Tyrosine nitration in prostaglandin H_2 synthase

Ruba S. Deeb, Matthew J. Resnick, Dev Mittar, Timothy McCaffrey,[1] David P. Hajjar, and Rita K. Upmacis[2]

Center of Vascular Biology, Weill Medical College of Cornell University, 1300 York Avenue, New York, New York 10021

Abstract **In this study, we investigated the effects of various nitrogen oxide (NO_x) species on the extent of prostaglandin H_2 synthase-1 (PGHS-1) nitration in purified protein and in vascular smooth muscle cells. We also examined PGHS-1 activity under these conditions and found the degree of nitration to correlate inversely with enzyme activity. In addition, since NO_x species are thought to invoke damage during the pathogenesis of atherosclerosis, we examined human atheromatous tissue for PGHS-1 nitration. Both peroxynitrite and tetranitromethane induced Tyr nitration of purified PGHS-1, whereas 1-hydroxy-2-oxo-3-(*N*-methyl-aminopropyl)-3-methyl-1-triazene (NOC-7; a nitric oxide-releasing compound) did not. Smooth muscle cells treated with peroxynitrite showed PGHS-1 nitration. The extent of nitration by specific NO_x species was determined by electrospray ionization mass spectrometry. Tetranitromethane was more effective than peroxynitrite, NOC-7, and nitrogen dioxide at nitrating a tyrosine-containing peptide (12%, 5%, 1%, and <1% nitration, respectively). Nitrogen dioxide and, to a lesser extent, peroxynitrite, induced dityrosine formation. Using UV/Vis spectroscopy, it was estimated that the reaction of PGHS-1 with excess peroxynitrite yielded two nitrated tyrosines/PGHS-1 subunit. Finally, atherosclerotic tissue obtained from endarterectomy patients was shown to contain nitrated PGHS-1. Thus, prolonged exposure to elevated levels of peroxynitrite may cause oxidative damage through tyrosine nitration.**—Deeb, R. S., M. J. Resnick, D. Mittar, T. McCaffrey, D. P. Hajjar, and R. K. Upmacis. **Tyrosine nitration in prostaglandin H_2 synthase.** ***J. Lipid Res.*** **2002.** 43: **1718–1726.**

Supplementary key words peroxynitrite • atherosclerosis • cyclooxygenase

The rapid, diffusion-limited reaction of superoxide anion and nitric oxide ($NO^{\bullet}$) (1) leads to the formation of peroxynitrite ($ONOO^-$), which can initiate a number of toxic reactions in vivo. For example, $ONOO^-$ is capable of initiating lipid peroxidation (2), reacting with sulfhydryl groups (3), and causing oxidative damage by introducing tyrosine nitration (4). When produced by inflammatory cells during the immune response, $ONOO^-$ has a beneficial role by destroying invading microorganisms, but under pathological conditions, $ONOO^-$ assumes a deleterious role, and may contribute to vascular disease (5).

We have previously demonstrated that specific nitrogen oxides (NO_x) have different modulatory effects on the enzyme prostaglandin H_2 synthase (PGHS; also known as cyclooxygenase) (6). This enzyme, which exists in both the constitutive (prostaglandin H_2 synthase-1; PGHS-1) and inducible (prostaglandin H_2 synthase-2; PGHS-2) forms, plays an important role in regulating vascular homeostasis. While PGHS-1 mediates housekeeping functions (7), PGHS-2 is a mediator of inflammation and has been closely associated with tumorigenesis and atherosclerosis (8, 9). Both isozymes function by rapidly converting arachidonic acid to the cyclic endoperoxide prostaglandin H_2 (PGH_2), via a PGG_2 intermediate. PGH_2 can subsequently be metabolized to prostacyclin (PGI_2) by prostacyclin synthase. PGI_2 is known to regulate processes such as vasodilation and inhibition of both platelet aggregation and smooth muscle cell proliferation (10, 11). Investigations of the effect of $NO^{\bullet}$ on purified PGHS-1 enzyme activity have been controversial with reports of activation (6, 12, 13) as well as inhibition (14–16). The reason for these reported differences may be due, in part, to the complexity of $NO^{\bullet}$ chemistry, with the result that various NO_x species are formed, that include (but are not limited to): nitrosonium ion (NO^+), nitroxyl anion (NO^-), nitrite (NO_2^-), nitrate (NO_3^-), and peroxynitrite ($ONOO^-$) (17). While $ONOO^-$ activates PGHS-1 in the presence of arachidonic acid substrate (6, 13, 18), $NO^{\bullet}$ has an inhibitory effect (6). Peroxynitrite most likely activates PGHS-1 in a manner similar to that described for peroxide activation of PGHS-1 (19). Thus, in the process of activation, the

Manuscript received 17 May 2002 and in revised form 27 June 2002.
DOI 10.1194/jlr.M200199-JLR200

Abbreviations: NO_x, nitrogen oxides; NOC-7, 1-hydroxy-2-oxo-3-(*N*-methyl-aminopropyl)-3-methyl-1-triazene; PGHS-1, prostaglandin H_2 synthase-1; PGHS-2, prostaglandin H_2 synthase-2; SIN-1, 3-morpholinosydnonimine hydrochloride; TNM, tetranitromethane.

[1] Present address: The George Washington University Medical Center, 2300 Eye Street, N. W. Rm. 542, Washington, D.C. 20037.
[2] To whom correspondence should be addressed.
e-mail: rupmacis@med.cornell.edu

Fe(III) porphyrin in PGHS is converted to an Fe(IV)=O porphyrin π cation radical. Indeed, $ONOO^-$ reacts with Fe(III) porphyrins on a very fast timescale (5×10^7 $M^{-1}s^{-1}$) (20).

While $ONOO^-$ may stimulate PGHS activity via its reaction with the heme moiety, its presence may also lead to tyrosine nitration, a reaction that is up to 100-fold slower than its reaction with Fe of hemoproteins (18). In this regard, we have examined the nitrating ability of various NO_x reagents and have measured the extent of purified PGHS-1 nitration by $ONOO^-$. We have also examined the effect of $ONOO^-$ on PGHS-1 nitration in smooth muscle cells. Further, since $ONOO^-$ is thought to cause tissue damage during atherosclerosis (21, 22), we investigated the hypothesis that elevated levels of nitrated PGHS exist in human atheromatous tissue.

MATERIALS AND METHODS

Ram seminal vesicles, arachidonic acid, and phenol were obtained from Oxford Biomedical Research, Inc. Diethyldithiocarbamate (DDC), hematin, and a Tyr-containing peptide (Thr-Tyr-Ser) (minimum 95% purity; HPLC grade) were obtained from Sigma. Sodium peroxynitrite (33–48 mM in 0.3 M sodium hydroxide) was obtained from Cayman Chemical Company (≥90% purity; the balance is nitrate/nitrite); 1-hydroxy-2-oxo-3-(*N*-methyl-aminopropyl)-3-methyl-1-triazene (NOC-7) (99.9% purity) and 3-morpholinosydnonimine hydrochloride (SIN-1) from Alexis Corporation; tetranitromethane (TNM), sodium nitrite ($NaNO_2$) and ^{15}N-labeled nitrogen dioxide ($^{15}NO_2^{\bullet}$) from Aldrich. Monoclonal PGHS-1 and PGHS-2 antibodies and purified PGHS-2 protein were purchased from Cayman Chemical Company; monoclonal (clone 1A6) and rabbit polyclonal 3-nitrotyrosine antibody from Upstate biotechnology; anti-mouse and anti-rabbit horseradish peroxidase conjugated IgG was from Amersham Life Science Inc. Protein A and G agarose beads were purchased from Santa Cruz. Tween-20, nitrocellulose and PVDF membranes were obtained from BioRad Laboratories; nonfat dry milk was from Santa Cruz. ECL and ECL Plus were obtained from Amersham.

Isolation and purification of PGHS-1 enzyme from ram seminal vesicles

The isolation and purification of PGHS-1 from ram seminal vesicles was performed according to previously published literature methods (23).

