Regulation of Prostaglandin H_2 Synthase Activity by Nitrogen Oxides[†]

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Received December 28, 1998; Revised Manuscript Received June 15, 1999

ABSTRACT: Nitric oxide and its derivatives have been shown to both activate and inhibit prostaglandin H_2 synthase 1 (PGHS-1). We set out to determine the mechanisms by which different nitrogen oxide derivatives modulate PGHS-1 activity. To this end, we show that 3-morpholinosydnonimine hydrochloride (SIN-1), a compound capable of generating peroxynitrite, activates purified PGHS-1 and also stimulates PGE_2 production in arterial smooth muscle cells in the presence of exogenous arachidonic acid. The effect of SIN-1 in smooth muscle cells was abrogated by superoxide and peroxynitrite inhibitors, which supports the hypothesis that peroxynitrite is an activating species of PGHS-1. Indeed, authentic peroxynitrite also induced PGE_2 production in arachidonic acid-stimulated cells. In contrast, when cells were exposed to the nitric oxide-releasing compound 1-hydroxy-2-oxo-3-[(methylamino)propyl]-3-methyl-1-triazene (NOC-7), PGHS-1 enzyme activity was inhibited in the presence of exogenous arachidonic acid. Finally, in lipid-loaded smooth muscle cells, we demonstrate that SIN-1 stimulates arachidonic acid-induced PGE_2 production; albeit, the extent of activation is reduced compared to that under normal conditions. These results indicate that formation of peroxynitrite is a key intermediary step in PGHS-1 activation. However, other forms of NO_x inhibit PGHS-1. These results may have implications in the regulation of vascular function and tone in normal and atherosclerotic arteries.

It is well established that oxygenated metabolites of arachidonic acid play an important role in regulating vascular homeostasis (*1*). For instance, prostaglandin E_2 (PGE_2)[1] has been shown to stimulate vasodilation (*2*) as well as vasoconstriction (*3*), and to inhibit proliferation of smooth muscle cells (*4*). PGE_2 and other eicosanoids may also be important in regulating cellular cholesterol metabolism and in preventing the onset of atherosclerosis (*4*−*6*). Interestingly, changes in the levels of eicosanoids produced may promote the development of this disease state (*7*−*9*).

Since eicosanoids are involved in key cellular processes, there is considerable interest in understanding the factors that affect their synthesis. Eicosanoid biosynthesis is initiated by the enzyme prostaglandin H_2 synthase (PGHS), which metabolizes arachidonic acid to the cyclic endoperoxides, prostaglandin (PG) G_2, and PGH_2 by one of two PGHS enzymes. The first, PGHS-1, is constitutively expressed, and the second, PGHS-2, is an inducible form. The precise mechanism of oxygenation of arachidonic acid is unknown. The current literature suggests that catalysis is initiated by oxidation of the heme Fe(III) center by a peroxide, resulting in the formation of an Fe(IV)═O porphyrin π cation radical. This promotes electron transfer from a tyrosine residue (Tyr 385) located in the binding channel to generate a tyrosyl cation radical which abstracts a hydrogen atom from C-13 of arachidonic acid. Other aromatic amino acids that lie near the heme moiety may provide alternative pathways for electron abstraction (*10*). Oxygenation and cyclization of arachidonic acid leads to PGG_2 formation, which is subsequently reduced to PGH_2 by the enzyme's peroxidase activity (*11*, *12*). PGH_2 is the immediate substrate for prostacyclin synthase, which converts PGH_2 to prostacyclin (PGI_2). Alternatively, PGH_2 is converted to PGE_2 and PGD_2 by the appropriate isomerase enzymes.

Several lines of evidence suggest that the nitric oxide ($NO^{\bullet}$) and eicosanoid pathways may be linked (*13*−*20*). Investigations of the direct effect of $NO^{\bullet}$ or other nitrogen oxides (NO_x) on purified PGHS-1 enzyme activity have produced dichotomous results of activation (*14*, *21*−*23*), as well as inhibition (*24*−*26*). The reason for these reported differences may be due, in part, to the fact that the chemistry of $NO^{\bullet}$ under physiological conditions is complex where the formation of various NO_x species can occur (*27*). It is plausible that while one particular form of NO_x may stimulate PGHS-1 enzymatic activity, another form may be capable of inhibition, depending on the conditions of the experiment.

[†] This study was made possible by grants from the NIH (HL-46403 and HL-49666) awarded to D.P.H. R.K.U. was supported, in part, by an American Heart Association Scientist Development Award (9630223N), by a NIH training grant awarded to D.P.H. (HL-07423), and through a fellowship program sponsored by The Norman and Rosita Winston Foundation.

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[1] Abbreviations: PGHS-1, prostaglandin H_2 synthase 1; SIN-1, 3-morpholinosydnonimine hydrochloride; NOC-7, 1-hydroxy-2-oxo-3-[(methylamino)propyl]-3-methyl-1-triazene; NO_x, nitrogen oxides, including but not limited to nitric oxide ($NO^{\bullet}$), nitrosonium cation (NO^+), and peroxynitrite ($ONOO^-$); PGE_2, prostaglandin E_2; PGD_2, prostaglandin D_2; GSH-Px, glutathione peroxidase; GSH, reduced glutathione; SNAP, *S*-nitroso-*N*-acetylpenicillamine; CuDips, copper-(II) 3,5-diisopropylsalicylate hydrate; Tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid; SOD, superoxide dismutase; cat-LDL, cationized low-density lipoprotein.

10.1021/bi983049e CCC: $18.00
Published on Web 08/27/1999

In this paper, we set out to clarify the dichotomy in the variable impact of NO_x compounds on PGHS-1 activity. We monitored purified enzyme activity, as well as its activity in normal and in cholesterol-loaded vascular smooth muscle cells (an in vitro model system that can mimic atherosclerosis). The NO_x compounds that we used included 3-morpholinosydnonimine hydrochloride (SIN-1), which is often used to provide a continuous source of peroxynitrite (or a peroxynitrite-related species) (*23*, *28*, *29*), authentic peroxynitrite, and 1-hydroxy-2-oxo-3-[(methylamino)propyl]-3-methyl-1-triazene (NOC-7), which releases $NO^{\bullet}$ upon decomposition (*30*). Both SIN-1 and NOC-7 are vasodilators. SIN-1 is a metabolite of molsidomine (*N*-carboxy-3-morpholinosydnonimine ethyl ester) which is used in the clinical treatment of ischemic coronary heart disease (*31*), and NOC-7 has been shown to have vasodilatory effects (*32*). This work demonstrates that various forms of NO_x can have different modulatory effects on PGHS-1 activity. We have identified one form of NO_x, peroxynitrite, that is responsible for PGHS-1 activation and another form, $NO^{\bullet}$, that is inhibitory.

