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Physical Evidence for Substrate Binding in Preventing Cyclooxygenase Inactivation under Nitrative Stress

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Abstract: Prostaglandin biosynthesis is catalyzed by two spatially and functionally distinct active sites in cyclooxygenase (COX) enzymes. Despite the crucial role of COXs in biology, molecular details regarding the function and regulation of these enzymes are incompletely defined. Reactive nitrogen species, formed during oxidative stress, produce modifications that alter COX functionalities and prostaglandin biosynthesis. We previously established that COX-1 undergoes selective nitration on Tyr385 via a mechanism that requires the presence of bound heme cofactor. As this is a critical residue for COX-1 catalysis, nitration at this site results in enzyme inactivation. We now show that occupancy of the COX-1 active site with substrate protects against Tyr385 nitration and redirects nitration to alternative Tyr residues on COX-1, preserving catalytic activity. This study reveals a novel role for the substrate in protecting COX-1 from inactivation by nitration in pathophysiological settings.

Introduction

Cyclooxygenases (COXs) 1 and 2 are dimeric integral membrane proteins that catalyze the stereospecific conversion of arachidonic acid (AA) into biologically active prostaglandins (PGs). Importantly, PGs play a critical role in mammalian physiology and are intimately linked to the pathophysiology of numerous inflammatory conditions, such as cancer, cardiovascular, neurodegenerative, and rheumatoid diseases. The diverse and often opposing biological effects of PGs have propelled structure-based pharmacological studies for the design of safe and effective COX inhibitors, namely, nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors that obstruct the binding of substrate and consequently suppress PG biosynthesis.[1−4]

Mechanisms of COX catalysis remain incompletely understood. At present, it is widely accepted that COXs catalyze two spatially separate and independent reactions that couple through Tyr385, a residue that is strategically positioned to synchronize catalysis between the two active sites.[5−7] The cyclooxygenase reaction catalyzes the conversion of AA into prostaglandin G_2 (PGG_2), while the heme peroxidase reaction reduces PGG_2 to the unstable endoperoxide PGH_2, the substrate for downstream enzymes in the PG biosynthetic pathway.[8−10] In addition to substrate bioavailability,[11] PG biosynthesis appears to be dependent on the cellular oxidant content, a component that is required for oxidizing Fe^{3+} into a catalytically competent Fe^{4+} in COX.[12,13] This catalytic profile of COX is consistent with a coordinated movement of substrate between the cyclooxygenase and peroxidase sites. As a result, structural methods that seek to reveal subtle dynamic changes in protein environment associated with catalysis or ligand binding have been difficult as catalysis is dependent on peroxidase turnover and is set in motion by very small levels of peroxide and/or hydroperoxide formed in either aerobic or anaerobic settings.[14] In fact, high-resolution crystallographic depictions of COX as stable substrate-bound or inhibitor-bound COX complexes have shed little light on the sequence of molecular events that culminate in catalysis.[3,15,16] Although solution-state nuclear magnetic resonance (NMR) is an ideal method for obtaining dynamic measurements of proteins in solution, the applicability of this method to COXs has been precluded by the fact that these

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(1) Moncada, S. *Arterioscler., Thromb., Vasc. Biol.* **1982**, *2*, 193–207.
(2) Kurumbail, R. G.; Kiefer, J. R.; Marnett, L. J. *Curr. Opin. Struct. Biol.* **2001**, *11*, 752–760.
(3) Garavito, R. M.; Malkowski, M. G.; DeWitt, D. L. *Prostaglandins Other Lipid Mediators* **2002**, *68−69*, 129–152.
(4) McGiff, J. C. *Pharmacol. Rep.* **2006**, *58*, 47–51.
(5) Shimokawa, T.; Kulmacz, R. J.; De Witt, D. L.; Smith, W. L. *J. Biol. Chem.* **1990**, *265*, 20073–20076.
(6) Smith, W. L.; Song, I. *Prostaglandins Other Lipid Mediators* **2002**, *68−69*, 115–128.
(7) Kozak, K. R.; Prusakiewicz, J. J.; Rowlinson, S. W.; Prudhomme, D. R.; Marnett, L. J. *Biochemistry* **2003**, *42*, 9041–9049.
(8) Dietz, R.; Nastainczyk, W.; Ruf, H. H. *Eur. J. Biochem.* **1988**, *171*, 321–328.
(9) Smith, W. L.; Marnett, L. J. *Biochim. Biophys. Acta* **1991**, *1083*, 1–17.
(10) Tsai, A. L.; Palmer, G.; Kulmacz, R. J. *J. Biol. Chem.* **1992**, *267*, 17753–17759.
(11) Marshall, P. J.; Kulmacz, R. J.; Lands, W. E. *J. Biol. Chem.* **1987**, *262*, 3510–3517.
(12) Landino, L. M.; Crews, B. C.; Timmons, M. D.; Morrow, J. D.; Marnett, L. J. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 15069–15074.
(13) Upmacis, R. K.; Deeb, R. S.; Hajjar, D. P. *Biochemistry* **1999**, *38*, 12505–12513.
(14) Smith, W. L.; Eling, T. E.; Kulmacz, R. J.; Marnett, L. J.; Tsai, A. *Biochemistry* **1992**, *31*, 3–7.
(15) Picot, D.; Loll, P. J.; Garavito, R. M. *Nature* **1994**, *367*, 243–249.
(16) Malkowski, M. G.; Theisen, M. J.; Scharmen, A.; Garavito, M. R. *Arch. Biochem. Biophys.* **2000**, *380*, 39–45.

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membrane proteins are stable homodimers of 70 kDa subunits and exceed the maximal size limit for effective determination of intact protein structures. Therefore, there is a continuing effort to elucidate structural features in COX that can explain fundamental, yet unresolved, aspects of COX catalysis, including the basis for sensitivity, selectivity, and affinity of isoform-specific inhibitors,[17−19] requirements for substrate recognition,[7,20] interactions between monomeric subunits,[19,21,22] and the coupling of cyclooxygenase and peroxidase activities via Tyr385.[23,24]

Another unique characteristic of COXs is their capacity to undergo irreversible self-inactivation during catalysis,[25] likely via a process following the formation of the catalytically competent ferryl−oxo porphyrin intermediate at the heme peroxidase site and a tyrosyl radical at nearby Tyr385.[8,26] Radicals at Tyr385 and alternate sites can also form during oxidative stress.[27,28] In fact, an oxidative milieu is known to generate reactive nitrogen species (RNS), e.g., peroxynitrite ($ONOO^-$), which engender NO-dependent modifications of Cys and Tyr residues that impact COX activities and overall PG production.[29−32] Numerous proteins undergo NO-dependent modifications of Cys and Tyr residues in a highly selective manner.[33−36] While the basis of selectivity remains largely hypothetical and poorly defined, chemical reactivity and the concentration of NO and NO-derived species, as well as structural features of the target protein, likely play a dominant role in dictating the functional impact of these modifications. To this end, we have demonstrated that $ONOO^-$-induced COX-1 nitration is selectively targeted to Tyr385 by a mechanism that requires bound heme cofactor[37] and results in loss of catalytic function.[5,37,38] Interestingly, $ONOO^-$-induced nitration occurs in the absence of Fe^{3+} whereby Tyr385 is left unscathed and thus catalytic activity preserved.[37] We have also shown that Tyr nitration in COX-1 occurs in vivo and is elevated in human and murine atherosclerotic blood vessels.[29,30,32] To this end, reactions between RNS and COX in its dynamic state may constitute a previously unrecognized pathway to alter PG production in inflammatory disease.