Measurement of cyclooxygenase activity by oxygen monitor

Cyclooxygenase activity was measured using an oxygen electrode (YSI Model 53) attached to a recorder (Hitachi 561) according to previously published methods (24). One unit of cyclooxygenase activity represents a peak velocity of 1 nmol of oxygen/min.

Preparation of samples for mass spectrometric and UV/Vis spectroscopic experiments

The reaction of several NO_x compounds ($^{15}NO_2^{\bullet}$, $ONOO^-$, TNM, and NOC-7) with a Tyr-containing peptide (Thr-Tyr-Ser; mw = 369.4) was monitored by mass spectrometry and UV/Vis spectroscopy. These reactions were conducted in ammonium bicarbonate buffer (20 mM; pH 7.4) at ambient temperature for 1 h. For the reaction with $^{15}NO_2^{\bullet}$, the peptide was deaerated in a Thunberg cuvette using a vacuum/argon gas manifold and exposed to $^{15}NO_2^{\bullet}$ gas (1 atm). When using $ONOO^-$, we added $ONOO^-$ from a stock solution directly to the peptide; for experiments using TNM, we prepared a stock solution (200 mM) in ethanol; and when using NOC-7, we prepared a stock solution (10 mM) in deionized water. Unless otherwise indicated, 5 mM peptide was reacted with equimolar concentrations of the NO_x compound (in the case of $^{15}NO_2^{\bullet}$, we exposed 5 mM peptide to 1 atm $^{15}NO_2^{\bullet}$ gas). Following the addition of either $ONOO^-$ or NOC-7 to the peptide, the pH of the solution was adjusted to pH 7.4, if necessary.

Preparation of PGHS-1 samples for UV/Vis spectrometric analysis involved adding $ONOO^-$ (1 mM) to purified PGHS-1 (7–13 μM) in ammonium bicarbonate for 1 h at ambient temperature. The mixture contained residual Tris buffer from the protein purification process. Final concentrations of Tris and ammonium bicarbonate were typically 5 mM and 15 mM, respectively.

Mass spectrometry

Electrospray ionization mass spectra were obtained on a Quattro II triple quadrupole mass spectrometer (Micromass, Beverly, MA) fitted with a standard electrospray probe. Samples from the nitration experiments (described above) were diluted (~50 μM) in 50:50:2 (v/v/v) methanol-water-acetic acid mixtures and infused (5 μl/min) via a syringe pump (Harvard Apparatus, Natick, MA). The capillary voltage, cone voltage, and source temperature were kept at 3.5 kV, 18 V, and 80°C, respectively. In tandem mass spectrometric experiments, argon (3.6×10^{-4} mbar) was used as the collision gas (20 V collision energy). To take advantage of the selective detection capability of the tandem mass spectrometer, each sample was analyzed in the Neutral Loss scanning mode as well as in the Single Quadrupole scanning mode of the instrument. In the collision induced decomposition (CID) of the singly protonated Thr-Tyr-Ser peptide ion, a facile channel is the cleavage of the peptide bond between Tyr and Ser residues. The loss of a neutral serine molecule results in a b_2 ion (25), which is 105 Da lighter than its precursor. When the two quadrupoles bracketing the collision chamber are scanned in tandem offset by 105 Da (Neutral Loss 105 mode), the Thr-Tyr-Ser peptide and species that are potentially modified on Tyr are selectively detected. Entirely similar results were observed in Single Quadrupole scans (spectra not shown), but the product peaks were difficult to interpret at low conversions when not well separated from unavoidable background.

The extent of nitration was calculated by taking the peak intensity of the nitrated peptide and dividing it by the total intensity of peaks and multiplying by 100. In total, ten peaks were observed ascribed to *i*) unreacted peptide (m/z = 370.2), *ii*) Na^+ adduct of unreacted peptide (m/z = 392.2), *iii*) nitrated peptide (m/z = 415.2), *iv*) Na^+ adduct of nitrated peptide (m/z = 437.2), *v*) non-covalent dimer (m/z = 739.3), *vi*) Na^+ adduct of non-covalent dimer (m/z = 761.3), *vii*) singly nitrated non-covalent dimer (m/z = 784.3), *viii*) covalent dimer (m/z = 737.3), *ix*) Na^+ adduct of covalent dimer (m/z = 759.3), and *x*) singly nitrated covalent dimer (m/z = 782.3). The control sample of the Thr-Tyr-Ser peptide showed 86.2% (*i*), 4.9% (*ii*), 7.1% (v), and 1.8% (*vi*); the $^{15}NO_2^{\bullet}$-reacted peptide showed 76.1% (*i*), 6.2% (*ii*), 0.3% (*iii*), 7.9% (*v*), 1.7% (*vi*), and 7.9% (*viii*); the $ONOO^-$-reacted peptide showed 71.3% (*i*), 12.9% (*ii*), 4.5% (*iii*), 0.4% (*iv*), 1.0% (*v*), 1.6% (*vi*), 1.2% (*vii*), 4.8% (*viii*), 1.7% (*ix*), and 0.7%(*x*); TNM-reacted peptide showed 70.5% (*i*), 7.0% (*ii*), 11.5% (*iii*), 6.0% (*v*), 1.5% (*vi*), 1.5% (*vii*), 1.7% (*viii*), and 0.4% (*x*); the NOC-7-reacted peptide 86.1% (*i*), 4.8% (*ii*), 0.7% (*iii*), 7.3% (*v*), and 1.0% (*vi*). We caution that these numbers are only

estimates of the relative efficiency of the various nitrating agents used. The calculations assume that *a*) the ionization efficiency of the Thr-Tyr-Ser peptide and its nitrated form are equal, *b*) the peptide and nitrated peptide are detected with equal efficiency, and *c*) the efficiency of Tyr-Ser peptide bond cleavage by collision induced decomposition (CID) is unaffected by the presence or absence of the NO_2 group.

UV/Vis spectroscopy

Experiments were performed using a Perkin Elmer Lambda 20 spectrophotometer. Matching quartz suprasil® precision cells (Hellma, 1 cm pathlength; 1 ml or 100 μl volume) were used. The concentration of $ONOO^-$ was determined by measuring its absorbance at 302 nm (ε = 1,670 M^{-1} cm^{-1}) (26). The extent of nitration of the Thr-Tyr-Ser peptide by specific NO_x reagents was calculated by measuring the absorption at 428 nm (ε = 4100 M^{-1} cm^{-1}) (27) at pH 9 after subtracting the unreacted peptide. The extent of apo- and holo-PGHS-1 nitration was calculated by measuring the absorption at 428 nm at pH 9, and subtracting any contribution at pH 6. Holo-PGHS-1 was prepared by optically titrating apo-PGHS-1 with hematin. A 1:1 hematin-protein complex was created by monitoring the Soret Band of PGHS-1 at 410 nm.

Treatment of purified PGHS-1 with nitrating agents

Purified holo-PGHS-1 (0.1 M Tris buffer) was exposed to a nitrating agent for 1 h at 37°C as follows. For experiments using $ONOO^-$, the appropriate amount of stock $ONOO^-$ (38–48 mM) was added directly to the holo-PGHS-1 solution. SIN-1 was prepared as an anaerobic stock solution (10 mM) in a Schlenk tube as described previously (6). The appropriate amounts of SIN-1 were directly added to the protein via a Hamilton syringe. For experiments using TNM, fresh stock solutions (200 mM) were prepared in ethanol. For the treatment of PGHS-1 with NOC-7 or nitrite, stock solutions (10 and 20 mM, respectively) were prepared in deionized water.

Smooth muscle cell culture

Smooth muscle cells were propagated from explants of rat thoracic aorta and cultured as previously described (6). Rat cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) glutamine as described previously (6). Cells between passage 8–15 were used in experiments. We did not see significant variation in our results in response to passage number.

Treatment of smooth muscle cells with $ONOO^-$, tetranitromethane, or NOC-7

Rat smooth muscle cells were grown to near-confluence in 10 cm diameter dishes (containing approximately 8.8×10^6 cells/dish). The cells were quiesced for 24 h using serum-free DMEM (quiescent medium) containing 1% (v/v) ITS to suppress PGHS-2 expression (6). The cells were then exposed to either quiescent medium or quiescent medium containing $ONOO^-$, TNM, or NOC-7 for 1 h at 37°C, as described below.

