Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 7240–7244, August 1993
Pharmacology

Nitric oxide activates cyclooxygenase enzymes

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Contributed by Philip Needleman, May 12, 1993

ABSTRACT We have evaluated the role of nitric oxide (NO) on the activity of the constitutive and induced forms of cyclooxygenase (COX; COX-1 and COX-2, respectively). Induction of NO synthase (NOS) and COX (COX-2) in the mouse macrophage cell line RAW264.7 by *Escherichia coli* lipopolysaccharide (1 μg/ml, 18 h) caused an increase in the release of nitrite (NO_2^-) and prostaglandin E_2 (PGE_2), products of NOS and COX, respectively. Production of both NO_2^- and PGE_2 was blocked by the NOS inhibitors N^G-monomethyl-L-arginine or aminoguanidine. The effects of N^G-monomethyl-L-arginine or aminoguanidine were reversed by coincubation with L-Arg, the precursor for NO synthesis, but not by D-Arg. RAW264.7 cells stimulated for 18 h with lipopolysaccharide in L-Arg-free medium (to reduce NO generation by the endogenous NOS pathway) failed to release NO_2^- and accumulated at least 4-fold less PGE_2 when compared to cells in the presence of L-Arg. PGE_2 production elicited by a 15-min arachidonic acid treatment of lipopolysaccharide-induced RAW264.7 cells in L-Arg-deficient medium was decreased 3-fold when compared to the release obtained with cells induced in medium containing L-Arg. To examine the NO activation of the induced form of COX in the absence of an endogenous L-Arg, human fetal fibroblasts were first stimulated for 18 h with interleukin 1β. These cells released PGE_2 but not NO_2^-, consistent with the induction of COX but not NOS in the fibroblast. Exogenous NO either as a gaseous solution or released by a NO donor, sodium nitroprusside or glyceryl trinitrate, increased COX activity in the interleukin 1β-stimulated fibroblasts by 5-fold; these effects were abolished by coincubation with hemoglobin (10 μM), which binds and inactivates NO, but not by methylene blue, an inhibitor of the soluble guanylate cyclase. Furthermore, sodium nitroprusside (0.25–1 mM) increased arachidonic acid-stimulated PGE_2 production by murine recombinant COX-1 and COX-2. These results demonstrate that NO enhances COX activity through a mechanism independent of cGMP and suggest that, in conditions in which both the NOS and COX systems are present, there is an NO-mediated increase in the production of proinflammatory prostaglandins that may result in an exacerbated inflammatory response. The data suggest that NO directly interacts with COX to cause an increase in the enzymatic activity.

Nitric oxide (NO) is formed from the terminal guanidino nitrogen atom of L-Arg; this reaction is catalyzed by the enzyme NO synthase (NOS; for review, see ref. 1). Two major forms of NOS have been identified to date. The constitutive calcium-dependent NOS isoform is present in some cells of the body, notably the endothelium and some neurons. The inducible calcium-independent NOS isoform is expressed after stimulation with *Escherichia coli* lipopolysaccharide (LPS), interleukin 1β (IL-1β), or tumor necrosis factor α in endothelial cells, smooth muscle cells, and macrophages (1). Release of NO is blocked by several analogues of L-Arg including N^G-monomethyl-L-arginine (L-NMMA; ref. 1) and the more recently described inducible NOS selective inhibitor aminoguanidine (AG; refs. 2 and 3).

Production of NO from constitutive NOS is a key regulator of homeostasis, whereas the generation of NO by inducible NOS plays an important role in the host–defense response (1). NOS shares a number of similarities with cyclooxygenase (COX). COX is the rate-limiting enzyme in the biosynthesis of prostaglandins (PGs), thromboxane A_2, and prostacyclin (PGI_2). In addition to the well-characterized constitutive form of COX (COX-1) (4), an inducible isoform of COX (COX-2) is found in endothelial cells (5), fibroblasts (6), and macrophages (7–9) after treatment with proinflammatory agents including LPS and IL-1β. Antiinflammatory steroids such as dexamethasone inhibit the induction of inducible NOS *in vitro* and *in vivo* but have no effect on the expression of constitutive NOS (1). In addition, dexamethasone inhibits IL-1β- and LPS-stimulated COX-2 protein synthesis *in vitro* (7, 8) and *in vivo* (8, 9) but has no effect on the constitutive form of COX.

