximal 815 bp of wild-type murine COX-2 promoter, which we had previously determined to mediate similar activity as the full-length promoter (9). Compared with vector-transfected
cells, basal COX-2 promoter-mediated luciferase activity was not elevated significantly; in contrast, SNAP significantly stimulated luciferase activity, which was reversed by SB-203580 (1.4 ± 0.2-, 8.3 ± 1.4-, and 0.4 ± 0.1-fold control, respectively, \( n = 4 \), \( P < 0.01 \)). SNAP did not stimulate luciferase activity in the NF-κB mutant COX-2 promoter construct (0.9 ± 0.1, 1.2 ± 0.1, and 1.0 ± 0.2, respectively, \( n = 4 \), NS; Fig. 4). EMSA indicated that SNAP stimulated NF-κB binding activity in cTAL (≈2 times the basal level by densitometric measurement), and this stimulation was inhibited by the p38 inhibitor (Fig. 5). Our previous supershift EMSA had indicated that the two bands represented p65 and p50 (9).

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Previously, we have demonstrated that low-chloride-induced increases in COX-2 expression from cultured cTAL cells (12). In cultured cTAL cells, basal COX-2 promoter-mediated luciferase activity was not elevated significantly; in contrast, SNAP significantly stimulated luciferase activity, which was reversed by SB-203580 (1.4 ± 0.2-, 8.3 ± 1.4-, and 0.4 ± 0.1-fold control, respectively, \( n = 4 \), \( P < 0.01 \)). SNAP did not stimulate luciferase activity in the NF-κB mutant COX-2 promoter construct (0.9 ± 0.1, 1.2 ± 0.1, and 1.0 ± 0.2, respectively, \( n = 4 \), NS; Fig. 4). EMSA indicated that SNAP stimulated NF-κB binding activity in cTAL (≈2 times the basal level by densitometric measurement), and this stimulation was inhibited by the p38 inhibitor (Fig. 5). Our previous supershift EMSA had indicated that the two bands represented p65 and p50 (9).

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cells, basal NO was found to be 3.2 ± 0.5 μM, whereas in cells exposed to the low-chloride medium for 16 h, there was a significant stimulation of NO production (7.8 ± 0.2 μM, n = 4, P < 0.01). We next examined whether inhibition of NOS would alter increased COX-2 expression in cultured cTAL in response to decreased extracellular chloride concentrations. The specific nNOS inhibitor 7-Ni (10⁻⁵ M) significantly decreased low-chloride-induced pp38 expression (2.2 ± 0.3- vs. 1.4 ± 0.2-fold control, n = 6, P < 0.05).

Increases in renal COX-2 by low salt were attenuated in nNOS−/− mice. We further investigated the role of nNOS in macula densa/cTAL COX-2 expression in vivo. Administration of a low-salt diet for 3 wk increased immunoreactive COX-2 in the kidneys of wild-type mice (3.3 ± 0.5-fold control, n = 6, P < 0.05), but not nNOS−/− mice (normal salt vs. low salt: 1.2 ± 0.2- vs. 1.5 ± 0.4-fold wild-type control, n = 6, NS). Immunohistochemical analysis confirmed that the increases in renal cortical COX-2 expression were confined to macula densa/cTAL. In contrast, renal immunoreactive nNOS expression was similar in COX-2−/− mice compared with the wild type (1.3 ± 0.2-fold, NS) and was comparably upregulated by salt restriction (wild-type vs. COX-2−/−: 2.9 ± 0.3- and 2.8 ± 0.3-fold of respective controls, n = 6, P < 0.05).

Fig. 3. SNAP stimulated COX-2 transcriptional regulation mediated by p38 via nuclear factor (NF)-κB. A: NF-κB mutation prevented the stimulation of SNAP on COX-2 promoter. Cultured cTAL cells were transiently transfected with vector (as control), a luciferase reporter construct driven by the wild-type COX-2 promoter, or the correspondent NF-κB mutant COX-2 promoter construct. Transfection, SNAP stimulation, and luciferase activity measurement were described in MATERIALS AND METHODS (n = 4 experiments). **P < 0.01. B: SNAP increased NF-κB binding in cTAL cells. After incubation as in Fig. 1, nuclear protein was extracted, and NF-κB binding activity was measured by electrophoretic mobility shift assay (representative of 3 separate experiments).

Fig. 4. The neuronal nitric oxide synthase (nNOS) inhibitor 7-nitroindazole (7-Ni) inhibited upregulation of COX-2 (A) and pp38 (B) expression in cultured cTAL by decreased extracellular chloride concentration (n = 6 experiments). *P < 0.05.
DISCUSSION

Our results indicated that the NO donor SNAP stimulates COX-2 expression and activity in primary cultured cTAL cells through a p38 MAP kinase-dependent pathway, mediated by NF-κB-dependent transcriptional regulation. In addition, the activation of p38 and upregulation of COX-2 induced by decreased extracellular chloride (9) were also significantly inhibited by NOS inhibition. In vivo studies demonstrated that increased renal cortical COX-2 seen in wild-type mice was blunted in nNOS null mice, further supporting a role for NO in COX-2 regulation in macula densa and cTAL.

