Nitric Oxide Enhances Prostaglandin-H Synthase-1 Activity by a Heme-Independent Mechanism: Evidence Implicating Nitrosothiols[†,‡]

David P. Hajjar,* Harry M. Lander, S. Frieda A. Pearce, Rita K. Upmacis, and Kenneth B. Pomerantz

Contribution from the Department of Biochemistry, Cornell University Medical College, 1300 York Avenue, New York, New York 10021

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Abstract: A mechanism by which nitric oxide (NO) enhances prostaglandin-H synthase-1 (PGHS-1) activity is described. Under aerobic conditions, NO stimulates the conversion of arachidonic acid to prostaglandin E_2 (PGE_2) and PGD_2 in a dose- (5−100 μM) and time- (over 5 min) dependent manner. PGHS-1 possesses several potential targets for NO interaction; heme and Tyr 385 (both of which are necessary for catalytic function) and three reduced cysteines (Cys 313, 512, and 540), all of which are located in the catalytic domain. Our data demonstrate that NO activated PGHS-1 independently of heme. The Soret band absorbance of PGHS-1 was unaffected by NO in air but was increased by carbon monoxide (CO). Heme-NO conjugates added to apo-PGHS-1 inhibited PGHS-1 activity relative to the addition of heme alone. PGHS-1 activity was also inhibited by H_2O_2. NO also activated PGHS-1 independently of Tyr 385, since tetranitromethane treatment of PGHS-1 did not block the enhancement of PGHS-1 by NO. However, NO promoted formation of nitrosothiols in a dose-dependent manner which plateaued at 3 mol nitrosothiol/mol PGHS-1. The kinetics of nitrosothiol formation directly correlated with measurements of PGHS-1 activity. The formation of nitrosothiols occurred concomitantly with a significant change in the secondary structure of PGHS-1. Our data suggest that enhancement of PGHS-1 activity by NO occurs by S-nitrosation of cysteine residues located in the catalytic domain, with subsequent alterations in secondary structure.

Introduction

The mechanisms by which NO interacts with heme- and non-heme-containing proteins to regulate protein function are diverse and incompletely understood.[1−4] NO binds heme in certain heme-containing enzymes, such as guanylyl cyclase, resulting in activation.[5,6] NO, acting either through nitrosonium ions or related species, may also interact with cysteine residues, forming nitrosothiols[7−9] or with vicinal thiols to generate disulfide bonds.[4] This may result in alterations in protein function. Examples of nitrosothiol-mediated alterations in activity include the *N*-methyl-d-aspartate subtype of the glutamate receptor,[10] calmodulin-stimulated adenylyl cyclase,[11] and tissue plasminogen activator.[12] NO can also interact with tyrosine residues or tyrosyl radicals[6] resulting in inhibition of enzyme activity, as in the case of ribonucleotide reductase.[13]

PGHS-1 catalyzes the oxidation of arachidonic acid to prostaglandins (PG)G_2 and PGH_2 as well as three incomplete products, 9-, 11-, or 15-hydroxyeicosatetraenoic acids (HETEs).[14] Catalysis is initiated by the oxidation of heme by a peroxide, which in turn promotes electron transfer from Tyr 385 to generate the tyrosyl radical, followed by the abstraction of the 13-proS hydrogen of arachidonic acid. The cyclooxygenase function of PGHS-1 promotes stereospecific olefinic rearrangement and insertion of molecular O_2 across the 9,11 region of arachidonic acid to yield PGG_2, which is subsequently reduced by the enzyme's peroxidase activity to PGH_2.[15−18] PGG_2 and PGH_2 are highly unstable in aqueous solution and spontaneously hydrolyze to PGE_2, PGD_2, and 7-hydroxyheptadecaenoic acid (7-HHT). PGHS-1 consists of an N-terminal epidermal growth factor (EGF)-like domain, a membrane-binding domain, and a catalytic domain which is similar in structure to other peroxidases.[19]

The effect of NO on PGHS-1 is controversial. NO has been reported to inhibit,[20] activate,[21−26] or have no effect[27,28] on

[†] Keywords: nitric oxide, cyclooxygenase, prostaglandin H synthase-1, eicosanoids, heme, nitrosothiols.

[‡] Abbreviations: prostaglandin H synthase-1, PGHS-1; nitric oxide, NO.

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PGHS-1 activity. Furthermore, the mechanism(s) by which NO may alter PGHS-1 activity are unknown. This study was undertaken to determine if, and how, NO alters PGHS-1 activity under aerobic conditions. We provide enzymatic, spectral, and biochemical evidence demonstrating that NO enhances PGHS-1 activity by inducing alterations in secondary structure commensurate with S-nitrosation of cysteine residues located in the catalytic domain and not by interaction with heme or Tyr 385.

Results

NO stimulated the conversion of arachidonic acid to PGE_2, PGD_2, and 7-HHT in a dose-dependent manner. However, NO did not alter the conversion rate of arachidonic acid to 9-, 11-, or 15-HETEs (Figure 1). NO increased the V_{max} (61−101 dpm $\times 10^3$ in PGE_2 and PGD_2/200 U PGHS-1/5 min) nearly 2-fold and decreased the K_m by the same amount (57 to 33 μM). Thus, NO stimulates PGHS-1 activity by increasing its catalytic efficiency. In addition, the influence of NO on enhancement of PGHS-1 activity is time-dependent, with linear accumulation of product over time (Figure 2).