MATERIALS AND METHODS

The PGHS-1 enzyme from ram seminal vesicles [in Tris buffer (pH 7.8) containing 300 μM diethyl dithiocarbamate and 0.1% Tween 20] was obtained from Oxford Biomedical Research, Inc. (lot no. 040997WC; 40 000 units/mg). Arachidonic acid and [1-^{14}C]arachidonic acid (55 mCi/mmol) were obtained from Oxford Biomedical Research, Inc., and New England Nuclear-Dupont, respectively. SIN-1 and NOC-7 were purchased from Alexis Biochemicals. Peroxynitrite was from Cayman Chemicals. Tiron, desferrioxamine mesylate, and superoxide dismutase (from bovine erythrocytes, 2500−7000 units/mg) were from Sigma. CuDips was from Aldrich Chemicals, and GSH-Px (from bovine erythrocytes, 104 units/mg) was from Fluka. SNAP was synthesized according to a literature method (*33*).

Preparation of Anaerobic Stock Solutions of SIN-1. Anaerobic stock solutions of SIN-1 (10 mM) were prepared in a Schlenk tube, by adding SIN-1 powder (under argon gas) to deionized water that was degassed by three cycles of vacuum and argon gas. After SIN-1 addition, the Schlenk tube was once again subjected to a cycle of vacuum and argon gas.

Preparation of Stock Solutions of NOC-7. NOC-7 was prepared aerobically as a 10 mM stock solution in sodium phosphate buffer (pH 8.5). Since NOC-7 has a half-life of 10.1 min at pH 7.4 (*30*, *34*), it was used within 1 min of preparation.

Purified PGHS-1 Activity. PGHS-1 activity was measured as the percent conversion of [1-^{14}C]arachidonic acid to PGE_2 or PGD_2. First, PGHS-1 (200 units) was added to sodium phosphate buffer (100 mM, pH 8.0) containing phenol (500 μM) with or without GSH-Px (24−48 units/mL)/GSH (0.25 mM). Next, either SIN-1, peroxynitrite, SNAP, or NOC-7 was added, immediately followed by arachidonic acid (100 μM) containing 0.1 μCi of [1-^{14}C]arachidonic acid. The final volume was 1 mL. The mixture was incubated at 37 °C for 5 min and the reaction terminated by the addition of HCl (6.0 N). PGE_2, PGD_2, and unreacted arachidonic acid were extracted, identified by thin-layer chromatography, and quantified by liquid scintillation counting as previously described (*21*, *35*).

UV−Vis Spectroscopy. The concentration of peroxynitrite was determined by measuring its absorbance at 302 nm (ϵ = 1670 M^{-1} cm^{-1}) using a Beckman DU-64 spectrophotometer.

Smooth Muscle Cell Culture and Treatment. Smooth muscle cells were propagated from explants of rat thoracic aorta and cultured as previously described (*35*). Rat cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) glutamine. All cells were incubated at 37 °C in 5% CO_2 in air. Cells between passage 3 and 15 were used in experiments. We did not see significant variation in the extent of eicosanoid formation in response to passage number.

Rat smooth muscle cells were grown to near confluence in six-well plates (containing approximately 1×10^6 cells/well). The cells were quiesced for 24−48 h using serum-free DMEM (quiescent medium) containing 1% (v/v) ITS. The cells were then placed on ice, washed with ice-cold PBS (without Mg^{2+} or Ca^{2+}), and then exposed to either quiescent medium or quiescent medium containing either SIN-1, peroxynitrite, NOC-7, arachidonic acid, inhibitors, or mixtures thereof, for 1 h at 37 °C.

To ensure the success of the experiment with SIN-1, preparing SIN-1 stock solutions (10 mM) anaerobically as described above proved to be critical. SIN-1 was added to the quiescent medium (with and without arachidonic acid or inhibitors, or mixtures thereof) via a Hamilton syringe, only when the cells were on ice and after they had been washed with PBS. To check that the SIN-1 stock solution remained active and did not significantly decompose while the cells were being treated, we performed the experiment described in Figure 4 three times using the same SIN-1 stock solution. The treatment portion of each experiment, in which increasing concentrations of SIN-1 were added to two six-well plates, took approximately 10 min. Our results from these separate experiments showed that the SIN-1 stock solution retained its activity over the 30 min required to treat these cells. Moreover, in concordance with the data presented in Figure 4, the level of PGE_2 increased significantly with increasing SIN-1 concentrations ($P < 0.001$) while in the presence of exogenous arachidonic acid (10 μM).

After treatment, the supernatants were removed, immediately frozen, and analyzed later for PGE_2 (by enzyme immunoassay using a kit from Amersham). Some variability in the absolute quantities of PGE_2 produced by the cells was noted between experiments. For this reason, the data given in the figures are taken from a single experiment in which duplicate or triplicate samples were prepared (as stated in the figure legends). Each sample was assayed twice for PGE_2 content.

When cells were treated with superoxide or peroxynitrite inhibitors, stock solutions of desferrioxamine mesylate (5 mM), Tiron (0.5 M), and CuDips (1 mM in diethyl ether) were first prepared. Their final concentrations in quiescent DMEM were 250 μM, 10 mM, and 20 μM, respectively. In the case of CuDips, the final amount of diethyl ether in DMEM (2% v/v) did not affect PGE_2 production as compared to our control samples. When using SOD, solutions (150 units/mL) were prepared in quiescent DMEM. In those

experiments in which cells were exposed to peroxynitrite, we added the appropriate amounts of peroxynitrite from a stock solution (48 mM) directly to the well, containing quiescent DMEM, to yield final concentrations of 200 and 500 μM. The supernatant liquid was gently triturated to provide a homogeneous dispersion of peroxynitrite. To ensure that control cells were exposed to the same conditions, we also gently triturated the quiescent medium in control wells receiving no peroxynitrite.

For the treatment of cells with NOC-7, we prepared a 10 mM stock solution in sodium phosphate buffer (pH 8.5). NOC-7 was diluted with quiescent medium to a final concentration of 200 μM and used to treat the cells within 1 min of preparation. After treatment, the supernatants were removed and assayed as described above.

