Supporting Information

Physical Evidence for Substrate in Preservation of Cyclooxygenase Function under Nitrative Stress

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Supporting Methods:

Activity assays. A peroxidase assay was used to measure COX-1 activity. This assay monitors the TMPD oxidation by changes in absorbance at 611 nm following enzyme activation. Peroxidase assays in this study utilize the COX substrates (AA or EPA) as the activating species and thus, are referred to as coupled cyclooxygenase-peroxidase assays. At 25°C, the ratio of TMPD oxidation to oxygen consumption by COX in 60 s is 1 mol TMPD/1mol O_2. COX-1 activity is expressed as the rate of change in TMPD oxidation in 1 m.

Western blot analysis. Following separation and protein transfer**,** membranes were blocked (in 5% non-fat milk; 1 h) followed by incubation with either a mouse monoclonal 3-NT antibody (1:1000 dilution with 1% non-fat milk; overnight) or with a custom-made rabbit polyclonal nitrated Tyr385 antibody (1:500 dilution with 1% non-fat milk; overnight) or with anti-mouse COX-1 antibody (1:1000 dilution with 1% non-fat milk; 1 h). Bands were revealed using enhanced chemiluminescence (ECL plus) and visualized on film or by Typhoon Trio+ (GE Healthcare).

Sample preparation for LC-MS/MS analysis. Proteins were precipitated with three volumes of ice-cold acetone, dried, then re-solubilized in ammonium bicarbonate (50 mM at pH 8) and 50 % trifluoroethanol. Following reduction by dithiothreitol (DTT; 4 mM, 1 h, 60°C), alkylation by iodoacetamide (16 mM, 1 h, RT, dark), proteins were digested with trypsin sequencing grade (Promega):COX-1 (1:25) for 16 h at 37°C. Acidified digests (0.2 % trifluoroacetic acid; TFA) were washed with 0.2 % TFA on C18 sorbent (Strata C18-T, Phenomenex) then eluted with 80% acetonitrile and 0.2% TFA.

Supporting Figures:

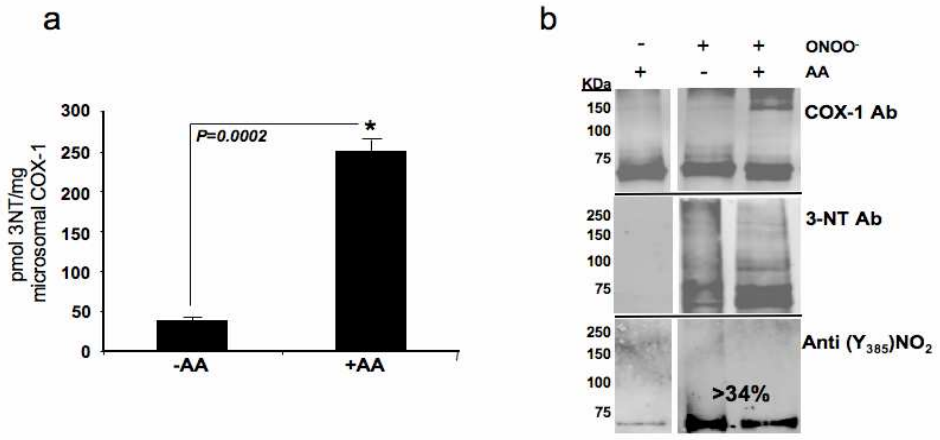

Figure S1a. Differential COX-1 nitration (with native endogenous heme) occurs in a complex biological mixture. (**a**) HPLC-EC quantification of total 3-NT in ovine microsomal homogenates (10.5 mg/ml) reacted with $ONOO^-$ (250 μM) in the presence and absence of arachidonic acid (AA; 100 μM). Data are averages ± SEM. *$p \leq 0.0002$; significantly different values between untreated and AA-treated microsomal homogenates. (**b**) Western blots for ovine microsomal homogenates (10.5 mg/ml) in the presence and absence of $ONOO^-$ (250 μM) and AA (100 μM). Blots were probed with monoclonal anti-COX-1 (top blot), monoclonal anti-nitrotyrosine (middle blot) and polyclonal anti-3-nitro-Tyr385-peptide from COX-1 (bottom blot). Relative $ONOO^-$ induced nitration of Tyr385 (bottom blot) was obtained by quantifying band density ratios between untreated and AA-treated microsomal homogenates.

Figure S1b. The specificity of polyclonal anti-3-nitro-Tyr385 antibody for nitrated Tyr385 peptide in COX is demonstrated by Western blotting for the reaction of ovine microsomal homogenates (10.5 mg/ml) ± $ONOO^-$ (250 μM) and AA (100 μM). The bottom blot shows that the anti-3-nitro-Tyr385 antibody is specific for nitrated COX out of a myriad of proteins abundant in microsomal homogenates. This is highlighted by the relative lack of immunoreactivity in the unnitrated sample.