Four lines of evidence suggest that NO enhances PGHS-1 activity in a heme-independent manner. Firstly, heme, when added to heme-depleted PGHS-1 (apo-PGHS-1), significantly increased PGHS-1 activity, and this was prevented by prior treatment of the heme with NO. This occurred with the heme-Fe in the +2 and +3 oxidation states (Table 1). Secondly, incubation of PGHS-1 with carbon monoxide (CO, a heme-specific ligand, 50 μM) significantly reduced the percentage of arachidonic acid converted 20 ± 2 to 9 ± 1 PGE_2/200 U PGHS-1/5 min, mean ± SEM, $p < 0.05$. Thus, under conditions where NO- and CO-heme interactions exist, net reductions in PGHS-1 activity are observed. Thirdly, H_2O_2 inhibited PGHS-1 activity (Figure 3) as has previously been demonstrated,[15,29] which is due principally to the quenching of the active site heme, further suggesting that NO is not enhancing PGHS-1 activity by binding to heme.[15,29] Fourthly, spectral data showed that activating concentrations of NO did not significantly alter the Soret band absorbance of PGHS-1, although evidence for NO-heme binding in myoglobin was demonstrated by the detection of a Soret band with decreased intensity (Figure 4). In contrast, formation of a CO-heme adduct in PGHS-1 generated a Soret band with increased absorbance at the same wavelength as PGHS-1 (Figure 4). Collectively, these data support the conclusion that NO activates PGHS-1 independently of heme.

The role of Tyr 385 in mediating NO-enhancement of PGHS-1 activity was examined. Tetranitromethane (TNM, a reagent which irreversibly nitrates tyrosines) inhibits PGHS-1 activity.[15,30] Furthermore, TNM-inhibition of PGHS-1 activity can be prevented by pretreatment of the enzyme with ibuprofen, a nonsteroidal antiinflammatory drug which reversibly occupies a region of the arachidonic acid binding site (and whose effect

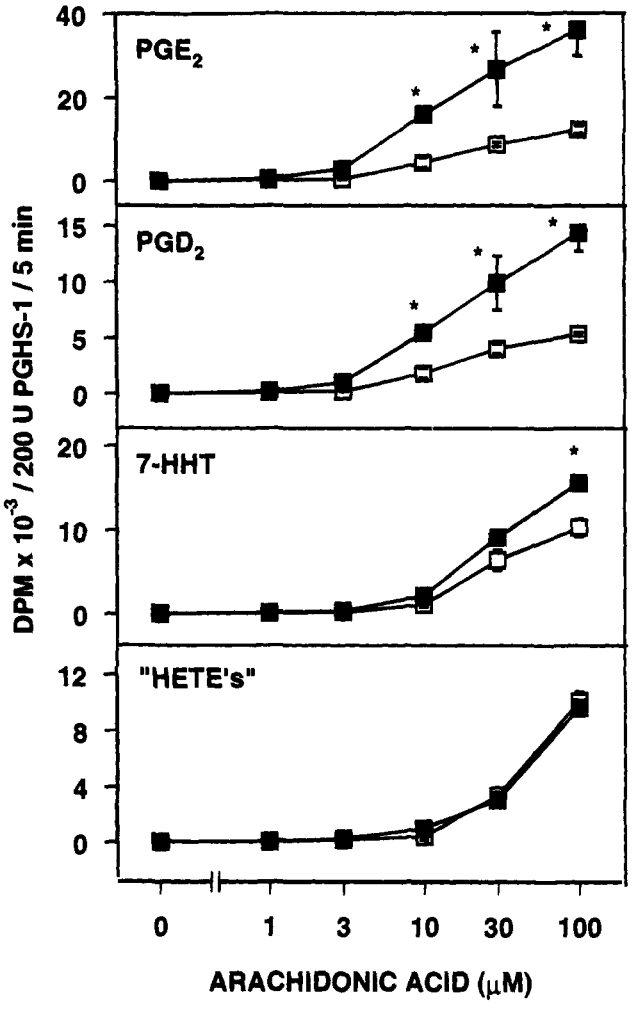

Figure 1. NO stimulates PGHS-1 activity—dose-response relationship: PGHS-1 (200 U in 100 mM Tris, pH 8.0) was exposed to Tris alone (□) or Tris containing 100 μM NO (■). After 5 min at 37 °C, samples were then exposed to increasing concentrations of [^{14}C]-arachidonic acid (0−100 μM) for 5 min at 37 °C, followed by extraction and quantitation of radiolabeled eicosanoids. Data are expressed as dpm $\times 10^{-3}$ of PGE_2, PGD_2, 7-HHT, and HETEs/200 U PGHS-1 /5 min (mean ± SEM). * = significantly different ($p < 0.05$).

is reversed upon dilution of the enzyme). This drug coordinates with Tyr 385 and thus inhibits catalysis. Ibuprofen thus prevents nitration of Tyr 385 by TNM through steric shielding.[15,30] As expected, TNM inhibited PGHS-1 activity, and the action of TNM on PGHS-1 was prevented by ibuprofen (Table 2). Moreover, NO increased activity of TNM-treated PGHS-1, whether or not the enzyme was pretreated with ibuprofen (Table 2). These data support the conclusion that NO activates PGHS-1 independently of Tyr 385.