We have employed a multifaceted approach in an attempt to detect subtle dynamic movements in COX-1 that may not be readily accessed using standard X-ray and NMR structure-based approaches. Herein, we used (i) $ONOO^-$, a reactive nitrogen species and source for Tyr nitration of COX, (ii) a pan-3-nitrotyrosine antibody to assess global Tyr nitration in COX, of which there are 27 Tyr residues per ovine COX-1 monomer (555 amino acid residues). (iii) a site-specific antibody that uniquely recognizes 3-nitro-Tyr385 in COX, (iv) HPLC with electrochemical detection (HPLC-EC) for absolute quantification of Tyr nitration in $ONOO^-$-treated COX complexes, (v) nanoflow liquid chromatography−tandem mass spectrometry (nLC−MS/MS) to identify specific sites of Tyr nitration in $ONOO^-$-treated COX-1, and (vi) circular dichroism (CD) spectroscopy to assess conformational changes in $ONOO^-$-treated COX-1 complexes. Our findings indicate that although the peroxidase heme plays an obligate role in targeting $ONOO^-$-induced Tyr385 nitration in COX-1, active-site channel occupancy allows for allosteric modifications that redirect nitration to alternative Tyr residues while protecting against heme-catalyzed Tyr385 nitration and enzyme inactivation. This paradigm shift suggests that working COX-1 is shielded from inactivation by nitrative modifications and provides new insight into enzyme function.

Methods

Sample Preparation. Ram seminal vesicles were obtained from Pel Freeze. Microsomal COX (~70% endogenous FePPCOX-1[39]) and purified COX-1 were prepared as previously described.[37,40] Tween-20, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), diethyldithiocarbamate (DEDTC), hemin (FePP), 3-nitrotyrosine (3-NT), sodium octanesulfonate, acetonitrile, proteinase K, AA (99% purity), eicosapentanoic acid (EPA; 99% purity), *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine (TMPD), and all other reagents were obtained from Sigma-Aldrich (minimum 95% purity, HPLC grade). AA and EPA were dissolved in ethanol and stored in aliquots (168 and 330 mM, respectively) at −80 °C. TMPD was prepared fresh at 1 mg/mL for each experiment. *N*-Octyl β-D-glucopyranoside (βOG) was from Anatrace. Cobalt(III) protoporphyrin IX chloride (CoPP) was purchased from Alexis Biochemicals. Aspirin, indomethacin, and monoclonal COX-1 antibody were purchased from Cayman Chemicals. Aspirin solutions (10 mM) were prepared fresh, dissolved in 70% ethanol and then diluted with 0.9% NaCl. Indomethacin solutions (70 μM) were prepared by solubilization in a minimum volume (~10 μL) of DMSO followed by dilution with 50 mM Tris, 150 mM NaCl, 0.01 mM EDTA, 20 mM CHAPS, pH 7.4. Sodium peroxynitrite

(17) Harman, C. A.; Turman, M. V.; Kozak, K. R.; Marnett, L. J.; Smith, W. L.; Garavito, R. M. *J. Biol. Chem.* **2007**, *282*, 28096–28105.
(18) Tosco, P.; Lazzarato, L. *ChemMedChem* **2009**, *4*, 939–945.
(19) Prusakiewicz, J. J.; Duggan, K. C.; Rouzer, C. A.; Marnett, L. J. *Biochemistry* **2009**, *48*, 7353–7355.
(20) Chubb, A. J.; Fitzgerald, D. J.; Nolan, K. B.; Moman, E. *Biochemistry* **2006**, *45*, 811–820.
(21) Yuan, C.; Rieke, C. J.; Rimon, G.; Wingerd, B. A.; Smith, W. L. *Proc. Natl. Sci. U.S.A.* **2006**, *103*, 6142–6147.
(22) Liu, J.; Seibold, S. A.; Rieke, C. J.; Song, I.; Cukier, R. I.; Smith, W. L. *J. Biol. Chem.* **2007**, *282*, 18233–18244.
(23) Lassmann, G.; Odenwaller, R.; Curtis, J. F.; DeGray, J. A.; Mason, R. P.; Marnett, L. J.; Eling, T. E. *J. Biol. Chem.* **1991**, *266*, 20045–20055.
(24) Tsai, A.; Hsi, L. C.; Kulmacz, R. J.; Palmer, G.; Smith, W. L. *J. Biol. Chem.* **1994**, *269*, 5085–5091.
(25) Smith, W. L.; Lands, W. E. M. *Biochemistry* **1972**, *11*, 3276–3285.
(26) Teruhiko, S.; Kulmacz, R. J.; DeWitt, D. L.; Smith, W. L. *J. Biol. Chem.* **1990**, *265*, 20073–20076.
(27) Hsi, L. C.; Hoganson, C. W.; Babcock, G. T.; Garavito, R. M.; Smith, W. L. *Biochem. Biophys. Res. Commun.* **1995**, *207*, 652–660.
(28) Shi, W.; Hoganson, C. W.; Espe, M.; Bender, C. J.; Babcock, G. T.; Palmer, G.; Kulmacz, R. J.; Tsai, A.-L. *Biochemistry* **2000**, *39*, 4112–4121.
(29) Boulos, C.; Jiang, H.; Balazy, M. *J. Pharm. Exp. Ther.* **2000**, *293*, 222–229.
(30) Deeb, R. S.; Resnick, M. J.; Mittar, D.; McCaffrey, T.; Hajjar, D. P.; Upmacis, R. K. *J. Lipid Res.* **2002**, *43*, 1718–1726.
(31) Kim, S. F.; Huri, D. A.; Snyder, S. H. *Science* **2005**, *310*, 1966–1970.
(32) Deeb, R. S.; Shen, H.; Gamss, C.; Gavrilova, T.; Summers, B. D.; Kraemer, R.; Hao, G.; Gross, S. S.; Laine, M.; Maeda, N.; Hajjar, D. P.; Upmacis, R. K. *Am. J. Pathol.* **2006**, *168*, 349–362.
(33) Iwakiri, Y.; Satoh, A.; Chatterjee, S.; Toomre, D. K.; Chalouni, C. M.; Fulton, D.; Groszmann, R. J.; Shah, V. H.; Sessa, W. C. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 19777–19782.
(34) Szabo, C.; Ischiropoulos, H.; Radi, R. *Nat. Rev. Drug Discovery* **2007**, *6*, 662–680.
(35) Derakhshan, B.; Hao, G.; Gross, S. S. *Cardiovasc. Res.* **2007**, *75*, 210–219.
(36) Deeb, R. S.; Upmacis, R. K.; Lamon, B. D.; Gross, S. S.; Hajjar, D. P. *Hypertension* **2008**, *51*, 1–7.
(37) Deeb, R. S.; Hao, G.; Gross, S. S.; Laine, M.; Qiu, J. H.; Resnick, B.; Barbar, E. J.; Hajjar, D. P.; Upmacis, R. K. *J. Lipid Res.* **2006**, *47*, 898–911.
(38) Goodwin, D. C.; Gunther, M. R.; Hsi, L. C.; Crews, B. C.; Eling, T. E.; Mason, R. P.; Marnett, L. J. *J. Biol. Chem.* **1998**, *273*, 8903–8909.
(39) Takeguchi, C.; Kono, E.; Sih, C. J. *Biochemistry* **1971**, *10*, 2372–2376.
(40) Van der Ouderaa, F. J.; Buytenhek, M.; Nugteren, D. H.; Van Dorp, D. A. *Biochim. Biophys. Acta* **1977**, *487*, 315–331.

(100−200 mM in 4.7% sodium hydroxide) was obtained from Calbiochem, aliquoted, stored at −80 °C, and used fresh for each reaction. Monoclonal 3-nitrotyrosine antibody was obtained from Upstate Biotechnology. Anti-Mouse horseradish peroxidase conjugated IgG and ECL Plus reagents were obtained from GE Healthcare. Materials for SDS−PAGE, Western blotting, and anti-Rabbit horseradish peroxidase conjugated IgG were obtained from Bio-Rad Laboratories. A custom-made antibody (New England Peptide, Inc.) was raised in rabbits that recognizes nitrated Tyr385 in the sequence (NRIAMEFNHLY$_{NO_2}$HWHPLMPNSF) with >90% sequence homology in COX-1 across species (humans, mice, rat, and sheep). This polyclonal anti-3-nitro-Tyr385 COX-1 antibody was found to be selective for 3-nitro-Tyr385 in COX-1 when used for Western blotting and to not recognize other proteins (nitrated or non-nitrated) under the conditions employed herein. Furthermore, the antibody preferentially binds the nitrated COX-1 peptide antigen relative to the non-nitrated peptide homologue. The selectivity of this reagent is documented in the Supporting Information (Figure S1b).