Preparation of Cat-LDL and Cat-LDL-Enriched Smooth Muscle Cells. LDL (1.019−1.063 g/mL) was isolated by the preparative ultracentrifugation of pooled donor human plasma using NaBr to adjust the density (*36*). LDL was cationized by covalently attaching *N*,*N*-dimethyl-1,3-propanediamine to aspartate and glutamate residues of LDL using 1-ethyl-3-[3-(dimethlyamino)propyl]carbodiimide hydrochloride as a catalyst at pH 6.5 (*37*, *38*). Agarose gel electrophoresis was used to demonstrate the successful derivitization of LDL to cat-LDL. Lipoproteins were dialyzed against NaCl (0.154 M), EDTA (0.3 mM), and $NaHPO_4$ (0.1 M, pH 7.4) for 48 h prior to concentration by ultrafiltration and added to DMEM just prior to the initiation of experiments. Fresh preparations of native and cat-LDL typically contain less than 2 nmol of thiobarbituric acid-reactive substances/mg of protein (*39*).

Near-confluent cell cultures were incubated with cat-LDL (50 μg of protein/mL) in DMEM containing 5% fetal bovine serum for 4−6 days. Fresh medium containing cat-LDL was added every 2−3 days. In control experiments, cells were exposed to DMEM containing 5% fetal bovine serum, which was replaced on the same days that medium containing cat-LDL was added to the cat-LDL-treated cells. The cells used as control cells and those that were loaded with cat-LDL were from the same passage (from the same rat) and were grown in parallel. Smooth muscle cells require several days of pretreatment (6 days minimum) before the lipid incorporated in the cells can be visualized using Oil Red O (*38*). The cell viability of cat-LDL treated cells was assessed by in situ staining with trypan blue, and the cells were found to exclude trypan blue to the same extent as untreated smooth muscle cells (*38*). Cells were rendered quiescent, prior to treatment with SIN-1.

Miscellaneous. Cellular and lipoprotein protein contents were measured by the method of Lowry (*40*), using bovine serum albumin as the standard. Cell viability was assessed by in situ staining with trypan blue. The number of cells was determined with a hemocytometer. Unless otherwise stated, data are reported as the average ± the standard error of the mean (SEM), with significant differences determined by a single-factor analysis of variance (ANOVA) or by the *t* test.

RESULTS

SIN-1 and Peroxynitrite Activate Purified PGHS-1 in the Presence of a Peroxide Scavenger. Figure 1 shows the effect of SIN-1 on purified PGHS-1 enzyme activity, which was measured as the percent conversion of arachidonic acid to (a) PGE_2 and (b) PGD_2. In our control experiment, arachidonic acid-induced PGHS-1 activity was similar with or without SIN-1 (Figure 1, black bars), with no significant changes in the response of either PGE_2 or PGD_2 to increasing SIN-1 concentrations (750−1000 μM). The observed activation is attributed to an initiator hydroperoxide (*41*), and was independent of SIN-1 concentration. The same experiment was then performed in the presence of a peroxide scavenging system comprising glutathione peroxidase (GSH-Px, 24−48 units/mL) and its substrate, glutathione (GSH, 0.25 mM) (*23*). GSH-Px is a selenocysteine-containing enzyme that catalyzes the reduction of hydroperoxides by GSH. Addition of GSH-Px/GSH destroys peroxide species that initiate PGHS-1 activity and reduces the extent of conversion of arachidonic acid to eicosanoids (*42*). In the presence of GSH-Px (24 units)/GSH (Figure 1, diagonally striped bars), SIN-1 significantly increased PGHS-1 activity ($p < 0.0005$ for both PGE_2 and PGD_2 production). Next, we repeated the experiment using a higher concentration of GSH-Px (48 units) (Figure 1, horizontally striped bars). Again, in the presence of GSH-Px (48 units)/GSH, SIN-1 significantly increased PGHS-1 activity ($p < 0.0005$ for both PGE_2 and PGD_2 production). However, increasing the concentration of GSH-Px resulted in an overall reduction in the extent of PGE_2 and PGD_2 production in the presence of SIN-1. The decrease in the extent of PGE_2 and PGD_2 production with increasing GSH-Px concentrations may be due to GSH-Px inactivation of peroxynitrite (*43*). Overall, the results show that SIN-1 activates purified PGHS-1 in the presence of GSH-Px/GSH which results in increased levels of PGE_2 and PGD_2.

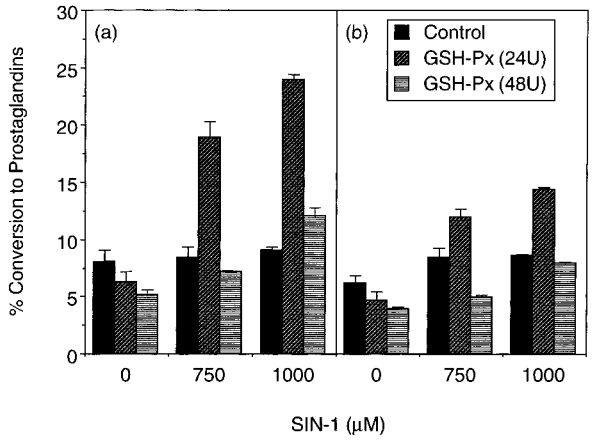

FIGURE 1: SIN-1 activates purified PGHS-1 in the presence of a peroxide scavenger. The levels of (a) PGE_2 and (b) PGD_2 observed in the absence of the peroxide scavenging system (black bars), in the presence of GSH-Px (24 units/mL) and GSH (0.25 mM) (diagonally striped bars), and in the presence of GSH-Px (48 units/mL) and GSH (0.25 mM) (horizontally striped bars). In each case, PGHS-1 (200 units) in sodium phosphate buffer (100 mM, pH 8.0) containing phenol (500 μM) was incubated with SIN-1 (0−1000 μM). The reaction was initiated by the addition of arachidonic acid (100 μM) containing 0.1 μCi of [1-^{14}C]arachidonic acid and terminated after 5 min. The eicosanoid products were extracted, separated, and quantified as described in Materials and Methods. The results are expressed as the percent of arachidonic acid converted to PGE_2 or PGD_2 per 200 units of PGHS-1 over the course of 5 min at 37 °C. The results are the average ± the standard error of the mean (SEM) of three replicates per treatment, and are representative of one experiment performed three times.