Many effectors of NO production lead to the simultaneous release of mediators (such as PGE_2 and PGI_2) from the COX pathway. This is true for the rapidly acting agonists such as bradykinin (10–12) and for the longer acting agents such as LPS or IL-1β (13, 14). NO, PGI_2, or PGE_2 increase the levels of cGMP or cAMP in effector cells (e.g., platelets). This synergistic effect may be one mechanism(s) through which the NOS and COX systems operate to amplify a physiological or pathological response.

Another possible interaction is at the level of the enzyme. In this respect, the COX enzymes are potential targets for NO because they contain an iron–heme center at their active site (15–17), and indeed, the vast majority of effects mediated by NO are a consequence of its interaction with iron or iron-containing enzymes. For example, the ability of NO to inhibit platelet aggregation and to relax vascular smooth muscle is the result of NO binding to the heme–Fe^{2+} prosthetic group of the soluble guanylate cyclase leading to its stimulation and subsequent increase in the levels of cGMP (18, 19). In the same way, NO interacts with hemoglobin (Hb) (20) or can exert its cytotoxic effects by interacting with iron–sulfur centers in key enzymes of the respiratory cycle and DNA synthesis (for review, see ref. 21). Thus, these observations raise the possibility that NO modulates the activity of COX.

MATERIALS AND METHODS

Materials. The mouse macrophage cell line RAW264.7 was obtained from the American Type Culture Collection. Human fetal fibroblasts (HFFs) were prepared from foreskin and cultured as described (22, 23). IL-1β was purified at Monsanto. Glyceryl trinitrate (GTN) was obtained from SoloPak Labo-

Abbreviations: NO, nitric oxide; LPS, lipopolysaccharide; L-NMMA, N^G-monomethyl-L-arginine; GTN, glyceryl trinitrate; SNP, sodium nitroprusside; AG, aminoguanidine; HFF, human fetal fibroblast; NOS, NO synthase; COX, cyclooxygenase; PG, prostaglandin; MeB, methylene blue; Hb, hemoglobin; IL-1β, interleukin 1β; r, recombinant; AA, arachidonic acid.
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ratories (Franklin Park, IL). NO solutions (2 mM) were prepared by bubbling NO into argon-saturated water at room temperature and stored on ice for the entire duration of the experiments.

Cell Culture. Cells were grown at 37°C in a humidified atmosphere (5% CO_2/95% air) in minimum essential medium (RAW264.7) or Dulbecco's modified essential medium (HFFs) containing 10% (vol/vol) fetal calf serum. RAW264.7 cells were plated at 2×10^4 cells per well and HFFs were plated at 1×10^4 cells per well in a 96-well microtiter plate. The concentration of L-Arg in the culture medium was 0.5 mM.

Induction of NOS and COX Activity. The production of NO_2^-, a breakdown product of NO, or PGE_2, one of the mediators released after activation of COX, was used, respectively, as markers for NOS or COX activity. All induction procedures were performed in medium that lacked fetal calf serum. The total incubation volume in the wells was 200 μl. Induction of NOS and COX was achieved by stimulating the RAW264.7 cells with LPS (1 μg/ml; serotype 0111:B4) for 18 h in the presence or absence of the drug under investigation. To better evaluate the effects of exogenous NO on PGE_2 production, experiments were performed with a cell type that did not possess an endogenous L-Arg to NO pathway. HFFs do not release measurable NO_2^- or PGE_2 in response to LPS stimulation (unpublished observations), but they do release substantial amounts of PGE_2 from endogenous and exogenous arachidonic acid (AA) upon induction (18 h) with IL-1β (1 unit/ml).

