Tyrosine nitrination in prostaglandin H$_2$ synthase

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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) nitrination in purified protein and in vascular smooth muscle cells. We also examined PGHS-1 activity under these conditions and found the degree of nitrination 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 nitrination. Both peroxynitrite and tetranitromethane induced Tyr nitrination 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 nitrination. The extent of nitrination 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 nitrination.—Deeb, R. S., M. J. Resnick, D. Mittar, T. McCaffrey, D. P. Hajjar, and R. K. Upmacis. Tyrosine nitrination in prostaglandin H$_2$ synthase. J. Lipid Res. 2002. 43: 1718–1726.

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The rapid, diffusion-limited reaction of superoxide anion and nitric oxide (NO$^+$) (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 nitrination (4). When produced by inflam-
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 × 10⁷ M⁻¹s⁻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 NOx 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 the Oxford Biomedical Research, Inc. Diethylthiocarbamate (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,N,N-methylaminopropyl)-3-methyl-1-triazene (NOC-7) (99.9% purity) and 3-morpholinopropionimine hydrochloride (SIN-1) from Alexix Corporation; tetranitromethane (TNM), sodium nitrite (NaNO₂) and 15N-labeled nitrogen dioxide (15NO₂) 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. Monoclonal PGHS-1 and PGHS-2 antibodies and purified PGHS-2 protein were purchased from Cayman Chemical Company.

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 spectrophotometric experiments

The reaction of several NOx compounds (15NO₂, 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 15NO₂, the peptide was deaerated in an Thunberg cuvette using a vacuum/argon gas manifold and exposed to 15NO₂ 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 NOx compound (in the case of 15NO₂, we exposed 5 mM peptide to 1 atm 15NO₂ 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 × 10⁻⁶ 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 b₂ 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 nitratived 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 15NO₂-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 NO₂ 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 NOₓ 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.

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

Protein (5–10 µg) was separated on a 10% acrylamide gel and transferred onto a nitrocellulose membrane. The membrane was immobilblotted 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 agitation 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.

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 Mg²⁺ or Ca²⁺), 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).

Preparation 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.

Treatment of smooth muscle cells with ONOO⁻, tetraniromethane, or NOC-7

Rat smooth muscle cells were grown to near-confluence in 10 cm diameter dishes (containing approximately 8.8 × 10⁶ cells/dish). The cells were quiesced for 24 h using serum-free DMEM (quiescent medium) containing 1% (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 ONO⁻₂, tetraniromethane, or NOC-7

Rat smooth muscle cells were grown to near-confluence in 10 cm diameter dishes (containing approximately 8.8 × 10⁶ 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 ONO⁻₂, TNM, or NOC-7 for 1 h at 37°C, as described below.

For experiments in which cells were exposed to ONO⁻₂, we added the appropriate amount of ONO⁻₂ 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).
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 NOₓ compounds

In order to establish the nitrating efficiency of specific NOₓ agents, a Tyr-containing peptide (Thr-Tyr-Ser, mw = 369.4) was reacted with ¹⁵NO₂⁻, ONOO⁻, TNM (27), and the NO•-generator, NOC-7 (29), and analyzed by electrospray ionization mass spectrometry (ESI-MS) (Fig. 1). The presence of nitrotyrosine (Tyr-NO₂) is indicated by an increase of 45 Da (m/z = 415) (or m/z = 416 for ¹⁵N-labeled nitrotyrosine in the case of ¹⁵NO₂⁻). Addition of either ¹⁵NO₂⁻ (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 ¹⁵NO₂⁻ 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.

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• and nitrite are not.

Fig. 1. ONOO⁻ nitrates a simple Tyr-containing peptide more efficiently than 1-hydroxy-2-oxo-3-(N-methyl-aminopropyl)-3-methyl-1-triazene (TNM). A Tyr-containing peptide (Thr-Tyr-Ser; mw = 369.4) was reacted with specific NOₓ 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) ¹⁵N-labeled NO₂⁻ (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.

Fig. 2. ONOO⁻ nitrates purified prostaglandin H₂ 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 nitrination by ONOO in purified PGHS-1

UV/Vis spectroscopy was used to quantify the extent of Tyr nitrination in peptide and purified PGHS-1 by ONOO\textsuperscript{--}. Tyr nitrination 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\textsuperscript{--} (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\textsuperscript{--} 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\textsuperscript{--} and apo- and holo-PGHS-1. The peak intensities reveal that 2.4 and 1.5 Tyr per subunit 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\textsuperscript{--}. Regardless, incubation of both apo- or holo-PGHS-1 with excess ONOO\textsuperscript{--} led to changes in the UV/Vis spectrum consistent with Tyr nitrination.

