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Differential Modulation of Prostaglandin H Synthase-2 by Nitric Oxide-Related Species in Intact Cells†

Aniruddha S. Vidwans,‡,§ Tracy F. Uliasz,§ James A. Hewett,§ and Sandra J. Hewett*,§

Department of Neuroscience, University of Connecticut Health Center, Farmington, Connecticut 06030-3401, and Department of Pediatrics, Division of Neonatology, University of Connecticut Health Center, Farmington, Connecticut 06030-2948

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ABSTRACT: Nitrogen monoxide (NO) has been reported to both activate and inhibit prostaglandin (PG) biosynthesis. This apparent paradox might be explained by the production/action of distinct NO-related species formed as a result of the prevailing redox states of different cellular systems. As such, the effect of NO donors with different redox characteristics on the modulation of prostaglandin H synthase-2 (PGHS-2) in primary mouse cortical astrocytes and COS-7 cells engineered to overexpress PGHS-2 was assessed. In general, compounds that released $NO^{\bullet}$ or NO^{-} enhanced, while a peroxynitrite ($OONO^{-}$) generator inhibited, PGHS-2-dependent prostaglandin production. While the possibility of altered gene transcription was eliminated in the COS-7 system as PGHS-2 was maximally expressed, in primary astrocytes where PGHS-2 expression was induced by lipopolysaccharide (LPS), effects on protein expression were detected. Compounds that released $NO^{\bullet}$ synergistically enhanced LPS-mediated PGHS-2 protein synthesis. None of these effects were mediated by cGMP. All donors lost their ability to modulate PGHS-2 expression and function when decayed. These results indicate that the ultimate effect of NO on PGHS-2 enzyme activity and expression is dictated by the prevalent NO-related species formed, suggesting that important interactions which may exist between NO and prostanoid pathways in vivo will be highly dependent on the inherent redox environment.

Nitric oxide ($NO^{\bullet}$)[1] and prostaglandins (PGs) are important mediators of inflammation and other physiological and pathophysiological processes. Cyclooxygenase (COX) or prostaglandin G/H synthase (PGHS) catalyzes the oxidation of arachidonic acid (AA) to prostaglandin H_2, which is subsequently isomerized and reduced to the major biologically active eicosanoids, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2), and thromboxane A_2 (*1*). NO is produced from the oxidation of L-arginine by the catalytic action of nitric oxide synthase (NOS) (*2*). In general, bioactive metabolites produced by constitutive isoforms of these enzymes, namely, PGHS-1, NOS-1, and NOS-3, regulate various physiological processes, whereas the inducible isoforms, PGHS-2 and NOS-2, are synthesized upon gene induction following inflammatory insults in vivo or by proinflammatory mediators in vitro (*3*).

A vast majority of the physiological functions of NO are a consequence of its interaction with iron or iron-containing enzymes (*4*). Thus, PGHS enzymes are potential targets for NO as they contain an iron−heme center at their active site (*5*). Indeed, numerous studies support a link between NO and PGHS; however, the data are contradictory. For instance, a significant enhancement of PG production from primary rat microglia (*6*, *7*), rat kupffer cells (*8*), and rat peritoneal macrophages (*9*) occurred upon pharmacological inhibition of endogenously generated NO species, suggesting that the NO produced by these cells negatively regulated PGHS-2 activity in these cells. In contrast, a decrease in cellular prostanoid production from mouse cortical astrocytes (*10*), human cultured epithelial and microglial cells (*11*, *12*), and rat islet and vascular smooth muscle cells (*13*, *14*) was observed when endogenous generation of NO was suppressed. This indicated that endogenously produced NO positively regulated PGHS-2 activity and/or expression in these cell types. Similar results were reported with the murine macrophage cell lines, ANA-1 and RAW 264.7 (*15*, *16*).

Results from ex vivo and in vivo studies mirror the complexity of interactions found in the in vitro studies discussed above. Prostanoid production from LPS-treated rats was suppressed when endogenous production of NO was inhibited (*17*, *18*), supporting the conclusion that NO enhanced PGHS-2 activity. Further, Salvemini and colleagues demonstrated that intravenous infusion of NO donors in-

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* To whom correspondence should be addressed: Department of Neuroscience, MC-3401, University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030-3401. Phone: (860) 679-2871. Fax: (860) 679-8766. E-mail: shewett@neuron.uchc.edu.

‡ Department of Pediatrics, Division of Neonatology.

§ Department of Neuroscience.

[1] Abbreviations: NO, nitrogen monoxide; $NO^{\bullet}$, nitric oxide; COX, cyclooxygenase; NOS, nitric oxide synthase; PG, prostaglandin; PGHS, prostaglandin H synthase; MS, medium stock; FBS, fetal bovine serum; FCS, fetal calf serum; AA, arachidonic acid; DETA/NO (NOC-18), (*Z*)-1-[(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate; SIN-1, 3-morpholinosydnonimine HCl; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; AS, Angeli's salt; SOD, superoxide dismutase; LPS, lipopolysaccharide; CPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide K; 8-bromo-cGMP, 8-bromoguanosine 3′,5′-cyclic monophosphate; $OONO^{-}$, peroxynitrite; SNP, sodium nitroprusside; GSNO, *S*-nitrosoglutathione; PLA_2, phospholipase A_2; SEM, standard error of the mean.

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creased prostanoid production from normal animals (*18*). However, more recent results of experiments with a similar paradigm do not support this conclusion (*19*). Finally, mice deficient in NOS-2 were found to synthesize significantly more PGHS-1-derived thromboxane B_2 following platelet aggregation, yet stimulated macrophages elicited from these same animals produced significantly less PGHS-2-derived PGE_2 than that measured from cells cultured from wild-type control animals (*20*).

Numerous reasons for the discrepancies in the response of PGHS enzymes to endogenously produced NO can be entertained. Species and cell type differences may account for some of the reported discrepancies. Alternatively, different cell culture methods and/or experimental conditions may influence the outcome. Finally, the redox state of each cellular system could influence the character of the NO-related species (NOx) that is produced. As such, it is possible that one particular form of NOx ($NO^{\bullet}$, NO^+, NO^-, $OONO^-$, or $NO_2^{\bullet}$) could stimulate PGHS-2 activity while another could inhibit or have no effect under essentially identical experimental conditions (*21*). To test this latter hypothesis, we assessed the effect of NO donors with different redox characteristics on the modulation of PGHS-2-dependent prostaglandin (PG) production from primary mouse cortical astrocytes as well as from COS-7 cells overexpressing PGHS-2. The results are consistent with the contention that the chemical form of NO (NOx) released and/or produced under experimental conditions does indeed make a substantial difference in its ability to modulate PGHS-2 function. Part of this work has been published in abstract form (*22*).

MATERIALS AND METHODS

Materials. Arachidonic acid (AA), 8-bromoguanosine 3′,5′-cyclic monophosphate (8-bromo-cGMP), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CP-TIO), and NS-398 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Flurbiprofen was from Sigma (St. Louis, MO). Catalase and superoxide dismutase were from Calbiochem (La Jolla, CA). Leupeptin, Nonidet P-40, and aprotinin were obtained from Boehringer Mannheim (Mannheim, Germany). Uric acid was purchased from Aldrich Chemical Co. Lipopolysaccharide (LPS) was from Difco Laboratories (Detroit, MI). Murine polyclonal PGHS-2 antibody was purchased from Cayman Chemical (Ann Arbor, MI). The NOx donors, (*Z*)-1-[(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA/NO, NOC-18), 3-morpholinosyndnonimine hydrochloride (SIN-1), and *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), were purchased from Alexis Biochemicals. Angeli's salt (AS) was a kind gift from D. Wink (National Cancer Institute, Bethesda, MD).