In some experiments, induction was carried out in L-Arg-free medium using Hepes-buffered Krebs (HBK) solution of the following composition: 120 mM NaCl/4.7 mM KCl/1.2 mM $MgSO_4 \cdot 7H_2O$/2.5 mM $CaCl_2 \cdot 2H_2O$/1.2 mM KH_2PO_4/25 mM $NaHCO_3$/10 mM dextrose/10 mM Hepes. After the 18-h induction period, the cell supernatants were removed and assayed for NO_2^- and PGE_2. Cells in each well were then washed twice with fresh culture medium or HBK and allowed to equilibrate in the absence or presence of various compounds for 30 min. The cells were thereafter incubated with 30 μM AA for 15 min; the supernatants were removed and assayed for NO_2^- and PGE_2. All incubations were terminated by placing the plates on ice. Indomethacin was dissolved in dimethyl sulfoxide. The concentration of dimethyl sulfoxide in the final incubation mixture did not exceed 0.25%. Alone at this concentration, the solvent did not modify PGE_2 release ($n = 5$).

Protein concentrations in each well were determined by the Bradford method (24) using bovine serum albumin as a standard and PGE_2 levels were determined by specific radioimmunoassay (25). NO_2^- analysis in cell culture medium was performed as described (26) using sodium nitrite as a standard. All determinations were performed in duplicate.

cGMP Determination. These experiments were performed with HFFs that were not stimulated with IL-1β. Concentrations of cGMP were determined by radioimmunoassay (27) after acetylation of the samples with acetic anhydride (28). Cells were grown to confluence in 48-well plates and stimulated at 37°C for 30 min with sodium nitroprusside (SNP) or GTN (50–200 μM) in the absence or presence of Hb (10 μM) or methylene blue (MeB) (50 μM). Isobutylmethylxanthine (1 mM) was present in the incubation mixture to inhibit phosphodiesterase activity. The incubation medium was aspirated and the cGMP was extracted by the addition of ice-cold HCl (0.1 M). After 10 min, the samples were transferred to a new plate, dried, and reconstituted in 5 mM sodium acetate (pH 4.75) for cGMP determination. Results are expressed as fmol of cGMP per μg of protein, and each experiment was performed in duplicate.

Preparation of Recombinant (r) COX Baculoviruses. A 2.0-kb fragment containing the coding region of murine COX-1 or COX-2 was cloned into a *Bam*HI site of the baculovirus transfer vector pVL1393 (Invitrogen) to generate the baculovirus transfer vector pMON23905 and pMON23904 for COX-1 and COX-2, respectively. Recombinant baculoviruses were isolated by transfecting 4 μg of baculovirus transfer vector DNA into 2×10^8 SF9 cells along with 200 ng of linearized baculovirus plasmid DNA by the calcium phosphate method (29). Recombinant virus was purified by three rounds of plaque purification, and high-titer (10^7–10^8 plaque-forming units/ml) stocks of virus were prepared. For large-scale production, Sf9 insect cells were infected in a spinner flask at 0.5×10^6 cells per ml with the recombinant baculovirus stock such that the multiplicity of infection was 0.1. After 72 h the cells were centrifuged, and the cell pellet was homogenized in 50 mM Tris·HCl, pH 8.0/25% (wt/vol) sucrose. rCOX-1 or rCOX-2 (10 μg of protein) was preincubated (room temperature, 30 min) with SNP (0.25–1 mM) before stimulation with AA (10 μM). The reaction was stopped with indomethacin (25 μM)-containing buffer. Baculovirus membranes prepared from nontransfected SF9 or from cells transfected with a non-COX construct have no detectable COX activity.

Viability Test. Cell viability after overnight incubations in medium with or without L-Arg was determined by trypan blue exclusion and exceeded 99%.

Statistics. Results are expressed as mean ± SEM. The results were analyzed by Student's unpaired *t* test and a *P* value of <0.05 was taken as significant.

RESULTS

Effects of L-NMMA, AG, or Indomethacin on NO_2^- and PGE_2 Production from RAW264.7 cells. RAW264.7 cells stimulated with LPS (1 μg/ml) for 18 h released 21 ± 6 μM NO_2^- and 93 ± 5 pg of PGE_2 per μg of protein per 18 h (n = 8). The release of NO_2^- and PGE_2 was inhibited in a concentration-dependent manner by coincubation with L-NMMA or AG (1–300 μM; Fig. 1 *A* and *B*); the effects of either L-NMMA or AG were reversed in a stereospecific manner by coincubation with L-Arg but not D-Arg (1 mM, n = 8; data not shown). Indomethacin (0.01–10 μM) inhibited PGE_2 but not nitrite production (Fig. 1 *C* and *D*). Nonstimulated cells did not release measurable NO_2^- or PGE_2 (n = 8; data not shown).

