Published in final edited form as:

J Inorg Biochem. 2011 March 1; 105(3): 238–247. doi:10.1016/j.jinorgbio.2010.11.013.

Cyclooxygenase Reaction Mechanism of PGHS ----- Evidence for a Reversible Transition between a Pentadienyl Radical and a New Tyrosyl Radical by Nitric Oxide Trapping

Jian-Ming Lü^a, Corina E. Rogge^b, Gang Wu^a, Richard J. Kulmacz^a, Wilfred A. van der Donk^c, and Ah-lim Tsai^{a,*}

^aDepartment of Internal Medicine, University of Texas Medical School at Houston, Houston, Texas 77030

^bConservation Science Department of Art Conservation, Buffalo State College, Buffalo, NY 14222

^cDepartment of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801

Abstract

Incubation of prostaglandin H synthase-1 (PGHS-1) under anaerobic conditions with peroxide and arachidonic acid leads to two major radical species: a pentadienyl radical and a radical with a narrow EPR spectrum. The proportions of the two radicals are sensitive to temperature, favoring the narrow radical species at 22 °C. The EPR characteristics of this latter radical are somewhat similar to the previously reported narrow-singlet tyrosine radical NS1a and are insensitive to deuterium labeling of AA. To probe the origin and structure of this radical, we combined EPR analysis with nitric oxide (NO) trapping of tyrosine and substrate derived radicals for both PGHS-1 and -2. Formation of 3-nitrotyrosine in the proteins was analyzed by immunoblotting, whereas NO adducts to AA and AA metabolites were analyzed by mass spectrometry and by chromatography of ¹⁴C-labeled products. The results indicate that both nitrated tyrosine residues and NO-AA adducts formed upon NO trapping. The NO-AA adduct was predominantly an oxime at C11 of AA with three conjugated double bonds, as indicated by absorption at 275 nm and by mass spectral analysis. This adduct amounted to 10% and 20% of the heme concentration of PGHS-1 and -2, respectively. For PGHS-1, the yield of NO-AA adduct matched the yield of the narrow radical signal obtained in parallel EPR experiments. High frequency EPR characterization of this narrow radical, reported in an accompanying paper, supports assignment to a new tyrosyl radical, NS1c, rather than an AA-based radical. To reconcile the results from EPR and NO-trapping studies, we propose that the NS1c is in equilibrium with an AA pentadienyl radical, and that the latter reacts preferentially with NO.

1. Introduction

Prostaglandin H synthase-1 and -2 (PGHS-1 and -2) are membrane-bound hemoproteins that catalyze the first committed step in prostanoid biosynthesis, namely the conversion of arachidonic acid to PGG₂/PGH₂ [1,2]. Both PGHS isoforms have a histidine-ligated heme

*To whom correspondence should be addressed: Division of Hematology, Department of Internal Medicine, University of Texas Health Science Center, 6431 Fannin St., Houston, TX 77030. Tel: 713-500-6771, FAX: 713-500-6810, ah-lim.tsai@uth.tmc.edu.

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group that reacts with peroxides to form Intermediate I (or Compound I), an intermediate containing an iron(IV)oxo species and a porphyrin cation radical [3,4]. Intermediate I can undergo an intra-molecular electron transfer, oxidizing the nearby Tyr385 residue to a tyrosyl radical [5,6]. This radical abstracts the 13(*S*) hydrogen atom of protein-bound AA substrate to initiate cyclooxygenase catalysis [7,8]. This radical-based mechanism has wide support from crystallographic [9-11] and spectroscopic data [7,8]. In particular, extended studies with PGHS-2 and specifically deuterated AA established the formation of a pentadienyl radical spanning C11-C15 of AA during cyclooxygenase catalysis [12-14].

The evidence for formation of a pentadienyl AA radical in PGHS-1 is less clear-cut. Results from the accompanying paper indicate that the predominant radical produced when PGHS-1 is reacted with a mixture of AA and EtOOH under anaerobic conditions is not a pentadienyl radical but rather a radical with narrow EPR linewidth (22-24 G), which is much less than the ~80 G observed for a model pentadienyl radical [15]. These observations suggested that the narrow EPR radical species is an allyl radical, as this would decrease the number of strongly interacting protons and the overall EPR linewidth. A reasonable simulation for the narrow PGHS-1 species can be obtained by enrichment of the spin concentration at C13 in the allyl radical [14], but the EPR lineshape of this narrow radical was found to be insensitive to deuteration of AA at C11, C13 or C15, where the unpaired electron was expected to reside (Tsai et al., accompanying paper). Thus, it appeared that most of the EPR signal attributed to an AA-derived allyl radical in PGHS-1 was not on AA but on the protein itself.

Radicals can form at two distinct tyrosine residues in PGHS-2: a catalytically competent radical with a wide doublet EPR spectrum (WD2) at Tyr385, and a catalytically inert radical with a narrow singlet spectrum (NS2) at Tyr504 [16]. Similar results were obtained in a recent study of human PGHS-1, with the radicals termed WD1 and NS1 [17]. Earlier EPR and ENDOR studies found that ovine PGHS-1 can also generate two other radical species: NS1a, observed when the enzyme is complexed with some cyclooxygenase inhibitors before reaction with peroxide; and NS1b, observed with high substrate levels (and consequent tendency for self-inactivation); NS1b has less-defined hyperfine features than NS1a [18,19]. The recently-observed narrow radical generated in anaerobic reactions of PGHS-1 with AA has an EPR spectrum similar to NS1b, but was generated at a higher temperature, 22 vs. 0 °C, and accordingly was named NS1c (Tsai et al, accompanying paper). In the present study, we turned to spin trapping with NO as an independent approach to assessing the contributions of tyrosine and AA to this NS1c radical and the rationale for the insensitivity of the EPR lineshape to site-specific deuteration of the substrate.