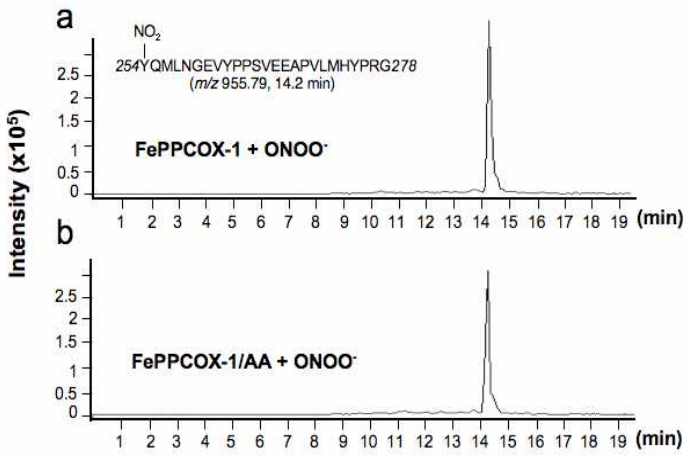

Figure S2. $ONOO^-$-induced nitration of Tyr254 in FePPCOX-1 is non-selective. FePPCOX-1 and AA-bound FePPCOX-1 (0.21 mg/ml each) were incubated with $ONOO^-$ (500 μM) for 1 h at RT. Trypsinized reaction mixtures were analyzed by nLC-MS/MS as described in Methods. (**a**) and (**b**) Extracted ion chromatograms that monitor the elution of the tryptic peptide fragment (*m/z* 955.79) representing the triply-charged peptide ion containing nitrated Tyr254, eluting at ~14.2 min.

