A Mechanism for the Hydroperoxide-Mediated Inactivation of Prostacyclin Synthetase

By Stephen J. Weiss, John Turk, and Philip Needleman

Hydroxyl radical generation was demonstrated during heme-catalyzed decomposition of 15-hydroperoxy arachidonic acid. The hydroperoxide-mediated inactivation of prostacyclin synthetase seems to be related to the generation of this highly reactive species. A relationship between hydroperoxides and the hydroxyl radical may be important in the modulation of prostacyclin synthesis in diseased states.

PROSTACYCLIN (PGI₂) SYNTHETASE is located in blood vessel endothelium, and its product, PGI₂, is a potent vasodilator and inhibitor of platelet aggregation. The enzyme may play a role in maintaining blood vessel patency and protecting endothelial cells from platelet-mediated injury. PGI₂ synthetase can be inhibited in vitro by the lipid hydroperoxide 15-hydroperoxy arachidonic acid. The demonstration of similar compounds in diseased blood vessels has led to the suggestion of in vivo enzyme inhibition and thus increased susceptibility to thrombus formation and atherosclerosis. The mechanism of inactivation of PGI₂ synthetase by hydroperoxides has remained unclear, but a possible role for free radicals appears tenable.

The hydroxyl radical (OH⁻) is the most powerful oxidant known, and it appears to play an important role in radiation damage, inflammation, and the bactericidal function of the leukocyte. Generation of this highly reactive species by lipid hydroperoxides would be expected to result in rapid reaction with susceptible target molecules.

OH⁻ generation may be investigated by studying the generation of ethylene gas (C₂H₄) as a product of the oxidation of 2-keto-4-thiomethylbutyric acid (KMBA) by OH⁻:

\[ \text{OH}^- + \text{CH}_3\text{SCH}_2\text{CH}_2\text{COCOOH} \rightarrow \text{C}_2\text{H}_4 + \frac{1}{2}(\text{CH}_3\text{S})_2 + \text{HCOOH} + \text{CO}_2 \]

By employing this system we have studied the generation of OH⁻ by 15-hydroperoxy arachidonic acid (15-HPAA) and its possible role in the inactivation of PGI₂ synthetase.

MATERIALS AND METHODS

Superoxide dismutase (3000 U/mg), catalase (type C-40), hemoglobin, methemoglobin, cytochrome c, hematin, L-tryptophan, L-histidine, KMBA, and soybean lipoxygenase were obtained from Sigma Chemical, St. Louis, Mo. Arachidonic acid was obtained from Nuchek Preparations, Elysian.
OH\textsuperscript{-} generation was studied according to the method of Weiss et al.\textsuperscript{11} Briefly, 15-HPAA was incubated with 1-mM KMBA in 50-mM potassium phosphate buffer (pH 7.8) in sealed vessels (final volume 1 ml) for indicated periods of time. 15-HPAA was incubated alone or in the presence of bovine aorta microsomes, 2.5 \times 10^{-3}-M hemoglobin, methemoglobin, catalase, cytochrome c, or hematin. In some experiments the generation of C\textsubscript{2}H\textsubscript{4} by the 15-HPAA-methemoglobin system was studied in the presence of ethanol (20 mM, 40 mM), butanol (20 mM, 40 mM), histidine (10 mM, 20 mM), superoxide dismutase (100 \mu g/ml), or catalase (100 \mu g/ml). Ethylene was quantitated in the gas phase with a GC-725 flame ionization gas chromatograph (Beckman Instruments, Fullerton, Calif.) as previously described.

PG\textsubscript{J} synthetase activity was studied in bovine aorta microsome preparations as previously described. Briefly, 500 ng of [1-\textsuperscript{14}C] PG\textsubscript{H}\textsubscript{2} (50,000 cpm) and 1 \mu g of cold PG\textsubscript{H}\textsubscript{2} were added to the bovine aorta microsomes (5 mg protein) and incubated at 37\textdegree C for 15 min. The prostaglandins were extracted, and the products were examined by radiochemical assay as described previously.\textsuperscript{11} In some experiments the microsomes were preincubated with 0.5 \mu g of 15-HPAA and/or 5-mM tryptophan for 10 min at 25\textdegree C before the addition of labeled prostaglandins.