FePPCOX-1 and CoPPCOX-1 were generated by reconstituting apoCOX-1 with metalloporphyrin (from a 500 μM solution) to a 1:1 molar ratio and measuring the absorbance at 412 nm ($\varepsilon = 1.42 \times 10^5$ M^{-1} cm^{-1}) using a Perkin-Elmer Lambda 20 spectrophotometer as described previously.[37]

Desired concentrations for COX inhibitors (described above) were reacted with COX samples for 40 min prior to the performance of further reactions. COX reactions with $ONOO^-$ were conducted at pH 8 in buffer (100 mM Tris or 20 mM $NaHCO_3$, 0.3 mM DeDTC, 5 mM EDTA, 0.1% Tween, or 0.3% βOG) using concentrated $ONOO^-$ (110 mM). The concentration of $ONOO^-$ used for each experiment was spectroscopically determined ($\varepsilon_{302nm} = 1670$ M^{-1} cm^{-1}).

Activity Assays. A peroxidase assay was used to measure COX-1 activity as previously described.[37,41,42] A detailed description of this assay is provided in the Supporting Information.

Quantification of 3-NT Using HPLC with Electrochemical (HPLC-EC) Detection. The protocol for 3-NT quantification by HPLC-EC was previously described[43] with modifications. A sample (30 μg of protein) is digested with proteinase K (150 U/mg, 8 h at 55 °C). The cooled digest is precipitated with 3 volumes of ice-cold buffer (0.1 M phosphoric acid and 0.23 M TCA), vortexed, incubated on ice for 5 min, and centrifuged at 12000*g* for 15 min at 4 °C. The resulting supernatant is concentrated at room temperature (rt; SpeedVac) followed by extraction with 2 volumes of chloroform. The aqueous fraction containing 3-NT is dried at rt (SpeedVac) and then reconstituted with vacuum-filtered (0.2 μm nylon membrane) and degassed HPLC mobile-phase buffer containing 90 mM sodium acetate, 35 mM citric acid, 130 μM EDTA, and 460 μM sodium octanesulfonate (pH 4.35) prepared in 18 MΩ resistance water. An isocratic HPLC system with a multichannel electrochemical (EC) CoulArray detector and EC cell (ESA, Inc.) is used to resolve 3-NT (+700 mV, retention time = 15 min) from other species using a 100 mm C18 column (Microsorb-MV, Varian) and flow rate of 0.75 mL/min. For pure COX-1 complexes, results are represented as pmol of 3-NT/mg of COX, i.e., pmol of 3-NT/14×10^3 pmol of COX.

Western Blot Analysis. A 2−3 μg portion of protein/sample was treated with SDS/mercaptoethanol, separated on a 10% acrylamide gel, and transferred onto a nitrocellulose membrane (Biorad). Conditions for probing with antibodies and band visualization are described in the Supporting Information.

LC−MS/MS Analysis and Database Search. nLC−MS/MS was used to identify sites of Tyr nitration in peptides from FePPCOX-1 and AA-bound FePPCOX-1 treated with $ONOO^-$ (0.5 mM). Analyses were performed using a 6520 accurate-mass quadrupole time-of-flight (Q-TOF) mass spectrometer with a chip cube and C18 column on-chip (Agilent). The mobile phases were 0.1% formic acid in water (solvent A) and 0.1% formic acid in 90% acetonitrile (solvent B). An 8 μL volume of the protein digest (preparation described in the Supporting Information) was injected onto a 4 mm 40 nL Zorbax 300SB-C18 enrichment column at a flow rate of 5 μL/min, and peptides were resolved on a 0.075 × 43 mm Zorbax 300SB-C18 analytical column (3.5 μm particle size) at a flow rate of 0.3 μL/min with a gradient of 3−50% solvent B for 20 min and 50−90% solvent B for 2 min. Mass spectra were acquired in the automated MS/MS mode, in which MS/MS scans were performed on the four most intense ions from each MS scan. The MS/MS spectra were used to identify the sites of Tyr nitration in COX-1 by a database search using SpectrumMill software (Agilent). Searching parameters were a minimum matched peak intensity of 50%, a precursor mass tolerance of 20 ppm, and a product mass tolerance of 50 ppm. The program was instructed to account for Tyr nitration when matching peptide fragments to COX-1.

CD Spectroscopy. Structural perturbations in COX-1 mixtures (0.2 mg/mL) were recorded in the UV region by CD spectroscopy using a Jasco J-720 spectropolarimeter optically reconditioned and upgraded electronically to the J-715 model. Spectral differences in the far-UV reflect contributions from the protein peptide backbone (i.e., α-helices, β-sheets, β-turns, or random coils); therefore, this region is a gauge of protein structure and conformation. Thus, the CD spectrum of COX-1, which has a very high helical content and almost no β-pleated sheets,[15] is characterized by two minima of almost similar intensity at ∼222 and ∼208 nm. Each spectrum represents data accumulated from 190 to 270 nm at 26 °C over a 10 min scanning period in a 0.01 cm cuvette (Helma). Data were recorded with a 1 nm bandwidth, 0.1 nm pitch, 1 s response time, and 100 nm/min scan speed. All results are reported as molar ellipticities and baseline corrected from buffer background and dilution effects using Jasco Spectra Manager software.

Statistical Analysis. All results were reproduced at least three times. Where appropriate, data are reported as averages ± SEM with significant differences determined by Student's *t* test. A $p < 0.05$ is statistically significant. Image J (version 1.34s, NIH) was used to quantify Western blot band densities in Figures S1b and S3a (Supporting Information).

Results

$ONOO^-$-Induced Structural Perturbations in COX-1 Are Driven by Heme. We previously demonstrated that Tyr385 in COX-1 undergoes heme-dependent nitration.[37] Accordingly, incubation of FePP-containing COX-1 with $ONOO^-$ abolishes enzyme activity, whereas incubation of apoCOX-1 (FePP-free) with $ONOO^-$, prior to reconstitution with FePP, does not alter AA catalysis. To investigate whether this phenomenon is facilitated by steric perturbations in COX-1, the FePP moiety of COX-1 was substituted with cobalt protoporphyrin (CoPP) and metalloporphyrin binding characteristics and the structural susceptibility of these complexes to $ONOO^-$ was examined. Notably, CoPPCOX-1 is structurally intact but catalytically incompetent.[16] As shown in Figure 1a, the absorbance spectrum of stoichiometrically reconstituted FePPCOX-1 (∼1:1 molar ratio) includes a characteristic Soret band at ∼412 nm. Likewise, reconstituted CoPPCOX-1 (Figure 1b) gives rise to a dominant Soret absorbance band (shifted to ∼425 nm) that closely resembles native-like reconstituted FePPCOX-1 in its spectral properties. However, the resulting change in the heme electronic environment when Fe^{3+} is substituted with Co^{3+} prevents CoPPCOX-1 from initiating catalysis.

(41) Kulmacz, R. J. *Prostaglandins* **1987**, *34*, 225–240.
(42) Loll, P. J.; Sharkey, C. T.; O'Connor, S. J.; Dooley, C. M.; O'Brien, E.; Devocelle, M.; Nolan, K. B.; Selinsky, B. S.; Fitzgerald, D. J. *Mol. Pharmacol.* **2001**, *60*, 1407–1413.
(43) Nuriel, T.; Deeb, R. S.; Hajjar, D. P.; Gross, S. S. *Methods Enzymol.* **2008**, *441*, 1–17.

