Peroxy nitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis

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ABSTRACT Peroxy nitrite activates the cyclooxygenase activities of constitutive and inducible prostaglandin endoperoxide synthases by serving as a substrate for the enzymes’ peroxidase activities. Activation of purified enzyme is induced by direct addition of peroxynitrite or by in situ generation of peroxynitrite from NO coupling to superoxide anion. Cu,Zn-superoxide dismutase completely inhibits cyclooxygenase activation in systems where peroxynitrite is generated in situ from superoxide. In the murine macrophage cell line RAW264.7, the lipophilic superoxide dismutase-mimetic agents, Cu(II) (3,5-diisopropylsalicylic acid)2, and Mn(III) tetrakis(1-methyl-4-pyridyl)porphyrin dose-dependently decrease the synthesis of prostaglandins without affecting the levels of NO synthase or prostaglandin endoperoxide synthase or by inhibiting the release of arachidonic acid. These findings support the hypothesis that peroxynitrite is an important modulator of cyclooxygenase activity in inflammatory cells and establish that superoxide anion serves as a biochemical link between NO and prostaglandin biosynthesis.

Prostaglandins and thromboxanes are important mediators of inflammation, hyperalgesia, cell growth, and hemostasis inter alia. The committed step in prostaglandin biosynthesis is the oxygenation of arachidonic acid (AA) by prostaglandin endoperoxide (PGH) synthase, a bifunctional, membrane-bound hemeprotein (1–4). Inhibition of the cyclooxygenase activity of PGH synthase is the basis for the pharmacological action of nonsteroidal antiinflammatory drugs (5, 6). Two different PGH synthases exist in vertebrates—PGH synthase-1, which is expressed constitutively and occurs in many tissues, and PGH synthase-2, which is inducible and expressed transiently (7–10). PGH synthase-2 is present at high levels in monocytes/macrophages, where it appears to play a major role in the production of inflammatory prostaglandins (7, 11).

Several recent reports demonstrate that NO stimulates prostaglandin biosynthesis in vivo, in perfused organs, and in macrophages (12–17). The stimulatory effect of NO is rapid and appears to be the result of direct activation of cyclooxygenase activity (16). However, conflicting reports exist regarding the ability of NO to stimulate purified PGH synthase, and it is possible that a derivative of NO is responsible for the activation in inflammatory cells (16, 18–20).

The principal mechanism described for direct activation of the cyclooxygenase activity of PGH synthase is reaction of fatty acid hydroperoxides with the heme prosthetic group to generate a protein radical. This protein radical (probably Tyr-385) serves as the catalytic oxidant of AA (21–23). The identity of the hydroperoxides that activate PGH synthase in different cell types is uncertain because fatty acid hydroperoxides are excellent substrates for glutathione peroxidase (GSH-Px)-catalyzed reduction by glutathione (GSH) (24). The potential for control of prostaglandin biosynthesis by GSH-Px/GSH reduction of hydroperoxide activators exhibits substantial tissue-to-tissue variation (25).

NO couples to superoxide anion (O2-) at a diffusion-controlled rate to produce peroxynitrite (Eq. 1) (26).

\[ \text{Nitric Oxide} + \text{Superoxide} \rightarrow \text{Peroxynitrite} \]

The occurrence of this reaction in macrophages contributes to their cytotoxic activity toward invading pathogens because peroxynitrite and its conjugate acid, peroxynitrous acid, are potent oxidizing agents (26–32). Because peroxynitrite is an inorganic hydroperoxide, it is a potential substrate for the peroxidase activity of PGH synthase and an activator of the enzyme’s cyclooxygenase activity. Therefore, we investigated the interaction of peroxynitrite with PGH synthase and its effect on cyclooxygenase and peroxidase catalysis. The results indicate that peroxynitrite is an efficient peroxidase substrate and cyclooxygenase activator. Both PGH synthase-1 and PGH synthase-2 are activated by peroxynitrite added directly to the enzymes or generated in situ from NO and O2-. Activation in vitro by the combination of NO and O2- is inhibited by Cu,Zn-superoxide dismutase (SOD). Membrane-permeant SOD-mimetic agents reduce prostaglandin biosynthesis in a cultured macrophage-like cell line by up to 80%. Thus, O2- appears to link NO synthesis to prostaglandin synthesis in macrophages through the intermediacy of peroxynitrite.