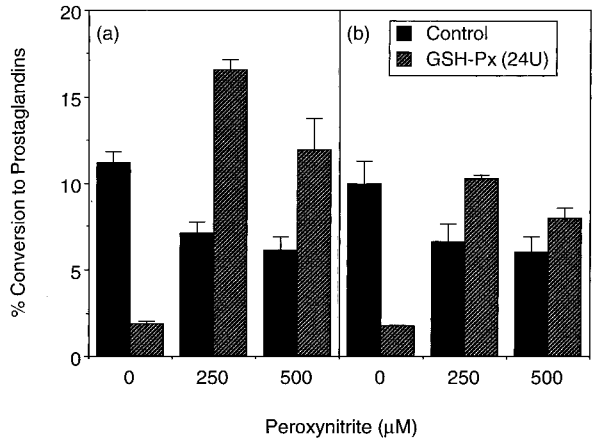

FIGURE 2: Peroxynitrite activates purified PGHS-1 in the presence of a peroxide scavenger. The levels of (a) PGE_2 and (b) PGD_2 observed in the absence of the peroxide scavenging system (black bars) and in the presence of GSH-Px (24 units/mL) and GSH (0.25 mM) (diagonally striped bars). In each case, PGHS-1 (200 units) in sodium phosphate buffer (100 mM, pH 8.0) containing phenol (500 μM) was incubated with peroxynitrite (0−500 μM). The reaction was initiated by the addition of arachidonic acid (100 μM) containing 0.1 μCi of [1-^{14}C]arachidonic acid and terminated after 5 min. The eicosanoid products were extracted, separated, and quantified as described in Materials and Methods. The results are expressed as the percent of arachidonic acid converted to PGE_2 or PGD_2 per 200 units of PGHS-1 over the course of 5 min at 37 °C. The results are the average ± SEM of three replicates per treatment, and are representative of one experiment performed three times.

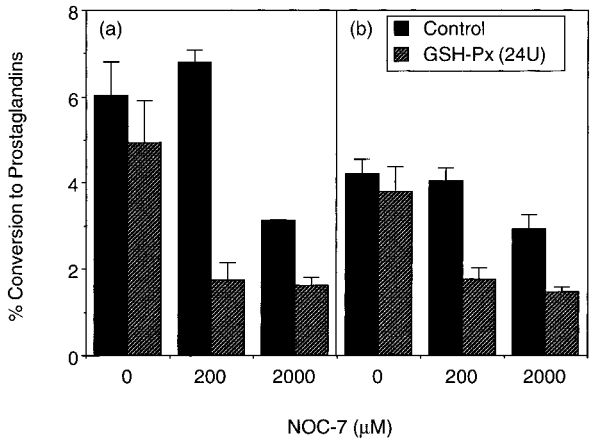

FIGURE 3: NO•-releasing compound NOC-7 inhibits purified PGHS-1 activity in the presence of a peroxide scavenger. The levels of (a) PGE_2 and (b) PGD_2 observed in the absence of the peroxide scavenging system (black bars) and in the presence of GSH-Px (24 units/mL) and GSH (0.25 mM) (diagonally striped bars). In each case, PGHS-1 (200 units) in sodium phosphate buffer (100 mM, pH 8.0) containing phenol (500 μM) was incubated with NOC-7 (0−2000 μM). The reaction was initiated by the addition of arachidonic acid (100 μM) containing 0.1 μCi of [1-^{14}C]-arachidonic acid and terminated after 5 min. The eicosanoid products were extracted, separated, and quantified as described in Materials and Methods. The results are expressed as the percent of arachidonic acid converted to PGE_2 or PGD_2 per 200 units of PGHS-1 over the course of 5 min at 37 °C. The results are the average ± SEM of three replicates per treatment, and are representative of one experiment performed three times.

To explore the hypothesis that peroxynitrite is the species responsible for activating purified PGHS-1 in the presence of GSH-Px/GSH, we repeated our experiment using authentic peroxynitrite (250−500 μM). Figure 2 shows the effect of peroxynitrite on (a) PGE_2 and (b) PGD_2 formation. In our control experiment (i.e., without GSH-Px/GSH), peroxynitrite decreased the extent of PGE_2 production ($p < 0.006$), but did not significantly alter the level of PGD_2 formation (Figure 2, black bars). As described above, the addition of GSH-Px (24 units)/GSH decreased arachidonic acid-induced PGHS-1 activity. However, addition of peroxynitrite to GSH-Px/GSH-treated PGHS-1 resulted in a significant increase in both PGE_2 and PGD_2 levels ($p \ll 0.0005$) (Figure 2, diagonally striped bars). Maximal PGE_2 formation was observed in the presence of 250 μM peroxynitrite and GSH-Px/GSH. Higher concentrations of peroxynitrite (>250 μM) had less of a stimulatory effect on PGE_2 production. It is interesting to note that in the presence of 250 μM peroxynitrite and GSH-Px/GSH, significantly more PGE_2 was produced compared to that in the control experiment without peroxynitrite and GSH-Px/GSH ($p < 0.005$). In summary, these data show that, under certain specific conditions, peroxynitrite enhances PGHS-1 activity.

S-Nitroso-N-acetylpenicillamine Does Not Alter PGHS-1 Activity. To determine whether NO_x compounds that are *S*-nitrosothiols can activate PGHS-1, we performed a PGHS-1 enzyme activity assay using *S*-nitroso-*N*-acetylpenicillamine (SNAP). SNAP is capable of taking part in transnitrosation reactions in which the nitrosonium ion (NO^+) is transferred to other thiols (e.g., Cys residues) (*44*). PGHS-1 contains three surface Cys residues (Cys 313, Cys 512, and Cys 540) that are potential targets for *S*-nitrosation. In the absence of GSH-Px/GSH, SNAP (100 μM to 8 mM) did not affect PGHS-1 activity (data not shown). Similarly, SNAP (100−750 μM) had no effect on PGHS-1 activity in the presence of GSH-Px (24 units)/GSH (0.25 mM) (data not shown). These data demonstrate that SNAP, an *S*-nitrosothiol, does not activate PGHS-1.

NOC-7 Inhibits Purified PGHS-1 Activity. Next, we investigated the effect of the NO•-releasing compound NOC-7 on purified PGHS-1 enzyme activity in the presence and absence of GSH-Px (24 units)/GSH (0.25 mM). When NOC-7 was employed, no enhancement in either PGE_2 (Figure 3a) or PGD_2 (Figure 3b) production was observed, either in the control experiment (black bars) or when utilizing GSH-Px (24 units)/GSH (diagonally striped bars). Thus, unlike SIN-1, NOC-7 does not liberate the type of NO_x species that leads to enhanced PGHS-1 activity. In fact, with or without GSH-Px, NOC-7 induced PGHS-1 inhibition, although the effective inhibitory concentration of NOC-7 was different in the two cases. In the control experiment, PGE_2 levels were significantly suppressed ($p < 0.005$) at a relatively high concentration of NOC-7 (2000 μM). The effect on PGD_2 formation at this concentration of NOC-7 was not significant. When GSH-Px (24 units) was present, a lower dose of NOC-7 (200 μM) led to significant PGHS-1 inhibition ($p < 0.05$ for both PGE_2 and PGD_2). These results indicate that once initiating hydroperoxides are scavenged, the inhibitory effect of NOC-7 on PGHS-1 enzyme activity becomes apparent at lower NOC-7 doses.