For experiments in which cells were exposed to $ONOO^-$, we added the appropriate amount of $ONOO^-$ from a stock solution (38–48 mM) directly to the dish containing quiescent DMEM to yield the desired concentrations (6). For experiments using TNM, we prepared fresh stock solutions of TNM (200 mM) in ethanol and added the appropriate amounts to quiescent medium within 1 min of preparation. The final ethanol volumes (0.05−0.25%) did not affect cell viability. For the treatment of cells with NOC-7, we prepared a 10 mM stock solution in deionized water (6).

Preparation of smooth muscle cells for Western blotting

Following treatment with $ONOO^-$, TNM, or NOC-7, the cells were washed once with cold PBS (7 ml; without Mg^{2+} or Ca^{2+}), and then ice-cold lysis buffer (500 μl; containing 0.15 M NaCl, 100 mM Tris pH 8.0, 1% Tween-20, 1 mM EDTA, 1 mM PMSF, and 10 μg/ml aprotinin and 10 μg/ml leupeptin) was added to each dish. The cells were scraped, put on ice for 30 min, briefly sonicated (Branson Sonicator), and clarified by centrifugation for 10 min (9,500 *g*) at 4°C. The protein content of the supernatants was measured by a modified Lowry method (28).

Detection of 3-nitrotyrosine in NO_x-treated cells or in NO_x-treated purified PGHS-1 by Western blotting

Protein (5–40μg) was separated on a 10% acrylamide gel and transferred onto a nitrocellulose membrane. The membrane was immunoblotted with either a mouse monoclonal or a rabbit polyclonal 3-nitrotyrosine antibody according to manufacturer's instructions (Upstate Biotechnology). The bands were visualized using enhanced chemiluminescence (ECL or ECL^+). Blots were stripped by agitating at 50°C for 30 min in Tris buffer (1 M, containing 20% SDS and 100 mM β-mercaptoethanol at pH 6.7) and reprobed with an anti-mouse PGHS-1 antibody.

Immunoprecipitation of nitrated PGHS-1 from rat smooth muscle cells

Rat smooth muscle cells were lysed as above or in a RIPA lysis buffer containing protease inhibitors. Polyclonal 3-nitrotyrosine antibody (8 μg) was added to the lysate (1 mg/ml), which was incubated on a nutator at 4°C for 1 h. Protein A-agarose beads (40 μl of a 50% slurry in PBS) were added to the lysates, which were again incubated on a nutator at 4°C overnight. The beads were washed three times with RIPA buffer (1 ml), centrifuged (30 s at 16,000 *g*), aspirated, and finally boiled in Laemmli sample buffer (30 μl). Following a final centrifugation (30 s at 16,000 *g*), the samples were separated on an 8% SDS-PAGE gel and immunoblotted with a PGHS-1 monoclonal antibody.

Purification of atherosclerotic tissue obtained from endarterectomy patients for Western blotting

Human tissue was obtained from carotid endarterectomy procedures (five patients) under an IRB-approved protocol, and was dissected into sections representing the media, the fibrous cap, and the atheromatous necrotic core. The tissue sections were subjected to a series of purification steps equivalent to those used when isolating PGHS from sheep seminal vesicles, except that the buffers contained protease inhibitors (23).

Immunoprecipitation of nitrated PGHS-1 from human atherosclerotic tissue

Atheromatous tissue (three patients), surrounding media (two patients) and uninvolved tissue (two patients) were homogenized using a Tissue Tearor (Dremel) and sonicated for 30 s. The homogenate was centrifuged (16,000 *g* for 15 min). The resulting lysate (1 mg/ml in PBS) was subjected to pre-clarification with Protein G-agarose beads (50 μl of a 50% slurry in PBS for 30 min) to reduce non-specific binding. The lysate/bead mixture was centrifuged (5,000 *g* for 5 min, followed by 16,000 *g* for 1 min) to remove the beads. The clarified lysate was incubated with polyclonal nitrotyrosine antibody (3.6 μg overnight at 4°C) and then with Protein G-agarose beads (75 μl of a 50% slurry overnight at 4°C). The bound agarose beads were collected by centrifugation (5,000 *g* for 10 min, followed by 16,000 *g* for 1 min), washed three times with PBS and resuspended in Laemmli sample buffer (75 μl). The bead-immunocomplex was boiled (5 min), the sample (25 μl) separated by SDS-PAGE (10%) and immunoblotted with monoclonal PGHS-1 antibody as described above.

Miscellaneous

Cell viability was assessed by in-situ staining with trypan blue and cell number was determined by hemocytometer. No cell injury was observed using 200 and 500 μM $ONOO^-$, although some cell death was associated with using 1,000 μM $ONOO^-$. Scion Image software was used to quantify Western blot band densities (Scion Corporation).

RESULTS

Nitrating efficiencies of NO_x compounds

In order to establish the nitrating efficiency of specific NO_x agents, a Tyr-containing peptide (Thr-Tyr-Ser, mw = 369.4) was reacted with $^{15}NO_2^{\bullet}$, $ONOO^-$, TNM (27), and the $NO^{\bullet}$-generator, NOC-7 (29), and analyzed by electrospray ionization mass spectrometry (ESI-MS) (**Fig. 1**). The presence of nitrotyrosine (Tyr-NO_2) is indicated by an increase of 45 Da (m/z = 415) (or m/z = 416 for ^{15}N-labeled nitrotyrosine in the case of $^{15}NO_2^{\bullet}$). Addition of either $^{15}NO_2^{\bullet}$ (Fig. 1B) or NOC-7 (Fig. 1E) resulted in very little nitration of the peptide (0.3% and 0.7%, respectively), whereas the addition of $ONOO^-$ (Fig. 1C) and TNM (Fig. 1D) led to 4.5% and 11.5% Tyr nitration, respectively. The use of $^{15}NO_2^{\bullet}$ instead yielded a dipeptide (7.9%; m/z = 373.3), which is the coupling product of two Tyr radicals (30), and was also observed for $ONOO^-$ and TNM (4.8% and 1.7%, respectively) (data not shown). For $ONOO^-$ and TNM, low amounts of nitrated dipeptide (0.7% and 0.4%, respectively) were observed. From these data, one may generate a hierarchy of nitrating efficiency that may be tested in a biological setting.

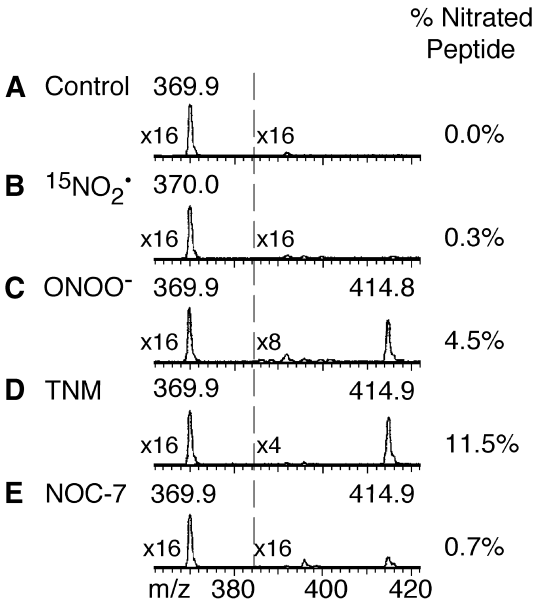

Fig. 1. $ONOO^-$ nitrates a simple Tyr-containing peptide more efficiently than 1-hydroxy-2-oxo-3-(*N*-methyl-aminopropyl)-3-methyl-1-triazene (NOC-7) and $NO_2^{\bullet}$, but less than tetranitromethane (TNM). A Tyr-containing peptide (Thr-Tyr-Ser; mw = 369.4) was reacted with specific NO_x compounds for 1 h in ammonium bicarbonate buffer (20 mM; pH 7.4) at ambient temperature, and then analyzed by electrospray ionization mass spectrometry (ESI-MS). The samples analyzed included: (A) the Tyr-containing peptide (labeled "control"; 5 mM) and the Tyr-containing peptide (5 mM) treated with either (B) ^{15}N-labeled $NO_2^{\bullet}$ (one atmosphere, under anaerobic conditions), (C) $ONOO^-$ (5 mM), (D) TNM (5 mM), or (E) NOC-7 (5 mM). Each spectrum was normalized to the peak of unreacted peptide (m/z = 370). Note that while the lower mass range of the spectra in each case is magnified × 16, the higher mass range, where nitration is expected to appear is magnified by different factors for individual samples.