NO, a free radical, can form adducts with alkyl radicals and with phenoxyl radicals on tyrosine residues [20-22]. Adducts of NO with AA-derived radicals should have distinctive chromatographic and mass spectrometric characteristics, and reaction of NO with protein-based tyrosyl radicals is known to form 3-nitrotyrosine derivatives detectable by Western blot [23]. The advantages of using NO as a trapping agent include its small size and its hydrophobic nature, which mimic properties of dioxygen and should allow NO to access substrate or tyrosyl radicals formed in PGHS during reaction with peroxide and AA. Further, NO trapping of AA radicals should form stable products, avoiding the complicated secondary reactions encountered with other spin trap reagents such as *tert*-nitrosobutane [24].

The results of the present experiments indicate that NO, as expected, trapped tyrosyl radical when added to anaerobic reactions of PGHS-1 with EtOOH and AA. However, NO also trapped AA-derived radical in yields comparable to the spin concentration of NS1c in parallel reactions. Much less NO trapping of AA-derived radical was anticipated from the

HFEPR results, which indicated that tyrosyl radical predominates in NS1c. The apparent discrepancy suggests that the tyrosyl radical responsible for NS1c equilibrates with an AA-derived radical in PGHS-1.

2. Experimental procedures

2.1 Material

AA was from NuChek Preps, Inc., Elysian, MN. d₈-AA, and anti-nitrotyrosine antibody were from Cayman Chemical. Syntheses of 10,10-d₂-, 11-d-, 13,13-d₂-, 15-d- and 16,16-d₂-AA have been described [13]. EtOOH was a gift from Dr. G. Barney Ellison (University of Colorado, Boulder, CO). 1-¹⁴C-AA was from American Radiolabeled Chemicals. NO gas was passed through a NaOH trap before use. Diethylamine NONOate (DEA/NO) from Calbiochem was diluted to 0.1 M in 0.01 M KOH and stored at -20 °C. All other reagents were from Sigma-Aldrich.

Ovine PGHS-1 and human PGHS-2 and the Y504F and Y385F mutants were purified as recombinant proteins [16,25] as detailed in the accompanying paper (Tsai et al., manuscript in review). PGHS-1 and -2 used in this study had specific activities of ~90 and ~15 μmol O₂/min/mg, respectively. Peroxidase activity was determined as described previously [26].

2.2 Anaerobic single-turnover reactions of PGHS with peroxide and arachidonate

The basic protocol was described previously [7]. PGHS-1 (15 μM) in 0.1 M KPi, pH 7.2, 30% glycerol and 0.1% Tween 20 was made anaerobic before addition of 10 eq. of EtOOH and 5 eq. of AA or deuterated AA and freeze trapping for EPR analysis.

2.3 Inactivation of PGHS-1 during reaction with EtOOH and AA

Aerobic samples of PGHS-1 (15 μM) were mixed sequentially with 2.4, 4.8 or 12 eq. of EtOOH and 2 eq. of AA and kept on ice for 5 min before cyclooxygenase and peroxidase assays.

2.4 NO trapping of PGHS-1 tyrosyl radical

An anaerobic solution of PGHS-1 (0.3 ml of 15 μM) on ice was reacted sequentially with 10 eq. of EtOOH for 10 s, 5 eq. of AA for 15 s and 2 eq. of NO (from a 1 mM stock solution prepared in 0.1 M KPi, pH 7.2, at 1 atm pressure) for 2 min. The reaction was quenched by vigorous mixing with 1 vol of ethyl acetate containing 1% TFA. After phase separation, the aqueous layer was removed and centrifuged to collect the precipitated protein which was re-dissolved in sample buffer containing 1% SDS and 1% dithiothreitol and heated at 100 °C for 5 min before electrophoresis and Western blot analysis.

For NO trapping of EPR samples, the tubes were thawed at 0 °C before adding 6 μl of 100 mM DEA/NO in 150 mM KOH, incubating 20 s on ice, and refreezing for EPR analysis. This approach efficiently traps tyrosyl radicals [20].

2.5 Nitric oxide trapping of AA radical

An anaerobic solution of PGHS-1 or -2 (15 μM, 0.3 ml) at either 0 or 22 °C was reacted sequentially with 5-10 eq. of EtOOH for 10 s, 2-5 eq of AA for 15 s, and 2 eq. of NO (from a 1 mM stock solution in 0.1 M KPi, pH 7.2) for 2 min. The mixture was quenched by vigorous mixing with 1 volume of ethyl acetate containing 1% TFA. The layers were separated and the organic layer was removed, concentrated by evaporation with a stream of N₂ gas and either kept on ice or stored at -20 °C until HPLC analysis. The aqueous layer

was centrifuged to collect the precipitated protein for electrophoresis and Western blot analysis.

2.6 Quantitation of 3-nitrotyrosine in NO-treated PGHS

Protein samples (5 – 20 µg/lane) were separated by electrophoresis on duplicate 10% polyacrylamide gels. One gel was stained with Coomassie Blue staining. Protein bands in the other gel were transferred to a nitrocellulose membrane and probed with antibody against 3-nitrotyrosine according to the supplier's instructions before immunoreactive bands were visualized with the BioRad Opti-4CN kit.

2.7 HPLC analysis

Organic extracts were separated on an Agilent 1100 Series chromatograph with a C18 column (250 × 4.6 mm, Phenomenex), using a linear gradient of acetonitrile (40-100% over 50 min) in water with 0.1% acetic acid. The eluate was monitored by diode-array detector at 205 nm (AA and products without conjugated double bonds), 235 nm (products with conjugated dienes, such as HETEs) and 275 nm (products with conjugated trienes). In experiments with 1-¹⁴C-AA, fractions were collected and their radioactivity determined with a Packard Tri-Carb 2100 Liquid Scintillation Analyzer.

2.8 Mass spectrometry

Samples were analyzed with a QSTAR Elite electrospray tandem mass spectrometer (Applied Biosystems) operating in negative ion mode with a capillary exit voltage of -40 V, a collision energy of -20 V, and a gas temperature of 300 °C.

2.9 EPR spectroscopy

Spectra were recorded at 110-120 K as described [7].