MATERIALS AND METHODS

Materials. Unlabeled AA was from Nu Chek Prep (Elysian, MN). Hematin, reduced GSH, xanthine, xanthine oxidase grade III, catalase (from bovine liver), SOD (from bovine erythrocytes), NADPH, butylated hydroxyanisole, oxidized glutathione (GSSG) reductase type IVB, S-nitroso-glutathione and S-nitroso-N-acetyl-penicillamine (SNAP) were from Sigma. 3-Morpholinosydnonimine (SIN-1) was from Molecular Probes. Phenol and hydrogen peroxide were from Fisher. Guaiacol and Cu(II) (3,5-diisopropylsalicylate)2 (CuDips) were from Aldrich. Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) was from Porphyrin Products (Logan, UT). Aromatic Probes. Phenol and hydrogen peroxide were from Fisher. Guaiacol and Cu(II) (3,5-diisopropylsalicylate)2 (CuDips) were from Aldrich. Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) was from Porphyrin Products (Logan, UT).

Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; GSH-Px, glutathione peroxidase; SOD, Cu,Zn-superoxide dismutase; CuDips, Cu(II) (3,5-diisopropylsalicylate)2; MnTMPyP, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin; SNAP, S-nitroso-N-acetyl-penicillamine; SIN-1, 3-morpholinosydnonimine; 15-HPETE, 15-hydroxyeicosatetraenoic acid; PGH synthase, prostaglandin endoperoxide synthase; LPS, lipopolysaccharide; IFN-γ, interferon γ; AA, arachidonic acid.

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UT). Nitrate reductase from Aspergillus, glutamate dehydrogenase from beef liver and α-ketoglutarate were from Boehringer Mannheim. Bovine erythrocyte GSH-Px and lipopolysaccharide (LPS) serotype 0111:B4 from Escherichia coli were obtained from Calbiochem. S.S′-1,4-phenylene-bis-(1,2-ethanediyl) bis-isothiourea (di-hydrobromide) was obtained from Cayman Chemical (Ann Arbor, MI). DMEM, minimal essential medium, and mouse recombinant interferon γ (IFN-γ) were obtained from GIBCO/BRL. Fetal bovine serum was from HyClone. Antibody to PGH synthase-2 was kindly provided by David Dewitt (Michigan State University). The enhanced chemiluminescence Western detection kit was from Amersham Life Science (Cleveland). [1-14C]AA (35 mCi/mmol; 1 Ci = 37 GBq) was from DuPont/NEN. 15-Hydroperoxyeicosatetraenoic acid (15-HPETE) was synthesized from AA using soybean lipoxygenase as described (33). apoPGH synthase-1 was purified from rat seminal vesicles as described and stored at −80°C (34, 35). Recombinant human PGH synthase-2 was expressed in SF9 cells and purified as described above.

Synthesis of Peroxynitrite. Peroxynitrite was synthesized in a quenched-flow apparatus as described with modifications (37). The concentration of H₂O₂ used in the synthesis was decreased to 0.2 M and the concentration of sodium nitrite was increased to 1 M. The concentration of peroxynitrite was determined by measuring the absorbance at 302 nm in 1.2 M NaOH (ε₃₀₂ = 1670 M⁻¹ cm⁻¹). Before use, peroxynitrite solutions were assayed for residual H₂O₂ as described (38). The concentration of H₂O₂ in peroxynitrite stocks was <1%.

Guaiacol Peroxidase Activity. PGH synthase peroxidase assays contained 100 nM apoPGH synthase, reconstituted with 100 nM hematin and 500 μM guaiacol in 0.1 M NaPO₄ (pH 8.0). The peroxidase reaction was initiated by the addition of peroxide substrate. Guaiacol oxidation was monitored at 436 nm (ε₄₃₆ = 6390 M⁻¹ cm⁻¹) (39). The peroxidase of PGH synthase undergoes rapid inactivation which makes rate determination difficult (40). Vₘₐₓ represents the maximal number of turnovers catalyzed by the peroxidase at saturating concentrations of peroxide. Previous studies have shown this to be an accurate reflection of Vₘₐₓ (41). The units of Vₘₐₓ are mol guaiacol oxidized per mol PGH synthase. The rates of guaiacol oxidation were determined over a range of peroxide concentrations, and control assays were performed in the absence of enzyme to correct for nonenzymatic oxidation.

