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Nitric Oxide Synthase/COX Cross-Talk: Nitric Oxide Activates COX-1 But Inhibits COX-2-Derived Prostaglandin Production

Robert Clancy, Branko Varenika, Weiqing Huang, Les Ballou, Mukundan Attur, Ashok R. Amin, and Steven B. Abramson

It is recognized that there is molecular cross-talk between the inflammatory mediators NO and PGs that may regulate tissue homeostasis and contribute to pathophysiological processes. However, the literature is divided with respect to whether NO activates or inhibits PG production. In this study, we sought to determine whether conflicting observations could be accounted for by divergent effects of NO on the two cyclooxygenase (COX) isoforms. Exposure of resting macrophages to NO (30 μM) enhanced PGE2 release by 4.5-fold. This enhancement was inhibited by indomethacin but not by the COX-2 selective inhibitor NS398. To separate the activation of phospholipase A2 and COX, we performed experiments using fibroblasts derived from COX-1-deficient or COX-2-deficient mice. These cells exhibited increased basal PG production, which is due to a constitutively stimulated cytosolic phospholipase A2 and enhanced basal expression of the remaining COX isozyme. The exposure of COX-2-deficient cells to exogenous NO (10 μM) resulted in a 2.4-fold increase of PGE2 release above controls. Further studies indicated that NO stimulated PGE2 release in COX-2-deficient cells, without altering COX-1 mRNA or protein expression. In contrast, NO inhibited COX-2-derived PGE2 production in both LPS-stimulated macrophages and COX-1 knockout cells. This inhibition was associated with both decreased expression and nitration of COX-2. Thus, these studies demonstrate divergent effects of NO on the COX isoforms. The regulation of PGE production by NO is therefore complex and will depend on the local environment in which these pleiotropic mediators are produced.

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Nitric oxide synthase (NOS) and cyclooxygenase (COX) produce important mediators of tissue homeostasis and pathophysiological processes. The regulation of these enzymes and their capacity to participate in molecular cross-talk is an important focus of investigation. Both NOS and COX have constitutive and inducible isoforms which are encoded by two unique genes, located on different chromosomes (1, 2). COX-2 is the inducible form of the COX enzyme, the synthesis of which is triggered by those cytokines that also induce NOS-2, or inducible NOS (reviewed in Refs. 3 and 4). COX-1 is the constitutive isoform that is expressed by most human cells and tissues (5). COX-1 and COX-2 are 60% identical within a species (6) with the conservation of a tyrosine (Tyr385) located in the active site. Tyr385 contributes to enzyme activity; in addition, the tyrosyl radical species is involved in suicide inactivation (7). In cell-free systems, NO has been reported to trap the tyrosyl radical of the COXs, which leads to tyrosine iminoxyl radical formation and inactivation of the enzyme (8, 9).

The PG-biosynthetic pathway is initiated by activation of phospholipase A2 (PLA2), which are primarily responsible for agonist-induced arachidonic acid release from membrane phospholipids (10). Conversion of arachidonic acid to PGH2, the committed step in prostanoioid biosynthesis, is mediated by both COX-1 and COX-2. The PGH2 is subsequently converted to a variety of eicosanoids depending on the downstream enzymatic machinery present in a particular cell type. The COX enzymes are thought to be the primary target enzymes for nonsteroidal antiinflammatory drugs, which block their ability to convert arachidonic acid to PGH2 (11).

Although it is recognized that there is “cross-talk” between products of the NOS and COX pathways, the literature is divided with respect to whether NO activates or inhibits PG production. For example, nitroglycerin (NO surrogate) is reported to inhibit platelet activation in vivo, which occurs via the stimulation of PG synthesis by endothelial cells (12). Salvemini and Masferrer (13) reported that NO stimulates COX activity in RAW 264.7 murine macrophages, possibly via reaction with the heme component which binds to the active site of the COX enzyme. In contrast, we and others have reported that endogenous NO inhibits PG synthesis in chondrocytes and LPS-stimulated macrophages (14, 15). In this study, we sought to determine whether these conflicting observations could be accounted for by divergent effects of NO on the two COX isoforms. In addition, the capacity of NO to promote NO modifications, such as tyrosine nitration, was investigated. Our studies indicate that NO exerts divergent effects on the constitutive and inducible COX isoforms, activating COX-1 but inactivating COX-2. Mechanisms by which NO exerts these effects on COX are explored.