3. Results

3.1 Effects of temperature on radicals produced in anaerobic reactions of PGHS-1 with peroxide and AA

Previous limited-turnover, manual, freeze-trap experiments with PGHS-1 were run at 0 °C [7,8]. To extend the temperature range, we first conducted similar experiments at room temperature (22 °C). The EPR spectra of samples from sequential reaction of PGHS-1 with EtOOH (2 – 5 eq.) and then 2-3 eq. of either AA (Fig. 1, spectrum **a**), 10,10-d₂-AA (spectrum **b**), 13,13-d₂-AA (spectrum **c**) or d₈-AA (spectrum **d**) all have narrow radical signals with a linewidth of 22-24 G at 0.1 - 0.15 spin/heme. The three deuterated AAs were included as probes to detect AA-derived radical species. The observed EPR signals are quite similar to the NS1c signal attributed to a tyrosyl radical (Tsai et al., accompanying manuscript) with little or no apparent contribution from a pentadienyl AA radical. Further, the EPR signal was not appreciably changed by deuterium substitution of the AA. Thus, there was no indication of significant amounts of an AA-based radical, either allyl or pentadienyl, when PGHS-1 was reacted with peroxide and AA at room temperature.

The absence of appreciable AA-based radical in the 22 °C reactions (Fig. 1) contrasted with the results from similar PGHS-1 reactions at 0 °C, where a distinct contribution from pentadienyl AA radical was evident with d₈-AA [7,8]. This discrepancy was examined by EPR analysis of reaction products at several temperatures between -10 and 22 °C (Fig. 2). For convenience in recognizing the pentadienyl radical EPR, either AA or d₈-AA was equilibrated with PGHS-1 under anaerobic conditions before reaction with EtOOH. As expected, control incubations of PGHS-1 with either AA or d₈-AA in the absence of EtOOH

produced no EPR signal (Figs. 2A and 3A black lines). When the mixture of PGHS-1 and d₈-AA was reacted with EtOOH at temperatures below 5 °C, the resulting EPR was a mixture of a 5-line pentadienyl radical signal and the NS1c signal, with a total intensity of ~0.1 spin/heme (Fig. 2A) [7,8,27]. In contrast, performing the same experiment at 22 °C produced only the NS1c signal (~0.1 spin/heme) (Fig. 2A). The change in observed reaction product from a mixture of a pentadienyl radical and NS1c signals to only NS1c signal occurred between ~5 - 15 °C (Lü, J. data not shown) when d₈-AA was the substrate. Assuming a temperature-dependent equilibrium exists between the pentadienyl radical and NS1c, the free energy change of the equilibrium would be < 0.25 kcal/mol for d₈-AA between 22 and 0 °C¹. When PGHS-1 was mixed with unlabeled AA before reaction with EtOOH, only the NS1c was observed at all temperatures tested from -10 to 22 °C (Fig. 3A).

When the control samples of PGHS-1/AA without EtOOH were thawed, exposed to air, and refrozen, all gave a wide doublet tyrosyl radical (WD1) EPR spectrum. In contrast, aerobic exposure of samples with a pentadienyl radical EPR component produced a mixture of wide doublet (WD1) and wide singlet (WS1) EPR signals (black lines in Fig. 2B). A slight increase in the spin/heme ratio also occurred, as we previously observed when a pentadienyl d₈-AA radical converted back to WD1/WS1 upon mixing with air [7,8,13,27]. On the other hand, the d₈-AA derived NS1c radical generated at 22 °C did not convert to a WD1 or WS1 after a thawing cycle in air and both its lineshape and spin/heme ratio remained unchanged (Fig. 2B). Other than a small increase in the signal intensity, the lineshape of AA derived NS1c radical remained the same or slightly widened after thawing and refreezing cycle (Fig. 3B). Thus, the results shown in Figs. 1 - 3 and the EPR study in the accompanying paper indicate a temperature-dependent EPR line shape transition from that of a pentadienyl AA radical to that of NS1c, with a different transition temperature range for different AA analogs

3.2 PGHS-1 inactivation during reaction with EtOOH and AA

One potential concern was that the NS1c signal might result from reaction of EtOOH and AA with PGHS-1 carrying unsuspected prior protein damage. To estimate the possible extent of inactivation, the surviving cyclooxygenase and peroxidase activities were assayed after reacting PGHS-1 aerobically at 24 °C with 2.4-12 eq. of EtOOH, followed by 2 eq. of AA. The fractions of surviving cyclooxygenase (peroxidase) activity were 93% (89%) at 2.4 eq. of EtOOH, 86% (83%) at 4.8 eq. EtOOH and 74% (82%) at 12 eq. EtOOH. These PGHS-1 samples, although kept on ice, were assayed aerobically, thus the extent of inactivation may be larger than for the anaerobically prepared RFQ-trapped EPR samples as more catalytic turnovers are possible under aerobic conditions. In any case, the majority of the PGHS-1 catalytic activities were retained even at the highest level of EtOOH/AA, suggesting that inactivated protein was unlikely to contribute significantly to generation of the NS1c signals observed in anaerobic reactions of PGHS-1 with EtOOH and AA.