NOx Donors. SNAP, DETA/NO, and SIN-1 were prepared as 10 mM stock solutions in medium stock (MS) immediately prior to experimentation. AS was prepared as a 100 mM stock solution in 0.1 N NaOH and diluted into MS immediately prior to experimentation. The reported $t_{1/2}$ values for these compounds at 37 °C (pH 7.4) are as follows: ≈20 h for DETA/NONOate (*23*), ≈6−8 h for SNAP (*24*), ≈2.8 min for Angeli's salt (17 min at 25 °C) (*25*), and ≈8 h for SIN-1 (*26*). MS is composed of modified Eagle's medium (Earle's salts, Gibco) supplemented with 2 mM glutamine and 20 mM glucose.

Expression of PGHS-2 in Primary Astrocytes. PGHS-2 expression was induced by exposure of murine astrocyte cultures to lipopolysaccharide (LPS, 2 μg/mL) (*27*). Astrocyte cultures were prepared from the cerebral hemispheres of 1−3-day-old Swiss Webster mice (CD-1, Charles River Laboratories, Wilmington, MA) as described previously (*28*, *29*). Briefly, cerebral cortices were obtained by aseptic dissection. After enzymatic digestion (0.025% trypsin, 20 min), cells were collected (770*g* for 5 min) and resuspended in MS containing 10% (v/v) fetal bovine serum (FBS), 10% (v/v) calf serum (CS), 10 ng/mL epidermal growth factor, 50 IU/mL penicillin, and 50 μg/mL streptomycin. Astrocytes were plated on 15 mm 24-well plates (Primaria, Falcon) and grown at 37 °C in a humidified atmosphere containing 6% CO_2. Once a confluent astrocytic monolayer was formed (generally 8−10 days in vitro), cytosine arabinofuranoside (8 μM) was added to prevent growth of microglia/macrophages and oligodendrocytes. Two days later, the medium was changed to MS containing 10% CS and antibiotics. The purity of astrocyte cultures is routinely >97% (*28*, *30*).

Expression of PGHS-2 in COS-7 Cells. The murine PGHS-2 cDNA (TIS10) was provided by H. R. Herschman (University of California, Los Angeles, CA) in the pGEM7 vector (Promega, Madison, WI). This was digested with *Bam*HI and *Xho*I to release a ∼2.3 kb fragment containing the entire coding region of PGHS-2 cDNA from the vector polylinker. After separation by electrophoresis in low-melting temperature agarose, the cDNA fragment was purified (Glassmax, Gibco BRL, Grand Island, NY) and ligated into the corresponding dephosphorylated sites of the mammalian expression vector, pcDNA3 (Invitrogen, Carlsbad, CA). The resulting plasmid DNA (pcDNA3-mPGHS-2) was amplified in *Escherichia coli* (DH5α, Life Sciences, St. Petersberg, FL) and purified for transfection using a commercially available kit (Promega). The DNA purity was generally >90% as determined spectrophotometrically.

COS-7 cells (originally obtained from American Type Tissue Culture) were cultured at 37 °C in a 6% CO_2-containing humidified incubator. Growth medium consisted of DMEM (Gibco BRL) supplemented with 5% FCS, 2 mM glutamine, and antibiotics (50 units/mL penicillin and 50 μg/mL streptomycin). Cells grown to approximately 90% confluence in 100 mm culture plates were transiently transfected with pcDNA3-mPGHS-2 by a standard calcium phosphate−DNA precipitation method employing HEPES-buffered saline (HBS) (*31*). Plasmid DNA (27 μg) was added to a final volume of 0.55 mL of calcium chloride solution (250 mM). After <5 min at room temperature, 0.525 mL of this solution was added dropwise to an equal volume of 2× HBS; the solution was mixed and immediately added to the cells (1 mL per 10 mL of growth medium per 100 mm dish). After overnight incubation with the DNA precipitate, the medium was changed and cells were allowed to recover for several hours. To ensure equal PGHS-2 protein expression between different experimental conditions, bulk transfectants were split into 6- or 12-well culture dishes and used 24 h later. PGHS-2 protein expression was assessed via Western blot analysis.

Measurement of the Amount of PGE_2. (1) Accumulation. Astrocyte cultures were rinsed twice with MS containing 2% FBS (MS-2%) and exposed to 400 μL of MS-2% alone (basal PGHS activity) or to MS-2% containing LPS (2 μg/mL) with

or without various NOx donors as indicated, and then placed back in the incubator. Supernatants were collected at the indicated times (24−40 h) and frozen at −80 °C. We have previously determined that the PGE_2 secreted from primary mouse astrocytes following LPS exposure is produced solely by newly expressed PGHS-2 protein (*27*).

(2) Activity. For a more direct measurement of COX activity, astrocyte cultures were treated under various conditions for 5 or 12 h and washed, and then exogenous arachidonic acid (30 μM) was added for 30 min (37 °C). The supernatants were removed for measurement of the amount of PGE_2. COS-7 cells were washed twice with MS and then donors added for 4 h (37 °C) with the exception of Angeli's salt (AS) where the exposure period was 1 h (26 °C). Arachidonic acid (30 μM) was then added to each well during the last 15 min of drug exposure. Cell culture supernatants were collected and stored at −80 °C. The amounts of PGE_2 from astrocyte and COS-7 cell cultures were measured at room temperature by EIA according to the manufacturer's instructions (Amersham, Arlington Heights, IL, or Caymen Chemicals).

Measurement of NOS-2 Activity. Production of NO from stimulated astrocytes was assessed indirectly by measurement of the amount of nitrite, a stable oxidation breakdown product of NO (*32*). Nitrite levels were determined by mixing 100 μL portions of culture medium with 100 μL of Greiss reagent (1 part 0.1% sulfanilamide in 60% acetic acid plus 1 part 0.1% naphthylenediamine dihydrochloride in distilled H_2O). After 10 min, the absorbance at 550 nm was determined on a microtiter plate reader (Dynatek).

Western Blot Analysis. Astrocyte cultures were washed twice with HBSS and harvested in 200 μL of HBSS containing 0.05% trypsin and 0.2 mM EDTA (Gibco BRL). Cells from four to six identically treated wells were combined in 2 mL of ice-cold MS containing 10% CS and pelleted by centrifugation (300*g* for 5 min at 4 °C). After being washed once with 3 mL of ice-cold PBS, pellets were resuspended in 100−200 μL of ice-cold PBS containing 1% Nonidet P-40, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM phenylmethanesulfonyl fluoride, and 5 mM iodoacetamide. Lysates were incubated on ice for 30 min, after which nuclei were pelleted by centrifugation (10000*g* for 5 min at 4 °C) and cleared lysates collected. COS-7 cell lysates were prepared in a similar manner except that cultures were washed twice with ice-cold PBS without trypsin and incubated on ice for 10 min prior to harvesting by gentle trituration. The contents of two to three wells per identical treatment condition were combined and pelleted (1000*g* for 5 min at 4 °C). Cells were lysed as described above. The protein concentration in the lysates was determined via the BCA assay (Pierce Chemical, Rockford, IL).