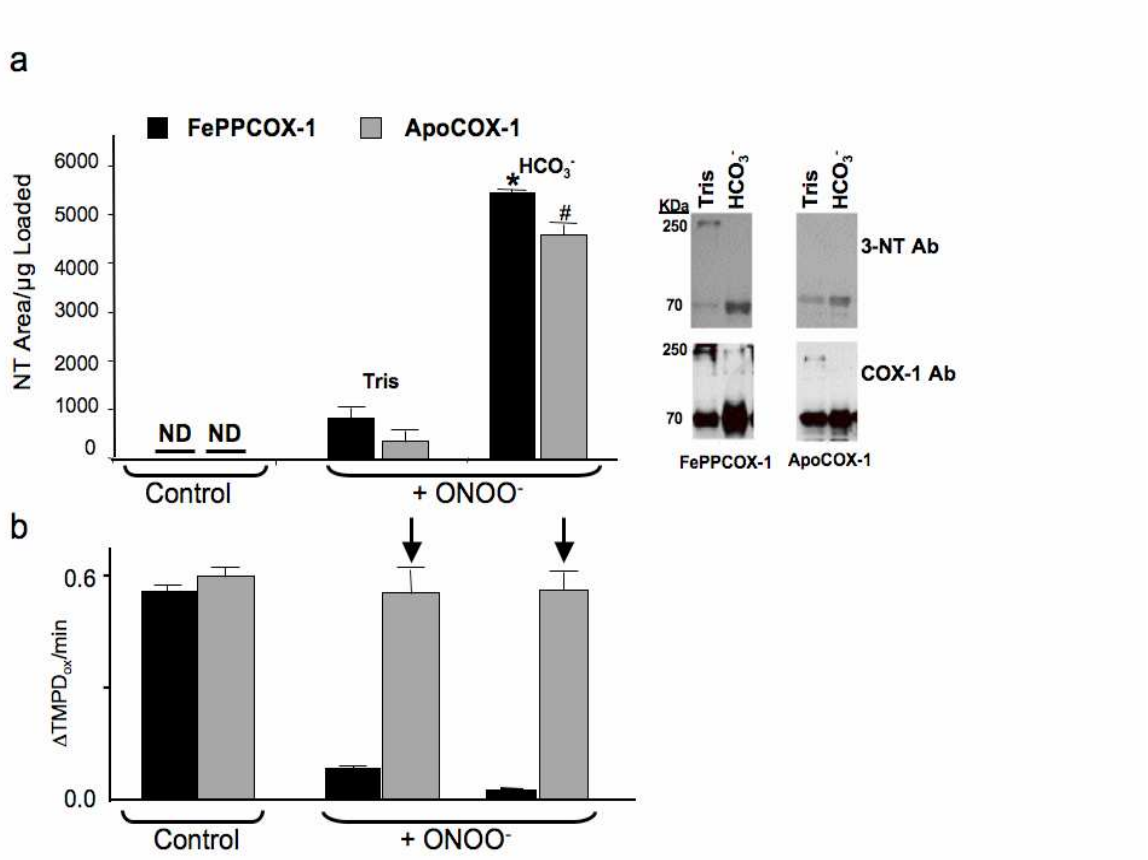

Figure S3. Selective COX-1 nitration determines enzymatic function. (**a**) Western blots for the reaction of FePPCOX-1 and ApoCOX-1 (0.21 mg/ml) with $ONOO^-$ (250 µM) in the presence of AA (100 µM). Reactions were performed at pH 8 in 100 mM Tris buffer or 20 mM $NaHCO_3$ buffer (described in methods). Blots were probed with monoclonal anti-3-NT and monoclonal anti-COX-1. Relative nitration was obtained by quantifying band density ratios between nitrated COX-1 and total amount of pure COX-1 loaded in each lane. Data are averages ± SEM. $*p < 0.04$ for FePPCOX-1 in HCO_3^- relative to Tris. $\#p < 0.05$ for ApoCOX-1 in HCO_3^- relative to Tris. ND = Not Detected. Arrows point to corresponding enzymatic activity for each reaction mixture. (**b**) Cyclooxygenase-coupled peroxidase activity measured for the reaction of $ONOO^-$ with FePPCOX-1 and ApoCOX-1 following the immediate addition of arachidonic acid (100 µM). For apoCOX-1, Hemin (1 µM) was added at the time of activity measurement. Results are monitored as ΔA_{611} during a 60 s interval.

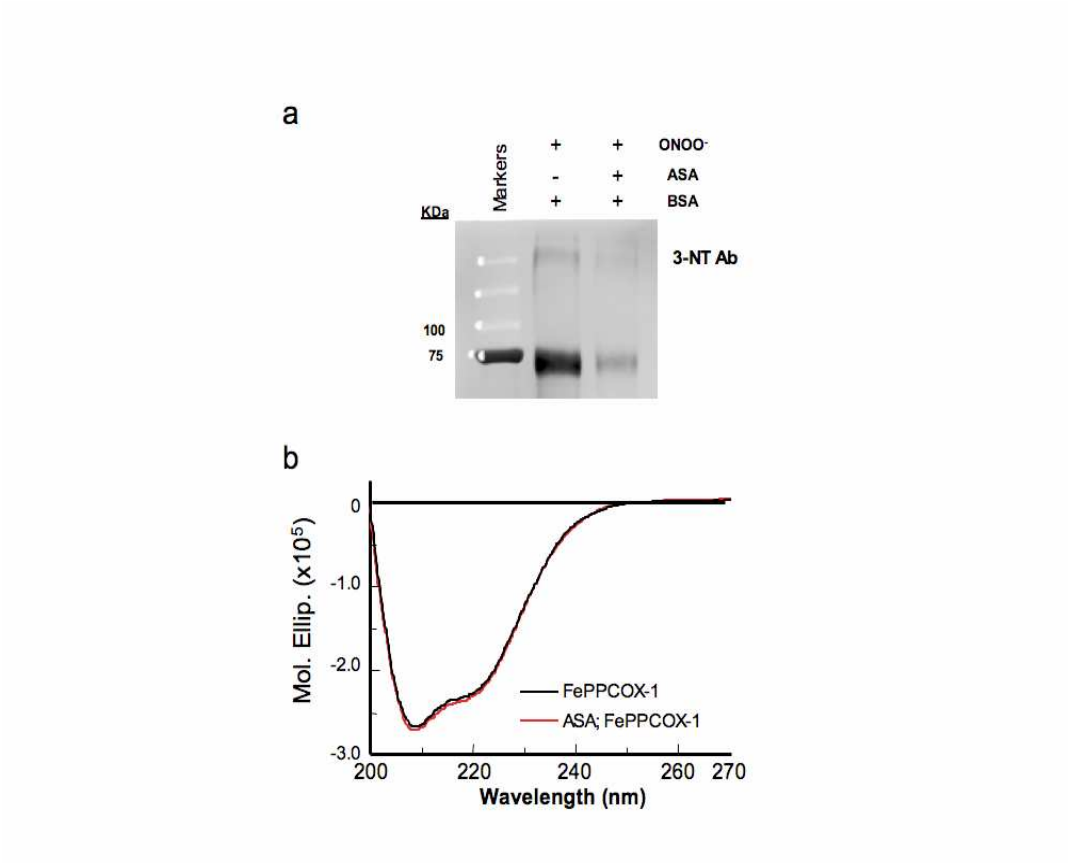

Figure S4a. Non-specific Suppression of Tyrosine Nitration by the COX inhibitor Aspirin. Western blots for the reaction of purified bovine serum albumin (0.21 mg/ml) with $ONOO^-$ (250 μM) in the presence and absence of aspirin (70 μM). Equally loaded blots were probed with monoclonal anti-nitrotyrosine (anti-3-NT).

Figure S4b. Aspirin-binding does not induce conformational changes in COX-1. CD spectra recorded in the far UV region at 25°C compare the structure of FePPCOX-1 (0.21 mg/ml; black spectrum) to aspirin-treated FePPCOX-1 (0.21 mg/ml; red spectrum).