Because of the colocalization of nNOS and COX-2 in renal cortex and the parallel increase of renin, COX-2, and nNOS gene expression in response to salt restriction, a number of previous studies have investigated their potential interactions (27, 31, 34, 39, 42). In cultured cTAL cells and macula densa cells, the same stimulus for macula densa mediation of increased renin secretion, namely low extracellular chloride, also stimulated COX-2 expression (12, 47). The induced nNOS was predominantly shown in macula densa by immunostain (Fig. 5), although the immunoreactive nNOS was detectable (11) and could be stimulated by low-chloride medium in cultured cTAL cells. Recently, Paliege et al. (31) reported that absence of either COX-2 or nNOS is associated with suppressed renin secretion, and COX-2-derived PGE2 inhibited nNOS expression.

Consistent with our previous studies (11), the current studies indicated that, in cultured cTAL, the NO donor SNAP stimulated COX-2 expression and production of vasodilatory PGs, and the nNOS specific inhibitor 7-Ni blocked increased COX-2 expression by low extracellular chloride, suggesting that NO may modulate COX-2 expression in these cells. This result was further confirmed by in vivo studies with nNOS knockout mice. However, previous immunohistochemical studies in nNOS knockout mice by Theilig et al. (40) did not confirm a requirement for active nNOS to detect stimulation of COX-2 expression by short- and long-term unilateral renal artery stenosis, low salt, or furosemide. Although we do not know the underlying reasons for the discrepancy, we note that mice were subjected to a longer period of salt restriction in our studies than in the previously reported studies of Theilig et al., although 1 or 2 wk of salt restriction have shown the effect to induce COX-2 expression. Furthermore, immunohistochemical studies are very helpful for the localization of antigen expression but may have certain limitations for quantitation, especially when the number of immunoreactive-positive cells is the only indicator without additional information concerning the relative level of expression (density) within a positive cell. In
our studies, immunoreactive COX-2 was measured by quantitative immunoblot analysis in addition to immunohistochemical analysis. With these complementary approaches, we failed to detect significant upregulation of cortical COX-2 expression by salt restriction in nNOS null mice.

There are multiple potential mechanisms by which NO may mediate activity and expression of COX-2-derived metabolites. It has been suggested that peroxynitrite produced by the reaction of NO with superoxide can initiate lipid peroxidation, thereby releasing arachidonic acid from the cell membrane and potentially increasing COX activity (15, 24). Additionally, NO may directly react with, and thereby remove, free radicals that can inactivate COX-2 (18, 36) or bind to the heme-Fe2⁺ group of COX-2, thus directly activating the enzyme (35, 43).

Previous studies have indicated parallel regulation of COX-2 and nNOS proteins in response to administration of diets of varying salt content (29, 42). Our previous studies demonstrated that decreased extracellular salt/chloride upregulated COX-2 expression in cTAL cells through a p38 MAP kinase pathway via NF-κB activation, which was correlated with increased inhibitory factor κB phosphorylation (9). The current results suggested that, in cultured cTAL, NO stimulates COX-2 expression by a similar signaling mechanism. NO has previously been shown in other cell types to activate p38 (26, 33), and, in the present studies, we determined that the NO donor SNAP could activate p38.

The mechanisms by which NO or low intracellular chloride activates p38 were not elucidated in the current studies. Because cell-permeable cGMP analogs stimulate COX-2 expression in cultured cTAL (11), one possible mechanism by which NO might increase COX-2 expression is by decreasing intracellular chloride concentrations by cGMP-dependent inhibition of the Na⁺-K⁺-Cl⁻ cotransporter (29, 30). NO may activate p38 MAP kinase (6) and increase NF-κB (p50/p65) DNA binding activity (22) in a cGMP-dependent fashion. Alternatively, low chloride could directly increase NO production, which then directly activates p38. There was a partial but significant inhibition of low-chloride-induced p38 activation and COX-2 expression in response to nNOS-specific NOS inhibitors. Further studies will be required to determine the mechanisms that underlie this effect.

Both NO and PGs are modulators of vascular tone, cell growth, inflammation, hormone release, renal hemodynamics, renin production, and tubuloglomerular feedback, and previous studies have suggested that prostanooids derived from COX-2 may serve as mediators or modulators of at least some of the biological actions of nNOS (5, 23, 28, 44). Our in vivo and in vitro studies indicating that inhibition of nNOS significantly inhibits increases in cTAL/macula densa COX-2 expression in response to low salt implicates macula densa-derived NO as a modulator of COX-2 expression in these cells.

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