To evaluate the possibility that NO increased PGHS-1 activity as a result of nitrosothiol formation, nitrosothiol content was measured under acidic conditions following a 5-min exposure of PGHS-1 (15 μM) to NO under aerobic conditions at pH 7.0. Glutathione, which contains 1 mol free cysteine/mol, was used as a positive control. Nitrosothiols were undetectable in untreated PGHS-1 or glutathione. After 5 min of exposure to NO, PGHS-1 nitrosothiol content dose-dependently increased and plateaued at 3 mol nitrosothiol/mol PGHS-1 (Figure 5). Nitrosothiol formation on glutathione plateaued at approximately 1 mol nitrosothiol/mol substrate (Figure 5). The dose-response kinetics of NO-induction of nitrosothiols paralleled the dose-response kinetics for enhancement of PGHS-1 catalytic activity (Figure 5). The observation that there were 3 mol nitrosothiol

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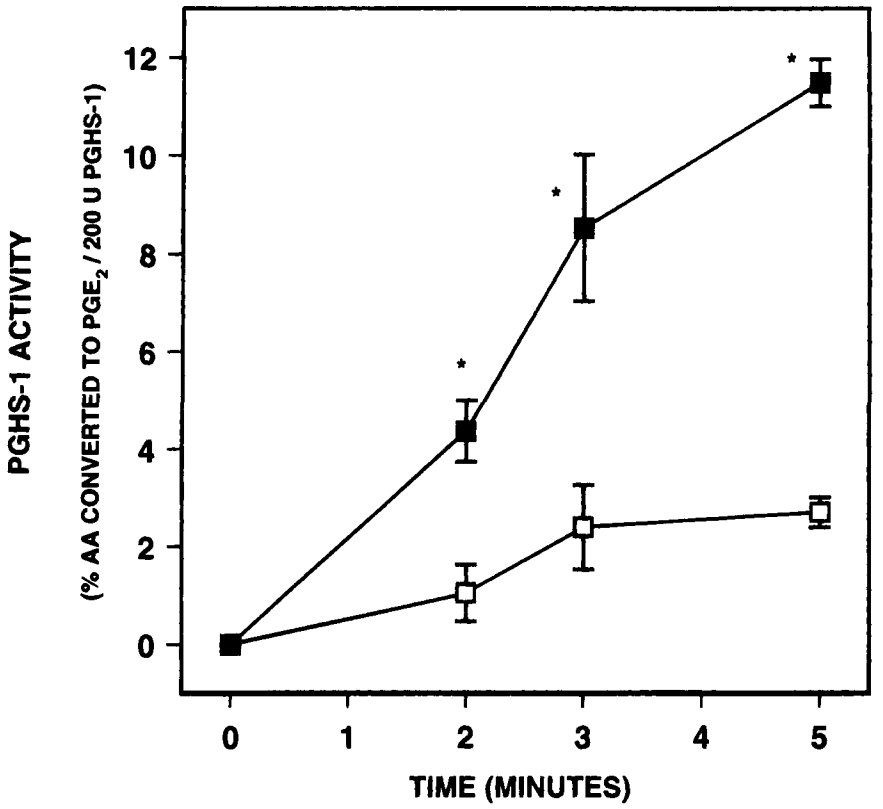

Figure 2. NO enhances PGHS-1 activity—time-course: PGHS-1 (200 U in 100 mM Tris, pH 8.0) was exposed to Tris alone (□) or Tris containing 100 μM NO (■). [^{14}C]arachidonic acid (100 μM) was then added, and the samples were then incubated at 37 °C for selected time-intervals. Eicosanoids were then extracted and quantified as described in the Experimental Section. Results are expressed as the percent of arachidonic acid converted to PGE_2/200 U PGHS-1 (mean ± SEM). * = significantly different ($p < 0.05$).

Table 1. Influence of NO-Substituted Heme on PGHS-1 Activity[a]

PGHS-1 FORM	native (Fe-III)	reduced (Fe-II)
APO-PGHS-1	1.9 ± 0.4	0.9 ± 0.4
APO-PGHS-1 + HEME	3.2 ± 0.3*	2.6 ± 0.1*
APO-PGHS-1 + NO-TREATED HEME	1.8 ± 0.1#	1.1 ± 0.2#

[a] PGHS-1 was washed with 100 mM Tris, pH 8.0. Hematin (Fe-III) (114 μM) was reduced to heme (Fe-II) using dithiothreitol (DTT, 1.0 mM). Fe-II and Fe-III hemes were gas-treated by sequential sparging with N_2 (10 min), NO (1 min), and N_2 (1 min). Apoprotein (3.57 μM) was reacted with DTT alone (25 μM) or with Fe-III or Fe-II hemes (2.86 μM) for 10 min at 25 °C. Aliquots (200 U) were then assayed for PGHS-1 activity using 100 μM arachidonic acid as substrate. Data (mean ± SEM) are expressed as percent of arachidonic acid converted to PGE_2/200 U PGHS-1/5 min (* = significantly different, $p < 0.05$ vs APO-PGHS-1, # = $p < 0.05$ vs heme).

equiv/mol PGHS-1 suggests that NO promoted formation of nitrosothiols with the three reduced cysteine residues which reside in the catalytic domain.