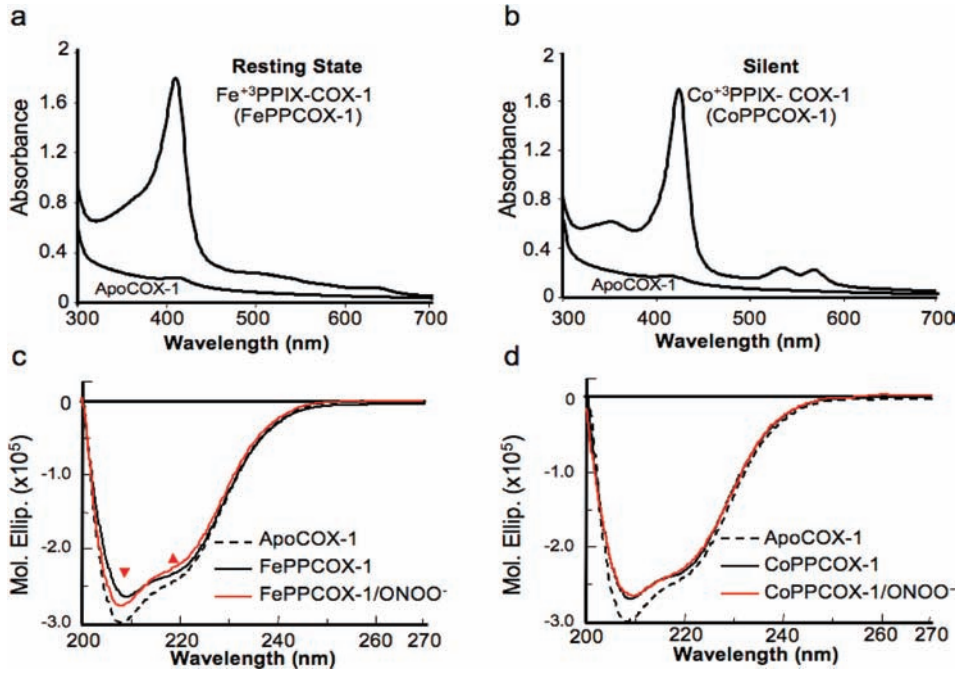

Figure 1. ONOO$^-$-induced structural perturbations in COX-1 are driven by heme. Absorbance spectra were acquired for the reconstitution of apoCOX-1 (0.21 mg/mL) at 25 °C with (a) Fe^{3+}PPIX (heme, FePP) and (b) Co^{3+}PPIX (CoPP) from stock solutions (500 μM each). (c) CD spectra acquired in the far-UV region at 25 °C compare the structure of apoCOX-1 (dashed) to that of FePPCOX-1 (solid black). The red CD spectrum was acquired following incubation of FePPCOX-1 with ONOO$^-$ (250 μM). (d) CD spectra were acquired as in (c), but for COX-1 reconstituted with CoPP.

COX-1 complexes in the absence of substrate were further analyzed by CD spectroscopy in the far-UV region to gauge secondary structural movements associated with ligand binding. As shown in Figure 1c, CD spectral characteristics of FePP-COX-1 are markedly different from those of apoCOX-1 at equimolar concentrations. While the shapes of both spectra, with two minima of almost identical intensities at 208 and 222 nm, suggest a dominant α-helical motif in both apoCOX-1 and FePPCOX-1, the increased total signal intensity in apoCOX-1 (a more negative signal) and the sharper minimum at 208 nm relative to 222 nm suggest helical disorder in the absence of bound FePP. ONOO$^-$ treatment of FePPCOX-1 induced a CD spectral change more closely resembling that of pure apoCOX-1 with predominant spectral band differences marked in Figure 1c by red arrows. Of note, spectral characteristics of apoCOX-1 were unaltered following exposure to ONOO$^-$. In contrast to that of FePPCOX-1, the CD spectrum for ONOO$^-$-treated CoPPCOX-1 was indistinguishable from that of untreated CoPPCOX-1 (Figure 1d), clearly indicating that global ONOO$^-$-induced secondary structural perturbations in COX-1 are heme-dependent (i.e., require Fe^{3+} rather than Co^{3+} occupancy of protoporphyrin IX).

Substrate Identity Dictates ONOO$^-$-Induced Structural Changes in FePPCOX-1. AA (ω-6-eicosatetraenoic acid) and EPA (ω-3-eicosapentanoic acid) are 20-carbon fatty acids that differ only in their double bond content (four vs five, respectively). Of note, mediators derived from ω-3 fatty acids (such as EPA) are endogenous attenuators of inflammation and elicit cardioprotective actions in humans.[44] Whereas AA is the principal substrate of COX-1, EPA is a relatively poor substrate, owing to the double bond between C17 and C18 which creates a poor fit within the COX-1 active-site channel[45] (Figure 2a). Furthermore, EPA oxygenation by COX-1 requires more hydroperoxide for the initiation of catalysis than does AA.[46] To this end, COX-1 peroxidase activity assays indicated that, at the same concentration, EPA is 3-fold less active as a substrate compared with AA, using either purified FePPCOX-1 or native microsomal COX-1 (Figure 2a).

Far-UV CD spectral characteristics for substrate-bound FeP-PCOX-1 complexes were compared and analyzed for secondary structural changes following ONOO$^-$ incubation. Despite its initiation of catalysis, binding of AA to FePPCOX-1 caused very slight spectral changes relative to unbound FePPCOX-1 over a 20 min scanning period (Figure 2b). While incubation of AA-bound FePPCOX-1 with ONOO$^-$ caused an additional attenuation in signal intensity (Figure 2b), the observed change in AA-bound FePPCOX-1 was minimal compared to that in AA-free FePPCOX-1 (Figure 1c). Interestingly, neither EPA binding nor ONOO$^-$ treatment of EPA-bound FePPCOX-1 generated a marked spectral perturbation relative to substrate-free FePPCOX-1 (Figure 2c). We confirmed that the observed spectral changes were not an artifact of negligible dilution arising from substrate addition and that substrate occupancy of the COX-1 active site indeed determines the extent of ONOO$^-$-induced structural alterations.

Arachidonic Acid Binding Redirects ONOO$^-$-Induced COX-1 Nitration. Conversion of AA to eicosanoid products by COXs requires both cyclooxygenase and peroxidase functionalities. Previously, we demonstrated that ONOO$^-$ promotes heme-catalyzed Tyr385 nitration and subsequent inactivation of FePPCOX-1.[37] While global COX-1 nitration was also observed in the absence of heme (apoCOX-1), the catalytically

(44) Dona, M.; Fredman, G.; Schwab, J. M.; Chiang, N.; Arita, M.; Goodarzi, A.; Cheng, G.; von Andrian, U. H.; Serhan, C. N. *Blood* **2008**, *112*, 848–855.
(45) Malkowski, M. G.; Thuresson, E. D.; Lakkides, K. M.; Rieke, C. J.; Micielli, R.; Smith, W. L.; Garavito, R. M. *J. Biol. Chem.* **2001**, *276*, 37547–37555.
(46) Smith, W. L. *Curr. Opin. Cell Biol.* **2005**, *17*, 174–182.

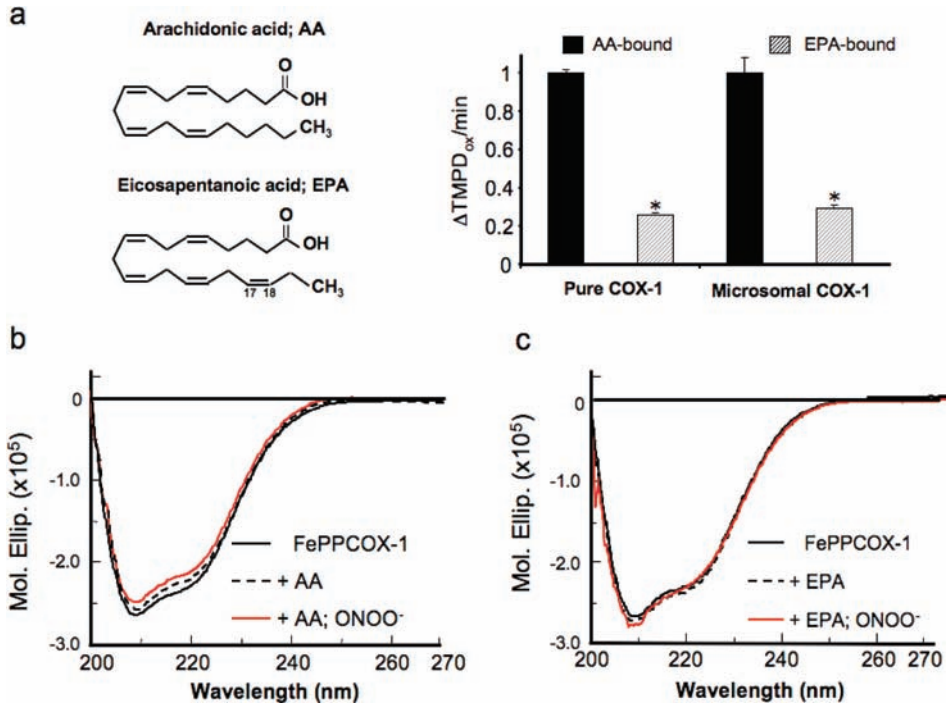

Figure 2. Substrate identity dictates $ONOO^-$-induced structural changes in FePPCOX-1. (a) Cyclooxygenase-coupled peroxidase activity measured in purified FePPCOX-1 (2 μg) and microsomal COX-1 (10.5 μg) following the immediate addition of AA (100 μM) and EPA (100 μM) at pH 8. Results are monitored as ΔA_{611} during a 60 s interval. Data are averages $\pm$ SEM. The asterisk indicates $p < 0.0002$ for EPA activation relative to AA activation. (b) CD spectra acquired in the far-UV region at 25 °C compare the structure of FePPCOX-1 (0.21 mg/mL, solid black) to that of AA-bound FePPCOX-1 (dashed). The red CD spectrum was acquired following incubation of AA-bound FePPCOX-1 with $ONOO^-$ (250 μM). (c) CD spectra were acquired similarly to those in (b) but for EPA-bound FePPCOX-1.