### RESULTS

The generation of C\textsubscript{2}H\textsubscript{4} from 15-HPAA was dramatically increased by the addition of a variety of heme compounds (Table 1). All of the agents tested were effective in generating C\textsubscript{2}H\textsubscript{4}, but hemoglobin and methemoglobin were most active. Elucidation of the mechanism of catalyst action with hemoglobin is complicated by its ease of auto-oxidation and subsequent formation of superoxide anion.\textsuperscript{13} Thus, in the remainder of experiments, methemoglobin was employed as catalyst.

The production of C\textsubscript{2}H\textsubscript{4} from the methemoglobin-hydroperoxide system increased with time and reached a plateau near 120 min (Fig. 1). Little C\textsubscript{2}H\textsubscript{4} was generated in the absence of methemoglobin, and omission of 15-HPAA or KMBA resulted in no production of C\textsubscript{2}H\textsubscript{4} over this time period. If the 15-HPAA was replaced with an equimolar concentration of arachidonic acid, no C\textsubscript{2}H\textsubscript{4} was detected. C\textsubscript{2}H\textsubscript{4} generation was directly related to 15-HPAA concentration over a range of 0–15 \mu g (Fig. 2), and it increased with KMBA concentration until a plateau was reached near 1 mM. Methemoglobin was most effective at a final concentration of 2.5 \times 10^{-3} M, with higher concentrations proving inhibitory.

During the incubation of 15-HPAA with methemoglobin the characteristic absorption peak of the hydroperoxide at 233 nm was markedly reduced, whereas the adsorption at 280 nm was increased (Fig. 3). These changes reflect a decrease

### Table 1. Effects of Various Heme Compounds on C\textsubscript{2}H\textsubscript{4} Generation by 15-HPAA

<table>
<thead>
<tr>
<th>Additive</th>
<th>C\textsubscript{2}H\textsubscript{4} Generated\textsuperscript{\dagger}</th>
<th>\textsuperscript{\dagger}Control system contained 15-HPAA (5 \mu g/ml) and 1-mM KMBA. Results are expressed as means \times 10\textsuperscript{10} moles \pm 1 SD/60 min (n = 4).</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.2 \pm 0.1</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>7.1 \pm 0.4</td>
<td></td>
</tr>
<tr>
<td>Methemoglobin</td>
<td>6.3 \pm 0.9</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>4.2 \pm 0.6</td>
<td></td>
</tr>
<tr>
<td>Hematin</td>
<td>2.7 \pm 0.6</td>
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</tr>
<tr>
<td>Cytochrome C</td>
<td>2.2 \pm 0.5</td>
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\textsuperscript{\dagger}Final concentration of all additives was 2.5 \times 10^{-3} M.
in the diene conjugation of 15-HPAA and the formation of products with trienone conjugation.\textsuperscript{14}

Superoxide anion (O\textsubscript{2}\textsuperscript{−}) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) have been demonstrated to interact in a variety of systems to generate OH\textsuperscript{−}\textsuperscript{9} In order to investigate their roles in C\textsubscript{2}H\textsubscript{4} generation in this system, experiments were performed in the presence of superoxide dismutase or catalase. In four experiments, neither superoxide dismutase nor catalase had an inhibitory effect. However, compounds known to scavenge OH\textsuperscript{−}\textsuperscript{15} were able to inhibit C\textsubscript{2}H\textsubscript{4} generation in the 15-HPAA system (Table 2). Although ethanol and butanol diminished C\textsubscript{2}H\textsubscript{4} production by 50\%, histidine and tryptophan almost completely inhibited C\textsubscript{2}H\textsubscript{4} production.
PGI₂ synthetase activity in bovine aortic microsomes can be measured by incubation with PGH₂, resulting in the generation of 6-keto-PGF₁α, the stable isomerization product of PGI₂ (Fig. 4A). Addition of 15-HPAA to the microsomes produced an inhibition of enzyme activity with little or no production of 6-keto-PGF₁α (Fig. 4C). The addition of tryptophan, the most effective scavenger for OH⁻ in this system, was able to protect the PGI₂ synthetase activity of the bovine aorta-microsomes incubated with 15-HPAA (Fig. 4D). In addition, coincubation of 15-HPAA with bovine aorta microsomes produced a 220 ± 10% (n = 3) increment in C₂H₄ generation, as compared with 15-HPAA alone, suggesting that OH⁻ was generated during the incubation.