$ONOO^-$ nitrates purified PGHS-1

Purified PGHS-1 was exposed to $ONOO^-$, SIN-1 (an $ONOO^-$ generator) (31), or TNM, and the presence of 3-nitrotyrosine was probed by Western blotting (**Fig. 2**). All three reagents caused nitration of PGHS-1, although in the case of SIN-1 (Fig. 2B), high concentrations were required (500 μM and above). Nitration was not observed in the presence of nitrite (200–5,000 μM), a degradation byproduct of $ONOO^-$, or NOC-7 (data not shown). As predicted by those agents tested by mass spectrometry, these data demonstrate that $ONOO^-$, SIN-1, and TNM are effective PGHS-1 nitrating agents whereas $NO^{\bullet}$ and nitrite are not.

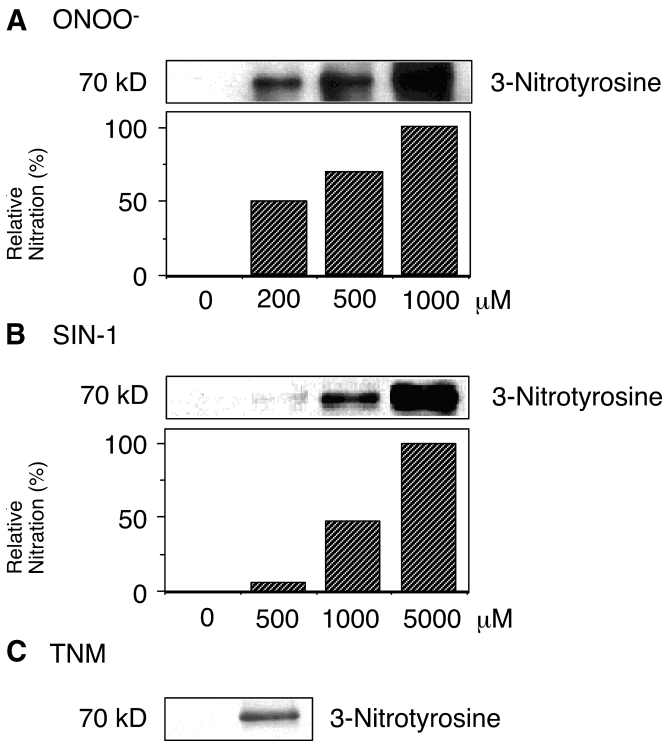

Fig. 2. $ONOO^-$ nitrates purified prostaglandin H_2 synthase-1 (PGHS-1). Holo-PGHS-1 (10 μg; 290 U) in Tris buffer (500 μl; 100 mM) was incubated at 37°C for 1 h with (A) $ONOO^-$ (0, 200, 500 and 1,000 μM), (B) 3-morpholinosydnonimine hydrochloride (SIN-1) (0, 500, 1,000, and 5,000 μM), or (C) TNM (0 and 200 μM). The samples were analyzed by Western blotting using a monoclonal anti-mouse 3-nitrotyrosine antibody. Nitration was quantified in A and B by measuring band densities with the darkest band representing 100%. Stripping the blots and re-probing with PGHS-1 antibody revealed that all lanes were loaded equally with protein (data not shown). These data are representative of one experiment performed three times.

Quantification of tyrosine nitration By $ONOO^-$ in purified PGHS-1

UV/Vis spectroscopy was used to quantify the extent of Tyr nitration in peptide and purified PGHS-1 by $ONOO^-$. Tyr nitration is characterized by an absorbance in the visible region of the UV/Vis spectrum which at pH > 8 occurs above 400 nm, but at pH 6 shifts to lower wavelengths (32). Spectra were generated at pH 6 and 9 upon the treatment of peptide, apo-PGHS-1 (heme-free), or holo-PGHS-1 (heme-containing) with $ONOO^-$ (**Fig. 3**). Under these conditions, we calculate ~5% nitration of the peptide (Fig. 3A), which was also observed by mass spectrometry (Fig. 1). Further, it was calculated that, approximately 2 Tyr/apo-PGHS-1 subunit are nitrated (Fig. 3B). Nitration of holo-PGHS-1 by $ONOO^-$ was also measured by this method. Since heme absorbs very close to the region where nitrated tyrosines absorb, it is not very informative to show a direct comparison of the spectra obtained for nitrated apo- and holo-PGHS-1. It is essential to employ difference spectra to minimize absorbance contributions arising from the Soret band of the heme group. Figure 3C shows difference spectra with maxima at 438 nm for the reaction between $ONOO^-$ and apo- and holo-PGHS-1. The peak intensities reveal that 2.4 and 1.5 Tyr per sunbunit were nitrated for apo-PGHS-1 and holo-PGHS-1, respectively. The 38% reduction in nitration of holo-PGHS-1 may be attributed to the scavenging reaction of heme with $ONOO^-$. Regardless, incubation of both apo- or holo-PGHS-1 with excess $ONOO^-$ led to changes in the UV/Vis spectrum consistent with Tyr nitration.

$ONOO^-$ nitrates PGHS-1 in vascular smooth muscle cells

Immunoprecipitation experiments were performed to determine the ability of specific NO_x reagents to nitrate PGHS-1 in vascular smooth muscle cells. After their exposure to $ONOO^-$ or TNM, proteins containing 3-nitrotyrosine were immunoprecipitated from cells using a polyclonal 3-nitrotyrosine antibody. The immunoprecipitated samples were loaded onto a gel and immunoblotted with a PGHS-1 monoclonal antibody (**Fig. 4**). While very little or no PGHS-1 was detected in the absence of $ONOO^-$, it was detected in the presence of $ONOO^-$, indicating that it is nitrated (Fig. 4A). TNM also induced a dose-dependent increase in PGHS-1 nitration (Fig. 4B). PGHS-1 nitration was not observed when using NOC-7 (data not shown). In parallel experiments, we determined the activity of PGHS-1 following $ONOO^-$ treatment. After incubation with different concentrations of $ONOO^-$, cells were treated with arachidonic acid and the supernatant media analyzed for PGE_2 production (Fig. 4A, top). The data indicate that PGHS-1 activity is inversely related to PGHS-1 nitration.

In separate experiments, Western blots of cell lysates obtained from treatment of smooth muscle cells with sodium nitrite (200–1,000 μM) revealed no nitration at 70 kDa. These data indicate that nitrite, a degradation byproduct of $ONOO^-$, is not the cause of nitration in cells under our experimental conditions. The 70 kDa band observed as a result of nitrated PGHS-1 was not visible when