Circular dichroism (CD) spectroscopy was then performed to test the hypothesis that nitrosothiol formation of PGHS-1 promoted alterations in its secondary structure. Native PGHS-1 contains 39% α-helix and less than 1% β-pleated sheet (Figure 6).[19] NO induced a significant and dramatic increase in the percentage of β-pleated sheet conformation in PGHS-1, with a corresponding reduction in the percentage of α-helical and random coil conformation (Figure 6). Neither ibuprofen nor CO changed the secondary structure of PGHS-1. Thus, NO affected the secondary structure of PGHS-1 independently of its potential interaction with either heme or Tyr 385 (Figure 6).

Discussion

Here, we demonstrate that NO increased the catalytic efficiency of PGHS-1 under aerobic conditions in a dose- (Figure 1) and time- (Figure 2) dependent manner. In mechanistic studies, we systematically determined that the ability of NO to stimulate PGHS-1 activity occurred independently of putative targets for NO in the PGHS-1 active site, which include heme (Figures 3 and 4 and Table 1) and Tyr 385 (Table 2). Rather, our data suggest that NO stimulated PGHS-1 activity by the covalent modification of the protein through the generation of nitrosothiols (Figure 5), with subsequent alteration in secondary structure (Figure 6).

The observation that the enhancement of PGHS-1 activity by NO is heme-independent deserves additional comment. Heme is a constituent of the active site in PGHS-1 and is essential for catalytic activity. As shown in previous reports, NO will bind to PGHS-1 heme with high affinity, as determined by EPR.[28,31] However, these experiments were performed under anaerobic conditions, conditions where PGHS-1 is inactive, and where activation of PGHS-1 by NO was not observed.[28] Lack of PGHS-1 activity under anaerobic conditions may be due to lack of O_2, which is a necessary substrate for PGHS-1. The role of heme in PGHS-1 is thus clearly different from the role of heme in guanylyl cyclase, which is activated by both NO and CO following heme-binding.[32] In guanylyl cyclase, the heme is an allosteric regulator and not a component of the active site.[5] Thus, the known differences in the mechanisms by which NO enhances the activities of these two enzymes, coupled with the results reported herein, suggest that the *enhancement* of PGHS-1 activity by NO occurs independently of a NO–heme interaction under aerobic conditions.

The use of combined inhibitor studies (TNM in the presence and absence of ibuprofen) were originally designed to define

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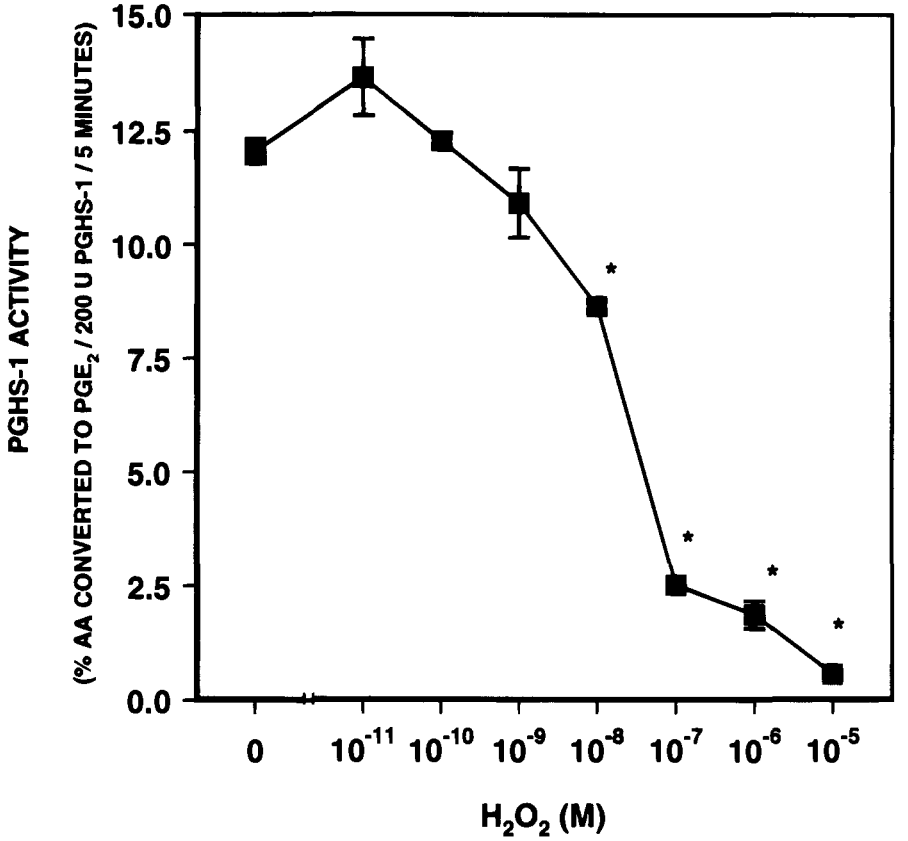

Figure 3. H_2O_2 inhibits PGHS-1 activity—dose response relationship: PGHS-1 (200 U in 100 mM Tris, pH 8.0) was exposed to Tris alone or Tris containing increasing concentrations of H_2O_2. After 5 min at 37 °C, PGHS-1 activity was measured by the conversion of [^{14}C]arachidonic acid (100 μM) to PGE_2. Data are expressed as the percent of arachidonic acid converted to PGE_2/200 U PGHS-1/5 min (mean ± SEM). * = significantly different, $p < 0.05$.