To detect NOS-2 or PGHS-2 in cell lysates, five to 20 μg of total cellular protein were separated by SDS−PAGE (7.5 to 8% polyacrylamide gel) and electrophoretically transferred to nitrocellulose (0.2 μm; Bio-Rad). Membranes were then placed in PBS (>70 °C) for 2 min, washed twice with H_2O (20 mL, 5 min), and then blotted with either an anti-PGHS-2 polyclonal antibody (400 ng/mL; Cayman Chemical) or an anti-NOS-2 polyclonal antibody (1 μg/mL; UBI, Lake Placid, NY) using a commercially available kit per the manufacturer's instructions (Western Breeze Chemiluminescent Immunodetection Protocol, Novex, San Diego, CA). Results were recorded on X-ray film (Hyperfilm, Amersham).

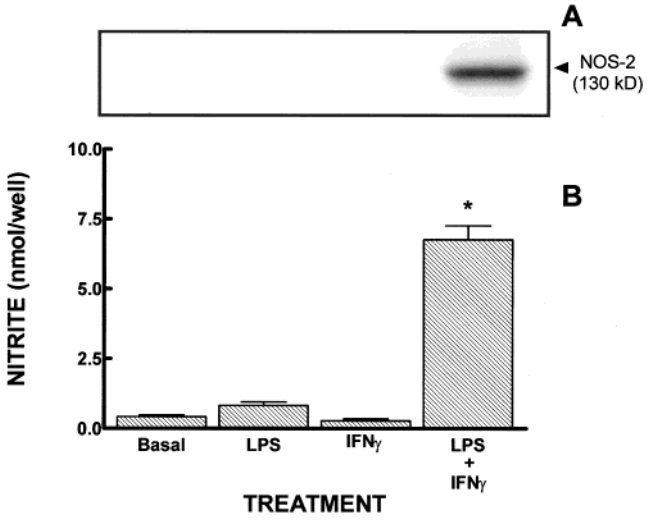

FIGURE 1: LPS is an insufficient stimulus for NOS-2 induction in primary mouse astrocytes. Astrocyte cultures were exposed to medium (MS with 2% FBS) alone (basal) or that containing LPS (2 μg/mL), IFNγ (1 ng/mL), or LPS and IFNγ. (B) Thirty hours later, cell culture supernatants were collected and nitrite accumulation was assessed as a measurement of NOS-2 catalytic activity ($n = 12$). An asterisk indicates a value significantly greater than basal levels of nitrite as determined by ANOVA followed by the Dunnett's t test for multiple comparisons ($P < 0.01$). (A) Total cellular protein from the same cells as used for panel B was isolated and 20 μg separated by SDS−PAGE. Western blot analysis was performed using a polyclonal antibody to NOS-2 as described in Materials and Methods. Results are representative of three independent experiments.

RESULTS

Effect of NOx Compounds on PG Synthesis from Primary Mouse Astrocytes. PGHS-2-dependent astrocytic PGE_2 production was induced by exposure of murine astrocyte cultures to lipopolysaccharide (2 μg/mL) (*27*). It is important to note that while LPS is a potent and sufficient stimulus for PG production, it alone is incapable of inducing NOS-2 protein (Figure 1A) or NO formation (Figure 1B) from mouse cortical astrocytes (also see refs *27* and *28*). Thus, the NOx compounds used [DETA/NO, which releases $NO^{\bullet}$ upon decomposition (*33*); Angeli's salt, which releases NO^- upon decomposition (*24*); SNAP, an *S*-nitrosothiol (NO^+ equivalent) (*34*); and SIN-1, which provides a continuous source of NO and $O_2^{\bullet -}$ (*35*) and presumably $OONO^-$] provided the only source of NOx species to these cells.

To mimic conditions as they occur in vivo, where NOS-2 and PGHS-2 are co-induced and NO has prolonged contact with PGHS-2, we exposed astrocyte cultures for 30−40 h to LPS with or without donors. Exposure of astrocytes to DETA/NO or SNAP (300 μM) resulted in an enhancement in the accumulation of PGE_2 induced by LPS (Figure 2A,C). By contrast, SIN-1 (300 μM) inhibited PGE_2 accumulation (Figure 2B). Unfortunately, we were unable to test the effect of AS, a NO^- donor, in this experimental paradigm. At 37 °C, AS has an extremely short half-life (≈2.3 min), while at 25 °C, a temperature at which AS has a $t_{1/2}$ of 17 min, LPS did not cause consistent induction of PGHS-2. None of the donors tested had an effect on the basal levels of PGE_2 that accumulated in the absence of LPS (data not shown).

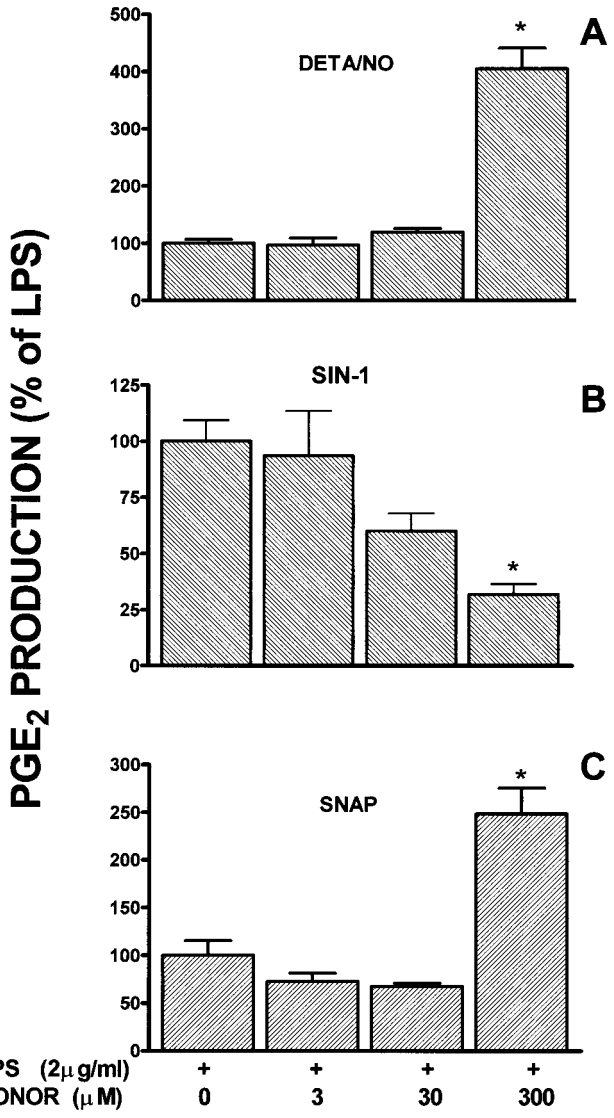

FIGURE 2: Effect of NO donors on LPS-stimulated PGE_2 production in primary mouse astrocytes. Cultures were exposed to medium alone (not shown), donor alone (not shown), or medium containing LPS (2 μg/mL) in the absence or presence of the indicated concentrations of DETA/NO ($n = 3-4$) (A), SIN-1 ($n = 8-10$) (B), or SNAP ($n = 3-4$) (C). Thirty-forty hours later, culture supernatants were collected for measurement of the PGE_2 level. To facilitate comparison between compounds, the amount of accumulated PGE_2 was scaled to the mean value obtained following exposure to LPS alone (set at 100%). Data represent the mean ± SEM. The asterisk denotes values significantly different from LPS-stimulated levels as determined in each experiment by ANOVA followed by the Student-Newman-Keul's test for multiple comparisons. Significance was assessed at the $P < 0.05$ level. Basal PGE_2 accumulation was unaffected by exposure to donors alone (not shown).