¹ Assuming 90 % pentadienyl radical at 273K (T₁) and 90 % NS1c at 298K (T₂) and defining the equilibrium constant (K) as [pentadienyl AA radical]/[NS1c]:

$$\Delta G_1 = -R \times T_1 \times \ln K_1; \Delta G_2 = -R \times T_2 \times \ln K_2$$

$$\begin{aligned} \Delta(\Delta G) &= -R \times (T_1 - T_2) \times 2.303 \times \log(K_1/K_2) \\ &= -2.0 \text{ cal/K/mol} \times (-25 \text{ K}) \times 2.3 \times (\sim 2 \text{ or larger}) \\ &= 0.23 \text{ kcal/mol} \end{aligned}$$

3.3 Nitric oxide trapping of tyrosyl and AA-derived radicals formed in PGHS-1

To assess the reactivity of NO with individual PGHS-1 radical species, aliquots of PGHS-1 were reacted with various combinations of peroxide, fatty acid, and cyclooxygenase inhibitor to generate distinct WD1, WS1, NS1a, NS1c, or pentadienyl radical EPR signals, and then incubated with the NO donor, DEA/NO. Comparison of the EPR spectra recorded before and after addition of DEA/NO (Fig. 4) indicates that the NO donor diminished the intensity of all radical signals, as expected for radical trapping by NO. For PGHS-1 with a WD1 signal (Fig. 4A), addition of DEA/NO removed ~80% of the original 34 G signal intensity within 30 s, leaving a residual WD with more pronounced central inflection and a slightly wider linewidth (36 G); this residual signal disappeared when incubation with DEA/NO was extended to 1 min (data not shown). Reaction of DEA/NO with PGHS-1 showing a WS1 EPR spectrum (a mixture of WD1 from Tyr385 radical and NS1b) produced a rapid and selective loss of the NS component, leaving a 36 G WD signal (Fig. 4B). Only ~30% of the original WS1 signal intensity remained 10 s after addition of DEA/NO; the remaining 36 G WD signal disappeared after 1 min (data not shown). The NS1a signal completely disappeared within 10 s after mixing with DEA/NO (Fig. 4C). The unresolved pentadienyl radical signal in PGHS-1 reacted with EtOOH and d₈-AA (Fig. 4D) and the NS1c signal formed by reaction of PGHS-1 with EtOOH and AA (Fig. 4E) essentially disappeared after 10 s of incubation with DEA/NO. In the negative control without DEA/NO, thawing under argon on ice for 20 s did not change the NS1c EPR intensity (Fig. 4F). Overall, the DEA/NO trapping results indicate that NO reacts more quickly with radical at Tyr504 (NS1a) than radical at Tyr385 (WD1). The location of the tyrosine residue responsible for the NS1c has not been identified. It remains possible that NS1c is at Tyr385, in a structural form different from the WD radical at that position.

3.4 Tyrosyl radical trapping by NO in PGHS-1 and -2

Western blot analysis for 3-nitrotyrosine formation provides convenient and direct assessment of tyrosyl radical trapping by NO. Addition of NO to control PGHS-1 produced very little 3-nitrotyrosine (Fig. 5 A-C, lane 1). Resting PGHS-1 has no detectable tyrosyl radical EPR, so the immunoreactive material in the control may reflect a low level of endogenous 3-nitrotyrosine in the protein. Reaction of PGHS-1 with EtOOH before reaction with NO for either 1 or 12 min resulted in a 20-fold increase in nitrotyrosine levels in the protein (Fig. 5 B, C, lanes 2 and 3), reflecting the trapping by NO of tyrosyl radical generated by peroxide. However, when AA was added after the peroxide and before the NO, (conditions expected to generate the NS1c tyrosyl radical) the 3-nitrotyrosine levels were only 2-3 times above the control value, regardless of the NO reaction time (Fig. 5 B, C, lanes 4-6). This indicates that the presence of AA decreased the concentration of tyrosyl radical or its accessibility to NO. When the delay between addition of AA and NO was shortened from 20 s to 10 s, the nitrotyrosine level increased to 7-fold over the control value (Fig. 5 B and C, lane 7 vs. 8), indicating that the suppression by AA of tyrosyl radical trapping by NO increased with time. These results are consistent with changes in active site structure near Tyr385 upon cyclooxygenase site ligand binding, as we observed recently with cyclooxygenase inhibitors [28].

NO trapping of the tyrosyl radical formed during the reaction of EtOOH with PGHS-2 and its mutants yielded additional interesting results (Fig. 5D). NO trapping of EtOOH-treated PGHS-2 led to a high level of 3-nitrotyrosine formation (Fig. 5D, lane 2), while addition of the PGHS-2 selective inhibitor nimesulide decreased the 3-nitrotyrosine level by ~25% (Fig. 5D, lane 3). This inhibitor prevents formation of the catalytic radical at Tyr385 [27].

The Y504F mutant of PGHS-2, when reacted with EtOOH, gave a stronger 3-nitrotyrosine signal than wild type PGHS-2 (Fig. 5D, lane 5 vs. lane 4); pretreatment by nimesulide led to

a ~60% reduction of the 3-nitrotyrosine level (Fig. 5D, lane 6). Inclusion of phenol, a commonly used peroxidase cosubstrate, further reduced the 3-nitrotyrosine level (Fig. 5D, lane 7). EtOOH-treated Y385F PGHS-2 (radical restricted to Tyr504) showed an increase of 3-nitrotyrosine level over the control (Fig. 5D, lane 9 vs. lane 8); pre-treatment of Y385F PGHS-2 with nimesulide reduced the 3-nitrotyrosine level by ~30% (Fig. 5D, lane 10) although nimesulide does not bind in a position likely to affect Tyr504 radical reactivity with NO.

3.5 Structural analysis of NO adducts of AA-derived radicals in PGHS-1 and -2

Radioactive tracer and HPLC analysis were used to characterize NO adducts formed by trapping of AA radical intermediates. The A_{205} and A_{275} chromatograms of lipid extracts from anaerobic reactions of PGHS-1 and -2 with EtOOH, AA, and NO revealed peaks at 10.5, 15.2, 23.5, 34.3, 38.4 and 43.6 min (Fig. 6). The peak at 38.4 min (unreacted AA) was found to contain the great majority of the radioactivity from reactions of ^{14}C -AA for PGHS-1 and PGHS-2 (~95% and ~90%, respectively). The radioactive peak at 23.5 min had significant A_{275} and thus represented an AA derivative containing a conjugated triene. The 23.5 min peak was not seen in chromatograms of extracts from PGHS-1 reactions with EtOOH and AA that were not treated with NO (data not shown), indicating that the 23.5 min species was probably an AA-derived NO adduct. The amount of ^{14}C radioactivity in the 23.5 min peak indicated adduct yields of 0.1/heme for PGHS-1 and 0.2/heme for PGHS-2. These yields of NO-AA adduct are comparable to those of NS1c determined by EPR in PGHS-1 reactions with EtOOH and AA that were not trapped with NO (Figs. 1 and 2).