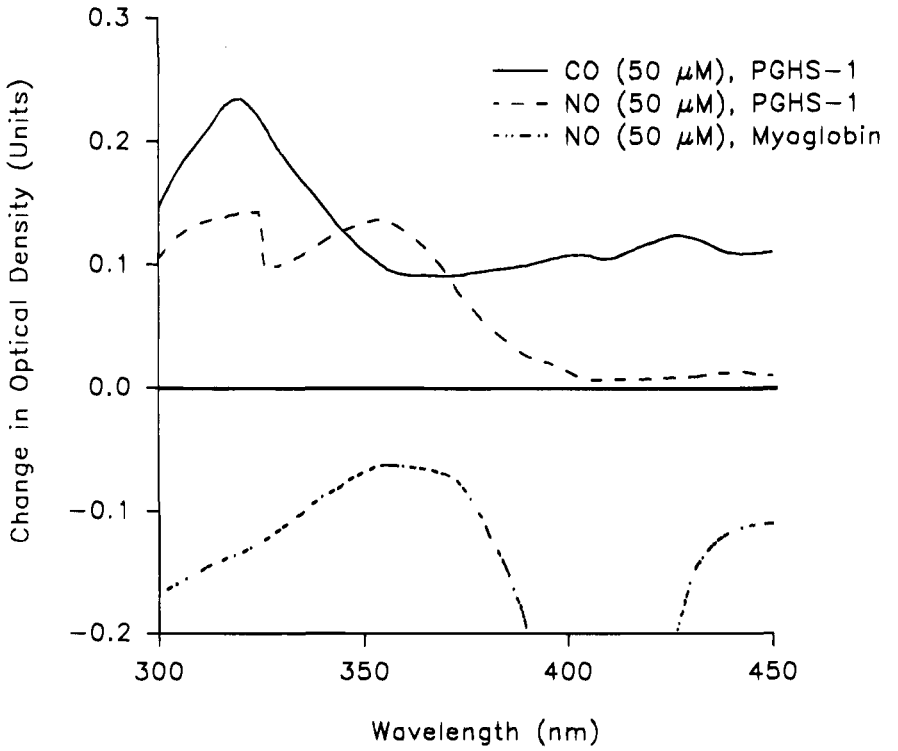

Figure 4. NO does not alter PGHS-1 heme (Soret) absorbance: UV spectroscopy was performed using a Perkin Elmer Lambda 2B spectrophotometer. Difference spectra were obtained using split cuvettes containing NO or CO and PGHS-1 or myoglobin. PGHS-1 (5 μM) or myoglobin (5 μM) were incubated with either NO or CO (50 μM) in 100 mM Tris, pH 8.0.

the obligatory role for Tyr 385 in PGHS-1 catalysis.[30] These observations were corroborated using site-directed mutagenesis studies in which Tyr 385 of PGHS-1 was replaced by Phe, which rendered the enzyme inactive.[30] Using the former method, our data suggest that the activation of PGHS-1 by NO is not due to interaction of NO with Tyr 385, since activation of PGHS-1