Finally, all effects were lost when the donors were allowed to decay for 24 h prior to experimentation, indicating that a short-lived reactive intermediate derived from each compound mediated the change in PGHS-2 function and that this was not due to stable metabolic breakdown products (data not shown).

Since LPS-induced PGHS-2 activity in astrocytes requires induction of PGHS-2, mRNA, and protein (*27*), the actions of the NOx donors could be due to an effect on PGHS-2 catalytic activity or to an alteration in PGHS-2 expression, or both. Thus, we next examined the effects of NOx donors on PGHS-2 protein expression 5 and 12 h following exposure. These time points were chosen as LPS-mediated PGHS-2 expression occurs within 3−5 h and continues to increase up to 12 h (*27*). Astrocyte cultures exposed to NOx donors in the absence of LPS did not express PGHS-2 protein as determined by Western blot analysis (data not shown). However, both SNAP and DETA/NO increased the level of LPS-mediated PGHS-2 protein expression (Figure 3A,B) at 5 h with a statistically significant increase measured at 12 h (Figure 3B). Consistent with this finding, PGE_2 production from SNAP- or DETA/NO-treated (both at 300 μM) astrocyte cultures supplied with exogenous arachidonic acid was significantly elevated (Figure 4). This suggests that the increase in PGE_2 synthesis that occurred following exposure to these compounds could be mediated, at least in part, by an increase in the level of protein synthesis. SIN-1 (300 μM) had no effect on LPS-stimulated PGHS-2 protein expression at either time point (Figure 3A,B), thus eliminating inhibition of protein synthesis as a mechanism by which it inhibited LPS-induced accumulation of PGE_2. The SIN-1-mediated decrease in LPS-induced PGE_2 production from astrocytes was instead likely due to a direct decrease in PGHS activity as PGE_2 production from SIN-1-treated astrocyte cultures supplied with exogenous arachidonic acid was inhibited by approximately 50% (Figure 4B).

A well-documented target of $NO^{\bullet}$ is soluble guanylate cyclase, the enzyme that synthesizes cGMP. As it is possible that the changes in PGHS-2 protein levels occur by a cGMP-dependent mechanism (*36*), we next examined the effect of 8-bromo-cGMP, a membrane permeant analogue of cGMP, on PGHS-2 protein expression to determine whether cGMP would mimic the effect of the $NO^{\bullet}$ donor compounds. 8-Bromo-cGMP did not induce PGHS-2 synthesis on its own (data not shown), nor did it alter LPS-induced PGHS-2 protein production (Figure 5A). In agreement with this, 8-bromo-cGMP alone did not alter basal PGE_2 levels, nor did it significantly affect LPS-mediated PGE_2 production (Figure 5B).

Effect of NOx Compounds on PG Synthesis in Transfected COS-7 Cells. To separate the effects of various NOx compounds on PGHS-2 activity from those on PGHS-2 protein expression, we overexpressed PGHS-2 in COS-7 cells. COS-7 cells were chosen specifically as they have little endogenous PGHS activity (M. K. O'Banion, personal communication; Figure 6). Cells transfected with the plasmid vector DNA (pcDNA3) lacking the PGHS-2 cDNA secreted negligible amounts of PGE_2 into the cell culture medium in response to exogenous addition of arachidonic acid (Figure 6). In contrast, cells transfected with pcDNA3-mPGHS-2 expressed an abundant amount of PGHS-2 protein (Figure 6 inset) and made copious amounts of PGE_2 when challenged with exogenous arachidonic acid. This PGE_2 production was completely inhibited by a 1 h pretreatment with the selective PGHS-2 antagonist, NS398 (100 μM), as well as by the nonselective PGHS inhibitor, flurbiprofen (100 μM) (Figure 6).

Arachidonic acid-mediated PGE_2 production from COS-7 cells engineered to overexpress PGHS-2 was enhanced by DETA/NO (300 μM; Figure 7A) and SNAP (300 μM; Figure 7C) but inhibited by SIN-1 (300 μM; Figure 7B). Interestingly, the NO^{-} donor, Angeli's salt, also increased PGHS-2 activity (AS; 300 μM; Figure 7D). It is important to note

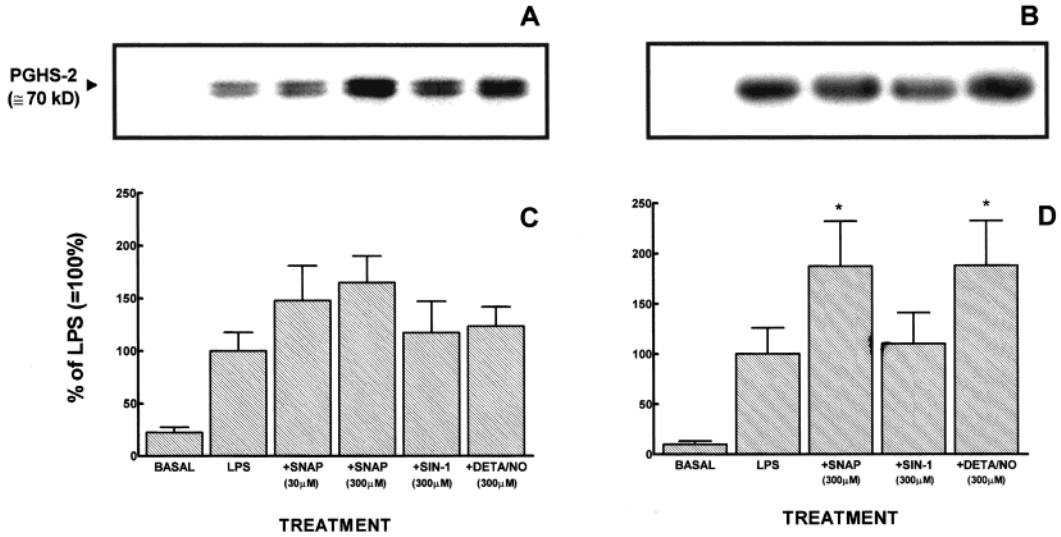

FIGURE 3: Effect of nitric oxide donors with different redox characteristics on PGHS-2 protein levels. Astrocyte cultures were treated as indicated. Five (A) or 12 (B) hours later, total cellular protein was isolated and 15 μg separated by SDS−PAGE and transferred to nitrocellulose, and Western Blot analysis was performed using a polyclonal PGHS-2 antibody as described in Materials and Methods. For each, results are representative of three separate experiments. (C and D) Gel films were scanned using an Epson Expression 800 scanner, and densitometry was performed using Scion (NIH) Image software. Relative protein levels were normalized to the mean level obtained following exposure to medium alone (basal; set at 1). Results are means ± SEM of five independent experiments.

that PGHS-2 protein levels were unaltered by exposure to the NOx donors, nor did decayed donor compounds have any effect on arachidonic acid-mediated PGE_2 production from COS-7 cells (data not shown).