GSH-Px Activity. GSH-Px activity was measured in a coupled enzyme assay containing 0.25 unit GSH-Px, 1 mM GSH, 1 unit GSSG reductase, 250 μM NADPH, and 150 μM peroxide substrate in 0.1 M NaPO₄ (pH 8.0). The oxidation of NADPH was monitored at 340 nm for 60 sec. Parallel experiments were conducted in the absence of GSH-Px, and the extent of NADPH oxidation was subtracted from the value determined in the presence of GSH-Px.

Activation of Cyclooxygenase Activity by Peroxynitrite. Purified PGH synthase-1 (22 nM) was incubated at 37°C with 8 units GSH-Px, varying concentrations of GSH, and 10 μg catalase in 0.1 M NaPO₄ (pH 8.0) containing 500 μM phenol (200 μl reaction volume). Following addition of 50 μM [1-14C]AA, 150 μM SNAP, and/or 100 μM xanthine plus 0.2 unit xanthine oxidase were added. Aliquots (200 μl) of the reaction mixture were removed at varying time points, terminated, and analyzed as described above.

Inhibition of AA Metabolism in RAW264.7 Cells. Stocks of RAW264.7 cells were maintained at low passage number in DMEM plus 10% fetal bovine serum. Cells (3.5 × 10⁵ cells/T25 flask) were activated with 500 ng/ml LPS and 10 units/ml IFN-γ in serum-free supplemented minimal essential medium (SMEM) for 7 hr. The composition of SMEM was 1 liter of minimal essential medium containing 3.5 g glucose, 110 mg pyruvate, 584 mg L-glutamine, and 3.57 g Hepes (pH 7.4); this medium did not contain phenol red. The cell monolayers were washed with PBS at t = 7 hr, then incubated in fresh PBS containing 0–10 μM CuDips for 60 min or 0–10 μM Mn(II-1)TmPyP for 30 min. The dimethyl sulfoxide vehicle was kept constant at 1% in all flasks. At t = 8 hr of activation, the PBS was removed and analyzed for prostaglandin content by gas chromatograph/negative ion chemical ionization mass spectrometry (42, 43). For determination of effects on exogenous AA metabolism, [1-14C]AA (20 μM) was incubated for 15 min at 25°C with LPS/IFN-γ-activated RAW264.7 cells that were preincubated for 30–60 min in 1 ml PBS with or without CuDips or Mn(III-TmPyP. An aliquot of the PBS was removed and mixed with an equal volume of ethyl ether/methanol/1 M citric acid (pH 4.0) (30:4:1) containing 10 μg butylated hydroxyanisole and 10 μg unlabeled AA. The organic layer was analyzed for radiolabeled metabolites as described above.

Nitrite/Nitrate Measurement. Total nitrite/nitrate in the culture medium were measured using the Griess reagent as described (44). Medium samples were treated with 0.1 unit nitrate reductase and 100 μM NADPH for 10 min to reduce nitrate to nitrite. NH₄Cl (500 mM), 4 mM Mn(II), and 20 μg glutamate dehydrogenase were then added to oxidize any remaining NADPH that can interfere with the Griess reaction. After 3 min, 500 μl of Griess reagent was added (total volume = 1 ml), and the absorbance at 540 nm was measured.

RESULTS

Purified rat seminal vesicle PGH synthase-1 and human recombinant PGH synthase-2 were incubated with H₂O₂-free peroxynitrite to assay for guaiacol peroxidase activity. Parallel incubations were performed with H₂O₂ and 15-HPETE (Table 1). Peroxynitrite was an excellent substrate for the peroxidase of both PGH synthase-1 and PGH synthase-2 and exhibited the highest Vₘₐₓ of the three peroxide substrates. 15-HPETE exhibited the lowest Kₘ and the highest Vₘₐₓ/Kₘ.

The ability of peroxynitrite to serve as a substrate for GSH-Px was tested in a coupled enzyme assay in which the oxidation of NADPH was monitored spectrophotometrically at 340 nm in the presence of peroxide substrate, GSH, GSH-Px, and GSSG reductase (45). Control assays were performed in the absence of GSH-Px to assess nonenzymatic GSSG formation. Although peroxynitrite induced significant nonenzymatic GSSG formation, the data in Table 1 illustrate that peroxynitrite was not an efficient substrate for GSH-Px. Pretreatment of GSH-Px with peroxynitrite in the presence of GSH did not affect the ability of GSH-Px to react with H₂O₂, demonstrating that peroxynitrite did not inactivate GSH-Px under these conditions.