SIN-1 Enhances PGHS-1 Activity in Smooth Muscle Cells. We next performed experiments to determine whether PGHS-1 in smooth muscle cells isolated from rat aortas can be activated by SIN-1. Cells were grown to confluence and rendered quiescent so that they expressed PGHS-1, but not PGHS-2 (*45*). In this way, we were assured that any observed eicosanoid formation was due to the activity of the constitu-

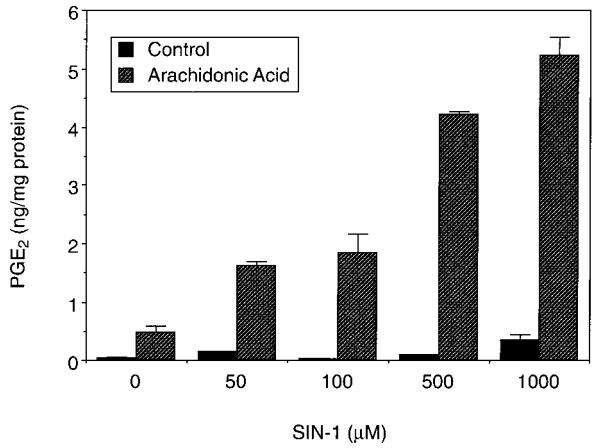

FIGURE 4: SIN-1 activates PGE_2 production in smooth muscle cells exposed to arachidonic acid. Aortic smooth muscle cells were grown to near confluence in six-well plates, quiesced for 42 h, and treated with serum-free DMEM containing either SIN-1 (0−1000 μM) (black bars) or SIN-1 (0−1000 μM) and arachidonic acid (10 μM) (diagonally striped bars). After incubation of the cells at 37 °C for 1 h, the supernatants were removed and assayed for PGE_2 formation. The results are the average ± SEM of two replicates per treatment, and are representative of one experiment performed more than three times.

tive form of the PGHS enzyme (PGHS-1), and not the inducible form (PGHS-2). Figure 4 shows that in the absence of exogenously added arachidonic acid, there was little or no stimulation of PGE_2 production by SIN-1 (0−1000 μM). However, in the presence of exogenous arachidonic acid (10 μM), PGE_2 levels increased significantly with increasing SIN-1 concentrations. Since smooth muscle cells possess a mechanism of regulating peroxide levels (*46*), it was not necessary to add the peroxide scavenging system comprising GSH-Px/GSH.

SIN-1-Enhanced PGHS-1 Activity Is Diminished by Superoxide and Peroxynitrite Inhibitors. We next attempted to suppress the effect of SIN-1 with suitable inhibitors of either superoxide or peroxynitrite. The following inhibitors were used: (a) desferrioxamine mesylate, a scavenger of peroxynitrite-derived oxidants (*47*−*49*), (b) copper(II) 3,5-diisopropylsalicylate hydrate (CuDips), which has superoxide dismutase-mimetic action, and is an effective quencher of oxygen radical generation (*23*, *50*, *51*), and (c) Tiron, a nonenzymatic cell-permeant chelator of superoxide (*52*−*57*). The addition of desferrioxamine mesylate, CuDiPs, or Tiron alone to smooth muscle cells did not affect PGE_2 formation compared with untreated cells (data not shown). The solid bars in Figure 5a−c show that co-incubation of smooth muscle cells with arachidonic acid (10 μM) and increasing concentrations of SIN-1 leads to a dose-dependent increase in the extent of PGE_2 formation, similar to that shown in Figure 4. However, the effect of SIN-1 was diminished by either desferrioxamine mesylate, CuDips, or Tiron (Figure 5a−c, striped bars).

Next, we repeated the above experiment using superoxide dismutase (SOD, 150 units/mL), which is known to react with both superoxide (*58*) and peroxynitrite (*59*). The results, shown in Figure 6, demonstrate that SOD abrogates the effect of SIN-1 on arachidonic acid-induced PGE_2 production in smooth muscle cells. These data are consistent with the hypothesis that SIN-1 first decomposes to peroxynitrite or a

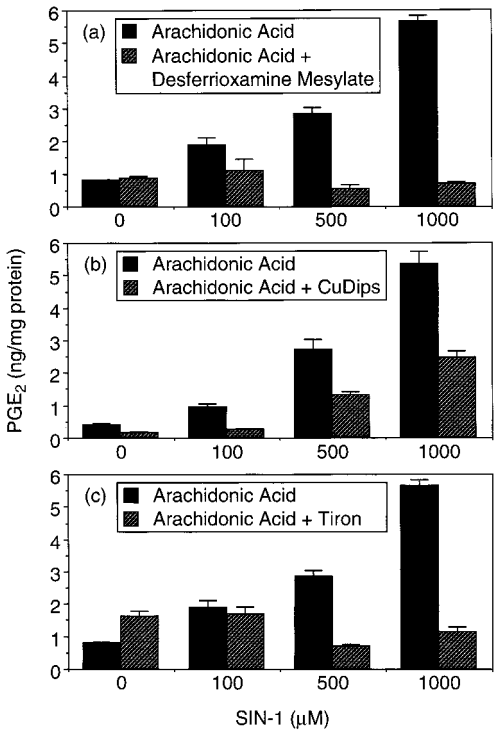

FIGURE 5: Inhibitors of peroxynitrite and superoxide diminish the level of SIN-1 activation of PGE_2 production in smooth muscle cells. Aortic smooth muscle cells were grown to near confluence in six-well plates, quiesced for 48 h, and incubated at 37 °C with serum-free DMEM containing arachidonic acid (10 μM) and SIN-1 (0−1000 μM) in the absence (black bars) and presence (diagonally striped bars) of an inhibitor. The following inhibitors were used: (a) desferrioxamine mesylate (250 μM), (b) CuDips (20 μM), and (c) Tiron (10 mM). The supernatants were removed after 1 h and assayed for PGE_2 formation. The results are the average ± SEM of two replicates per treatment, and are representative of one experiment repeated twice.