Finally, different radical scavenging systems were used to confirm that the effects of the NOx compounds on PGHS-2 activity were mediated via their purported release products. The stimulatory effect of DETA/NO and SNAP (300 μM) on PGHS activity in COS-7 cells overexpressing PGHS-2 was prevented by the $NO^{\bullet}$ scavenger, CPTIO (200 μM), indicating that $NO^{\bullet}$ was indeed mediating this increase (Figure 8A,B). Interestingly, not only was the $NO^{\bullet}$-mediated enhancement reversed by CPTIO, but the PGE_2 levels were significantly decreased in comparison to those in cultures receiving AA alone (Figure 8A,B). This could not be explained by a nonspecific effect of the scavenger as CPTIO had no effect on AA-stimulated PGE_2 accumulation alone (Figure 8A,B). Next, the antioxidant enzymes SOD and catalase were used in conjunction with SIN-1 to remove $O_2^{\bullet-}$ (and the subsequent formation of H_2O_2), thus preventing the formation of $OONO^-$ ($NO^{\bullet} + O_2^{\bullet-}$). The combination of SOD and catalase (500 units/mL each) suppressed the inhibitory effect of SIN-1 without affecting AA-mediated PGE_2 production alone (Figure 9A). Interestingly, removal of $O_2^{\bullet-}$ from SIN-1 resulted in an elevation of the level of PGE_2 as compared to those in cultures receiving AA alone (Figure 9A). This is consistent with our observation that donors with $NO^{\bullet}$ release characteristics activate PGHS-2 activity (Figures 7 and 8). To further verify that the inhibitory effect of SIN-1 was due to the action of $OONO^-$, we used the selective $OONO^-$ scavenger uric acid (*37*, *38*). Uric acid (300 μM) completely reversed the inhibitory effect of SIN-1 without altering AA-mediated PGE_2 levels.

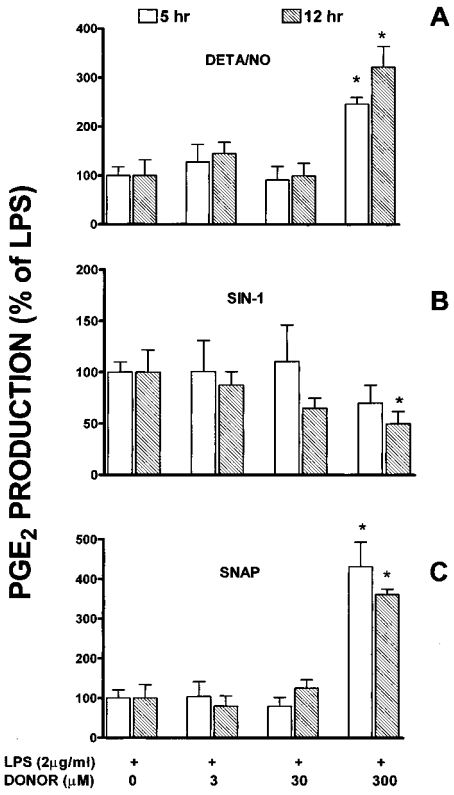

FIGURE 4: Effect of NO donor compounds on astrocyte PGHS-2 enzymatic activity. PGHS-2 enzymatic activity was assessed by measurement of the amount of PGE_2 produced from astrocyte cultures in the presence of exogenous arachidonic acid. Five or 12 hours following exposure to LPS with or without donors, arachidonic acid (30 μM) was added to the cultures (30 min at 37 °C) and supernatants were collected. To facilitate comparison between the compounds, the amount of PGE_2 was scaled to the mean value obtained following exposure to LPS alone (set at 100%). Data represent the mean ± SEM ($n = 3-7$ for each experiment). The asterisk denotes values significantly different from LPS-stimulated levels as determined in each experiment by ANOVA followed by the Student-Newman-Keul's test for multiple comparisons. Significance was assessed at the $P < 0.05$ level.

DISCUSSION

The goal of this study was test the hypothesis that different chemical forms of NO ($NO^{\bullet}$, NO^+, NO^-, and $OONO^-$) differentially modulate PGHS-2 function and/or expression.

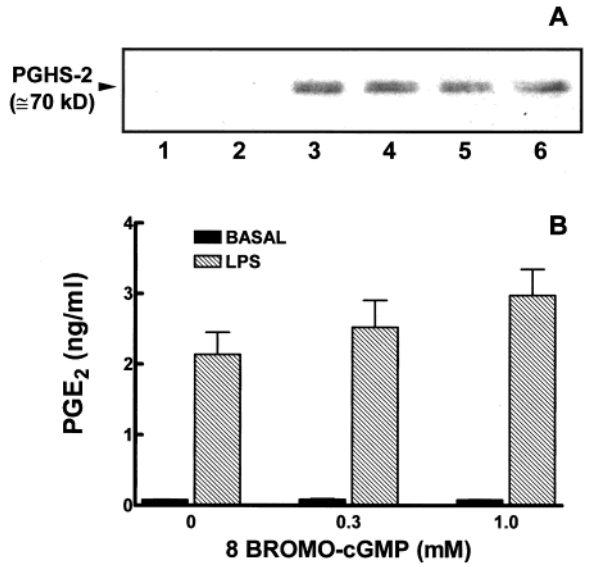

FIGURE 5: Effect of cGMP on LPS-stimulated PGHS-2 protein expression and PGE_2 production. (A) Astrocyte cultures were treated as follows: lane 1, medium alone; lane 2, 8-bromo-cGMP (1 mM); lane 3, LPS (2 μg/mL); and lanes 4−6, LPS with 0.01, 0.1, and 1 mM 8-bromo-cGMP, respectively. Five hours later, total cellular protein was isolated and 15 μg separated on a 7.5% polyacrylamide gel and transferred to nitrocellulose, and Western Blot analysis was performed using a polyclonal PGHS-2 antibody as described in Materials and Methods. Results are representative of three separate experiments. (B) Cultures were treated with 8-bromo-cGMP in the absence (basal) or presence of LPS (2 μg/mL). Thirty hours later, culture supernatants were collected for measurement of PGE_2 concentration (nanograms per milliliter). Data represent the means ± SEM (n = 8 from two separate experiments). cGMP had no effect on LPS-induced PGE_2 production as determined by ANOVA.