Because peroxynitrite was an excellent substrate for the PGH synthase peroxidase activities, we evaluated its ability to activate the enzymes’ cyclooxygenase activities. PGH syn-
Peroxynitrite is a potent oxidant and nitrosylating agent that is generated from the reaction of NO and superoxide (30). It has been shown to have a variety of effects on different biological systems, including the inhibition of cyclooxygenase and peroxynitrite synthase (PGH synthase) activity (31-35). In this study, we investigated the effects of peroxynitrite on the activity of PGH synthase and its inhibition by GSH-Px (glutathione peroxidase).

**Table 1. Substrate specificity of PGH synthase peroxidases and GSH-Px**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>PGH synthase-1</th>
<th>PGH synthase-2</th>
<th>GSH-Px†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxynitrite</td>
<td>140</td>
<td>100</td>
<td>0.08</td>
</tr>
<tr>
<td>15-HPETE</td>
<td>24</td>
<td>9</td>
<td>1.54</td>
</tr>
<tr>
<td>H2O2</td>
<td>287</td>
<td>109</td>
<td>1.69</td>
</tr>
</tbody>
</table>

*PGH synthase peroxidase assays were performed as described. The units of V_{max} are mol guaiacol oxidized per mol PGH synthase. The rates of guaiacol oxidation were determined over a range of peroxide concentrations, and control assays were performed in the absence of enzyme to correct for nonenzymatic oxidation.

†GSH-Px assays were conducted as described.

Peroxynitrite decomposes rapidly in physiological buffers with a half-life of \( \approx 1 \) sec; thus the ability of peroxynitrite added as a bolus to serve as a peroxidase substrate or to activate cyclooxygenase is limited (29). SIN-1, which decomposes to yield NO and O2\(_2\) in situ, was used to provide a continuous source of peroxynitrite (47). Incubation of GSH-Px/GSH-inhibited PGH synthase-1 with \([1-\text{14C}]\text{AA}\) and 750 \(\mu\)M SIN-1 resulted in substantial prostaglandin formation (Fig. 2). SIN-1 activation of GSH-Px/GSH inhibited PGH synthase-1 was observed even at 2.5 mM GSH although prostaglandin formation was greatest at 0.25 mM GSH. SIN-1 activation of prostaglandin formation was completely inhibited by 1 \(\mu\)M SOD, indicating that NO alone was unable to activate GSH-Px/GSH-inhibited PGH synthase.

PGH synthase also was activated by in situ generation of peroxynitrite by the combination of the NO donor, SNAP, with the superoxide-generating system, xanthine/xanthine oxidase. Addition of either SNAP or xanthine/xanthine oxidase alone did not activate PGH synthase (Fig. 3). Thus, three independent methods for generating and administering peroxynitrite—direct addition, gradual production from SNAP plus xanthine/xanthine oxidase—stimulated the cyclooxygenase activity of PGH synthase whereas the addition of NO or O2\(_2\) alone did not. The results summarized in Figs. 1–3 were obtained with PGH synthase-1 purified from ram seminal vesicles, but identical results were obtained with recombinant human PGH synthase-2. No enhancement of the cyclooxygenase activity of either enzyme was observed if the peroxynitrite decomposition products, nitrite or nitrate, were added instead of peroxynitrite. Also, S-nitrosoglutathione, a putative product of the reaction of GSH with peroxynitrite, did not stimulate product formation (48).