essential Tyr385 residue did not undergo nitration, and thus, activity was preserved. To determine whether substrate binding sterically hinders $ONOO^-$ access to Tyr385 for heme-catalyzed nitration, we compared the susceptibility of AA-incubated (active) and AA-free (resting) COX-1 to global and Tyr385-specific nitration by $ONOO^-$. HPLC-EC and Western blot analysis with an anti-nitrotyrosine (anti-3-NT) antibody were used to measure global levels of $ONOO^-$-induced protein Tyr nitration in the presence and absence of substrate, in two types of COX-1 complexes: (1) FePPCOX-1, which possesses both cyclooxygenase and peroxidase functionalities and is capable of metabolizing substrate, and (2) CoPPCOX-1, which possesses only peroxidase activity and is incapable of metabolizing substrate. Of note, the use of HPLC-EC for measurement of global protein 3-NT levels circumvents the detection of false signals that can arise from nonspecific interactions involving the anti-3-NT antibody or diminished signals that can arise from COX-1 multimer formation under certain reaction conditions (described in Figure 6). In the absence of substrate, global Tyr nitration was reduced by about 3-fold in CoPPCOX-1 relative to FePPCOX-1, as measured by HPLC-EC (Figure 3a). Consistent with this HPLC-EC finding, Tyr nitration was also observed by Western blotting of both $ONOO^-$-treated FePPCOX-1 and $ONOO^-$-treated CoPPCOX-1 and the levels were markedly reduced when Fe^{3+} was substituted with Co^{3+} (Figure 3b). Of note, Western blotting results indicate that AA binding enhanced total levels of nitration in $ONOO^-$-treated COX-1 (Figure 3b). Additional aliquots of these samples were probed by Western blotting with a selective anti-3-nitro-Try385 antibody. While AA binding to FePPCOX-1 was associated with a global increase in $ONOO^-$-induced FePPCOX-1 nitration, this was accompanied by a remarkable decrease in Tyr385-specific nitration (Figure 3c). Substitution of Fe^{3+} with Co^{3+} had little impact on the levels of Tyr385 nitration in AA-bound COX-1 (Figure 3c), emphasizing the fundamental role heme plays in directing this modification. Notably, HPLC-EC results showed an apparent slight decrease in global Tyr nitration of AA-bound CoPPCOX-1 relative to CoPPCOX-1 (Figure 3a), while Western blotting suggested the opposite result (Figure 3b). These fluctuations were not statistically significant.

Interestingly, substitution of AA with EPA in these complexes was associated with a reduced susceptibility to nitration (Figure 3d−f). Whereas AA binding elicited a ~2-fold increase in $ONOO^-$-induced FePPCOX-1 nitration, EPA binding significantly diminished $ONOO^-$-induced nitration of this complex (Figure 3d). This HPLC-EC finding of decreased total nitration of COX-1 by $ONOO^-$ when occupied by EPA, compared with AA, was further corroborated by Western blotting (Figure 3e). Importantly, both AA and EPA binding within the active-site channel of COX-1 were associated with specific protection of the catalytically essential Tyr385 from $ONOO^-$-induced nitration (Figure 3f). Thus, strong levels of global Tyr nitration are observed with AA as substrate (active state), while robust Tyr385 nitration is observed in the resting state of COX-1 when substrate does not occupy the active-site channel. These findings indicate that both AA and EPA binding to COX-1 may prevent access of the active-site Tyr385 to nitration, while concomitantly redirecting nitration to other Tyr residues in COX-1, presumably via a substrate-induced allosteric interaction. The ability of substrate occupancy of the active site to prevent Tyr385 from nitration (i.e., prevention of an inactivating process) and redirect nitration to other Tyr residues was similarly found in studies of crude native COX-1 (i.e., sheep microsomes), where the heme moiety is endogenously bound in the COX-1 peroxidase site (Supporting Information, Figure S1).

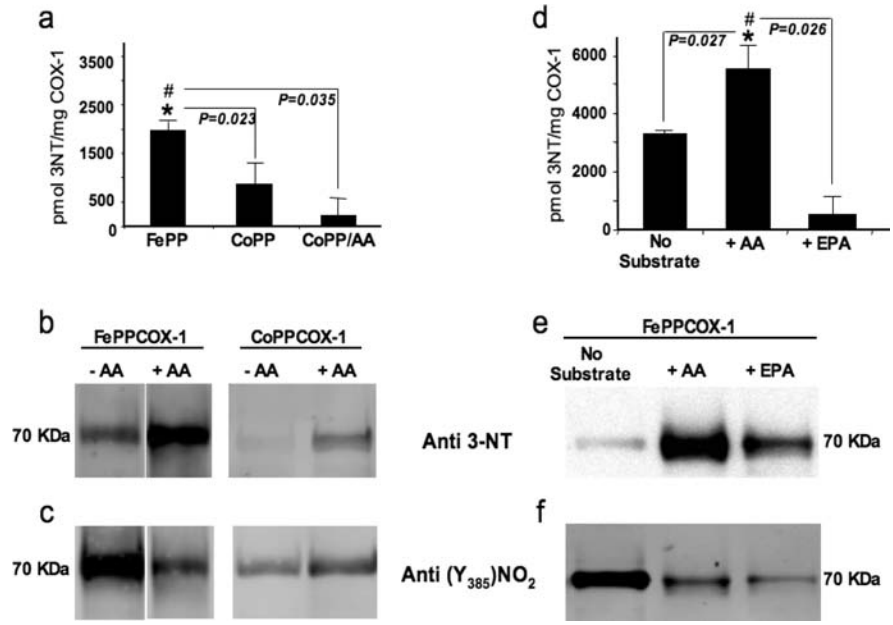

Figure 3. AA binding redirects $ONOO^-$-induced COX-1 nitration. In all reactions, COX-1 complexes were at 0.21 mg/mL, AA was at 100 μM, and $ONOO^-$ was at 250 μM. (a) Quantification of total 3-NT by HPLC-EC for the reaction of FePPCOX-1 and CoPPCOX-1 with $ONOO^-$. Total 3-NT for the reaction of AA-bound CoPPCOX-1 with $ONOO^-$ was also quantified. Data are averages ± SEM. The asterisk indicates $p \leq 0.023$ for FePPCOX-1 relative to CoPPCOX-1. The pound sign indicates $p \leq 0.035$ for AA-bound CoPPCOX-1 relative to FePPCOX-1. (b) Western blots compare the reactions of FePPCOX-1 and CoPPCOX-1 with $ONOO^-$ ± AA. Blots were probed with monoclonal anti-3-NT and with (c) polyclonal anti-3-nitro-Tyr385-peptide in COX-1. (d) Quantification of total 3-NT by HPLC-EC for the reaction of FePPCOX-1 with $ONOO^-$ ± AA and EPA (100 μM). Data are averages ± SEM. The asterisk indicates $p \leq 0.027$ for AA-bound FePPCOX-1 relative to substrate-free FePPCOX-1. The pound sign indicates $p \leq 0.026$ for EPA-bound FePPCOX-1 relative to AA-bound FePPCOX-1. (e) Western blots for the reaction of FePPCOX-1 with $ONOO^-$ ± AA and EPA. Blots were probed similarly to those in (b) and (c).