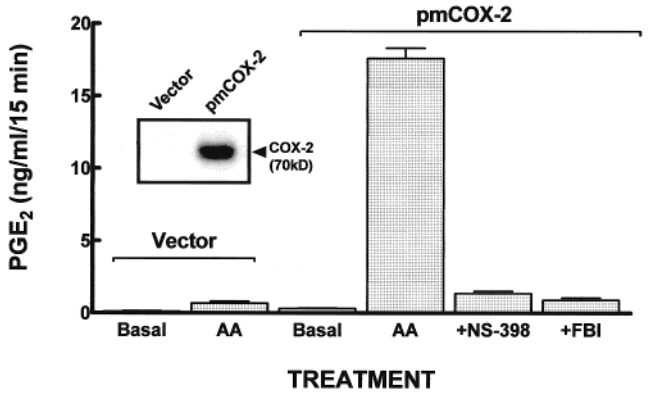

FIGURE 6: Pharmacological characterization of COS-7 cells transfected with an expression vector containing the murine PGHS-2 cDNA. COS-7 cells were transiently transfected with an expression plasmid (pcDNA3) containing the murine PGHS-2 cDNA (pmCOX-2). Sister cultures were transfected in parallel with plasmid lacking cDNA as a control (vector). pmCOX-2-transfected cells were treated for 1 h (37 °C) with NS-398 (PGHS-2 selective inhibitor; 100 μM) or flurbiprofen (FBI) (PGHS1/PGHS-2 inhibitor; 100 μM). During the last 15 min of exposure, arachidonic acid (30 μM) was added. Vector-transfected cells were exposed to AA (30 μM) for 15 min only. Culture supernatants were collected for measurement of PGE_2 levels. Values represent the means ± standard deviation from two separate transfections. The inset shows immunoreactivity for PGHS-2 in COS-7 cells following transfection with empty plasmid (vector) or plasmid containing PGHS-2 cDNA (pmCOX2). Forty-eight hours following transient transfection, total cellular protein was isolated and 5 μg of protein was separated on an 8% SDS−polyacrylamide gel and transferred to nitrocellulose, and Western blot analysis was performed using a polyclonal antibody to PGHS-2 as described in Materials and Methods.

The results obtained using donors with different redox characteristics favor the notion that the chemical state in which NO exists does indeed determine whether PGHS-2 will be activated, inhibited, or unaffected by NO. This was demonstrated in two separate cellular systems. For example, we found that DETA/NO, a compound reported to release pure $NO^{\bullet}$ (*33*), and SNAP (300 μM), a nitrosothiol and a putative NO^+ donor (*34*), enhanced LPS-induced PGHS-2 protein synthesis and PGE_2 production from mouse cortical astrocytes as well as PGHS-2 activity in COS-7 cells engineered to overexpress PGHS-2. The effect of both compounds was reversed by CPTIO, a direct scavenger of $NO^{\bullet}$ (*39*). As SNAP is a known NO^+ donor, this result might at first seem surprising. However, it has been reported previously that at high concentrations, nitrosothiols can homolytically cleave and release $NO^{\bullet}$ (*24*, *34*). In support of this, we found that the lower concentrations of SNAP utilized in this study (3 and 30 μM) produced little to no nitrite, an oxidation breakdown product of $NO^{\bullet}$, while 300 μM SNAP produced copious quantities (A. S. Vidwans and S. J. Hewett, unpublished observation). It is interesting to note that the lower concentrations of SNAP attenuated astrocyte PGE_2 accumulation, though this effect did not achieve statistical significance. However, inhibition of PGHS-2-mediated PG synthesis with low concentrations of SNAP has previously been reported in LPS-stimulated rat microglia and RAW 264.7 cells maintained in culture (*7*). Further, *S*-nitrosoglutathione (GSNO), another compound with NO^+ release characteristics, inhibited PGHS-2-mediated PG production in LPS-stimulated rat peritoneal macrophages (*9*). NO^+ can interact with cysteine residues, forming nitrosothiols, or with vicinal thiols to generate disulfide bonds (*24*). Interestingly, modification with maleimides or with site-directed mutagenesis of Cys^{313} and Cys^{540} in the catalytic domain of PGHS-1, cysteines that are conserved in the PGHS-2 molecule (*40*), causes near-complete enzyme inactivation (*41*). Thus, it is possible that the inhibitory effect of SNAP, a nitrosothiol that participates in transnitrosation reactions at low concentrations, is due to its reaction with catalytically important cysteine residues in the PGHS-2 molecule. However, the inhibition observed in the accumulation assay was not demonstrated in the astrocyte or COS-7 activity assays where exogenous AA was utilized. Thus, it is possible that an alteration in PLA_2 activity, and hence a decrease in substrate availability, could explain the inhibition of PGE_2 accumulation which follows SNAP treatment. In support of this, Thang and colleagues found that a different nitrosothiol, *S*-nitrosocysteine, irreversibly inhibited $cPLA_2$, possibly through S-nitrosation of Cys^{331} (*42*).

The enhanced production of PGE_2 seen following incubation with higher concentrations of SNAP and DETA/NO (both at 300 μM) is in agreement with a previous study in which sodium nitroprusside (SNP) was employed, which showed that PGE_2 production from LPS-stimulated astrocytes was enhanced by exposure to SNP (*10*). While SNP, like SNAP, is not the best donor of $NO^{\bullet}$ (*24*, *43*), its stimulatory effect was prevented by the addition of hemoglobin, a known $NO^{\bullet}$ scavenger, indicating that the enhancement of PGHS-2-dependent PGE_2 production from astrocytes was mediated by $NO^{\bullet}$ (*10*). Further, exogenous addition of $NO^{\bullet}$, either as a gaseous solution or by addition of donor compounds, increased PGHS-2 activity in IL1β-stimulated human fibro-