Relatively high concentrations of PGH synthase were used in the in vitro studies to facilitate cyclooxygenase and peroxidase assays. This necessitated the use of elevated concentrations of peroxynitrite, which makes extrapolation to cellular conditions difficult. To assess the potential cellular role of peroxynitrite as a mediator of prostaglandin biosynthesis, we employed the murine macrophage cell line, RAW264.7. This
CuDips (10–50 μM) or MnTMPyP (1–25 μM) effected dose-dependent inhibition of the formation of radiolabeled prostaglandins (mainly PGD₂). CuDips did not inhibit mobilization of AA as measured by the release of total radioactivity in prelabeled macrophages. Cells were labeled for 15 hr with 10 μM [1-14C]AA, washed, activated with LPS and IFN-γ for 7 hr, washed, and then stimulated with 100 ng/ml 12-O-
tetradecanoxyphlorbol-13-acetate in the presence or absence of 10 μM CuDips for 1 hr. Although CuDips inhibited the conversion of AA to PGD₂, it did not affect the release of total radioactivity from prelabeled phospholipids. The ability of CuDips to inhibit prostaglandin biosynthesis from endogenous or exogenous AA and its inability to inhibit the release of AA from cellular phospholipids suggests it does not inhibit phospholipase A₂. Finally, neither CuDips nor MnTMPyP at concentrations up to 50 μM decreased cell viability as judged by trypan blue exclusion.

**DISCUSSION**

We demonstrate in the present experiments that peroxynitrite activates the cyclooxygenase activity of PGH synthase by serving as a hydroperoxide substrate for the enzyme’s peroxidase activity. Cyclooxygenase activation was observed with pure peroxynitrite added as a bolus, with peroxynitrite generated in situ from SIN-1, or with peroxynitrite generated by production of NO in the presence of the O₂⁻-generating system xanthine/xanthine oxidase. Activation by in situ generation of peroxynitrite from NO and O₂⁻ was completely prevented by SOD. No activation was effected by NO or O₂⁻ alone, by the NO decomposition products nitrite or nitrate, or by S-nitrosoglutathione. Thus, the peroxidase-dependent activation of the cyclooxygenase activity of purified or microsomal PGH synthase is specific for peroxynitrite.

Peroxynitrite was shown previously to react with other heme peroxidases including myeloperoxidase, lactoperoxidase, and horseradish peroxidase (56). However, our experiments demonstrate that it does not react with the selenium-containing peroxidase, GSH-Px, under the conditions employed in the present studies. Peroxynitrite reacts directly and rapidly with cysteine (k = 6 × 10⁴ M⁻¹ sec⁻¹) (46) so GSH consumption was observed in the absence of GSH-Px. However, addition of GSH-Px did not increase the rate of GSSG formation. The lack of reactivity of peroxynitrite with GSH-Px may provide a basis for selective activation of the cyclooxygenase activity of PGH synthases by peroxynitrite relative to fatty acid hydroperoxides. Fatty acid hydroperoxides react with GSH-Px with a rate coefficient of 4 × 10⁷ M⁻¹ sec⁻¹ (24). The data in Fig. 2 indicate that gradual generation of peroxynitrite by coupling of NO and O₂⁻ can activate cyclooxygenase even in the presence of 8 units GSH-Px and 2.5 mM GSH. Under comparable conditions, no activation is detected following addition of fatty acid hydroperoxides.

Two structurally distinct SOD-mimetic agents, CuDips and MnTMPyP, effectively inhibited PGE₂ and PGD₂ production by RAW264.7 cells pretreated with LPS/IFN-γ. LPS/IFN-γ treatment was carried out for 7 hr before replacement of the medium with PBS containing the SOD-mimetic agents. By 7 hr, the induction of NO synthase and PGH synthase-2 was near maximal so the effects of the inhibitors were not on the induction of either enzyme. In fact, addition of CuDips prior to treatment of the cells with LPS/IFN-γ did not inhibit induction of NO synthase at any time up to 24 hr (L.M.L. and B.C.C., unpublished results). Tetsuka et al. (57) have reported that treatment of rat mesangial cells for 24 hr with scavengers of reactive oxygen species lowers the level of PGH synthase-2 protein. However, a similar effect does not appear to be responsible for inhibition of PGE₂ and PGD₂ biosynthesis by O₂⁻ in present experiments. Inhibition was seen after only a 30- to 60-min incubation of RAW264.7 cells with CuDips or MnTMPyP and the inhibitory effect could be abolished by washing the cells to remove the inhibitors prior to the addition of [1-¹⁴C]AA. Furthermore, control experiments indicated that neither compound lowered the level of PGH synthase-2 protein under the conditions of these experiments.

Strong inhibition of prostaglandin biosynthesis by CuDips or MnTMPyP was observed from either endogenous or exoge-

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**FIG. 6. Relation of NO, O₂⁻, and prostaglandin biosynthesis in inflammatory cells.**

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