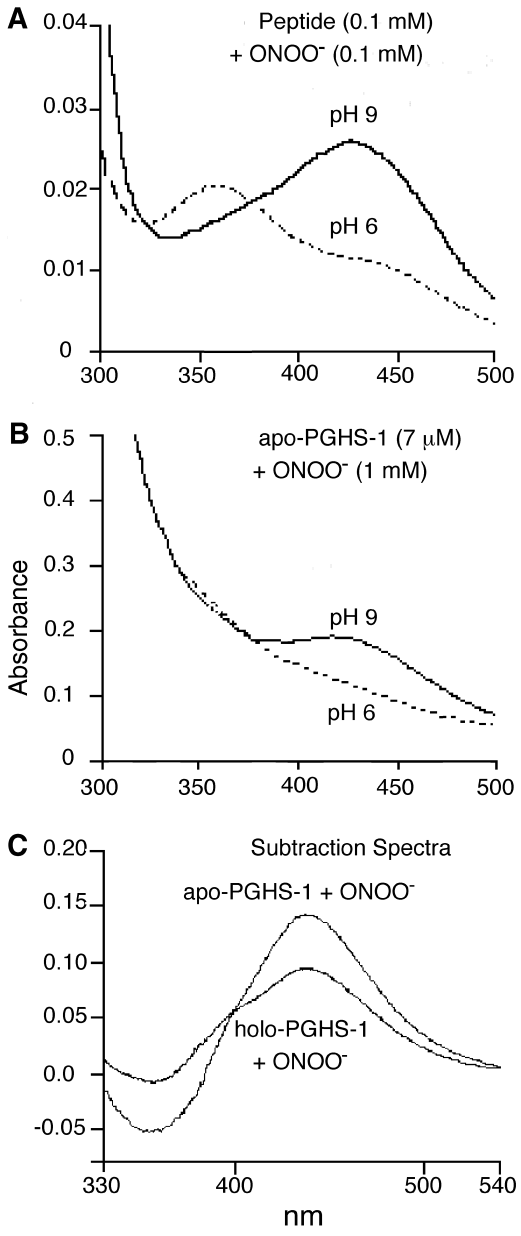

Fig. 3. UV/Vis spectroscopy demonstrates that $ONOO^-$ nitrates a simple Tyr-containing peptide and PGHS-1. A: A Tyr-containing peptide (Thr-Tyr-Ser; mw = 369.4, 0.1 mM) was exposed to $ONOO^-$ (0.1 mM) in ammonium bicarbonate (0.1 M; pH 7.4) at ambient temperature for 1 h, and then analyzed by UV/Vis spectroscopy. The pH of the solution was changed to six (by careful addition of 5N HCl) and then to nine (by careful addition of 5N NaOH). After each pH change, a spectrum was recorded. B: Apo-PGHS-1 (7 μM) was exposed to $ONOO^-$ (1 mM) in ammonium bicarbonate buffer (15 mM) (containing 5 mM Tris buffer) for 1 h at ambient temperature, and analyzed by UV/Vis spectroscopy at pH 6 and pH 9. C: Apo-PGHS-1 (13.4 μM) and holo-PGHS-1 (13.4 μM) were each exposed to $ONOO^-$ (1 mM) in Tris buffer (100 mM, pH 7.4) for 1 h at ambient temperature, and UV/Vis spectra were recorded at pH 6 and pH 9. The spectra shown represent difference spectra that were obtained upon subtracting the spectrum at pH 6 from that obtained at pH 9 and provide a direct comparison of the amount of $ONOO^-$-induced Tyr nitration in apo-PGHS-1 and holo-PGHS-1, respectively. These data are representative of one experiment performed three times.

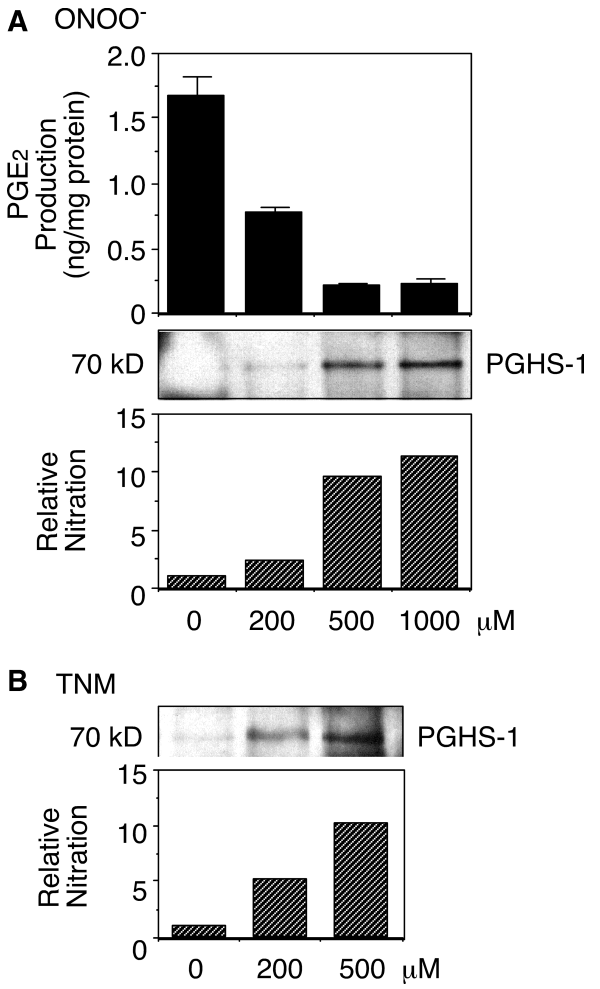

Fig. 4. $ONOO^-$ and TNM nitrate PGHS-1 in smooth muscle cells. A: Aortic smooth muscle cells were grown to near confluence in 10 cm dishes, quiesced for 24 h, and treated with serum-free DMEM containing $ONOO^-$ (0, 200 μM, 500 μM, and 1,000 μM). After incubating at 37°C for 1 h, the media was removed. Top: PGHS-1 activity was determined by measuring PGE_2 production in the supernatant media after arachidonic acid treatment (10 μM for 30 min at 37°C). Middle: Nitration of PGHS-1 was determined by immunoprecipitation using a polyclonal anti-rabbit 3-nitrotyrosine antibody and immunoblotting with a PGHS-1 monoclonal antibody. Bottom: Nitration was quantified by measuring band densities. B: Cells were grown as above and treated with TNM (0, 200 μM and 500 μM). Top: Nitration of PGHS-1 was determined by immunoprecipitation as above. Bottom: Nitration was quantified by measuring band densities and normalizing the control bands to one. These data are representative of one experiment performed twice.

using either secondary antibody alone or 3-nitrotyrosine antibody that had been rendered inactive by binding to 3-nitrotyrosine (data not shown). In summary, these data demonstrate that both $ONOO^-$ and TNM are effective at nitrating PGHS-1 in vascular smooth muscle cells.

Human atherosclerotic tissue contains nitrated PGHS-1

The media, necrotic core, and fibrous cap from human atherosclerotic lesions were each purified and probed by Western blotting for 3-nitrotyrosine, PGHS-1 and PGHS-2 (**Fig. 5**). A nitrated 70 kDa protein was present in the necrotic core and fibrous cap, with less in the surrounding media. In contrast, PGHS-1 levels were similar in all three sections. PGHS-2 was induced in the necrotic core and fibrous cap, with less appearing in the media (9). Thus, the immunoblotting data in Fig. 5 show that a 70 kDa protein (possibly PGHS-1 or PGHS-2) is nitrated in the fibrous cap and necrotic core of human atherosclerotic lesions.

Next, immunoprecipitation experiments were performed to determine whether nitrated PGHS-1 is found in human atherosclerotic tissue (**Fig. 6**). Nitrated proteins from atherosclerotic and uninvolved control tissue (from human mammary arteries) were immunoprecipitated using a polyclonal 3-nitrotyrosine antibody. The immunoprecipitated samples were separated by gel electrophoresis and immunoblotted with a PGHS-1 monoclonal antibody (Fig. 6A). While very little PGHS-1 nitration was detected in uninvolved tissue, substantial PGHS-1 nitration was present in atherosclerotic lesions. Next, we compared the degree of PGHS-1 nitration in the media proximate to the atherosclerotic lesion to the degree of nitration in the lesion (Fig. 6B). While minimal nitration was observed in the media, significant nitration was observed in the lesion ($P = 0.01$).