Evidence for Specificity in $ONOO^-$-Induced Nitration of COX-1. To confirm that AA binding is associated with a significant increase in $ONOO^-$-induced global nitration of FePPCOX-1, despite protection against Tyr385 nitration, we sequence-mapped Tyr nitration in reaction mixtures by nLC−MS/MS. We had previously demonstrated by mass spectral studies that $ONOO^-$ treatment of FePPCOX-1 caused selective heme-driven nitration of Tyr385 that was absent in apoCOX-1.[37] Herein, we have extended these studies to investigate the impact of AA binding in COX-1. Figure 4a depicts overlays of deconvoluted extracted ion chromatograms (EICs) with a mass-to-charge ratio (*m*/*z*) of 649.06 for $ONOO^-$-treated FePPCOX-1 in the presence and absence of AA. The identified peptide ion, which elutes at ~15 min, represents the 649.06 *m*/*z* peptide from substrate-free FePPCOX-1 which is nitrated at Tyr385 (377-IAMEFNQLY$_{NO_2}$HWHPLMPDSFR-396), establishing that Tyr385 is indeed susceptible to nitration when AA is absent (Figure 4a, top EIC). However, this peptide ion was not detected in $ONOO^-$-treated AA-bound FePPCOX-1 (Figure 4a, bottom EIC), indicating that AA selectively prevents Tyr385 nitration. Notably, identification of the *m*/*z* 649.06 peptide ion as containing nitrated Tyr385 was confirmed by collision-induced dissociation and analysis of y and b product ion fragments (Figure 4b). As expected, untreated FePPCOX-1 in the presence or absence of AA did not result in an *m*/*z* 649.06 peptide ion, in accord with a lack of Tyr385 nitration. Additionally, the peptide ion containing unmodified Tyr385 (i.e., Ile377-Arg396) could be detected at *m*/*z* 634.30 in all samples. Together, these results demonstrate that COX-1 occupancy with AA selectively protects against heme-catalyzed Tyr385 nitration, while promoting collateral nitration.

An additional COX-1-derived peptide, containing nitrated Tyr254 (254-Y$_{NO_2}$QMLNGEVYPPSVEEAPVLMHYPRG-278),

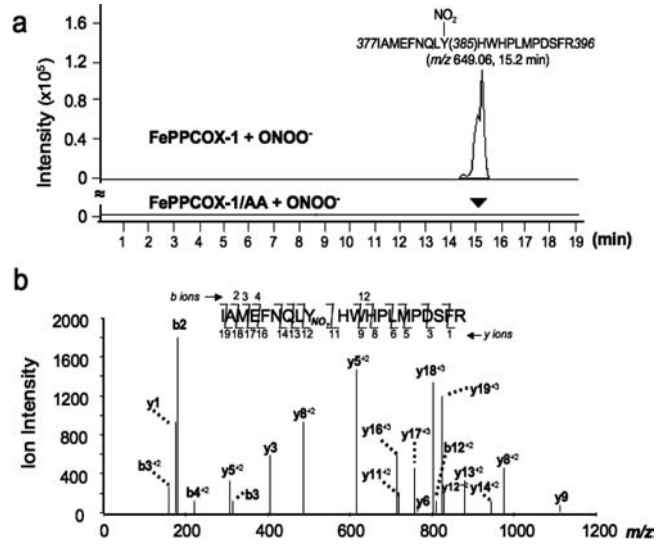

Figure 4. Evidence for specificity in $ONOO^-$-induced nitration of COX-1. FePPCOX-1 and AA-bound FePPCOX-1 (0.21 mg/mL each) were incubated with $ONOO^-$ (500 μM) for 1 h at room temperature. Trypsinized reaction mixtures were analyzed by nLC−MS/MS as described in the Methods. (a) Overlays of deconvoluted extracted ion chromatograms that monitor the elution of the tryptic peptide fragment (*m*/*z* 649.06) representing the quadruply charged peptide ion containing nitrated Tyr385. The arrow at ~15 min in the lower elution profile denotes the missing peptide of *m*/*z* 649.06 for AA-bound FePPCOX-1 treated with $ONOO^-$ that is present in the top elution profile of $ONOO^-$-treated FePPCOX-1. (b) MS/MS analysis of the peptide ion eluting at ~15 min from $ONOO^-$-treated FePPCOX-1 for confirmation of peptide identity. Daughter ions that arise from the parent ion *m*/*z* 649.06 are depicted.

was present at similar levels in the absence and presence of AA in $ONOO^-$-treated FePPCOX-1, eluting at ~14 min with

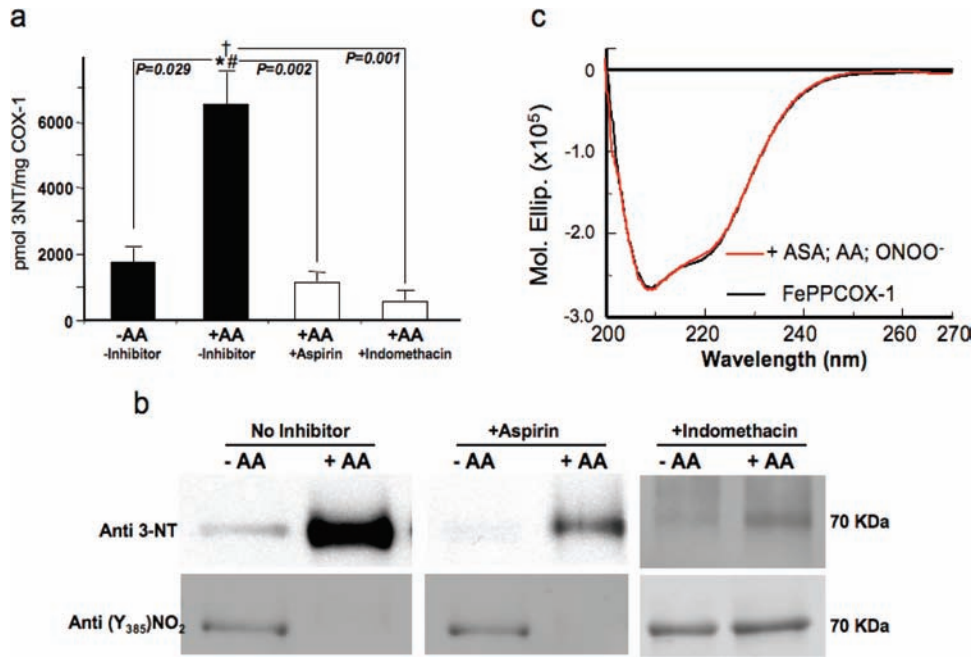

Figure 5. COX inhibitors attenuate $ONOO^-$-induced nitration and prevent conformational changes in COX-1. In all reactions, COX-1 complexes were at 0.21 mg/mL and $ONOO^-$ was at 250 μM (a) Quantification of total 3-NT by HPLC-EC for the reaction of FePPCOX-1 with $ONOO^-$ ± AA (100 μM) and the COX inhibitors aspirin (70 μM) and indomethacin (14 μM). Data are averages ± SEM. The asterisk indicates $p \leq 0.029$ for AA-bound FePPCOX-1 relative to substrate-free FePPCOX-1. The pound sign indicates $p \leq 0.002$ for aspirin-treated FePPCOX-1 relative to AA-bound FePPCOX-1. The dagger indicates $p \leq 0.001$ for indomethacin-treated FePPCOX-1 relative to AA-bound FePPCOX-1. (b) Western blots for the reaction of FePPCOX-1 with $ONOO^-$ ± AA (100 μM) and the COX inhibitors aspirin (70 μM) and indomethacin (14 μM). Blots were probed with monoclonal anti-3-NT (top) and with polyclonal anti-3-nitro-Tyr385 peptide in COX-1 (bottom). (c) CD spectra recorded in the far-UV region at 25 °C compare the structure of FePPCOX-1 (black) to that of aspirin-treated FePPCOX-1 following reaction with $ONOO^-$.

m/z 955.79 (Supporting Information, Figure S2). The nonselectivity of $ONOO^-$-induced Tyr254 nitration is not surprising given the fact that it is surface exposed as compared to Tyr385, which is buried in the enzyme core. Of note, treatment of AA-bound FePPCOX-1 with increasing concentrations of $ONOO^-$ (0.5−2 mM) enhanced levels of methionine oxidation (detected residues include Met256, Met273, Met522, and Met525) and Tyr254 nitration relative to substrate-free FePPCOX-1.