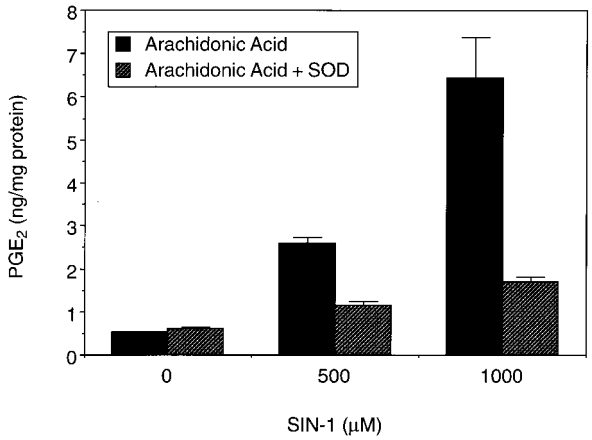

FIGURE 6: Superoxide dismutase diminishes the level of SIN-1 activation of PGE_2 production in smooth muscle cells. Aortic smooth muscle cells were grown to near confluence in six-well plates, quiesced for 48 h, and incubated at 37 °C with serum-free DMEM containing arachidonic acid (10 μM) and SIN-1 (0−1000 μM) in the absence (black bars) and presence (diagonally striped bars) of superoxide dismutase (SOD, 150 units/mL). The supernatants were removed after 1 h and assayed for PGE_2 formation. The results are the average ± SEM of three replicates per treatment, and are representative of one experiment repeated twice.

peroxynitrite-related species, which then activates PGE_2 formation.

Peroxynitrite Enhances PGHS-1 Activity in Smooth Muscle Cells. We next tested the effect of peroxynitrite on PGE_2

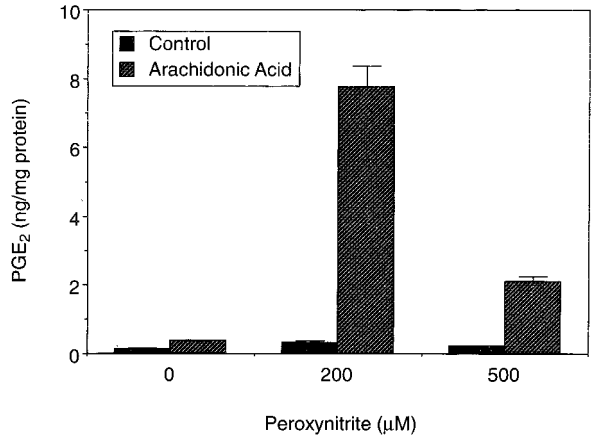

FIGURE 7: Peroxynitrite activates PGE_2 production in smooth muscle cells exposed to arachidonic acid. Aortic smooth muscle cells were grown to near confluence in six-well plates, quiesced for 48 h, and treated with serum-free DMEM containing either peroxynitrite (0−500 μM) (black bars) or peroxynitrite (0−500 μM) and arachidonic acid (10 μM) (diagonally striped bars). After incubation of the cells at 37 °C for 1 h, the supernatants were removed and assayed for PGE_2 formation. The results are the average ± SEM of three replicates per treatment, and are representative of one experiment repeated twice.

production in smooth muscle cells in the presence and absence of exogenous arachidonic acid. Figure 7 shows that in the absence of exogenously added arachidonic acid, the addition of peroxynitrite (200−500 μM) had a smaller effect on PGE_2 production as compared to the arachidonic acid group, over 1 h. However, when arachidonic acid (10 μM) was present, peroxynitrite significantly activated PGE_2 production ($p \ll 0.0005$). The level of PGE_2 production was higher using 200 μM rather than 500 μM peroxynitrite, implying that higher levels of peroxynitrite have less of a stimulatory effect on PGHS-1 activity. These levels of peroxynitrite did not cause cell death over the time course of the experiment, as determined by trypan blue exclusion.

Nitric Oxide-Releasing Compounds Inhibit PGHS-1 Activity in Smooth Muscle Cells. Smooth muscle cells were exposed to the NO•-releasing compound NOC-7 in the presence and absence of exogenous arachidonic acid. The levels of PGE_2 in the supernatants were measured after incubation for 1 h. Figure 8 shows that with or without exogenous arachidonic acid, NOC-7 failed to increase the level of PGE_2 formation in smooth muscle cells over basal levels. In fact, NOC-7 inhibited PGE_2 production when arachidonic acid was present ($P < 0.001$). Under these conditions, there was no significant cell death, as determined by trypan blue exclusion. Thus, unlike SIN-1, the NO•-releasing compound NOC-7 does not activate PGHS-1 in smooth muscle cells exposed to arachidonic acid.

SIN-1-Enhanced PGHS-1 Activity in Smooth Muscle Cells Is Altered under Atherogenic Conditions. Finally, we examined the effect of SIN-1 on PGE_2 formation in smooth muscle cells loaded with lipid in amounts that approximate the level seen in smooth muscle cells isolated from atherosclerotic rabbits fed a high-cholesterol diet (*60*). Cells were lipid-loaded with low-density lipoprotein (LDL) that was rendered polycationic by covalent attachment of *N*,*N*-dimethyl-1,3-propanediamine residues to the lipoprotein by the method of Goldstein et al. (*37*). This was done to increase the uptake of LDL by cells. In our hands, this derivitized LDL

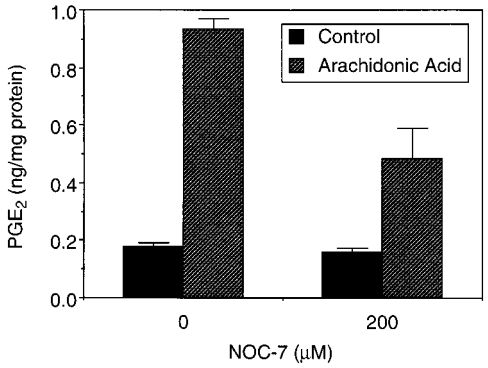

FIGURE 8: NO•-releasing compound NOC-7 inhibits PGHS-1 activity in smooth muscle cells. Aortic smooth muscle cells were grown to near confluence in six-well plates, quiesced for 48 h, and incubated at 37 °C with serum-free DMEM containing NOC-7 (0−200 μM) in the absence (black bars) and presence (diagonally striped bars) of arachidonic acid (10 μM). The supernatants were removed after 1 h and assayed for PGE_2 formation. The results are the average ± SEM of three replicates per treatment, and are representative of one experiment repeated twice.