As illustrated in Scheme 1, nitroso adducts are the expected initial products of NO attack on a pentadienyl or allyl carbon-centered radical. Nitroso compounds with a tautomerizable hydrogen spontaneously convert to the more stable oximes (=NOH) [29]. AA oximes were thus the prime candidates for NO radical trapping adducts, and indeed EI-MS analysis of material from the 23.5 min peak from reactions with PGHS-1 revealed a molecular ion at m/z 332, consistent with an oxime derivative of AA (Fig. 7A). Further MS/MS fragmentation analysis showed a pattern consistent with location of the oxime at either C11 or C15: m/z 332 (M^-), 314 ($\text{M}-\text{H}_2\text{O}^-$), 301 ($\text{M}-\text{NOH}^-$), 288 ($\text{M}-\text{CO}_2^-$), 270 ($\text{M}-\text{H}_2\text{O}-\text{CO}_2^-$), 261 ($\text{M}-\text{C}_5\text{H}_{11}^-$), and 148 ($166-\text{H}_2\text{O}^-$). Note that an oxime on C11 or C15 of AA would have three conjugated double bonds (Scheme 1), consistent with the A_{275} peak at 23.5 min (Fig. 6). The m/z 301 ion indicates that the molecular ion lost an =NOH instead of an -NO group. The lack of a molecular ion of m/z 317 eliminated the possible hydrolysis of oxime to form a ketone at C11 or C15.

MS/MS analyses of parallel reactions with 11-d-AA, 13,13-d₂-AA and 15-d-AA were used to identify the location of the oxime group in the NO-AA adduct. MS analysis of the oxime product from 11-d-AA showed a major molecular ion at m/z 332 (D lost) and a minor species at m/z 333 (D retained) m/z , indicating that the oxime was predominantly located on C11 (Fig. 7A). In contrast, MS analysis of the oxime products from 13,13-d₂-AA or 15-d-AA showed only one molecular ion at m/z 333 (D retained) (the spectrum for the 15-d-AA oxime is shown in Fig. 7B), indicating that the oxime was not at C13 or C15, and confirming its location at C11. Note that one of the two deuteriums at C13 is lost during formation of the AA radical. This indicates that NO added to the AA radical at the same position as the first addition of molecular oxygen during cyclooxygenase catalysis [30], suggesting that the enzyme's positional specificity was the same for NO as for O₂ (Scheme 1, pathway framed in red). Finally, the fragment ion with m/z of 149 ($167-\text{H}_2\text{O}$) resulting from fragmentation of the parent ion at m/z 333 of oximo-15-d-AA confirms the assignment of the fragment ion with m/z 148 in unlabeled AA (Figure 7A) to the ω -end of the AA-derived oxime.

4. Discussion

4.1 Temperature- dependent radical EPR lineshape change in the reaction of PGHS-1 with EtOOH and AA

We have conducted more than hundred anaerobic single-turnover EPR experiments for both PGHS isozymes to characterize the structures of carbon-centered radical intermediates [7,8,14]. These earlier experiments were carried out at lower temperatures (0 – 4 °C) to slow the reactions to increase the chances of observing early intermediates and to minimize loss of enzyme activity. For PGHS-2, we reproducibly observed a pentadienyl AA radical EPR signal, either alone or mixed with WS2 signal radicals [13]. On the other hand, most low-temperature anaerobic reactions of PGHS-1 with EtOOH and AA did not produce a detectable level of pentadienyl radical signal. In most cases the major EPR component is the NS1c signal that arises mainly from a tyrosyl radical whose location has yet to be established (Tsai et al, accompanying paper). This unexpected and variable reaction outcome for PGHS-1 was very perplexing and prompted the present exploration of reaction temperature as a potential controlling factor, using d₈-AA as the substrate. The results (Fig. 2) revealed that the proportions of pentadienyl radical and NS1c signals was a function of temperature, with NS1c dominating at 22 °C and major contributions from pentadienyl radical seen only at temperatures of 5 °C and lower. One possible explanation for this behavior is a temperature-dependent equilibrium between pentadienyl and NS1c radicals. As discussed below, the results of the NO trapping experiments also pointed to equilibration between these two radical species.

4.2 Tyrosyl radical trapping by NO

Quenching of a tyrosyl radical by nitric oxide is very rapid, a rate of 10⁹ M⁻¹s⁻¹ estimated by pulse radiolysis measurements [31,32]. Initial formation of an unstable tyrosyl radical-nitric oxide adduct is expected to produce an EPR-silent nitrosocyclohexadienone species that can reversibly regenerate the tyrosyl radical and nitric oxide or rearrange to form 3-nitrosotyrosine and then 3-nitrotyrosine [21,31,32]. The tyrosyl radicals in PGHS-1 were indeed rapidly converted to EPR silent species by NO, with their EPR signals completely gone within 1 min after addition of NO donor (Fig. 4), and maximal 3-nitrotyrosine formation within 1 min after addition of NO itself (Fig. 5).

As expected, NO trapping of PGHS-1 samples treated with combinations of EtOOH and AA was found to generate WD1, WS1, and NS1c EPR signals attributed to tyrosyl radicals (Fig. 4) and produced 3-nitrotyrosine detected by immunoblotting (Fig. 5). What was quite unexpected, however, was the finding that NO trapping of NS1c samples also produced AA oxime in ~10% yield (Figs. 6 and 7), comparable to the tyrosyl radical content in NS1c before NO addition (Figs. 1 - 3). NO trapping of an AA radical is expected to produce AA oxime, so it is apparent that NO trapped considerable amounts of AA radical in PGHS-1 samples that initially contained mostly tyrosyl radical and little AA radical. A reasonable interpretation is that the tyrosyl radical of NS1c equilibrates with AA radical, so that NO trapping of the AA radical initially present pulls the equilibrium from tyrosyl radical towards AA radical (and eventually AA oxime) by mass action. Such equilibration between tyrosyl radical and AA radical is also consistent with the observation that the proportions of NS1c and pentadienyl radical signals vary with temperature for reaction of PGHS-1 with EtOOH and d₈-AA (Fig. 2).