A body of work exists on the chemical reactivity of nitrosocarbonate, the reaction product of $ONOO^-$ with carbon dioxide and a powerful nitrating agent that can form under physiological bicarbonate levels.[47,48] Thus, we repeated experiments in the presence of millimolar bicarbonate levels to determine whether nitration patterns could be modified under these conditions. Western blotting results demonstrate a remarkable increase in nitration levels of apoCOX-1 as well as FePPCOX-1 in bicarbonate buffer relative to Tris buffer. Importantly, the increase in apoCOX-1 nitration in the presence of bicarbonate did not interfere with the enzyme's ability to reconstitute heme and regain full catalytic function (Supporting Information, Figure S3). These results support our findings that, despite extensive increases in COX-1 nitration and oxidation under certain conditions, only nitration at Tyr385 results in loss of catalytic function. Additionally, substrate occupancy and ensuing allosteric consequences determine the sites and extent of COX-1 nitration by $ONOO^-$.

COX Inhibitors Attenuate $ONOO^-$-Induced Nitration and Prevent Conformational Changes in COX-1. We then investigated whether COX-1 active-site occupancy by pharmacological inhibitors alters global COX-1 nitration and protects against heme-catalyzed Tyr385 nitration. Levels of $ONOO^-$-induced FePPCOX-1 nitration were compared following AA addition, in the presence and absence of aspirin or indomethacin. HPLC-EC quantification of global COX-1 nitration levels showed a ∼6-fold reduction in aspirin-pretreated FePPCOX-1 and a ∼10-fold reduction in indomethacin-pretreated FePPCOX-1, relative to FePPCOX-1 in the presence of AA alone (Figure 5a). Consistent with HPLC-EC measurements, global nitration levels were markedly reduced in aspirin- and indomethacin-pretreated COX-1 in both the presence and absence of AA, as shown by Western blotting (Figure 5b). Notably, these reagents suppressed tyrosine nitration in a nonspecific manner as demonstrated by their ability to alter $ONOO^-$-induced nitration levels in bovine serum albumin, an unrelated protein (Supporting Information, Figure S4a). Interestingly, while aspirin pretreatment of FePPCOX-1 protected Tyr385 from $ONOO^-$-induced nitration, indomethacin pretreatment did not attenuate nitration of Tyr385 (Figure 5b).

Structural evaluation by CD spectroscopy showed that aspirin-pretreated FePPCOX-1 is resistant to $ONOO^-$-induced structural perturbations, relative to untreated FePPCCOX-1 (Figure 5c). Binding of aspirin to FePPCOX-1 in the absence of $ONOO^-$ treatment did not result in detectable spectral changes relative to untreated FePPCOX-1 (Supporting Information, Figure S4b). Solutions containing indomethacin contributed interference in the far-UV region, which prevented the acquisition of CD spectra. Nevertheless, COX-1 occupancy by aspirin appears to

(47) Lymar, S. V.; Jiang, Q.; Hurst, J. K. *Biochemistry* **1996**, *35*, 7855–7861.
(48) Radi, R.; Denicola, A.; Freeman, B. A. *Methods Enzymol.* **1999**, *301*, 353–367.

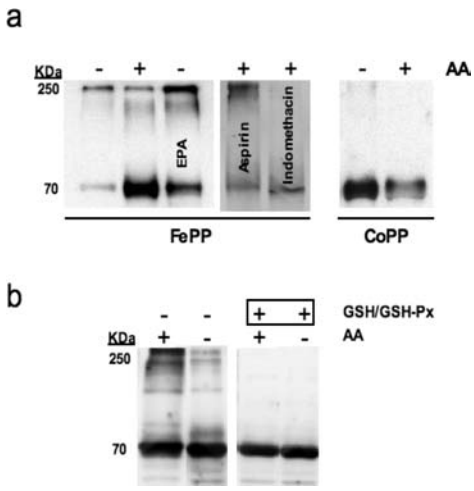

Figure 6. $ONOO^-$ is a stimulus for heme-dependent COX-1 multimer formation. Western blots for the reaction of reconstituted COX-1 (0.12 mg/mL) with $ONOO^-$ (250 μM) in the presence (+) and absence (−) of (a) AA (100 μM), EPA (100 μM), aspirin (70 μM), and indomethacin (14 μM) and (b) AA (100 μM) and glutathione (GSH; 0.25 mM) in combination with glutathione peroxidase (GSH-Px; 25 U/mL). Blots were probed with monoclonal anti-3-NT.

limit the access of $ONOO^-$ into the active-site channel, thereby protecting against heme-catalyzed Tyr385 nitration.

$ONOO^-$ Is a Stimulus for Heme-Dependent COX-1 Multimer Formation. We previously demonstrated that treatment of purified and reconstituted FePPCOX-1 with $ONOO^-$ results in the appearance of high molecular mass bands at ∼200−250 kDa (when probed with an anti-3-NT Ab), suggesting the presence of nitrated COX-1 multimers which were absent in $ONOO^-$-treated apoCOX-1.[37] Furthermore, substrate binding to FePPCOX-1 amplified nitration levels at an apparent mass of 70 kDa as well as in higher molecular mass COX-1 species.[37] Although EPA binding to FePPCOX-1 attenuated total nitration by $ONOO^-$ relative to AA binding (at 70 kDa), the presence of nitrated COX-1 multimers was similarly observed with EPA and AA (Figure 6a). On the other hand, aspirin, and to a greater extent indomethacin, reduced the levels of AA-engendered nitrated COX-1 multimers (Figure 6a). Interestingly, treatment of reaction mixtures with a hydroperoxide scavenging system (glutathione and glutathione peroxidase) abolished the formation of COX-1 multimers (Figure 6b). Collectively, these findings suggest that exposure of FePPCOX-1 to peroxide, generated from catalysis or by the direct addition of $ONOO^-$, promotes COX-1 multimer formation. Notably, nitrated multimers were absent in $ONOO^-$-treated FePPCOX-1 when using bicarbonate buffer (Supporting Information, Figure S3a). Furthermore, substitution of Co^{3+} for Fe^{3+} in protoporphyrin IX of $ONOO^-$-treated COX-1 abrogated the formation of nitrated COX-1 multimers in the presence and absence of AA (Figure 6a), consistent with the absence of multimers in apoCOX-1,[37] thereby implicating heme as a mediator of COX-1 multimer formation.

Discussion

RNS-dependent posttranslational modifications of proteins are increasingly appreciated for their potential to alter protein and cellular functions. The results presented herein demonstrate that COX nitration occurs in a manner in which substrate binding determines the modification site and degree to which catalytic function is consequently lost. Furthermore, we demonstrate the use of nitration as a probe for allosteric changes in COX-1 protein structure.

The heme moiety in the solvent-exposed peroxidase site of COXs can be easily replaced with other metalloporphyrins, e.g., CoPP, to yield structurally native like complexes but with altered functionality.[16] Additionally, the cyclooxygenase active-site channel can accommodate an array of chemically distinct fatty acid substrates and NSAIDs that produce COX complexes with or without productive catalysis.[2,3] It is likely that some degree of molecular motion is associated with ligand binding regardless of whether the end result is activation or inhibition. Although crystallographic images do not reveal tangible structural variations among the various COX complexes, it is inferred that the entry site to the hydrophobic channel may undergo conformational changes that allow for substrate binding and product exit, or may exhibit structural rigidity as a result of inhibitor binding.[3,49]

By using a transient pulse of $ONOO^-$ as a reactive probe that accesses unhindered sites on COX-1 and can be detected as stable covalent modifications, we could distinguish very subtle changes in secondary structure with CD spectroscopy. Notably, in the absence of ligand binding, $ONOO^-$ resulted in heme-dependent structural perturbations that were undetectable when COX-1 heme was substituted with CoPP (Figure 1). The observed spectral distortions in resting-state heme-bound COX may indicate helical flexibility at the peroxidase site that could arise from heme alterations by the oxidant $ONOO^-$.[37] Interestingly, COX-1 adopted a more rigid helical structure with ligand binding in the active-site channel (Figures 2 and 5). Whereas aspirin inhibition as well as binding of a poorly metabolized substrate (EPA) resulted in no apparent $ONOO^-$-induced structural perturbations, binding of AA, the preferred COX substrate, resulted in a very modest but reproducible spectral change that may suggest protein aggregation following catalysis. Thus, the identity of the COX-1 ligand, either substrate or inhibitor, confers rigidity to the molecule that is detectable by CD spectroscopy following exposure to $ONOO^-$.