Consequences of L-Arg Removal on NO_2^- and PGE_2 Production from RAW264.7 Cells. RAW264.7 stimulated for 18 h with LPS (1 μg/ml) in Arg-free medium failed to release NO_2^- and produced at least 4-fold less PGE_2 when compared to their release in normal culture medium (n = 8; Fig. 2 *A* and *B*); the addition of exogenous L-Arg but not D-Arg (both at 1 mM) to the Arg-free medium during the 18-h stimulation with LPS restored their ability to release NO_2^- and PGE_2.

RAW264.7 cells stimulated with LPS in normal culture medium responded to AA with a 9-fold increase in PGE_2 release (from 15 ± 1.2 in the absence to 167 ± 13 pg of PGE_2 per μg of protein per min in the presence of AA). This release was decreased 3-fold in those cells that were induced with LPS in Arg-free medium (from 167 ± 13 to 60 ± 9 pg of PGE_2 per μg of protein per min). COX-2 activity was blocked in both cases by 10 μM indomethacin (6 ± 3 and 3 ± 1 pg of PGE_2 per μg of protein per min, respectively; n = 8). Fig. 3*A* shows that a 30-min addition of 1 mM L-Arg but not 1 mM D-Arg increased PGE_2 production in response to AA (30 μM) stimulation. These effects were reversed by L-NMMA (1 mM) or Hb (10 μM) (Fig. 3*A*). Under these conditions, 1 mM L-Arg but not 1 mM D-Arg released 3 μM NO_2^- and this was totally abolished by coincubation with 1 mM L-NMMA (n = 8). SNP or GTN at 200 μM but not sodium nitrite potentiated the production of PGE_2 in response to AA (Fig. 3*B*). The effects of SNP or GTN were abolished by Hb (10 μM) but not with L-NMMA (1 mM). Production of PGE_2 in the absence of AA (15 ± 1.2 pg per μg of protein per min) was not affected by L-Arg (14 ± 0.9 pg per μg of protein per min), L-NMMA