In PGHS-2 the 3-nitrosotyrosine can convert to an EPR-detectable tyrosine iminoxyl radical [20,21], but the iminoxyl radical was not observed in PGHS-1 even though 3-nitrotyrosine was detected by Western blot analysis [20,22] (Figs. 4 and 5). This difference between the

PGHS isoforms may reflect differences in the local protein environment that affect the stability of the iminoxyl radical intermediate.

Another surprising observation in the present study is that NO addition to PGHS-1 exhibiting the WS1 EPR signal removed the NS1 component faster than the WD1 component (Fig. 4A and B). The NS1a signal in indomethacin-treated PGHS-1 also was quickly lost (Fig. 4C). These results indicate that NO reacts more quickly with the Tyr504 radical (NS EPR) than with the Tyr385 radical (WD EPR). This relative reactivity is opposite the behavior expected from crystallographic data, which have Tyr385 exposed to solvent via the cyclooxygenase channel, whereas Tyr504 lacks surface exposure [10]. However, Tyr504 is ~4 Å closer to the surface than Tyr385 in PGHS-1 [10], and this may give NO faster access to Tyr504. An alternative explanation may be that the H-bonding interactions between Tyr385 and Tyr348 apparent from structural models [10] decrease the reactivity of a Tyr385 radical towards NO. Whatever its mechanistic basis, it is clear that the difference in NO reaction kinetics may be of practical use in selectively probing the Tyr385 and Tyr504 radicals in both PGHS isoforms.

The intensity of the NS1c tyrosyl radical was depleted quickly by NO, becoming almost completely EPR silent 10 s after addition of NO donor (Fig. 4E). However, the reaction conditions giving the NS1c radical (PGHS-1/EtOOH/AA) produced markedly less trapping of tyrosyl radical (as 3-nitrotyrosine) than observed for PGHS-1 treated with EtOOH alone (Fig. 5). Either the inherent reactivity of NO with AA pentadienyl radical is much higher than that with tyrosyl radical or the presence of AA impaired direct reaction of NO with the NS1c tyrosyl radical. This latter action of AA is difficult to envision if NS1c is located at Tyr504, which is some distance from the AA binding site [10]. However, if the NS1c radical is at Tyr385 then binding of AA in the cyclooxygenase channel might well limit access by NO, just as binding of competitive inhibitors decreased nitration of Tyr385 by tetranitromethane [33]. An NS1c radical at Tyr385 would presumably convert some bound AA to AA radical, whose rapid trapping by NO could account for the observed depletion of NS1c signal without accumulation of a pentadienyl AA radical signal (Fig. 4E) as well as the observed production of AA oxime (Figs. 6 and 7). The location of the oxime at C11 of AA, the same position as the first oxygen addition during cyclooxygenase catalysis, also supports NS1c being at Tyr385. Assignment of both NS1c and WD1 as Tyr385 radicals poses no conceptual problems because the spectroscopic differences between the two EPR signals can readily be explained by differences in tyrosyl sidechain conformation [34].

Based on the EPR studies of PGHS-1 and -2 with specific mutations, Tyr385 and Tyr504 are the only two sites found so far that form tyrosyl radicals during PGHS catalysis [16,35,36]. When PGHS is treated with EtOOH, it always forms a WD radical at Tyr385. However, if PGHS is treated with inhibitor first and then with EtOOH, a NS radical is formed at Tyr504 with a lower spin/heme ratio than that of the WD [6,27]. There are two possible pathways for electron transfer from tyrosine to Intermediate I: an electron can be transferred sequentially from Tyr385 to Intermediate I and then from Tyr504 to Tyr385, or via parallel pathways from Tyr385 and Tyr504 to Intermediate I.

Our previous RFQ-EPR kinetic measurements [37] and the Western blot results of PGHS-2 in this study seem to support the second, parallel pathway, mechanism. As shown in Fig 4D, PGHS-2 treated with EtOOH gave a high level of 3-nitrotyrosine, which probably includes 3-nitrotyrosine at both Tyr385 and Tyr504. Nimesulide treatment of PGHS-2 reduced the total 3-nitrotyrosine level because inhibitor binding allows formation of a radical (and NO adduct) only at Tyr504. Inhibitor treatment of Y504F PGHS-2 reduced the 3-nitrotyrosine level significantly because the combination of mutation and inhibitor binding prevent tyrosyl radical formation at either Tyr385 or Tyr504. When Tyr385 is mutated, radical forms only at

Tyr504, and in lower yields [16], consistent with the observed low level of 3-nitrotyrosine formation in this mutant and the lack of effect of cyclooxygenase inhibitor binding (Fig. 5D).

4.3 Reconciling the apparent contradiction between NO trapping of AA radical and the insensitivity of the AA-induced radical EPR to deuteration and the HFEPR results

The lack of change of the EPR spectra of NS1c upon deuteration of AA (Figs 1, 2), and the HFEPR results in the accompanying paper convincingly show NS1c to be a tyrosyl radical. In contrast, results of the present MS analysis show convincingly that NO traps an AA radical under conditions where NS1c is the main radical detected by EPR. To resolve this apparent paradox, we propose that an AA-derived radical bound in the cyclooxygenase active site equilibrates with the NS1c tyrosyl radical (Scheme 2, solid reversible arrows). The NS1c tyrosyl radical predominates in EPR observations whereas the AA radical is detected in NO-trapping experiments, either because NO reacts more quickly with AA radical than with tyrosyl radical, or because AA binding blocks access of NO to NS1c.

The results from the anaerobic limited-turnover EPR experiments at various temperatures (Fig. 2) further support an equilibrium between the pentadienyl radical and NS1c radical, with the latter being favored at high temperature.