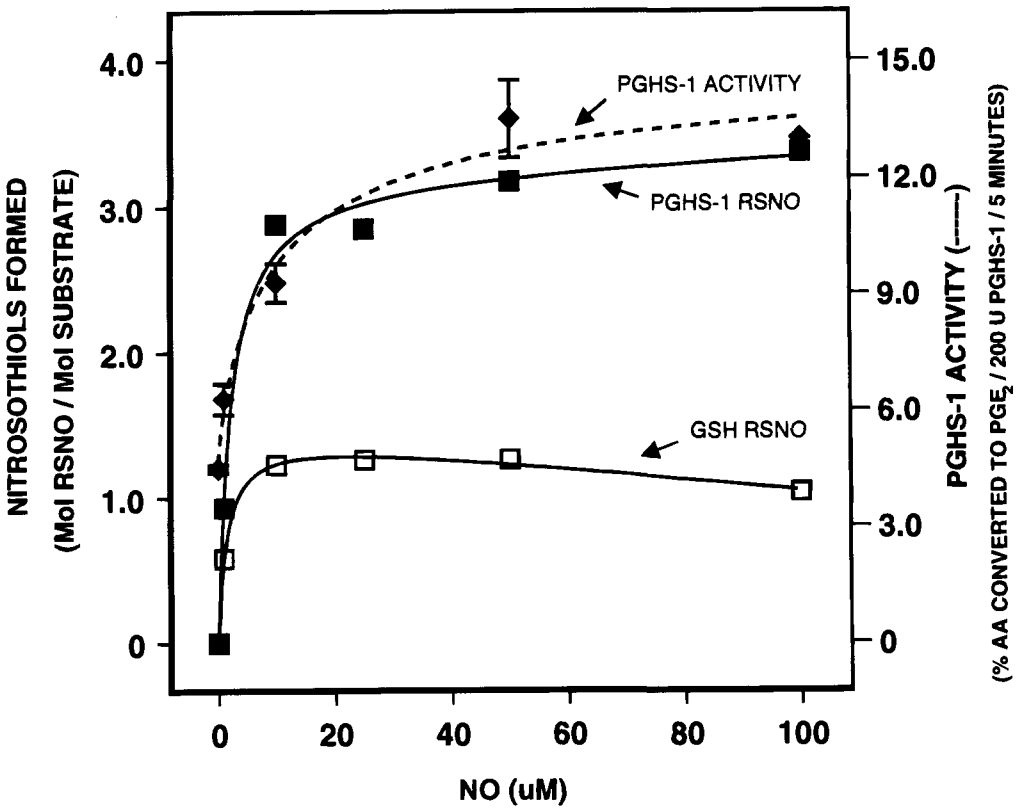

Figure 5. NO promotes nitrosothiol formation in PGHS-1—relationship to enzyme activity. Left axis: PGHS-1 (15 μM, ■), or glutathione (□, 31 μM) in 100 mM $KHPO_4$ buffer (pH 8.0) were exposed to increasing concentrations of NO (0−100 μM). After 5 min at room temperature, 20 μL aliquots were assayed for the presence of nitrosothiols as described in the Experimental Section. Data are expressed as mol nitrosothiol/mol substrate. Right axis: PGHS-1 (200 U of PGHS-1 was exposed to increasing concentrations of NO (0−100 μM) for 5 min. PGHS-1 activity was then determined using 100 μM arachidonic acid (- - ◆ - -). Data are expressed as percent of arachidonic acid converted to PGE_2/200 U PGHS-1/5 min (mean ± SEM).

Table 2. NO Activates PGHS-1 Independently of Tyr 385[a]

treatment	CTL	NO (50 μM)
PGHS-1	17.6	22.0
PGHS-1 + TNM	4.8	9.9
PGHS-1 + IBUPROFEN + TNM	18.2	23.0

[a] PGHS-1 (3.5 μM, 2000 U) was incubated with ibuprofen (200 μM, 30 min) prior to exposure to TNM (1.0 mM, 4 min) at 25 °C. PGHS-1 activity was then measured in duplicate 200 U aliquots upon dilution (which releases PGHS-1 from ibuprofen-inhibition[30]) using 100 μM arachidonic acid as substrate. Data are expressed as percent conversion of arachidonic acid to PGE_2/200 U PGHS-1/5 min.

was observed even in the presence of TNM. Systematic evaluation of NO-tyrosine interactions in PGHS-1 has not been performed. However, the EPR spectra of the tyrosyl radical of PGHS-1 has been analyzed and is essentially identical to the EPR spectra of the tyrosyl radical in ribonucleotide reductase.[17] Indeed, the activity of ribonucleotide reductase (like PGHS-1) is totally dependent upon the presence of its tyrosyl radical.[13] However, NO has been shown to *inhibit* ribonucleotide reductase activity by scavenging the tyrosyl radical.[13] This is in contrast to our findings, demonstrating that NO enhances PGHS-1 activity. Thus, while our data do not exclude the possibility that NO may be interacting with Tyr 385 under aerobic conditions, our data strongly suggest that an interaction between NO and Tyr 385 does not mediate the enhancement of PGHS-1 activity by NO.

Our results support the hypothesis that PGHS-1 is activated by NO through the generation of a nitrosothiol. Our data reveal that each of the three free cysteines in the catalytic domain of PGHS-1[19] are available for S-nitrosation (Figure 5). Indeed, the dose-response kinetics of the formation of nitrosothiols following exposure to NO are essentially similar to the dose-response kinetics for the enhancement of PGHS-1 activity by NO (Figure 5). At this time, it is unclear as to which cysteine(s) may mediate the enhancement of PGHS-1 activity by NO. However, it has been previously suggested that modifications of the free cysteine residues in PGHS-1 can influence its catalytic activity.[33] Cys 313 is located on α-helix H6, which traverses the entire length of the molecule and may be responsible for its structure. It is possible that alterations in S-nitrosation of Cys 313 may enhance the catalytic efficiency of PGHS-1 by either altering the position of Cys 313 relative to His 309 to facilitate catalysis or alter the secondary structure of PGHS-1 to render it more catalytically efficient. Cys 540 is located at the end of helix H17, which runs perpendicular to the proposed arachidonic acid binding channel. Cys 540 is located at a region which orients α-helix H17 to H5 and may thus be important as a structural element in PGHS-1. It is possible that S-nitrosation of Cys 540 may alter the geometry of the arachidonic acid binding channel to facilitate substrate entry and, hence, catalytic efficiency. We provide evidence that S-nitrosation of PGHS-1 significantly influences secondary structure commensurate with enhancement of its activity (Figure 6). There is precedent for this observation; Stamler et al. have demonstrated that NO stimulates tissue type plasminogen activator activity by promoting the S-nitrosation of a single cysteine (Cys 83).[12]