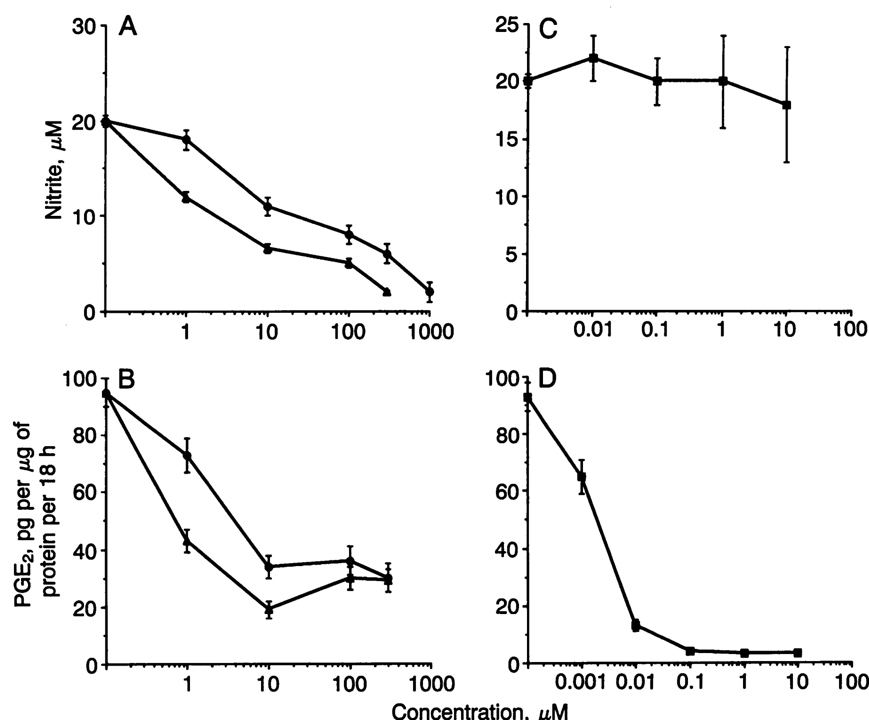

FIG. 1. Accumulation of NO_2^- (*A*) or PGE_2 (*B*) in medium from RAW264.7 cells stimulated with LPS (1 μg/ml) for 18 h was blocked by coincubation with L-NMMA (●) or AG (▲). Indomethacin (■) did not block NO_2^- production (*C*) but abolished PGE_2 release (*D*). Vertical bars represent the mean ± SEM for eight experiments.

(18.6 ± 1.2 pg per μg of protein per min), or Hb (22.2 ± 3 pg per μg of protein per min) (n = 8).

Effects of NOS Inhibitors and NO Donors on NO_2^- and PGE_2 Production from HFFs. Stimulation of HFFs with IL-1β at 1 unit/ml for 18 h led to a 2-fold accumulation of PGE_2 in the medium (from 90 ± 5 to 195 ± 6 pg per μg of protein per 18 h, n = 16), which was not affected by 1 mM L-NMMA or 1 mM AG (n = 16; data not shown). IL-1β-stimulated fibroblasts did not produce measurable NO_2^- (n = 16). The total capacity of the cells to synthesize PGE_2 in response to exogenous AA was increased ≈7-fold (from 25 ± 2 in the absence of AA to 169 ± 29 pg per μg of protein per min after AA stimulation, n = 7); this increase was reduced to 9 ± 0.3 pg per μg of protein per min by indomethacin (10 μM). COX-2 activity was not affected in HFF stimulated with IL-1β in Arg-free medium (from 20 ± 4 to 177 ± 7 pg per μg of protein per min in the absence or presence of AA, respectively; n = 8). A 30-min pretreatment of HFFs before the addition of AA with SNP, GTN, or NO (each at 10–200 μM) significantly increased the release of PGE_2 in response to 30 μM AA (Table 1). The effects of SNP or GTN were abolished by coincubation with 10 μM indomethacin or Hb but not by MeB (50 μM) (Fig. 4 *A* and *B*). These drugs had no effect on basal PGE_2 release (n = 7, data not shown). Sodium nitrite (200 μM) did not increase COX activity in HFFs (n = 4, data not shown).

Effects of GTN or SNP on cGMP Levels in HFFs. A 30-min stimulation of HFFs with GTN or SNP (50–200 μM) in the presence of 1 mM isobutylmethylxanthine led to a concentration-dependent increase in cGMP levels; the effects of GTN or SNP were blocked by coincubation with 10 μM Hb or 50 μM MeB (Fig. 4 *C* and *D*).

Effects of SNP on rCOX-1 and rCOX-2. SNP directly stimulated PGE_2 formation by rCOX-1 and rCOX-2. Thus, rCOX-1 activity was increased 3-, 3-, and 8-fold and rCOX-2 activity was increased 2-, 2-, and 4-fold by SNP at 0.25, 0.5, and 1 mM (n = 3).

DISCUSSION

Our results strongly suggest that endogenous or exogenous NO plays a critical role in the release of PGE_2 by direct activation of COX. Thus, inhibition of endogenous NO release in RAW264.7 cells (*i*) by the use of two inhibitors of NOS, namely L-NMMA and AG, or (*ii*) by inducing the cells in the absence of L-Arg, the precursor for NO synthesis, markedly attenuated PGE_2 release. Since L-Arg depletion did