Further experiments are needed to determine whether PGHS-2 undergoes a transition to the equivalent of NS1c under conditions different from those explored so far.

Our current PGHS model proposes that EtOOH generates Compound I, which in turn generates tyrosyl radicals at Tyr385 and Tyr504, with PGHS-1 showing less partitioning to Tyr504 than PGHS-2 (Scheme 2). In both enzymes, the Tyr385 radical abstracts a hydrogen atom from C13 of bound AA, forming an AA pentadienyl radical, which in PGHS-1 then oxidizes Tyr385 or Tyr348 nearby to generate NS1c (Scheme 2, in grey text). An AA radical in the cyclooxygenase site is unlikely to be in redox equilibrium with Tyr504 because of the much longer distance involved. If the NS1c radical resides on Tyr385, the side chain must occupy a different conformation than that of the initial radical (WD) at this position (Scheme 2, in grey text). Regardless of its location, NS1c is the major radical species at 22 °C, with only a small portion of AA radical evident by EPR spectroscopy. However, it is the C11 AA radical that is preferentially trapped by NO. In this connection, results in a recent report suggest that hydrogen atom abstraction during the cyclooxygenase reaction may also be reversible, with O₂ “trapping” at C11 resulting in productive catalysis [38].

4.4 Implications for substrate-induced protein conformational changes

When PGHS-1 was reacted simultaneously with AA and EtOOH in RFQ-EPR kinetic measurements, the AA pentadienyl radical appeared before NS1c (Tsai et al, accompanying paper). However, when PGHS-1 was pre-loaded with AA and then reacted with EtOOH, only NS1c was observed during the entire course of the reaction (Figs. 3, 4 and Gang et al, manuscript in review). This difference in outcome could indicate that a protein conformational change is induced by substrate binding, shifting the equilibrium in favor of the NS1c rather than the pentadienyl radical. The structural basis for such a shift is not obvious from PGHS-1 crystallographic data, where substrate and substrate analog binding produce minimal changes within the cyclooxygenase pocket [10,11,39,40]. However, of the ability of substrate analogs to decrease protease attack on the PGHS-1 apoenzyme near Arg277 clearly implies that a long-range conformational change occurs upon substrate/inhibitor binding [41,42]. Further, results from recent studies with heterodimeric PGHS-2 and investigations of inhibitor competition with AA and 2-arachidonoylglycerol oxygenation have suggested that ligand binding to the cyclooxygenase site in one subunit

changes the behavior of the other subunit [43–45]. This communication between the subunits is likely mediated through the dimer interface. Recent EPR characterization of tyrosyl radical in PGHS-2 also showed that structural differences in cyclooxygenase inhibitor caused dramatically different changes in tyrosyl radical EPR spectrum and lifetime [28]. Such protein dynamic changes induced by substrate (or inhibitor) binding may be relevant to cyclooxygenase catalysis, because large conformational changes occur in both substrate and protein as AA is converted to PGG₂.

5. Summary

The present results demonstrate that NO readily traps AA and tyrosyl radical intermediates in PGHS-1 and -2. This novel approach should be applicable to other enzyme systems that involve similar radical intermediates, such as lipoxygenases and the prostacyclin and thromboxane synthases. Addition of NO to PGHS-1 and -2 reacted anaerobically with peroxide and AA resulted in formation of an oxime at C11 of AA. This positional specificity of oxime formation indicates that NO traps a catalytic AA radical intermediate bound in the cyclooxygenase pocket. The C11 oxime of AA forms efficiently in PGHS-1 even under conditions where a tyrosyl radical (NS1c) is the major species and AA radical a minor component. This indicates that the NS1c tyrosyl radical equilibrates with the AA radical and suggests that hydrogen atom abstraction from AA by a tyrosyl radical can be reversible. NS1c was not observed with PGHS-2, pointing to a clear distinction between the isozymes in the proposed equilibrium between AA radical and tyrosyl radical. The results of this NO trapping study thus provide new mechanistic insights into the interconversions of tyrosyl and substrate radicals in PGHS-1 and -2. In the presence of NO, and likely O₂, the reaction is shifted towards AA radical generation. Oxygen could play a similar role as NO in regulating the dynamics of AA-induced radical intermediates. Furthermore, the effect of substrate binding sequence on the structures of the radicals observed by EPR provides new evidence for substrate-induced protein conformation changes of PGHS.

Acknowledgments

We thank Dr William P. Dubinsky for the mass spectrometric measurements. Supported by NIH GM44911 (A.-L.T), and GM52170 (R.J.K).

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Abbreviations

PGHS	prostaglandin H synthase
COX	cyclooxygenase
AA	arachidonic acid
EtOH	ethyl alcohol
HETE	hydroxyeicosatetraenoic acid
DEA/NO	diethylamine NONOate
NO	nitric oxide
WD	wide doublet tyrosyl radical
WS	wide singlet tyrosyl radical
NS	narrow singlet tyrosyl radical
MS	mass spectrometry
HFEPR	high-field EPR
TFA	trifluoroacetic acid

Figure 1.

EPR spectra of radicals in anaerobic limited-turnover reactions at 22 °C. PGHS-1 (15 μ M) was first reacted with 2-5 eq. of EtOOH for ~5 s, followed by addition of 2 eq. of either AA (a), 10,10-d₂-AA (b), 13,13-d₂-AA (c) or d₈-AA (d), and ~5 s of further reaction before samples were frozen for EPR analysis. Details are described in Experimental Procedures.

Figure 2.

Effects of temperature on EPR signatures of PGHS-1 reacted anaerobically with d_8 -AA and EtOOH. **A.** PGHS-1 (15 μ M) was equilibrated with 3 eq. of d_8 -AA at the indicated temperature before reaction with 5 eq. of EtOOH for 5-10 s (times indicated next to the spectra at left, red solid lines) and EPR analysis. EtOOH was omitted from the control (0 s) samples (black dashes). **B.** After recording the EPR spectra, each sample was thawed at 0 °C and exposed to air for ~10 s before re-freezing and acquisition of the EPR spectra shown in the right hand panels. EPR intensities (spin/heme) are shown.