Materials and Methods

Culture of murine macrophages

Mouse monocyte/macrophage cell line J774.A1 (ATCC TIB 67) was cultured in DMEM medium plus 10% FBS, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. The cells were activated by IFN-γ (100 U/ml) and LPS (5 μg/ml) in the presence and absence of N-t.-methylarginine.
Culture of COX-deficient pulmonary fibroblasts

We utilized COX-1−/− (COX-1) or COX-2−/− (COX-2) lung fibroblasts, which express an immortalized phenotype and a hygromycin resistance gene as described by Kirtikara et al. (16). Cells were seeded in DMEM medium plus 10% FBS, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 250 μg/ml hygromycin.

Viability measurements

Cell viability was determined using a lactic dehydrogenase kit (Sigma, St. Louis, MO), and measurements were determined following the recommendations of the manufacturer. Results are expressed as a percentage compared with values obtained after treatment with PBS plus 0.1% Triton X-100.

RNA extraction

RNA was extracted using a Promega kit (Promega, Madison, WI). Northern blots used a hybridization probe prepared from full length cDNA for COX-1, and the procedure was performed as previously described (23).

NO treatment

NO solutions were prepared by dissolving diethylamine (DEA)/NO in 10 mM NaOH. NO was quantitated by measuring the absorbance at 250 nm as described by the manufacturer.

PGE2 measurement

In the macrophage studies, PGE2 biosynthesis was measured in cell fluid recovered after treatment by NO or LPS (24 h). In studies using COX-1/COX-2 cells, NO treatment was varied. The treatment medium was then removed, and cells were washed twice with fresh medium. Cells were placed in fresh medium, and the interval to harvest PGE2 release was 10 or 3 min (as described in text). PGE2 in the medium was measured by ELISA using a commercial kit (Cayman Chemicals, Ann Arbor, MI) following the instructions of the manufacturer.

Western blot analysis

Laemmli buffer was directly added to the cells at the termination of the reaction. Proteins were separated by SDS-PAGE (10%), and proteins were transferred to nitrocellulose and analyzed using rabbit anti-nitrotyrosine (Upstate Biotechnology, Lake Placid, NY), rabbit anti-COX-2 (Transduction Laboratories, Lexington, KY) rabbit anti-actin (Sigma), rabbit anti-GAPDH (Sigma), rabbit anti-glucose 6 phosphate dehydrogenase (gift from Dr. E. Beutler), and rabbit anti-cytosolic PLA2 (Upstate Biotechnology). Images were evaluated after scanning of autoradiographs using the storage phosphor technique performed with the Molecular Dynamics 400A PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Two-dimensional gel electrophoresis and trypsin digestion

Two-dimensional electrophoresis was performed as previously described (17–19). Detection of anti-COX-2 was by ECL, and then the same blot was reprobed with anti-nitrotyrosine which was reported by an alkaline phosphatase method. Lysate (20 mg/ml) was treated with bead-conjugated trypsin (Pierce, Rockford, IL, Immobilized tosylphenylchloromethyl ketone trypsin, 1 volume sample to 1 volume resin) at 4°C or 37°C (10 min). The beads were removed at 10,000 × g (1 min), and SDS sample buffer was added to the supernatant fraction. Proteins were separated by SDS-PAGE (10%), and proteins were transferred to nitrocellulose and analyzed using rabbit anti-nitrotyrosine, rabbit anti-COX-2 (Transduction), or rabbit anti-actin.

Data variability

Data were analyzed as the mean and the SEM. The levels of significance were calculated using Student’s t test.

Results

NO increases PGE2 production in resting murine macrophages

Exposure of resting macrophages to NO enhanced PGE2 release (Fig. 1). The enhancement of PGE2 production was inhibited by indomethacin (nonselective COX inhibitor) but not by the COX-2 selective inhibitor NS398. The dose-response relationship between NO and PGE2 production by macrophages was obtained by combining the data from four separate experiments. The amount of PGE2 produced was significantly greater than control (p < 0.01) at NO concentrations of 30 μM and above. NO-dependent PGE2 release by unstimulated macrophages was not due to increase in expression of PLA2 or COX-1 as assessed by Western blot analysis (Fig. 1). These findings suggest that activation of PLA2 or COX-1 is necessary for NO-dependent PGE2 production. To further separate the activation of PLA2 and COX-1, we performed experiments using immortalized, nontransformed cells derived from COX-1-deficient or COX-2-deficient mice (16). These cells exhibit
increased basal PG production, which is due to a constitutively stimulated PLA₂ and enhanced basal expression of the remaining COX isozyme.