ONOO\textsuperscript{--} nitrates PGHS-1 in vascular smooth muscle cells

Immunoprecipitation experiments were performed to determine the ability of specific NO\textsubscript{x} reagents to nitrate PGHS-1 in vascular smooth muscle cells. After their exposure to ONOO\textsuperscript{--} 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\textsuperscript{--}, it was detected in the presence of ONOO\textsuperscript{--}, 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\textsuperscript{--} treatment. After incubation with different concentrations of ONOO\textsuperscript{--}, cells were treated with arachidonic acid and the supernatant media analyzed for PGE\textsubscript{2} production (Fig. 4A, bottom). 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 \muM) revealed no nitrination at 70 kDa. These data indicate that nitrite, a degradation byproduct of ONOO\textsuperscript{--}, is not the cause of nitrination in cells under our experimental conditions. The 70 kDa band observed as a result of nitrated PGHS-1 was not visible when

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**Fig. 3.** UV/Vis spectroscopy demonstrates that ONOO\textsuperscript{--} 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\textsuperscript{--} (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 \muM) was exposed to ONOO\textsuperscript{--} (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 \muM) and holo-PGHS-1 (13.4 \muM) were each exposed to ONOO\textsuperscript{--} (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 apoPGHS-1 and holoPGHS-1, respectively. These data are representative of one experiment performed three times.
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$^-$/H$_2$O$_2$ 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$^*$ and PGI$_2$ perform similar functions and their production can be stimulated by the same inflamma-
PGHS-2 is ONOO\(^{-}/H11002\) for PGHS activation by ONOO\(^{-}/H11002\) arachidonic acid and concentration of ONOO\(^{-}\) inhibit PGHS activity depending on the availability of.

There is “cross talk” between the NO\(^{•}\) and arachidonic acid pathways. A form of NO\(^{x}\) that activates PGHS-1 and inhibits PGHS-2 is ONOO\(^{-}/H11002\) for PGHS activation by ONOO\(^{-}/H11002\) arachidonic acid and concentration of ONOO\(^{-}\), which has been implicated in tyrosine nitrination 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 nitrination of a simple peptide was established to be TNM > ONOO\(^{-}/H11002\) > NOC-7 ≈ ONOO\(^{-}/H11002\). Both 15NO\(^{2-}/H11002\) and ONOO\(^{-}/H11002\) caused Tyr dimer formation, indicating that ONOO\(^{-}/H11002\) upon decomposition may form a variety of different NO\(^x\) species (32). Indeed, ONOO\(^{-}/H11002\) can homolytically decompose to NO\(^{•}/H11002\) (38) or may combine with carbon dioxide to form nitrosoperoxocarbonate (ONOOOCO\(^{2-}/H11022\)), heterolytic cleavage of which yields NO\(^{•}/H11002\), 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 nitrination. By UV/Vis spectroscopy, ONOO\(^{-}/H11002\) 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\(^{-}/H11002\). Oxine and human PGHS-1 possess 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 nitrination 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\(^{-}/H11002\) may contribute to PGHS-1 deactivation.

Levels of NO\(^x\) accumulation have been reported in some disease states to be as high as 36 \(\mu\)M (43). The concentrations of ONOO\(^{-}/H11002\) 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\(^{-}/H11002\) is \(~1\) s under physiological conditions (44). This means that 500 \(\mu\)M ONOO\(^{-}/H11002\) will decay to nanomolar concentrations in seconds (i.e., 500 \(\mu\)M in 10 s, and to 16 nm in 15 s). In addition, ONOO\(^{-}/H11002\) is consumed in reactions other than nitrination that will deplete the stock of added ONOO\(^{-}/H11002\). Thus, in order to generate ONOO\(^{-}/H11002\) 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\(_2\)O\(_2\)) as substrates (46). In addition, the combination of hypochlorous acid (HOCI) 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\(^+\) 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\(^+\) (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 vasodilator 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.

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Deeb et al. Prostaglandin H\(_2\) synthase nitration 1725