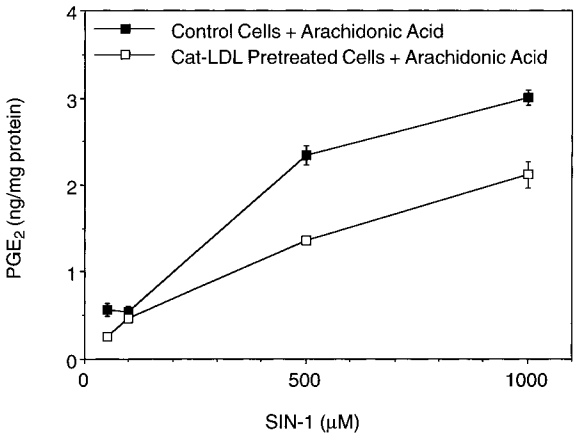

FIGURE 9: SIN-1 activates prostaglandin formation in cationized low-density lipoprotein-pretreated cells. Aortic smooth muscle cells were grown to near confluence in six-well plates, and grown in either DMEM (5% serum) (black squares) or DMEM (5% serum) containing 50 μg/mL cationized low-density lipoprotein (cat-LDL) for 4 days (□). The cells were rendered quiescent for 24 h with serum-free DMEM and then treated with serum-free DMEM containing arachidonic acid (10 μM) and SIN-1 (50−1000 μM) for 1 h at 37 °C. The supernatants were removed and assayed for PGE_2 formation. The results are the average ± SEM of two replicates per treatment, and are representative of one experiment repeated twice.

preparation was not cytotoxic to cells, nor did it induce apoptosis, protein, or DNA synthesis (*38*).

Figure 9 compares the effect of co-incubating smooth muscle cells with SIN-1 (50−1000 μM) and arachidonic acid (10 μM) for 1 h under normal conditions (control cells) and under conditions that model atherosclerosis (cells pretreated with 50 μg of protein/mL of cationized LDL for 4 days). In the presence of SIN-1, the level of arachidonic acid-induced PGE_2 production by cells pretreated with cat-LDL exhibited a dose-dependent increase, but these levels were reduced compared to those produced by control cells. In separate experiments, similar trends were obtained when the PGE_2 levels were normalized to cell count rather than to the total amount of protein (data not shown). This was done to exclude the possibility that the cat-LDL protein may be contributing to the overall protein measurement. Thus, although SIN-1

stimulates arachidonic acid-induced eicosanoid production in lipid-loaded smooth muscle cells, the extent of activation is reduced compared to that under normal conditions.

DISCUSSION

This paper demonstrates that different forms of NO_x have opposing effects on PGHS-1 enzyme activity. These opposing effects were observed either when using purified enzyme or when monitoring PGE_2 production in vascular smooth muscle cells. SIN-1 was found to activate PGHS-1 in the presence of arachidonic acid, while NOC-7 inhibited arachidonic acid-induced PGHS-1 activity. Thus, SIN-1 activation of PGHS-1 most likely occurs through the formation of a NO_x intermediate which is not formed during NOC-7 decomposition. SIN-1 is known to produce both superoxide and NO$^\bullet$ (*28*), which can combine at rates near the diffusion-controlled limit to form peroxynitrite (*61*). Peroxynitrite is likely the activating species, since the use of authentic peroxynitrite also stimulated increased PGE_2 production by purified PGHS-1 and in smooth muscle cells. NOC-7, on the other hand, is an NO$^\bullet$-containing zwitterionic polyamine that releases NO$^\bullet$ (*30*) with no inherent ability to form peroxynitrite.

To observe the stimulatory effects of SIN-1 and peroxynitrite and the inhibitory effect of NOC-7 on purified PGHS-1 enzyme activity, it was essential to add GSH-Px and its substrate, GSH. SIN-1 significantly increased purified PGHS-1 activity, once initiating fatty acid hydroperoxide species were removed by GSH-Px. However, when the GSH-Px concentration was increased, the effect of SIN-1 on PGHS-1 activity was reduced. We speculate that this is a consequence of GSH-Px metabolizing the active species produced upon SIN-1 decomposition. Indeed, GSH-Px has been shown to inactivate peroxynitrite in the presence of GSH (*43*). To test the hypothesis that peroxynitrite was the PGHS-1-activating species, we also used authentic peroxynitrite. In our hands, peroxynitrite increased purified PGHS-1 activity in the presence of GSH-Px and GSH, although high concentrations of peroxynitrite ($>250\ \mu$M) had less of a stimulatory effect. Interestingly, in the absence of GSH-Px and GSH, peroxynitrite had an inhibitory effect on purified PGHS-1 activity. The exact mechanism of peroxynitrite-induced PGHS-1 inactivation is not known, but may involve either oxidative inactivation (*62*) or modification of key amino residues in the polypeptide backbone (*63*). In separate experiments using NOC-7, we observed that high concentrations (2 mM) were required, in the absence of GSH-Px and GSH, before an inhibitory effect was observed. However, once the initiating fatty acid hydroperoxide species were removed by GSH-Px and GSH, much lower doses of NOC-7 (200 μM) led to PGHS-1 inhibition. This may demonstrate that NO$^\bullet$ released from NOC-7 is consumed by initiating hydroperoxide species. Indeed, NO$^\bullet$ is known to react with lipid peroxyl radicals at an almost diffusion-limited rate (*64*).

When using vascular smooth muscle cells, we observed that both SIN-1 and peroxynitrite stimulated PGE_2 production, while NOC-7 inhibited it. It was not necessary to add GSH-Px and GSH, since both components are found intracellularly (*65*). It is important to note that these cell experiments best represent the physiological response of PGHS-1 to the NO_x compounds, SIN-1, peroxynitrite, and NOC-7. The purified enzyme assay provides us with important information concerning the direct effects of certain components or mixtures of components on PGHS-1 activity, but it may be impossible to reproduce fully the levels of components that contribute to the overall redox state of the cell. Nevertheless, the purified enzyme assay results that best reflect our observations in smooth muscle cells are those in which GSH-Px and GSH were employed.

The concentrations of SIN-1, peroxynitrite, and NOC-7 that are effective at modulating PGHS-1 activity cannot be used as a direct measure of the amount of active species present. With respect to SIN-1, its decomposition yields relatively low levels of superoxide and NO$^\bullet$ under physiological conditions; e.g., 1 mM SIN-1 produces 7.02 μmol of superoxide/min and 3.68 μmol of NO$^\bullet$/min (*28*). Also, it is possible that SIN-1 and NOC-7 decomposition products are consumed in competing reactions. For instance, in addition to its reaction with GSH-Px (*43*), peroxynitrite also reacts with thiols (*23*, *66*) and other amino acid residues (*63*). NO$^\bullet$ released by NOC-7 is consumed by oxygen (*27*), and possibly in a reaction with GSH-Px which leads to its inactivation (*67*). With respect to our cell experiments, the concentrations of SIN-1, peroxynitrite, and NOC-7 were nontoxic in the time frame in which they were employed.