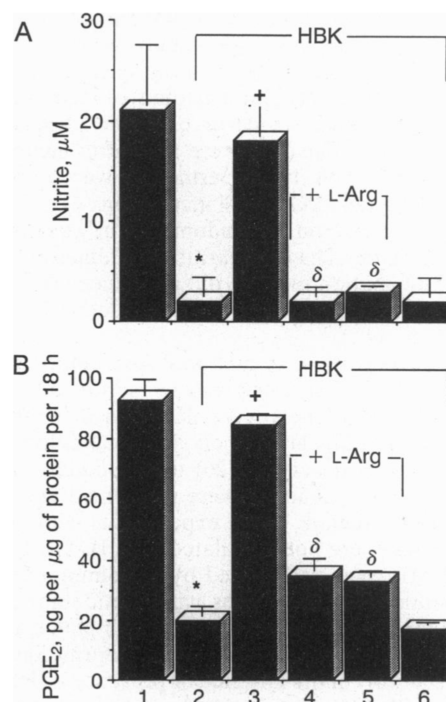

FIG. 2. Reduction in PGE_2 production by RAW264.7 cells in L-Arg-deficient medium. When compared to values obtained in culture medium, the induction of RAW264.7 cells with LPS (1 μg/ml) for 18 h in HBK (Arg-free) virtually abolished NO_2^- production (*A*) and attenuated PGE_2 release (*B*). Addition of HBK containing L-Arg but not D-Arg (1 mM) for 18 h restored this loss; the effects of L-Arg were prevented by coincubation with L-NMMA or AG (1 mM). Vertical bars represent the mean ± SEM of eight experiments. *, $P < 0.01$, compared to value obtained in culture medium; +, $P < 0.01$, compared to value obtained in HBK; δ, $P < 0.01$, compared to value obtained in HBK containing L-Arg. Bars: 1, culture medium; 2, HBK; 3, L-Arg; 4, L-NMMA; 5, AG; 6, D-Arg.

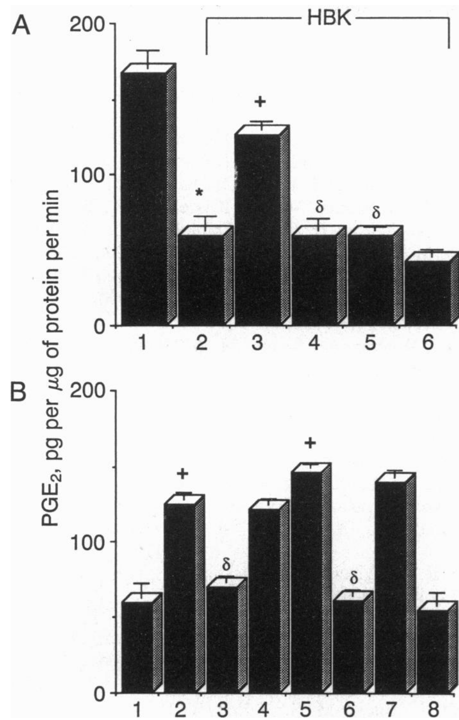

FIG. 3. Addition of 1 mM L-Arg (*A*) or 200 μM SNP or GTN (*B*) for 30 min to RAW264.7 cells that were prestimulated 18 h with LPS (1 μg/ml) in Arg-free medium (HBK) increased PGE_2 production in response to AA. These effects were abolished by 10 μM Hb. L-NMMA (1 mM) prevented the effects of L-Arg but not those of SNP or GTN. Vertical bars represent the mean ± SEM for eight experiments. *, $P < 0.01$, compared to release of PGE_2 obtained in normal culture medium; +, $P < 0.01$, compared to the value obtained with AA alone in HBK; δ, $P < 0.01$, compared to the value obtained with AA in HBK in the presence of L-Arg (*A*) or with AA in HBK in the presence of SNP or GTN (*B*). (*A*) Bars: 1, culture medium; 2, HBK; 3, HBK plus L-Arg; 4, HBK, L-Arg, plus L-NMMA; 5, HBK, L-Arg, plus Ag; 6, MBK plus D-Arg. (*B*) Bars: 1, HBK; 2, HBK plus SNP; 3, HBK, SNP, plus Hb; 4, HBK, SNP, plus L-NMMA; 5, HBK plus GTN; 6, HBK, GTN, plus Hb; 7, HBK, GTN, plus L-NMMA; 8, HBK plus $NaNO_2$.