NO increases PGE₂ production in COX-2-deficient (but not COX-1-deficient) cells

The above studies, which showed enhancement of PGE₂ production in unstimulated macrophages (not inhibited by NS-398), indicated that NO activates COX-1-dependent PGE₂ production. We next examined the effect of exogenous NO on PGE₂ release by COX-1 and COX-2-deficient cells. As shown in Fig. 2, treatment of COX-2-deficient cells with as low as 1 μM NO increased PGE₂ release to 155% control and NO stimulated PGE₂ synthesis in a dose-dependent manner. As expected, in COX-2-deficient cells, the NO-stimulated PGE₂ release was inhibited by indomethacin but not by the COX-2 selective agent NS-398. Exposure of COX-2-deficient cells to NO (1 μM) in the presence of exogenously added arachidonic acid (10 μM) also enhanced PGE₂ release by 180% (not shown). In contrast, and consistent with the conclusions drawn from studies of macrophages, exogenous NO failed to stimulate PGE₂ release in COX-1-deficient cells; NO treatment, in fact, inhibited PGE₂ release from these cells (Fig. 2).

Further studies were undertaken to define the manner in which NO stimulates PGE₂ production. COX-2-deficient cells were incubated with 10 μM NO for 20 and 60 min and protein and RNA were extracted to examine COX-1 expression. As shown in Fig. 2, NO treatment did not affect COX-1 expression of protein or mRNA. We next examined the kinetics of NO-dependent PGE₂ production in COX-2-deficient cells (Fig. 3). Exposure of COX-2-deficient cells to 10 μM NO resulted in a rapid PGE₂ release detectable at 1 min. After exposure to NO, PGE₂ release reached its peak at 10 min and returned to baseline by 60 min. NO treatment at 100 μM resulted in a similar profile with a more gradual decrease to baseline. A23187 exposure resulted in a rapid increase in PGE₂ release that was first measurable at 1 min and that, unlike the response to NO, was sustained during the 60-min observation period. Taken together, these results suggest that NO stimulates PGE₂ release due to COX-1 activation and not by altering COX-1 expression.

NO inhibits PGE₂ biosynthesis by COX-1-deficient cells or by LPS-stimulated macrophages in association with decreased COX-2 expression

Additional experiments were performed to further explore the effects of NO on COX-2 activity and expression. As shown in Fig. 4, the exposure of COX-1-deficient cells to exogenous NO for 1 h decreased basal PGE₂ release by >70%. In addition, we observed a 50% decrease in COX-2 protein expression compared with controls at 1 h. NO treatment did not affect cellular viability, nor did it reduce the expression of actin and GAPDH (control protein not shown). Similarly, in LPS-stimulated macrophages, NO exposure resulted in a decrease in PGE₂ release and total COX-2 protein (at 24 h, Western blot (Fig. 4)). In experiments using varying doses of NO, we observed a correlation between PGE₂ release and COX-2 expression (not shown).

NO inhibits PGE₂ biosynthesis by COX-1-deficient cells or by LPS-stimulated macrophages in association with COX-2 nitration

The inhibition of PGE₂ production in LPS-treated macrophages was accompanied by the formation of a 72-kDa nitrated protein (nitrotyrosine immunoblot (Fig. 5)). Coincubation of 3-nitrotyrosine (10 mM) prevented Ab binding to the 72-kDa protein (Western blot assay, not shown), confirming recognition by the Ab of a nitrated protein. To assess whether the 72-kDa protein could be identified as COX-2, we utilized two-dimensional gel electrophoresis and trypsin digestion. In lysates of LPS-stimulated macrophages exposed to 30 μM NO, the nitrated protein was separated by isoelectric focusing and by SDS-PAGE followed by transfer to nitrocellulose. As shown (Fig. 6), autoradiographic analysis revealed that COX-2 eluted at 72 kDa with an apparent pl of 7.5. The same blot was reanalyzed by Western blot using a rabbit anti-nitrotyrosine Ab. The analysis revealed that one of two nitrated proteins migrated with an identical molecular mass and apparent pl as COX-2. COX-2 is trypsin sensitive, and we next focused on the capacity of trypsin to digest the 72-kDa nitrated protein. The 72-kDa nitrated protein digestion was separated by SDS gels, transferred to nitrocellulose, and sequentially probed with anti-COX-2 and anti-nitrotyrosine. Treatment resulted in a loss of both immunodetectable COX-2 and the nitrated 72-kDa protein (Fig. 6); trypsin digestion did not affect G6PDH examined as a control (not shown).
Thus, these findings indicate that NO induces the nitration of COX-2 in LPS-stimulated macrophages. As has been shown in cell-free systems by Gunther et al. (9), it is likely that such nitration inhibits the catalytic activity of the enzyme, as those authors have reported (8, 9). Whether COX-2 nitration decreases the stability of the enzyme and accounts for decreased COX-2 expression in NO-treated cells remains to be determined.