The susceptibility of Tyr residues to nitration due to peroxidase metal and ligand identity was also determined (Figure 3). Substitution of Fe^{3+} with Co^{3+} significantly diminished global levels of Tyr nitration that were further reduced by substrate binding. The static nature of substrate-bound CoPPCOX-1 likely limits $ONOO^-$ access to Tyr sites. In contrast, occupancy of the COX-1 channel with AA, a catalytically productive complex, generates the highest levels of $ONOO^-$-induced Tyr nitration. A lower amount of Tyr nitration was observed with EPA-bound FePPCOX-1 relative to AA-bound FePPCOX-1, where the substrate is misaligned in the COX channel and the complex is catalytically incompetent.[46] Surprisingly, our results demonstrate that while Tyr385, residing in the conduit between peroxidase and cyclooxygenase functionalities, is a target for nitration in the absence of substrate binding, AA protects Tyr385 from nitration. We further found that AA binding protects COX-1 from inactivation by $ONOO^-$ by redirecting the nitrative insult away from Tyr385 and toward alternate Tyr residues on COX-1. This is evident from the increase in global Tyr nitration coupled with a specific decrease in Tyr385 nitration during AA-initiated catalysis (Figures 3 and 4). Additionally, while the powerful and physiological nitrating agent nitrosocarbonate

(49) Kurumbail, R. G.; Stevens, A. M.; Gierse, J. K.; McDonald, J. J.; Stegeman, R. A.; Pak, J. Y.; Gildehaus, D.; Miyashiro, J. M.; Penning, T. D.; Seibert, K.; Isakson, P. C.; Stallings, W. C. *Nature* **1996**, *384*, 644–648.

remarkably increased nitration levels in COX-1, it did not prevent heme reconstitution and regeneration of full catalytic function (Supporting Information, Figure S3). As expected, inhibition of COX function with aspirin and indomethacin attenuated global $ONOO^-$-induced nitration (Figure 5). While aspirin protected Tyr385 from nitration, this effect was not observed for indomethacin for reasons that are unclear. Of note, aspirin and NSAIDs have been previously shown to display antioxidant properties and possess the ability to scavenge reactive oxygen species.[50−52] Thus, a reduction in global COX-1 nitration in the presence of NSAIDs may be due to $ONOO^-$ scavenging, a phenomenon that was underlined by the ability of aspirin to reduce global nitration in bovine serum albumin, an unrelated protein (Supporting Information, Figure S4). Interestingly, aspirin, but not other NSAIDs, has been shown to trigger cell−cell interactions that can shunt arachidonic acid metabolism and form novel anti-inflammatory mediators.[53]

The specificity and differential nitration observed for $ONOO^-$-induced Tyr385 nitration, a residue that is buried in the enzyme core, was not observed for any of the other Tyr residues in COX-1. By mass spectrometry, we identified and determined that the surface-exposed Tyr254 is almost equally nitrated in substrate-bound and substrate-free COX-1 complexes (Supporting Information, Figure S2). Thus, the COX-1 conformation, Tyr residue environment, steric hindrance to nitrating agent, and degree of solvent exposure all emerge as physical parameters that determine the specificity of nitration in COX-1 and ultimately characteristics of enzymatic function.

Previously, we demonstrated that heme catalyzed the formation of $ONOO^-$-induced nitrated COX-1 multimers but their levels were increased with AA binding by a mechanism that remains unclear.[37] In this study, nitrated multimers were also observed with EPA binding and were abrogated when the heme moiety was replaced by CoPP (Figure 6). Aspirin, and to a greater extent indomethacin, also reduced the levels of nitrated multimers. Of note, heme-catalyzed COX-1 multimer formation was observed with bound substrate and in the absence of $ONOO^-$ treatment. We can rule out disulfide formation as the mechanism for this observed cross-linking in substrate-bound samples since all samples were treated with a thiol reductant prior to Western blotting. As treatment of reaction mixtures with a hydroperoxide scavenging system abolished the formation of COX-1 multimers, we can conclude that exposure of COX-1 to peroxide generated from either catalysis or by the direct addition of $ONOO^-$ may be a stimulus for COX-1 multimer formation in the presence of heme (Figure 6). An alternative process leading to aggregate formation could involve dityrosine cross-linking, which has been observed in proteins identified in neurodegenerative disorders.[54] Nevertheless, as the identity of substrate did not modify COX-1 aggregates and bicarbonate attenuated the capacity of these multimers to form, it remains unclear whether high molecular mass COX forms contribute to enzymatic deactivation or other pathophysiological events in vivo.

Conclusion

We and others have previously demonstrated that $ONOO^-$ can provide the peroxide tone for heme activation of COX.[12,13] Subsequent results suggested that $ONOO^-$ can inactivate COX-1 catalysis by nitration.[37] We now know that substrate can regulate the access of inactivating nitrating species to COX-1. While heme-driven nitration of the centrally located Tyr385 by $ONOO^-$ in substrate-free COX inhibits all catalytic function, AA-bound COX is structurally and functionally preserved with nitration directed away from Tyr385 and to alternate Tyr sites. EPA similarly protects Tyr385 nitration, albeit with attenuated catalytic activity and distinct product generation versus that observed with AA. Alternatively, NSAIDs inhibit catalysis and prevent Tyr385 nitration, yet these resultant COX−inhibitor complexes retain their peroxidase activity. During inflammatory conditions, $ONOO^-$ formation is enhanced, increasing the biological relevance of interactions between RNS and COX enzymes that appear to be directed by the bioavailability of substrate and/or the presence of inhibitors. A relevant paradigm has been shown in cardiovascular disease models whereby the bioavailability of functional NO is decreased and may be compensated for by an elevation in COX function and a concomitant increase in vasorelaxant prostanoid synthesis (i.e., prostacyclin).[55,56] It is known that blood vessels are prone to alterations in NO chemistry in the setting of chronic inflammation whereby reactive oxygen species (ROS; i.e., superoxide) production is elevated and may consume NO to generate RNS (e.g., N_2O_3, NO_2, and $ONOO^-$). This study reveals a novel role for substrate in protecting COX-1 from inactivation by nitration in pathophysiological settings.

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Supporting Information Available: An expansion of methods essential to experimental reproducibility as well as additional results that contribute to the overall conclusions of the study are provided as supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

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(50) Wolin, M. S. *Circ. Res.* **1998**, *82*, 1021–1022.
(51) Shi, X.; Ding, M.; Dong, Z.; Chen, F.; Ye, J.; Wang, S.; Leonard, S. S.; Castranova, V.; Vallyathan, V. *Mol. Cell. Biochem.* **1999**, *199*, 93–102.
(52) Petersen, A.; Carlsson, T.; Karlsson, J. O.; Zetterberg, M. *Ophthalmic Res.* **2008**, *40*, 77–85.
(53) Claria, J.; Serhan, C. N. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 9475–9479.
(54) Souza, J. M.; Giasson, B. I.; Chen, Q.; Lee, V. M.-Y.; Ischiropoulos, H. *J. Biol. Chem.* **2000**, *275*, 18344–18349.
(55) Osanai, T.; Fujita, N.; Fujiwara, N.; Nakano, T.; Takahashi, K.; Guan, W. P.; Okumura, K. *Am. J. Physiol.: Heart Circ. Physiol.* **2000**, *278*, H233–H238.
(56) Drelicharz, L.; Kozlovski, V.; Skorka, T.; Heinze-Paluchowska, S.; Jasinski, A.; Gebska, A.; Guzik, T.; Olszanecki, R.; Wojnar, L.; Mende, U.; Csanyi, G.; Chlopicki, S. *Basic Res. Cardiol.* **2008**, *103*, 417–430.