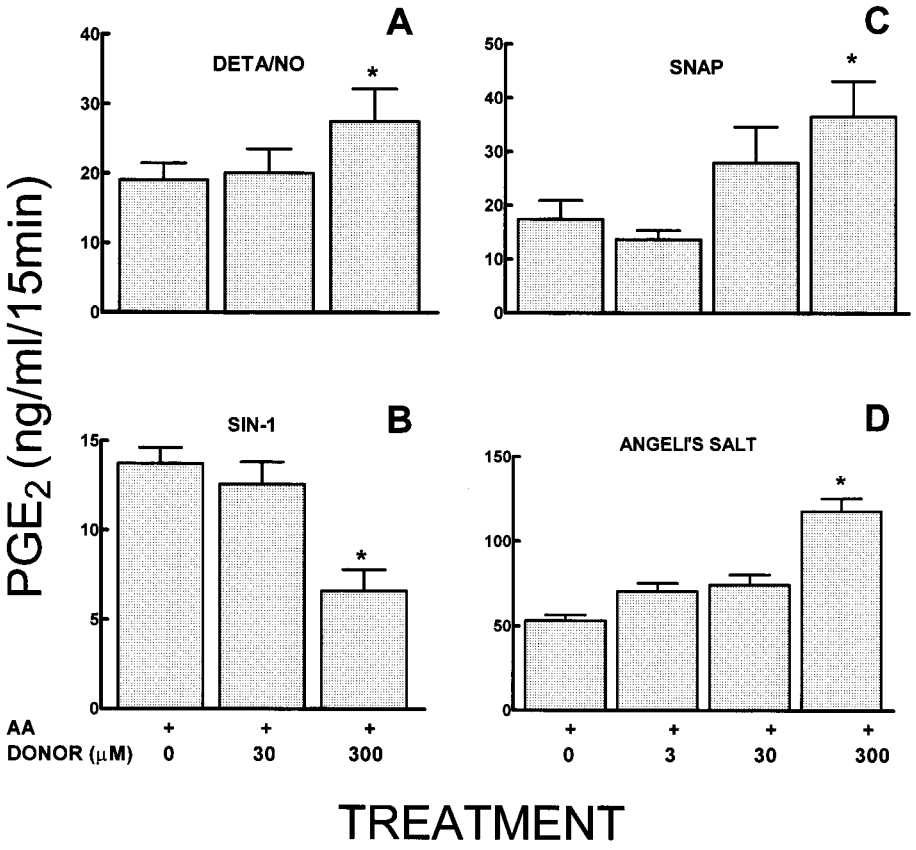

FIGURE 7: Effect of nitric oxide donors with different redox characteristics on PGHS-2 activity. PGHS-2 enzymatic activity was assessed by measurement of the amount of PGE_2 produced from COS-7 cells overexpressing murine PGHS-2 in the absence (basal; not shown) or presence of exogenous arachidonic acid. Three hours and 45 min following exposure to medium alone or medium containing (A) DETA/NO (37 °C; $n = 6$), (B) SIN-1 (37 °C; $n = 4-7$), or (C) SNAP (37 °C; $n = 4-5$), or 45 min following exposure to (D) Angeli's salt (26 °C; $n = 4$), AA (30 μM; 15 min) was added to the cultures, after which supernatants were collected and analyzed for PGE_2 content. Data are expressed as means ± SEM (nanograms per milliliter per 15 min). An asterisk represents a value significantly different from that with AA alone as determined by ANOVA followed by the Student-Newman-Keul's test for multiple comparisons ($P < 0.05$).

blasts (*16*) and in an IL1β-stimulated human airway epithelial cell line (*12*). It should be noted, however, that Curtis et al. (*44*) reported that NO$^\bullet$ neither enhanced nor inhibited PGHS-2 activity in the RAW 264.7 murine macrophage cell line (*44*).

The mechanism(s) by which NO$^\bullet$ or an NO$^\bullet$ reaction product promotes an increase in PGHS-2 activity and/or protein expression is likely complex. There are numerous studies which report that NO$^\bullet$ can function as a direct modulator of gene expression through its interactions with transcription factors (*45*). While the donors in our study were unable to stimulate transcription of the PGHS-2 gene on their own, they did effectively amplify PGHS-2 protein synthesis induced by LPS in murine astrocyte cultures. Similarly, Tetuska and colleagues (*36*) reported that NO$^\bullet$ or an NO$^\bullet$ reaction product derived from SNP and SNAP potentiated IL1β-induced PGHS-2 mRNA and protein expression in rat mesangial cells (*36*). However, no change in PGHS-2 protein expression with SNAP in the ANA-1 mouse macrophage cell line was reported (*15*). This result is consistent with the reported lack of an effect of NO$^\bullet$ donors on PGHS-2 activity in the RAW 264.7 murine macrophage cell line (*44*). Additionally, it is possible that the maximum concentration of SNAP used in the Perkins study (100 μM) (*15*) released an insufficient amount of NO$^\bullet$ to exert an effect (*29*).

Independent of its effects on protein synthesis, NO$^\bullet$ could possibly enhance PGHS-2 activity via interaction with the heme moiety, perhaps in the same way as it is known to activate guanylate cyclase, i.e., via the induction of a structural change (*46*−*48*). Indeed, it was reported that activation of purified PGHS-1 protein by NO$^\bullet$ was accompanied by a change in the absorption spectrum of the molecule in the region occupied by the heme moiety (*49*). However, Tsai and colleagues using stopped-flow spectrophotometry reported that the ferric heme in resting PGHS-1 has a weak affinity for NO$^\bullet$ (*50*). A heme-independent process of activation was also proposed by Hajjar and colleagues, calling into question whether the interaction of heme and NO$^\bullet$ is necessary for the activation of PGHS enzymes (*51*). Interestingly, the H_2O_2-mediated inhibition of PGHS activity caused by the oxidation of heme iron (*52*) can be reversed by NO$^\bullet$ (*49*). This suggests that NO$^\bullet$ can act either as a reducing agent thereby counteracting H_2O_2 by reducing Fe(IV) or -(V) to Fe(III) or by protecting PGHS from peroxide inactivation (*49*).

Another possibility is that NO$^\bullet$ exerts its effects indirectly via stimulation of soluble guanylate cyclase (sGC). Indeed, the NO$^\bullet$-mediated enhancement of IL1β-induced PGHS-2 activity, mRNA and protein expression in rat mesangial cells was demonstrated to occur by a cGMP-dependent mechanism as methylene blue, an sGC inhibitor, reversed the effect and 8-bromo-cGMP mimicked it (*36*). However, 8-bromo-cGMP did not alter astrocytic PGHS-2 protein expression, nor did it affect PGHS-2 activity in this study. Similar cGMP-

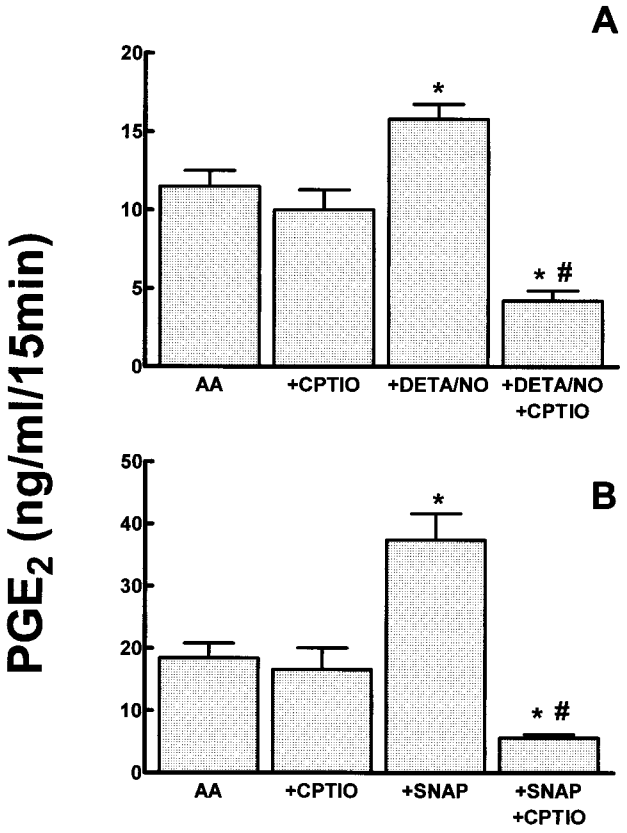

FIGURE 8: Nitric oxide scavenger CPTIO reverses the effect of NO donors on PGHS-2 activity. PGHS-2 enzymatic activity was directly assessed by measurement of the amount of PGE_2 produced from COS-7 cells overexpressing murine PGHS-2. (A) Cultures were treated for 4 h with medium alone, DETA/NO (300 μM), DETA/NO and CPTIO (300 μM), or CPTIO. AA (30 μM) was added to each well during the last 15 min. Thereafter, supernatants were collected and analyzed for PGE_2 content. Data are expressed as means ± SEM (nanograms per milliliter per 15 min) ($n = 6$). (B) Same as panel A except the NO donor was SNAP (300 μM) ($n = 6$). An asterisk represents a value significantly different from that of AA alone, whereas a pound sign indicates a significant reversal of the NOx donor response as determined by ANOVA followed by the Student-Newman-Keul's test for multiple comparisons. Significance was assessed at the $P < 0.05$ level.