DISCUSSION

PGI₂ is the most potent inhibitor of platelet aggregation yet studied and a powerful vasodilator.¹² The inhibition of PGI₂ synthetase by 15-HPAA, coupled
Table 2. Effects of Inhibitors on C2H4 Generation by 15-HPAA

<table>
<thead>
<tr>
<th>Additive</th>
<th>Percentage Inhibition</th>
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<tbody>
<tr>
<td>Ethanol (20 mM)</td>
<td>53 ± 2</td>
</tr>
<tr>
<td>Ethanol (40 mM)</td>
<td>54 ± 8</td>
</tr>
<tr>
<td>Butanol (20 mM)</td>
<td>55 ± 1</td>
</tr>
<tr>
<td>Butanol (40 mM)</td>
<td>57 ± 5</td>
</tr>
<tr>
<td>Histidine (10 mM)</td>
<td>85 ± 2</td>
</tr>
<tr>
<td>Histidine (20 mM)</td>
<td>92 ± 0</td>
</tr>
<tr>
<td>Tryptophan (2 mM)</td>
<td>89 ± 1</td>
</tr>
<tr>
<td>Tryptophan (5 mM)</td>
<td>94 ± 1</td>
</tr>
</tbody>
</table>

*Control system consisted of 15-HPAA (5 μg/ml), methemoglobin (2.5 X 10^-7 M), and 1-mM KMBA. Incubation time was 60 min. Results expressed as means ± 1 SD (n = 4).

with the demonstration of similar hydroperoxides in diseased blood vessels, may be responsible for the reduced PGI2 generation seen in atherosclerotic animals.16 Our findings indicate that the mechanism of inactivation of PGI2 synthetase by 15-HPAA involves OH' or a species of similar reactivity. The recent demonstration of PGI2 synthetase inhibition by a variety of hydroperoxides seems consistent with the generation of a common reactive species rather than any specific structural interaction.17

15-HPAA alone was incapable of generating C2H4 in our system, but the addition of heme-containing compounds stimulated the decomposition of the hydroperoxide and the formation of C2H4. The role of OH' in the formation of C2H4 from KMBA is reinforced by the inhibition of C2H4 production by a variety of OH' scavengers. The observed variability in inhibition caused by the scavengers may be caused by differences in the rates of interaction with OH' and/or differences in accessibility to sites of radical generation (i.e., the hydroperoxide-methemoglobin

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Fig. 4. A representative set of radiochemical scans of 6-keto-PGF1α formation by bovine aorta microsomes (full-scale deflection 25,000 cpm). Dark circles indicate migration of standards applied to same plate and located by iodine staining. A: Bovine aorta microsomes with PGH2. B: Bovine aorta microsomes preincubated with 5-mM tryptophan followed by PGH2. C: Bovine aorta microsomes pretreated with 15-HPAA and followed by incubation with PGH2. D: Bovine aorta microsomes preincubated with 5-mM tryptophan, treated with 15-HPAA, and followed by the addition of PGH2.
complex). However, we cannot dismiss the possibility that other powerful oxidants may be generated with reactivities similar to that of OH'. The mechanism of radical generation remains unclear, but it appears to be independent of O$_2^-$ and H$_2$O$_2$. Heme-containing compounds may be capable of catalyzing the generation of OH' directly from the hydroperoxide.

Tryptophan, the most potent OH' scavenger we studied, protected PGI$_2$ synthetase activity from 15-HPAA. We hypothesize that on the addition of 15-HPAA to the bovine aorta microsomes, endogenous microsomal components catalyze the formation of OH' or a species of similar reactivity that is responsible for inhibition of the enzyme.

Thus, evidence has been presented for the generation of OH' or a similar species during the heme-catalyzed decomposition of 15-HPAA. The 15-HPAA-mediated inactivation of PGI$_2$ synthetase seems to be related to the generation of this reactive species. Whether or not other potential sources of OH' in vivo (neutrophils, monocytes, platelets, etc.) are capable of mediating this effect is under investigation.

REFERENCES

2. Moncada S, Gryglewski RJ, Bunting S, Vane JR: A lipid peroxide inhibits the enzyme in blood vessel microsomes that generates from prostaglandin endoperoxides the substance which prevents platelet aggregation. Prostaglandins 12:715, 1976
A mechanism for the hydroperoxide-mediated inactivation of prostacyclin synthetase

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