Table 1. NO, SNP, or GTN enhance production of PGE_2 by AA

Drug, μM	PGE_2, pg per μg of protein per min		
	NO	SNP	GTN
0	166 ± 4	166 ± 4	166 ± 4
10	263 ± 22*	398 ± 40*	215 ± 18*
100	489 ± 45*	617 ± 29*	309 ± 32*
200	841 ± 28*	874 ± 46*	620 ± 65*

NO, SNP, or GTN (each at 10–200 μM) when incubated for 30 min with IL-1β (1 unit/ml, 18 h)-treated HFFs enhanced the production of PGE_2 by AA (30 μM, 15 min). Data are the mean ± SEM of seven experiments.

*$P < 0.01$ compared to control value.

not affect COX-2 activity in HFFs, the latter observation cannot be the result of decreased protein synthesis and thus enzyme induction.

Nitrovasodilators such as SNP or GTN act as NO donors. SNP releases NO spontaneously (30), whereas GTN releases NO after enzymatic metabolism in various cells, including fibroblasts (31) and macrophages (32). Using these two types of nitrovasodilators as a tool to deliver NO, we have demonstrated that in RAW264.7 cells with an inactive L-Arg–NO pathway, SNP or GTN enhanced the production of PGE_2 in response to AA and thus mimicked the effects of exogenous L-Arg. Similarly, NO activated COX in fibroblasts. These cells failed to release NO_2^- in response to IL-1β but did release PGE_2 from endogenous and exogenous AA. This together with a lack of effect of L-NMMA or AG or PGE_2 release indicates that IL-1β does not induce NOS in these cells. However, the addition of exogenous NO (e.g., by NO gas, SNP, or GTN) caused a marked enhancement of COX activity. Sodium nitrite had no effect on AA-induced PGE_2 release, excluding a possible role for NO_2^- in the action of NO. The increased production of PGE_2 release by HFFs in the presence of SNP or GTN was abolished by indomethacin, and the increase in PGE_2 production by L-Arg, NO, SNP, or GTN was abolished by Hb. Since indomethacin blocks COX-2 activity and since Hb binds NO and inactivates it by oxidizing it to NO_2^- and NO_3^- (33), these findings add further proof to the concept that NO is the active species involved in the stimulation of COX-2.

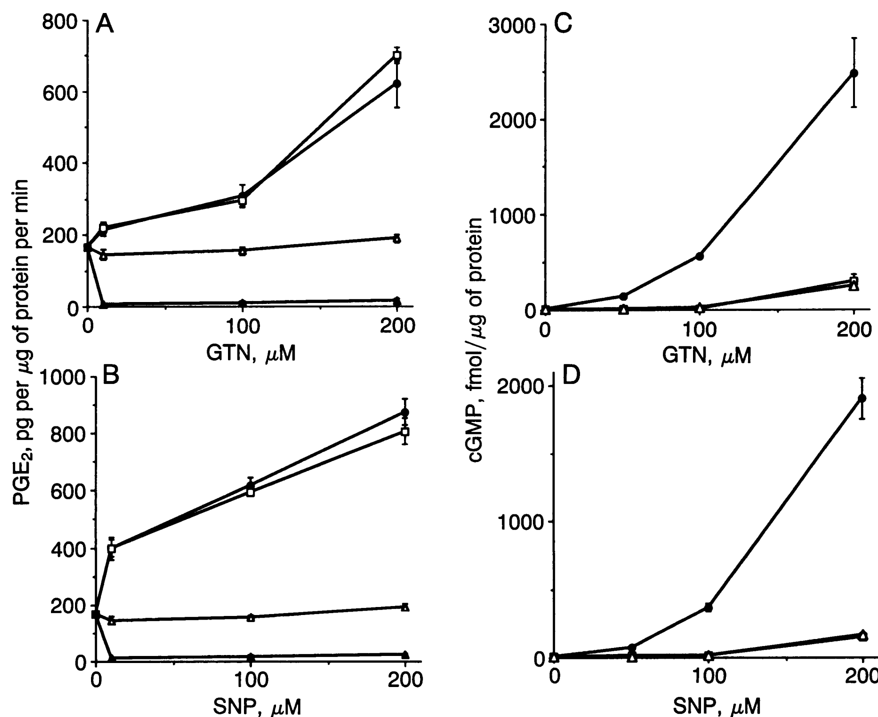

FIG. 4. Increased production of PGE_2 by HFFs after a 15-min incubation with 30 μM AA in the presence of GTN (*A*) (●) or SNP (*B*) (●) is blocked by 10 μM Hb (△) or by 10 μM indomethacin (▲) but not by 50 μM MeB (□). Each point is the mean ± SEM for seven experiments. A 30-min stimulation of HFFs with GTN (*C*) or SNP (*D*) led to a concentration-dependent increase in the levels of cGMP. MeB (50 μM) or Hb (10 μM) attenuated these effects. Each point is the mean ± SEM for five experiments.