DISCUSSION

Since $NO^\bullet$ and PGI_2 perform similar functions and their production can be stimulated by the same inflamma-

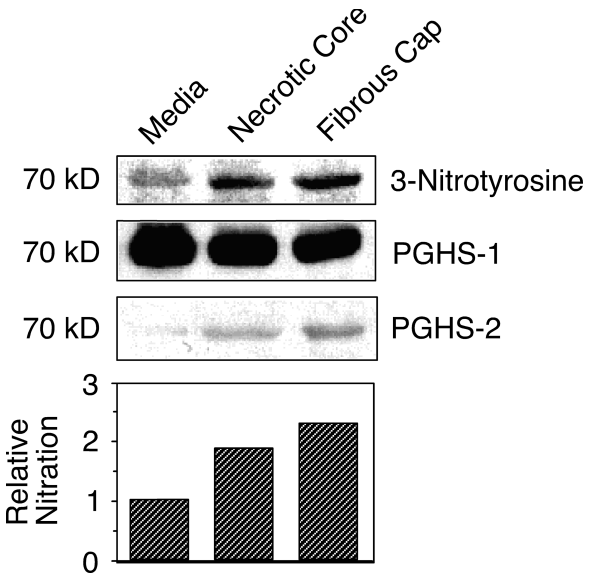

Fig. 5. Western blot of human atherosclerotic lesion comparing nitration levels in the media, fibrous cap, and necrotic core. Human atherosclerotic tissue (n = 5) was sectioned into the media, fibrous cap, and necrotic core, and subjected to a purification procedure designed to isolate the PGHS-rich microsomes (described in Experimental Procedures). Western blots of purified protein from each tissue section were probed with monoclonal 3-nitrotyrosine, PGHS-1, and PGHS-2 antibodies. Data for relative nitration was obtained by calculating a 3-nitrotyrosine/PGHS-1 band density ratio. The band density ratio was normalized to one in the control case.

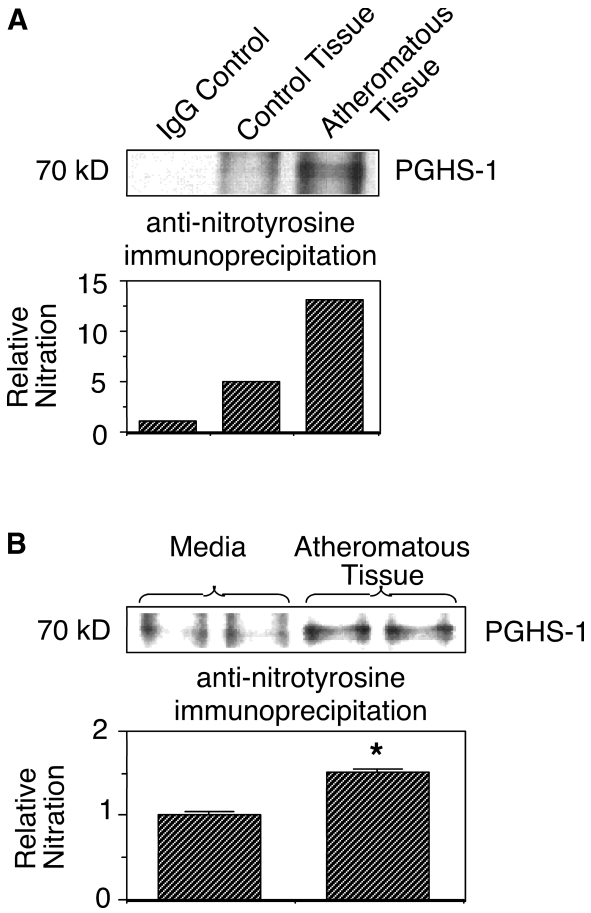

Fig. 6. PGHS-1 is nitrated in human atherosclerotic tissue. A: Atheromatous (n = 3) and uninvolved tissue (n = 2) was homogenized as described in Materials and Methods. Proteins containing 3-nitrotyrosine were immunoprecipitated using a polyclonal 3-nitrotyrosine antibody and then immunoblotted with a PGHS-1 monoclonal antibody. IgG control refers to rabbit anti-nitrotyrosine that was subjected to the same protocol as both the control and atheromatous tissue samples to ensure that the 70 kDa PGHS-1 immunoreactive band was not the result of secondary interactions. B: Atheromatous tissue (n = 3) and the surrounding media (n = 2) were homogenized and immunoprecipitated with polyclonal 3-nitrotyrosine antibody as described in Materials and Methods. Samples were separated by SDS-PAGE and immunoblotted with a monoclonal PGHS-1 antibody. $*P < 0.05$.

tory cytokines (33), it has previously been proposed that there is "cross talk" between the $NO^{\bullet}$ and arachidonic acid pathways. A form of NO_x that activates PGHS-1 and PGHS-2 is $ONOO^-$ (6, 13, 18). A necessary requirement for PGHS activation by $ONOO^-$ is the simultaneous addition of arachidonic acid and $ONOO^-$. If $ONOO^-$ is added prior to arachidonic acid, enzyme deactivation occurs which has been correlated with an increase in 3-nitrotyrosine levels (18). Taken together, these studies indicate that $ONOO^-$ has the potential to either stimulate or inhibit PGHS activity depending on the availability of arachidonic acid and concentration of $ONOO^-$.

This is the first study to demonstrate that, in the absence of exogenously-added arachidonic acid, $ONOO^-$ is capable of nitrating PGHS-1 in vascular smooth muscle cells. Immunoprecipitation experiments unequivocally demonstrate that PGHS-1 in smooth muscle cells is nitrated by $ONOO^-$ and TNM. In contrast, the NO-releasing compound, NOC-7, did not lead to any observable nitration. Indeed, $NO^{\bullet}$ cannot nitrate Tyr residues directly, but can couple with Tyr cation radicals, resulting in the eventual formation of 3-nitrotyrosine (34). Nitrite, which has been implicated in tyrosine nitration in a peroxidase-dependent pathway (35, 36), also had no effect. Similar results were observed with purified PGHS-1. Although SIN-1 was effective at nitrating PGHS, high doses were required. In light of SIN-1's limited ability to donate O_2^- in the presence of biological electron acceptors (e.g., heme proteins) (37), this result is not surprising.

By ESI-MS, the order of nitration of a simple peptide was established to be TNM > $ONOO^-$ > NOC-7 ~ $^{15}NO_2^{\bullet}$. Both $^{15}NO_2^{\bullet}$ and $ONOO^-$ caused Tyr dimer formation, indicating that $ONOO^-$ upon decomposition may form a variety of different NO_x species (32). Indeed, $ONOO^-$ can homolytically decompose to $NO_2^{\bullet}$ (38) or may combine with carbon dioxide to form nitrosoperoxocarbonate ($ONOOCO_2^-$), heterolytic cleavage of which yields NO_2^+, a highly reactive nitrating species (39). UV/Vis spectroscopic data corroborated ESI-MS results, indicating that UV/Vis spectroscopy is a reliable technique for monitoring nitration. By UV/Vis spectroscopy, $ONOO^-$ was demonstrated to nitrate ~2 Tyr residues per PGHS-1 subunit (18). TNM has previously been found to nitrate up to three Tyr residues per PGHS-1 subunit (using a 43:1 molar ratio of TNM-PGHS-1) (40).

It is not yet known which two Tyr residues in PGHS-1 are nitrated by $ONOO^-$. Ovine and human PGHS-1 posess 27 Tyr residues, and yet the significance of only a few is known. In particular, studies have been conducted to examine the importance of Tyr 355, Tyr 348, and Tyr 385. While the mutation of either Tyr355 or Tyr348 to Phe results in little or no effect, the mutation of Tyr385 results in a complete loss of cyclooxygenase activity (41, 42). As we have found that the degree of Tyr nitration in PGHS-1 inversely correlates with enzyme activity, it is possible that Tyr385 is nitrated. However, more conclusive studies must be conducted in order to corroborate this speculation. It is also important to consider that concomitant cysteine oxidation by $ONOO^-$ may contribute to PGHS-1 deactivation.

Levels of NO_x accumulation have been reported in some disease states to be as high as 36 μM (43). The concentrations of $ONOO^-$ employed in the study, although high, cannot be used as a direct measure of the amount of active species. It is important to recognize that the half-life of $ONOO^-$ is ~1 s under physiological conditions (44). This means that 500 μM $ONOO^-$ will decay to nanomolar concentrations in seconds (i.e., 500 nM in 10 s, and to 16 nM in 15 s). In addition, $ONOO^-$ is consumed in reactions other than nitration that will deplete the stock of added $ONOO^-$. Thus, in order to generate $ONOO^-$ concentrations found in local environments, it is necessary to add excessive amounts.