The chemical mechanism by which NO interacts with cysteines in PGHS-1 to enhance activity is currently unclear. NO itself will not nitrosate free thiols under anaerobic condi-

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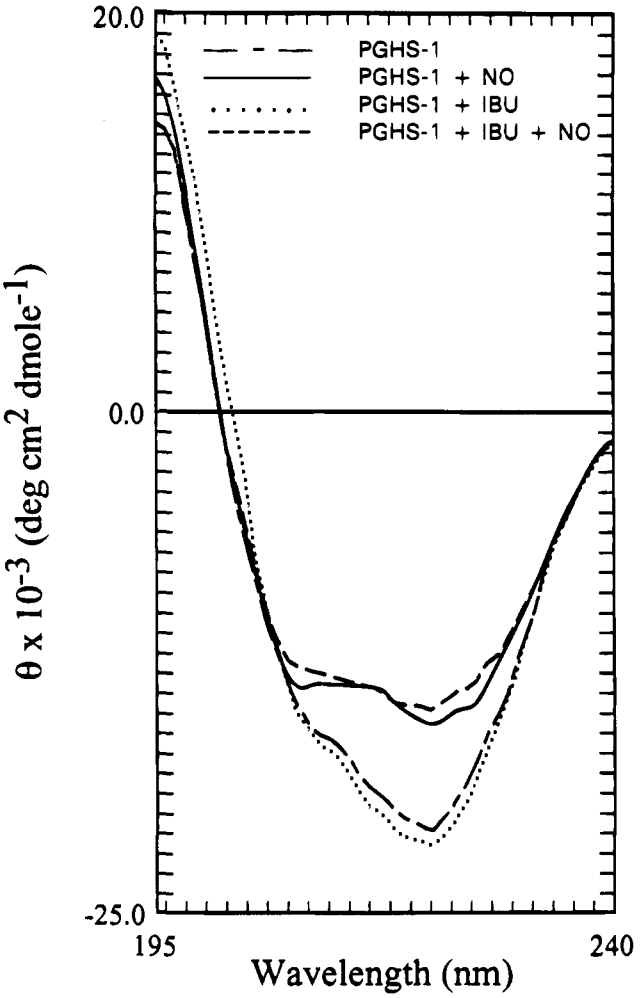

Figure 6. Influence of NO on secondary structure—CD analysis. Representative CD spectra of PGHS-1. CD-spectra of PGHS-1 (5 μM) were taken at 22 °C in 0.1 mm cuvettes in a Jasco 710 spectrophotometer. Samples were measured with a 0.2 nm bandwidth, a 0.2 nm step scan, and varying scan speeds of 60−100 nm/min. NO (50 μM) was added to control or ibuprofen (IBU)-treated (10 μM, 30 min) PGHS-1. Spectral characteristics were determined from 100 averaged scans. Quantitative analysis of data are shown below.

treatment	α-helix	β-sheet	turns & random coil
PGHS-1	39.2	0	58.7
PGHS-1 + NO	22.6	40.2	37.2
PGHS-1 + IBU	34.1	0	65.8
PGHS-1 + IBU + NO	20.5	40.9	38.5
PGHS-1 + CO	38.1	2.5	59.7

tions.[34] It has been suggested that higher oxides of NO mediate the generation of nitrosothiols, including NO^+, N_2O_4, N_2O_3, NO_2, HNO_2, or $ONOO^-$.[7,34−36] This raises the possibility that the enhancement of PGHS-1 activity observed with purified enzyme *in vitro* under aerobic conditions may reflect the ability of NO to enhance PGHS-1 activity in biological systems.[21,22,25,37]

In summary, we provide evidence to support the hypothesis that NO stimulates PGHS-1 activity through the formation of nitrosothiols and subsequent alterations in secondary structure which result in increased catalytic efficiency. We propose that the increase in catalytic activity induced by NO may be due to either increased efficiency of heme-binding (Cys 313) or increased substrate entry into the arachidonic acid binding channel (Cys 540).

Experimental Section

Materials. PGHS-1 (from ram seminal vesicles, specific activity of 50 000 U/mg), arachidonic acid, and phenol were purchased from Oxford Biomedical Corporation. The purity of the PGHS-1 preparations was monitored by SDS/PAGE electrophoresis; reduced PGHS-1 migrated as a single band at 70 kD, using Coomassie Blue staining. The enzyme was subjected to buffer exchange with 100 mM Tris (pH 8.0) to dilute diethyldithiocarbamic acid in the PGHS-1 preparation to a final concentration of less than 2.5 nM. PGHS-1 was then reconstituted to its original starting volume for further assays. 1-[^{14}C]-arachidonic acid (0.5 μCi/nmol) was obtained from New England Nuclear-Dupont (Boston, MA). Unlabeled prostaglandin standards (6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, TxB_2, PGE_2, and PGD_2) were purchased from Cayman Chemical (Ann Arbor, MI). Nitric oxide (NO) and carbon monoxide (CO) were supplied from Matheson. Saturated aqueous solutions of NO were prepared anaerobically by first sparging 100 mM Tris (pH 7.8) or 100 mM $NaHPO_4$ (pH 8.0) buffer in rubber-sealed tubes with N_2 (30 min) to remove O_2. NO was then directly bubbled into these buffers for 15 min without further treatment to remove higher oxides to achieve a final NO concentration of 1.25 mM.[38] The influence of these higher oxides was not evaluated. CO was similarly prepared to achieve a final concentration of 1.0 mM.[39] Organic solvents (reagent grade) were purchased from Fisher.