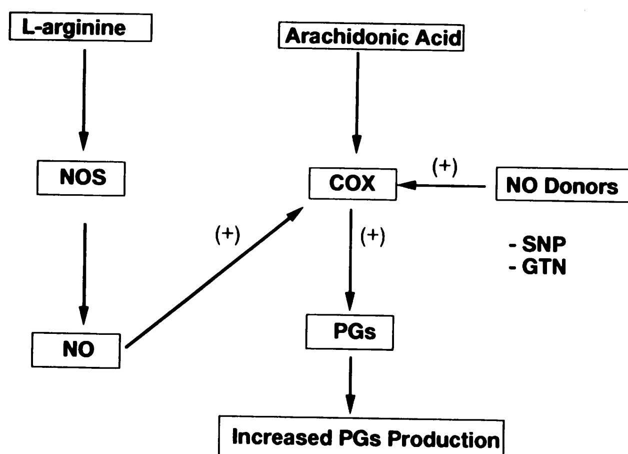

FIG. 5. Model of the regulation of COX activity by NO. We propose that NO produced either endogenously or exogenously (e.g., NO donors) acts on COX to increase the production of PGs from AA. The similarities of this model with the proposed mechanism of NO activation of soluble guanylate cyclase suggest that COX and soluble guanylate cyclase represent a class of enzymes regulated in a similar manner by NO.

It is now well established that NO acts in cGMP-dependent and independent ways. For example, the cytotoxic action of macrophage-derived NO on tumor cells and other tissues is the result of nitrosylation and subsequent inactivation of iron and iron-containing enzymes; these effects are cGMP-independent (21). On the other hand, the antiplatelet action of macrophage-derived NO release is a cGMP-dependent effect (34). MeB, an inhibitor of the soluble but not particulate guanylate cyclase (35), blocked the increase in cGMP levels in HFFs exposed to SNP or GTN but it did not affect their ability to augment PGE_2 release by AA. These findings suggest that the effects of NO on PGE_2 release and hence COX activity are independent of cGMP elevations. The pathway(s) leading to COX activation by NO are unknown but may involve an interaction at the iron–heme center of the enzyme. It is known that COX contains an iron–heme center at the active site (15–17) and that NO interacts with iron-containing enzymes (1, 39) leading to either a stimulation (i.e., soluble guanylate cyclase) or inhibition (i.e., aconitase) of the enzymatic activity. In summary, we have demonstrated using whole-cell systems that NO activates COX leading to an increased metabolism of AA. Furthermore, we have shown that when both pathways are present together, the endogenous production of NO from L-Arg is involved in the regulation of COX activity. This was supported further by demonstrating that SNP activated rCOX-1 and rCOX-2.

Increasing evidence is emerging that the link between the COX and NOS pathways may be of widespread importance in a number of systems. A stimulatory role for NO in PG release has been observed in the perfused rat kidney (36), LPS-stimulated chondrocytes (14), rat hypothalami (37), and the microcirculation of the rat (38). Thus, the NOS and COX enzymes may represent a key regulatory step at which interactions between pro- and anti-inflammatory mediators occur. Our model to illustrate these interactions is shown in Fig. 5. This interaction between NO and the COX pathway may be a critical determinant in the optimum function of COX. For example, in pathological conditions such as nephrosis, sepsis, or rheumatoid arthritis where both pathways are coexpressed, regulation of COX activity by NO may represent an important mechanism by which the initial inflammatory response can be amplified or attenuated. This could represent a therapeutic basis to manipulate the course of an inflammatory response.

We thank D. Gacioch for editorial help and B. Zweifel for culturing the HFFs.

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