Extensive protein nitration has been observed in atherosclerotic lesions (21, 22). To test the hypothesis that

PGHS nitration occurs, we obtained human atherosclerotic tissue from several endarterectomy procedures and purified it prior to immunoblotting with both 3-nitrotyrosine and PGHS antibodies. Our results show that a 70 kDa protein is indeed nitrated, which may be PGHS-1 or PGHS-2. Our results also demonstrate that inducible PGHS (PGHS-2) expression is augmented in human atherosclerotic lesions (9). Immunoprecipitation results showed conclusively that PGHS-1 is nitrated in atherosclerotic tissue. PGHS-2 was not detected by immunoprecipitation, and was only minimally detected by Western blotting. Thus, PGHS-2 nitration in atheromatous lesions cannot be ruled out.

While $ONOO^-$ has previously been invoked as the initial oxidant involved in the pathogenesis of atherosclerosis (45), other mechanisms may be involved. For instance, peroxidase enzymes (e.g., myeloperoxidase) can catalyze Tyr nitration in proteins when utilizing nitrite (NO_2^-) and hydrogen peroxide (H_2O_2) as substrates (46). In addition, the combination of hypochlorous acid (HOCl) and NO_2^-, which also yields 3-chlorotyrosine (47), can lead to nitrotyrosine formation in proteins (48). Thus, in atherosclerosis the nature of the species causing nitration is not known, but could involve $ONOO^-$.

Under atherosclerotic conditions, both $NO^\bullet$ and superoxide production are elevated (49) providing favorable conditions for $ONOO^-$ formation. While NADPH oxidation may provide a source of superoxide (50), recent data suggest that iNOS itself generates superoxide anion simultaneously with $NO^\bullet$ (51), indicating that iNOS alone is capable of generating $ONOO^-$ (52). Interestingly, iNOS has been found to co-localize with both PGHS-2 and nitrated proteins in macrophages/foam cells (8). This increases the probability that PGHS is affected by NO_x in atherosclerotic tissue.

In a biological setting, it is possible that peroxidase enzymes, such as PGHS, may initially play an important role in detoxifying $ONOO^-$, much in the way that PGHS detoxifies hydroperoxides. The fast reaction of the heme moiety with $ONOO^-$ forms a less harmful NO_x product, NO_2^-. As a consequence, eicosanoids are produced in the presence of arachidonic acid substrate that may or may not be beneficial to restoring vascular reactivity depending on the availability of downstream prostacyclin synthase. Indeed, prostacyclin synthase is nitrated and its activity is impaired in early atherosclerosis (53). While PGHS activity remains unaffected initially, it results in elevated PGH_2 and PGE_2 production. Since reduced prostacyclin synthase activity in arteries prevents the rapid use of PGH_2, it accumulates and acts as an agonist on the vasoconstrictive thromboxane receptor (53). In the presence of molar excesses of $ONOO^-$, we propose that nitration of PGHS will occur, leading to loss of function. Tyr dimer formation may also occur during atherosclerosis, but this has yet to be determined. Elucidation of the nitrating species, together with the identification of the relative amounts of PGH_2 and PGI_2 that are formed as the disease progresses, will lead to a better definition of the molecular basis of this vascular disease.

The authors are grateful to Dr. Menard Gertler (Weill Medical College of Cornell University), to Dr. Michael Silane (Beth Israel Medical Center), and to Dr. Wayne Isom (New York Presbyterian Hospital) for providing our laboratory with endarterectomy or uninvolved tissue; Dr. Gerard Parkin (Columbia University) for his assistance in the preparation of anaerobic solutions of $^{15}NO_2^\bullet$; Feng Xian Zhao for excellent technical assistance; and to Dr. Ivan Haller (Weill Medical College of Cornell University) for providing ESI-MS data. We are also grateful to Dr. Esther Breslow (Weill Medical College of Cornell University) for helpful discussions. This study was made possible by Grants from the National Institutes of Health awarded to D.P.H. (HL-46403 and HL-49666) and to T.M. (AG-12712). R.S.D. was supported by a National Institutes of Health T32 training Grant in Cardiovascular Biology awarded to D.P.H. R.K.U. was supported, in part, by an American Heart Association Scientist Development Award (9630223N) and an Atorvastatin Research Award (Pfizer Inc.).

REFERENCES

1. Huie, R. E., and S. Padmaja. 1993. The reaction of no with superoxide. *Free Radic. Res. Commun.* **18:** 195–199.
2. Radi, R., J. S. Beckman, K. M. Bush, and B. A. Freeman. 1991. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **288:** 481–487.
3. Radi, R., J. S. Beckman, K. M. Bush, and B. A. Freeman. 1991. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* **266:** 4244–4250.
4. Ischiropoulos, H., and A. B. al-Mehdi. 1995. Peroxynitrite-mediated oxidative protein modifications. *FEBS Lett.* **364:** 279–282.
5. Matheis, G., M. P. Sherman, G. D. Buckberg, D. M. Haybron, H. H. Young, and L. J. Ignarro. 1992. Role of L-arginine-nitric oxide pathway in myocardial reoxygenation injury. *Am. J. Physiol.* **262:** H616–H620.
6. Upmacis, R. K., R. S. Deeb, and D. P. Hajjar. 1999. Regulation of prostaglandin H2 synthase activity by nitrogen oxides. *Biochemistry.* **38:** 12505–12513.
7. Oshima, M., J. E. Dinchuk, S. L. Kargman, H. Oshima, B. Hancock, E. Kwong, J. M. Trzaskos, J. F. Evans, and M. M. Taketo. 1996. Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell.* **87:** 803–809.
8. Baker, C. S., R. J. Hall, T. J. Evans, A. Pomerance, J. Maclouf, C. Creminon, M. H. Yacoub, and J. M. Polak. 1999. Cyclooxygenase-2 is widely expressed in atherosclerotic lesions affecting native and transplanted human coronary arteries and colocalizes with inducible nitric oxide synthase and nitrotyrosine particularly in macrophages. *Arterioscler. Thromb. Vasc. Biol.* **19:** 646–655.
9. Schonbeck, U., G. K. Sukhova, P. Graber, S. Coulter, and P. Libby. 1999. Augmented expression of cyclooxygenase-2 in human atherosclerotic lesions. *Am. J. Pathol.* **155:** 1281–1291.
10. Moncada, S., and J. Vane. 1978. Unstable metabolites of arachidonic acid and their role in haemostasis and thrombosis. *Br. Med. Bull.* **34:** 129–135.
11. Libby, P., S. J. Warner, and G. B. Friedman. 1988. Interleukin 1: a mitogen for human vascular smooth muscle cells that induces the release of growth-inhibitory prostanoids. *J. Clin. Invest.* **81:** 487–498.
12. Salvemini, D., T. P. Misko, J. L. Masferrer, K. Seibert, M. G. Currie, and P. Needleman. 1993. Nitric oxide activates cyclooxygenase enzymes. *Proc. Natl. Acad. Sci. USA.* **90:** 7240–7244.
13. Landino, L. M., B. C. Crews, M. D. Timmons, J. D. Morrow, and L. J. Marnett. 1996. Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc. Natl. Acad. Sci. USA.* **93:** 15069–15074.
14. Kanner, J., S. Harel, and R. Granit. 1992. Nitric oxide, an inhibitor of lipid oxidation by lipoxygenase, cyclooxygenase and hemoglobin. *Lipids.* **27:** 46–49.
15. Tsai, A. L., C. Wei, and R. J. Kulmacz. 1994. Interaction between nitric oxide and prostaglandin H synthase. *Arch. Biochem. Biophys.* **313:** 367–372.