Figure 3.

Temperature and time dependence of AA-derived EPR signatures of PGHS-1. The AA-derived radicals were generated anaerobically at -10 to 22 °C. PGHS-1 (15 μM) was preincubated with or without 3 eq. of AA (A, black dashes,) and then reacted with 5 eq. of EtOOH for 5-10 s (indicated next to the spectrum, red solid lines) before freezing for EPR analysis. Samples from panel **A** were thawed, mixed with air and refrozen to obtain the EPR spectra shown in panel **B**.

Figure 4.

EPR spectra of PGHS-1 tyrosyl and AA radicals before (black dashes) and after (red solid lines) NO trapping. PGHS-1 (15 μ M) at 0 $^{\circ}$ C was reacted with 5 eq. of EtOOH for 6 s to produce WD1 (A) or for 15 s to produce WS1 (B). For NS1a (C), PGHS-1 was pretreated with 1 eq. of indomethacin before reaction with EtOOH for 60 s. For the pentadienyl AA radical (D), PGHS-1 was reacted with EtOOH first and then for 10 s with 3 eq. of d₈-AA; formation of NS1c (E and F) used AA instead of d₈-AA. NO trapping used incubation with 500 μ M DEA/NO at 0 $^{\circ}$ C for 10 s (30 s for the WD1 sample). DEA/NO was omitted for the control in panel F.

Figure 5.

Formation of 3-nitrotyrosine by NO trapping in PGHS-1 and -2 reacted anaerobically with EtOOH and AA. Panels A-C: PGHS-1 was reacted sequentially with the indicated combinations of EtOOH (10 eq.) and AA (5 eq.) before trapping with NO (2 eq.) and quenching with organic solvent. The precipitated protein fractions were separated by electrophoresis before the bands corresponding to PGHS-1 were visualized by staining with Coomassie Blue (panel A) or by immunoblotting with antibody against 3-nitrotyrosine (panel B). Panel C: the relative 3-nitrotyrosine contents of PGHS-1 samples were calculated from the ratio of densitometric values for the corresponding immunoblotted and Coomassie stained bands. Lane 1, PGHS-1 + NO; Lanes 2 & 3, PGHS-1 + EtOOH + NO; and Lanes 4-8, PGHS-1 + EtOOH + AA + NO (NO was added 10 s after AA addition for sample in Lane 8 and 20 s after AA for the others). The trapping reactions were stopped by chemical quench either 1 or 12 min after addition of NO, as indicated. Panel D: PGHS-2 nitrotyrosine

content determined by ratio of immunoblot and Coomassie densitometric values for enzyme samples trapped with NO (as 500 μ M DEA/NO, $\pm$ 50 μ M phenol) after pretreatment with EtOOH (10 eq.), and/or nimesulide (2 eq.). Lane 1, PGHS-2 + NO; Lane 2, PGHS-2 + EtOOH + NO; Lane 3, PGHS-2 + nimesulide + EtOOH + NO; Lane 4, Y504F PGHS-2 + NO; Lane 5, Y504F PGHS-2 + EtOOH + NO; Lane 6, Y504F PGHS-2 + nimesulide + EtOOH + NO; Lane 7, Y504F PGHS-2 + nimesulide + EtOOH + NO/phenol; Lane 8, Y385F PGHS-2 + NO; Lane 9, Y385F PGHS-2 + EtOOH + NO; and Lane 10, Y385F PGHS-2 + nimesulide + EtOOH + NO. The nitrotyrosine contents in panels C and D were scaled to set the highest value as 1.0.

Figure 6.

HPLC analyses of lipid products after NO trapping of PGHS-1 and -2 reacted anaerobically with EtOOH and AA at 0 °C. HPLC profiles of the main products from the reactions are presented: (A) PGHS-1/EtOOH/ ^{14}C -AA/NO and (B) PGHS-2/EtOOH/ ^{14}C -AA/NO. The eluate absorbance (mAU) was monitored at 205 nm and 275 nm. The vertical bars in both panels represent the radioactivity recovered in individual peaks. The error bars indicate the variation of the radioactivity counts of the fraction at the same retention time from three (PGHS-1) or two (PGHS-2) different samples.

Figure 7. Tandem MS analyses of NO adduct of AA-derived radical in PGHS-1. ESI MS/MS mass spectra (negative ions) showing fragmentation of 11-oximo-AA and 15-d-11-oximo-AA (bottom). Details are described in Experimental Procedures. The chemical structures of the oximes are shown.

Scheme 1.

Proposed mechanism for formation of oximo-AA by NO trapping of AA-derived radicals in PGHS. The chemical steps highlighted by red arrows are those proposed to lead to the 11-oximo-AA identified by MS analysis.

Scheme 2.

Proposed interconversions of tyrosyl and AA radical species in PGHS-1 and -2. Each of the tyrosyl radical species and their assigned location are given; the AA pentadienyl radical (AA•) is the only experimentally confirmed substrate radical. Parallel formation of the Tyr385 and Tyr504 radicals from Intermediate I after EtOOH reaction is indicated by the yellow arrows and electron transfer between Tyr385 and Tyr504 is indicated by the dashed maroon arrow. AA reacts with the Tyr385 radical to form the AA radical (AA•), which accumulates under anaerobiosis (double blue dashed lines). In PGHS-1, AA binding to protein with a Tyr385 radical (**WD1**) is proposed to transform the radical to either **NS1c** (radical at Tyr348 or at Tyr385 with altered conformation). The AA• reacts with O₂ to eventually form PGG₂. In trapping experiments, NO reacts with tyrosyl radicals to form 3-nitrotyrosine end product, and with AA• to form oxime. Steps specific to one isozyme or the other are indicated by color coding. Anticipated relative intensities of tyrosyl radicals are indicated by differences in font size.