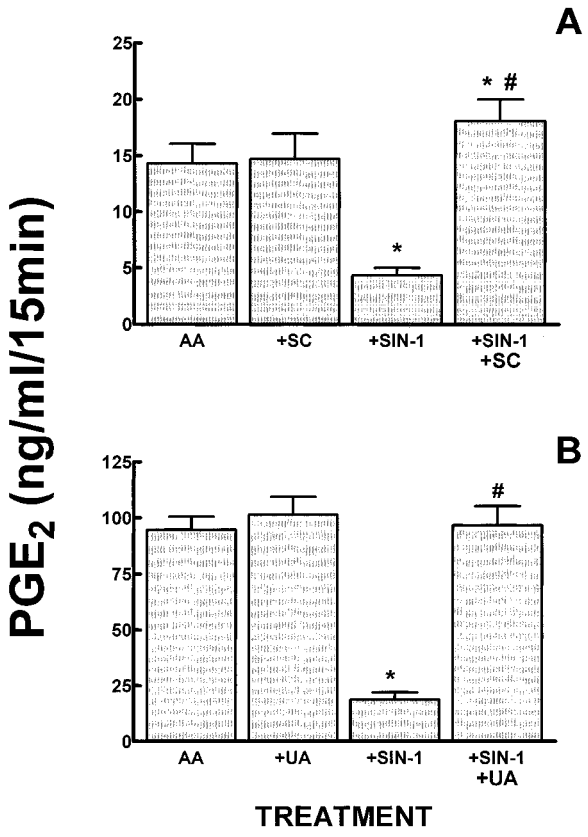

FIGURE 9: Free radical scavengers reverse the effect of SIN-1 on PGHS-2 activity. PGHS-2 enzymatic activity was directly assessed by measurement of the amount of PGE_2 produced from COS-7 cells overexpressing murine PGHS-2. (A) Cultures were treated for 4 h with medium alone, SIN-1 (300 μM), SIN-1 with SOD/catalase (SC; 500 units/mL each), or SC alone. AA (30 μM) was added during the last 15 min. Thereafter, supernatants were collected and analyzed for PGE_2 content. Data are expressed as means ± SEM (nanograms per milliliter per 15 min) ($n = 5$). (B) Same as panel A except the scavenger was uric acid (300 μM) ($n = 4$). An asterisk represents a value significantly different from that of AA alone, whereas a pound sign indicates a significant reversal of the SIN-1 response as determined by ANOVA followed by the Student-Newman-Keul's test for multiple comparisons. Significance was assessed at the $P < 0.05$ level.

independent effects of $NO^{\bullet}$ on PGHS enzymes have also been reported previously (*14*, *16*, *53*). Why this effect would be mediated by cGMP in one cell type and not another remains undetermined.

Finally, it is possible that the stimulation of PGHS-2 activity originally attributed to $NO^{\bullet}$ might occur via $OONO^-$ which can be formed from the diffusion-limited reaction of $NO^{\bullet}$ and $O_2^{\bullet -}$ (*54*). Utilizing a purified enzyme system, several investigators reported that SIN-1 and authentic $OONO^-$ enhanced PGHS-2 activity through its ability to act as an initiator hydroperoxide molecule (*55*, *56*). In support of this finding, SIN-1 was reported to enhance PGHS-2-dependent prostanoid production from RAW 264.7 cells (*7*) and from purified PGHS enzymes in the presence of a peroxide scavenger (*21*, *55*). However, we found that exposure to $OONO^-$, generated from SIN-1 decomposition, had an inhibitory effect on PGHS-2 activity in both of our culture systems. The reversal of the SIN-1-mediated inhibition of PGHS-2 activity by uric acid confirmed that $OONO^-$ was indeed the active species mediating this effect. Similar results with SIN-1 were reported in LPS-stimulated rat peritoneal macrophages (*9*) and rat microglia (*6*, *7*), strengthening the conclusion that $OONO^-$ can also have an inhibitory effect on PGHS-2 activity. These apparently paradoxical findings may be explained by the time of exposure of PGHS-2 to $OONO^-$ relative to the initiation of catalytic activity. In this regard, Boulos et al. (*57*) elegantly demonstrated that concomitant exposure to peroxynitrite or SIN-1 with AA (as in ref *55*) caused an enhancement of PG production while pre-exposure to $OONO^-$ or SIN-1 followed by AA (as in this study) suppressed prostanoid production (*57*).

Inhibition of PGHS-2 enzyme activity by $OONO^-$ could result from its ability to interact with critical tyrosine residues or with tyrosyl radicals, as is the case for ribonucleotide reductase (*58*). Spectral and biochemical data indicate that Tyr^{385} and Tyr^{371} are required for the cyclooxygenase reaction catalyzed by PGHS-1 and -2, respectively (*59*), and nitration of Tyr^{385} of PGHS-1 with tetranitromethane results in the

loss of cyclooxygenase activity (*59*−*61*). In fact, $OONO^-$ has been reported to inhibit platelet PGHS-1 activity by this exact mechanism (*57*). It is interesting to note that the removal of $NO^\bullet$ with CPTIO not only prevented the increase in PGHS-2 activity induced by DETA/NO and SNAP but also resulted in significant inhibition of PGHS-2 activity. As CPTIO works through stoichiometric conversion of $NO^\bullet$ to $NO_2^\bullet$ (*39*) and $NO_2^\bullet$, like $OONO^-$, is capable of nitrating protein tyrosine residues (*62*), it is intriguing to speculate that this inhibition resulted from nitration of Tyr^{371} as was suggested for $OONO^-$.

Finally, NO^- released by Angeli's salt caused stimulation of PGHS-2 activity in COS-7 cells transfected with murine PGHS-2. As already discussed, a substantial literature exists on the effects of other NOx species on PGHS-2 activity. This is to our knowledge the first report of the effect of NO^-. It is important to mention that NO^- is isoelectronic with oxygen and can thus also exist in a triplet ($^3NO^-$) or a singlet state ($^1NO^-$) (*63*). It has been reported that $^1NO^-$ tends to react with thiol groups whereas $^3NO^-$ favors reaction with $^3O_2$ to form $OONO^-$ (*64*, *65*). Evidence suggests that decomposition of AS results in the formation of $^1NO^-$ (*63*). The lack of an inhibitory effect of AS (should $^3NO^-$ and $^3O_2$ have formed $OONO^-$) on PGHS-2 activity in our system is consistent with this contention. However, given the ability of $^1NO^-$ to interact with thiols, one might suspect that AS might have inhibited the activity of PGHS enzymes as was discussed with regard to the NO^+ donors above. However, it is possible that NO^- was converted to $NO^\bullet$ by Cu/ZnSOD or another suitable physiological electron acceptor (*66*−*68*), and thus, it is $NO^\bullet$ and not NO^- that is responsible for the increase in PGHS-2 activity.

Taken in toto, our data demonstrate that the ultimate effect of NO on PGHS-2 enzyme activity and expression is predominantly dictated by the prevalent NO-related species ($NO^\bullet$, NO^+, NO^-, or $OONO^-$) formed and present. In a broader sense, our results suggest that the discrepant results reported, with regard to the effects of endogenously generated NO on PGHS-2 function, may be due to the fact that alternate NOx species were formed in different tissues. This may be directly related to the differential intracellular redox environment of each cellular system. As such, the cellular redox environment may establish the balance between NO and prostanoids, thus determining whether an inflammatory response is limited or sustained.

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