16. Curtis, J. F., N. G. Reddy, R. P. Mason, B. Kalyanaraman, and T. E. Eling. 1996. Nitric oxide: a prostaglandin H synthase 1 and 2 reducing cosubstrate that does not stimulate cyclooxygenase activity or prostaglandin H synthase expression in murine macrophages. *Arch. Biochem. Biophys.* **335:** 369–376.
17. Stamler, J. S., D. J. Singel, and J. Loscalzo. 1992. Biochemistry of nitric oxide and its redox-activated forms. *Science.* **258:** 1898–1902.
18. Boulos, C., H. Jiang, and M. Balazy. 2000. Diffusion of Peroxynitrite into the Human Platelet Inhibits Cyclooxygenase via Nitration of Tyrosine Residues. *J. Pharmacol. Exp. Ther.* **293:** 222–229.
19. Dietz, R., W. Nastainczyk, and H. H. Ruf. 1988. Higher oxidation states of prostaglandin H synthase. Rapid electronic spectroscopy detected two spectral intermediates during the peroxidase reaction with prostaglandin G2. *Eur. J. Biochem.* **171:** 321–328.
20. Lee, J., J. A. Hunt, and J. T. Groves. 1998. Mechanisms of Iron Porphyrin Reactions with Peroxynitrite. *J. Am. Chem. Soc.* **120:** 7493–7501.
21. Beckmann, J. S., Y. Z. Ye, P. G. Anderson, J. Chen, M. A. Accavitti, M. M. Tarpey, and C. R. White. 1994. Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biol. Chem. Hoppe Seyler.* **375:** 81–88.
22. Leeuwenburgh, C., M. M. Hardy, S. L. Hazen, P. Wagner, S. Ohishi, U. P. Steinbrecher, and J. W. Heinecke. 1997. Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. *J. Biol. Chem.* **272:** 1433–1436.
23. Mevkh, A. T., G. F. Sud'ina, N. B. Golub, and S. D. Varfolomeev. 1985. Purification of prostaglandin H synthetase and a fluorometric assay for its activity. *Anal. Biochem.* **150:** 91–96.
24. Kulmacz, R. J., and W. E. M. Lands. 1987. Prostaglandins and Related Substances: A Practical Approach. IRL Press, Washington, D.C.
25. Roepstorff, P., and J. Fohlman. 1984. *Biomed. Mass Spectrom.* **11:** 601.
26. Hughes, M. N., and H. G. Nicklin. 1968. The Chemistry of Pernitrites. Part I. Kinetics of Decomposition of Pernitrous Acid. *J. Chem. Soc.* (A): 450–452.
27. Sokolovsky, M., J. F. Riordan, and B. L. Vallee. 1966. Tetranitromethane. A reagent for the nitration of tyrosyl residues in proteins. *Biochemistry.* **5:** p3582–p3589.
28. Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87:** p206–p210.
29. Hrabie, J. A., J. R. Klose, D. A. Wink, and L. K. Keefer. 1993. New Nitric Oxide-Releasing Zwitterions Derived from Polyamines. *J. Org. Chem.* **58:** 1472–1476.
30. Prutz, W. A., H. Monig, J. Butler, and E. J. Land. 1985. Reactions of nitrogen dioxide in aqueous model systems: oxidation of tyrosine units in peptides and proteins. *Arch. Biochem. Biophys.* **243:** p125–p134.
31. Rosenkranz, B., B. R. Winkelmann, and M. J. Parnham. 1996. Clinical pharmacokinetics of molsidomine. *Clin. Pharmacokinet.* **30:** 372–384.
32. van der Vliet, A., J. P. Eiserich, H. Kaur, C. E. Cross, and B. Halliwell. 1996. Nitrotyrosine as biomarker for reactive nitrogen species. *Methods Enzymol.* **269:** 175–184.
33. Wu, K. K. 1995. Inducible cyclooxygenase and nitric oxide synthase. *Adv. Pharmacol.* **33:** 179–207.
34. Goodwin, D. C., M. R. Gunther, L. C. Hsi, B. C. Crews, T. E. Eling, R. P. Mason, and L. J. Marnett. 1998. Nitric oxide trapping of tyrosyl radicals generated during prostaglandin endoperoxide synthase turnover. Detection of the radical derivative of tyrosine 385. *J. Biol. Chem.* **273:** 8903–8909.
35. Pfeiffer, S., A. Lass, K. Schmidt, and B. Mayer. 2001. Protein tyrosine nitration in cytokine-activated murine macrophages. Involvement of a peroxidase/nitrite pathway rather than peroxynitrite. *J. Biol. Chem.* **276:** p34051–p34058.
36. Espey, M. G., S. Xavier, D. D. Thomas, K. M. Miranda, and D. A. Wink. 2002. Direct real-time evaluation of nitration with green fluorescent protein in solution and within human cells reveals the impact of nitrogen dioxide vs. peroxynitrite mechanisms. *Proc. Natl. Acad. Sci. USA.* **99:** p3481–p3486.
37. Singh, R. J., N. Hogg, J. Joseph, E. Konorev, and B. Kalyanaraman. 1999. The peroxynitrite generator, SIN-1, becomes a nitric oxide donor in the presence of electron acceptors. *Arch. Biochem. Biophys.* **361:** p331–p339.
38. Koppenol, W. H., and R. Kissner. 1998. Can O=NOOH undergo homolysis? *Chem. Res. Toxicol.* **11:** p87–90.
39. Lymar, S. V., and J. K. Hurst. 1995. Rapid Reaction Between Peroxonitrite Ion and Carbon Dioxide: Implications for Biological Activity. *J. Am. Chem. Soc.* **117:** 8867–8868.
40. Kulmacz, R. J., Y. Ren, A. L. Tsai, and G. Palmer. 1990. Prostaglandin H synthase: spectroscopic studies of the interaction with hydroperoxides and with indomethacin. *Biochemistry.* **29:** p8760–p8771.
41. Tsai, A. L., G. Palmer, and R. J. Kulmacz. 1992. Prostaglandin H synthase. Kinetics of tyrosyl radical formation and of cyclooxygenase catalysis. *J. Biol. Chem.* **267:** 17753–17759.
42. Hsi, L. C., C. W. Hoganson, G. T. Babcock, R. M. Garavito, and W. L. Smith. 1995. An examination of the source of the tyrosyl radical in ovine prostaglandin endoperoxide synthase-1. *Biochem. Biophys. Res. Commun.* **207:** 652–660.
43. Torre, D., G. Ferrario, F. Speranza, A. Orani, G. P. Fiori, and C. Zeroli. 1996. Serum concentrations of nitrite in patients with HIV-1 infection. *J. Clin. Pathol.* **49:** p574–p576.
44. Koppenol, W. H. 1998. The basic chemistry of nitrogen monoxide and peroxynitrite. *Free Radic. Biol. Med.* **25:** p385–p391.
45. Darley-Usmar, V. M., N. Hogg, V. J. O'Leary, M. T. Wilson, and S. Moncada. 1992. The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low density lipoprotein. *Free Radic. Res. Commun.* **17:** 9–20.
46. van der Vliet, A., J. P. Eiserich, B. Halliwell, and C. E. Cross. 1997. Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. *J. Biol. Chem.* **272:** 7617–7625.
47. Domigan, N. M., T. S. Charlton, M. W. Duncan, C. C. Winterbourn, and A. J. Kettle. 1995. Chlorination of tyrosyl residues in peptides by myeloperoxidase and human neutrophils. *J. Biol. Chem.* **270:** 16542–16548.
48. Hazen, S. L., J. R. Crowley, D. M. Mueller, and J. W. Heinecke. 1997. Mass spectrometric quantification of 3-chlorotyrosine in human tissues with attomole sensitivity: a sensitive and specific marker for myeloperoxidase-catalyzed chlorination at sites of inflammation. *Free Radic. Biol. Med.* **23:** 909–916.
49. Ohara, Y., T. E. Peterson, and D. G. Harrison. 1993. Hypercholesterolemia increases endothelial superoxide anion production. *J. Clin. Invest.* **91:** 2546–2551.
50. Olken, N. M., and M. A. Marletta. 1993. NG-methyl-L-arginine functions as an alternate substrate and mechanism-based inhibitor of nitric oxide synthase. *Biochemistry.* **32:** p9677–p9685.
51. Xia, Y., L. J. Roman, B. S. Masters, and J. L. Zweier. 1998. Inducible nitric-oxide synthase generates superoxide from the reductase domain. *J. Biol. Chem.* **273:** p22635–p22639.
52. Xia, Y., and J. L. Zweier. 1997. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proc. Natl. Acad. Sci. USA.* **94:** p6954–p6958.
53. Zou, M. H., M. Leist, and V. Ullrich. 1999. Selective nitration of prostacyclin synthase and defective vasorelaxation in atherosclerotic bovine coronary arteries. *Am. J. Pathol.* **154:** 1359–1365.