To show that the PGHS-1-activating species is likely peroxynitrite, in the presence of SIN-1, we used inhibitors of superoxide and peroxynitrite in our cell experiments. Desferrioxamine mesylate, CuDips, and Tiron strongly inhibited PGE_2 biosynthesis. While these studies with desferrioxamine mesylate, CuDips, and Tiron support the notion that peroxynitrite is the activating species of PGHS-1, it could be argued that these agents are nonspecific. For instance, desferrioxamine mesylate is known to chelate iron (*68*) and react with hydroxyl radicals (*69*). To overcome these potential problems, we also used SOD, which competes with NO$^\bullet$ for superoxide (*47*) and also deactivates peroxynitrite (*70*). SOD strongly inhibited arachidonic acid-induced PGE_2 production by SIN-1 in smooth muscle cells. The mechanism of this inhibition likely involves removal by SOD of superoxide or peroxynitrite produced during SIN-1 decomposition, which prevents subsequent PGHS-1 activation. Further evidence that peroxynitrite is the activating species is provided by the fact that authentic peroxynitrite stimulated PGE_2 production in cells. Taken together, these data are consistent with the hypothesis that peroxynitrite is the activating species.

It was recently demonstrated that peroxynitrite can serve as an excellent substrate for the peroxidase and cyclooxygenase activities of purified ram seminal vesicle PGHS-1 and human recombinant PGHS-2 (*23*). Our results are consistent with this previous study. We hypothesize that the mechanism by which peroxynitrite activates PGHS-1 likely involves oxidation of the heme moiety. Peroxynitrite, like most peroxides, has an O−O bond that is subject to attack, leading to the transfer of either OH^- or an oxygen atom (*71*). Peroxynitrite most likely activates PGHS-1 in a manner that is similar to peroxide activation of PGHS-1. Thus, in the process of activation, an Fe(IV)═O porphyrin π cation radical may be formed. Indeed, the reaction of peroxynitrite with myeloperoxidase, lactoperoxidase, and horseradish peroxidase results in the formation of short-lived intermediates involving perturbations of the heme moiety that are

detected by stopped-flow and rapid-scan UV−vis spectroscopy (*72*). The intermediates formed in the presence of peroxynitrite and PGHS-1 require characterization by these "fast" techniques.

We have previously reported that NO_x enhanced PGHS-1 activity, although the form of NO_x responsible for activating PGHS-1 was not identified (*21*). It is now clear from our latest work that peroxynitrite or a peroxynitrite-related species is the only activating species. We also provided evidence in our previous work that was consistent with NO_x causing *S*-nitrosation of three surface cysteine residues in PGHS-1 (Cys 313, 512, and 540), and proposed that this may be the mechanism of NO_x activation of PGHS-1. Although the three Cys residues in PGHS-1 are sites for *S*-nitrosation, the extent of *S*-nitrosation observed in the previous study may have been influenced by the acidic conditions of the *S*-nitrosothiol assay (*73*, *74*). In this study, we demonstrated that SNAP, an *S*-nitrosothiol capable of taking part in transnitrosation reactions, had no effect on purified PGHS-1 enzyme activity, either in the absence or in the presence of GSH-Px and GSH. Likewise, others have shown that both SNAP and *S*-nitrosated glutathione (GSNO, also an *S*-nitrosothiol) have no effect on PGHS-1 activity (*23*). Moreover, it is unlikely that the stimulatory effect of peroxynitrite is mediated by *S*-nitrosation, since it has recently been reported that the reaction between peroxynitrite and GSH yields *S*-nitroglutathione ($GSNO_2$), rather than GSNO (*75*). $GSNO_2$ decomposes either by spontaneously releasing $NO^\bullet$ or, if excess GSH is present, by forming disulfide (GSSG) and nitrite (*75*). Neither $NO^\bullet$ nor nitrite (*23*) is expected to activate PGHS-1. Thus, we conclude that PGHS-1 activation by either SIN-1 or peroxynitrite occurs by a mechanism other than *S*-nitrosation.

If peroxynitrite is indeed responsible for activating PGHS-1, it follows that NO_x compounds that do not possess the inherent ability to form peroxynitrite during their decomposition pathways are not expected to activate PGHS-1 directly. Thus, we found that the $NO^\bullet$-releasing compound NOC-7 did not activate PGHS-1, but actually has an inhibitory effect on purified PGHS-1 enzyme or when monitoring PGHS-1 activity in smooth muscle cells. Other investigators have also reported that $NO^\bullet$ has an inhibitory effect on PGHS activity. For instance, incubation of PGHS-1 with the $NO^\bullet$-releasing compound diethylamine nitric oxide (500 μM) resulted in some inhibition of cyclooxygenase activity (67% of control) (*26*). The mechanism of this inhibition may involve $NO^\bullet$ coupling with the Tyr 385 radical of PGHS-1 (*76*), leading to the eventual nitration of Tyr 385 (*77*), but could also involve loss and degradation of the heme moiety, as has been reported for other hemoproteins (*78*). In addition, $NO^\bullet$ may also interfere with eicosanoid production by reacting with fatty acid peroxyl radicals (*64*).

Finally, we demonstrate herein that under in vitro conditions that model lipid accumulation in vascular cells during atherosclerosis, SIN-1 increases PGE_2 levels in smooth muscle cells. Our laboratory has previously shown that the use of cat-LDL causes a 10−12-fold increase in the levels of cholesteryl ester (CE) stores in smooth muscle cells compared to cells exposed to LDL alone (*38*). Cells enriched with cat-LDL in this way resemble "foam cells" characteristic of foamy atherosclerotic lesions both morphologically and biochemically (*79*). Using this method, it was previously found that the levels of PGI_2 and PGE_2 were decreased in cat-LDL-enriched smooth muscle cells compared to those in control cells (*38*, *80*). The reduced PGI_2 and PGE_2 levels were found to be due, in part, to impaired transcription of mRNA for PGHS-1 (*39*). In this study, we demonstrate that SIN-1 is partially able to replenish PGE_2 levels in cat-LDL-enriched smooth muscle cells. Under these conditions, peroxynitrite may restore some of the impaired functions of atherogenic cells which result from reduced PGE_2 production. Therefore, peroxynitrite may be participating importantly in the arachidonic acid pathway in the vessel wall, but the cellular conditions for its production need to be further defined.

ACKNOWLEDGMENT

We are very grateful to Drs. Gerard Parkin, Jun Ho Shin, and Prasenjit Ghosh at Columbia University (New York, NY) for their assistance in the preparation of anaerobic solutions of SIN-1 and to Drs. Andrew C. Nicholson and Domenick Falcone at the Weill Medical College of Cornell University for helpful discussions and advice during the course of these investigations. We also thank Ms. Barbara Summers, Ms. Feng Xian Zhao, Mr. James Tauras, and Mr. Joel Rosenthal for expert technical assistance.

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BI983049E