PGHS-1 Activity. PGHS-1 activity was measured as the percent conversion of [^{14}C]arachidonic acid (100 μM) to PGE_2, using 200 U PGHS-1 in 1.0 mL of Tris buffer containing phenol (0.1 mM) in the absence or presence of either NO or CO at 37 °C. Specific reaction conditions are described in the figure legends. The immediate PGHS-1 products, PGG_2 and PGH_2, are unstable in aqueous solution ($t_{1/2}$ of 30 s and 3 min, respectively) and spontaneously hydrolyze to PGE_2 and, to a much lesser extent PGD_2 and 7-HHT.[14,40] Therefore, we measured the accumulation of PGE_2, PGD_2, and 7-HHT as an index of PGHS-1 activity, rather than measure the flux of radioactivity through their unstable precursors, PGG_2 and PGH_2. Typically, samples were spiked with cold eicosanoid standards, acidified to pH 2.5 with 6.0 N HCl, and then extracted into ethyl acetate.[41] Each sample was then washed with water to remove excess acid and then re-extracted into ethyl acetate. Eicosanoids were then developed by TLC using silica gel G in the organic phase of a modified A9 buffer (ethyl acetate/isooctane/acetic acid/water, 110/50/20/100 v/v/v/v) after humidification for 30 min.[41] Eicosanoids were visualized by exposure to phosphomolybdic acid, and the radioactivity in zones corresponding to authentic eicosanoid standards was quantified by liquid scintillation counting. The incomplete PGHS-1 products, 9-hydroxyeicosatetraenoic acid (9-HETE), 11-HETE, and 15-HETE, comigrate in this solvent system and thus are termed "HETEs" in those experiments where HETEs are measured. Kinetic parameters of PGHS-1 activity were calculated using Enzfit (Elsevier Biosoft).

We have observed lot-to-lot variations in PGHS-1 activity. We have based our assays on the stated specific activity of the commercial PGHS-1 preparation. The range of activation of PGHS-1 activity by NO was up to 4.0-fold. Apo-PGHS-1 protein has residual activity since our washing conditions remove only up to 80% of protein-bound heme.[42] However, the amounts of heme in our apo-PGHS-1 preparations are not detectable by UV spectroscopy (no Soret band).

UV-Spectroscopy. PGHS-1 (approximately 2000 U) was diluted to 300 μL with 130 μL of 100 mM Tris (pH 7.8) to yield a final

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concentration of 10 μM. No additional hematin was added since the native enzyme as commercially prepared is approximately 75% saturated with heme. After equilibration at room temperature, spectra from 190−600 nm were obtained using a Perkin Elmer λ-2B spectrophotometer. NO was then added to a final concentration of 50 μM, and spectral analysis was performed after 3 min. Spectral data were digitized using a MacIntosh digital conversion program.

Circular Dichroism. CD spectra of PGHS-1 (5 μM in 100 mM Tris, pH 8.0) were taken at 22 °C in 0.1 mm cuvettes in a Jasco 710 spectrophotometer. Samples were measured with a 0.2 nm bandwidth, a 0.2 nm step scan, and varying scan speeds of 60−100 nm/min. The data were analyzed by comparisons to theoretical data files using a convex constraint algorithm obtained from Dr. A. Perczel et al.[43,44] Data analyses resulted in a 5−10% error.

Nitrosothiol Assay. The molar nitrosothiol content of PGHS-1 was determined by the method of Saville,[45] adapted for microassay. Briefly, PGHS-1, glutathione, or *S*-nitroso-*N*-acetylpenicillamine (SNAP, used as a nitrosothiol standard from 0−200 μM, 20 μL each) was added to 1.5 mL Eppendorf tubes. NO was then added to PGHS-1 or glutathione to the desired final concentration (0−100 μM) and allowed to incubate for 5 min at 25 °C (preliminary experiments revealed that nitrosoglutathione was stable for approximately 30 min— data not shown). Samples were then acidified by the addition of 100 μL of 0.2 N H_2SO_4. After 2 min, 200 μL of 0.5% ammonium sulfamate was added and allowed to incubate for 3 min at 25 °C. This was followed by addition of 160 μL of a solution containing 0.25% $HgCl_2$, 2.55% sulfanilamide, in 0.4 N HCl. After 5 min incubation at 25 °C, replicate 200 μL aliquots were transferred to microtiter wells, and the OD_{550} was obtained. Data are expressed as the mol of nitrosothiol/mol substrate. Controls included NO alone in the absence of protein up to 100 μM, and PGHS-1 alone in the absence of NO, neither of which reacted in the Saville microassay.

Miscellaneous. Protein content of PGHS-1 preparations was determined according to the method of Lowry.[46]

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