As shown in Fig. 5, exposure of resting macrophages to NO did not lead to protein nitration. We next examined the effect of NO on nitration of protein in COX-1-deficient cells. The exposure of these cells to NO lead to the formation of a 72-kDa nitrated protein (Fig. 5). In contrast to observations made with COX-1-deficient cells, NO exposure did not lead to the formation of a 72-kDa nitrated protein in COX-2-deficient cells (Fig. 5). These data indicate that COX-2 (but not COX-1) undergoes protein nitration after exposure to NO. The percentage of the protein that is nitrated is not known; thus, it is impossible to equate nitration with enzyme inhibition or instability of COX-2 expression.

Discussion

In this study, the effect of NO on PG biosynthesis by murine macrophages and COX-1 or -2-deficient murine fibroblasts was examined. We found that exposure of resting macrophages to NO enhanced PGE2 release. This enhancement was inhibited by indomethacin (nonselective COX inhibitor) but not by the COX-2 selective inhibitor NS398. In contrast, exposure of LPS-stimulated macrophages to NO inhibited PGE2 release. To test whether these divergent effects of NO depend on the COX isoform, we studied PGE2 production in immortalized, nontransformed cells derived from COX-1-deficient or COX-2-deficient mice. These cells release PGE2 due to increased basal expression of the remaining
our studies demonstrate that the capacity of NO to inhibit PGE₂ but inhibited COX-2-derived PG production. In addition, using COX-deficient cell lines, we confirmed that NO activated COX-1 by NO could be linked to the effect of NO on glutathione as suggested by Goodwin et al. (28).

FIGURE 5. COX-2 undergoes protein nitration after exposure to NO. A, control macrophages (as in Fig. 1) and LPS-stimulated macrophages (as in Fig. 4) were incubated with or without NO (NO varied, 1 h). B, COX-1-deficient cells were exposed to NO (1 mM, time varied). After treatments, proteins were separated on SDS-PAGE, transferred to nitrocellulose, and probed with an anti-nitrotyrosine Ab. As shown, a 72-kDa nitrated protein was detected in LPS-stimulated and COX-1-deficient cells, but not in controlled macrophages or COX-2-deficient cells.

COX isozyme as well as the elevated expression of PLA₂ (16). Using COX-deficient cell lines, we confirmed that NO activated COX-1 but inhibited COX-2-derived PG production. In addition, our studies demonstrate that the capacity of NO to inhibit PGE₂ release is associated with a decrease in COX-2 expression as well as nitrination of the enzyme. The NO dependent decrease in COX-2 expression has been previously reported and may be, in part, due to the attenuation of cytokine elicited transcription factors which regulate COX-2 expression such as NF κ B (4, 15, 20–22). Recently, we have reported that NO also inhibits the translocation of COX-2 to a cytosolic compartment which favors enzyme activity (23).

As noted, our studies indicate that the inhibition of PGE synthesis by NO is accompanied by nitration of COX-2. Although it has been previously demonstrated that NO converts tyrosine to nitrotyrosine in cell-free cyclooxygenases (8, 9), this is the first report to show that the reaction occurs in cells and that COX-2 is more sensitive to nitration than COX-1. In these studies, we found that micromolar NO stimulated COX-2 nitration in LPS-stimulated macrophages and in COX-1-deficient cells. COX-2 nitration has been shown to inhibit the catalytic activity of the enzyme (8, 9). Whether nitration also decreases COX-2 protein stability remains to be determined.

In contrast to these effects of NO on COX-2, we did not find evidence for nitration of COX-1. However, the activation of COX-1 by NO may occur via an allosteric effect subsequent to 3-nitrosylation that stimulates enzymatic activity as reported in a cell-free system by Hajjar et al. (24). Alternatively, the activation of COX-1 by NO could be linked to the effect of NO on glutathione metabolism. Our laboratory has demonstrated that neutrophils exposed to NO convert intracellular glutathione to a nitrosylated adduct (25). This has been termed “nitrosative stress” and had been implicated in the activation of signaling proteins such as p21ras and the transcription factor OxyR (26, 27). NO may therefore serve as a potent peroxide activator of COX-1 due to the depletion of reduced glutathione as suggested by Goodwin et al. (28).

In summary, NO activates COX-1 but inhibits COX-2-derived PG production. Because a variety of cells (e.g., endothelium, macrophages, chondrocytes) produce NO and PGs simultaneously in response to cytokines and other activators (3, 29), we speculate that enhanced PG biosynthesis by COX-1 in the presence of elevated levels of NO may contribute to inflammatory mitogenic and angiogenic processes. The studies here reported help resolve a lingering controversy in the literature regarding NOS/COX cross-talk by demonstrating divergent effects of NO on the COX isozymes. The regulation of PGE production by NO is therefore complex and will depend on the local environment in which these pleiotropic mediators are produced.

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References


