THE POSSIBLE INVOLVEMENT OF A PEROXIDASE IN PROSTAGLANDIN BIOSYNTHESIS

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Received April 5,1976

Summary: Sheep vesicular gland microsomes have been found to have an unusual peroxidase activity with a wide peroxide specificity and capable of oxidizing cofactors of prostaglandin synthetase. The peroxidase was also similar to the synthetase in its cellular location, its activation by hemin, inhibition by heme ligands and its inactivation by different peroxides. The inhibition by 2,7-naphthalenediol (K_i = 2 μM) also suggests that the peroxidase is an integral part of the synthetase complex.

The recent discovery of endoperoxide intermediates in prostaglandin biosynthesis with more biological activity than prostaglandins (1,2) have led to the concept that arachidonic acid exerts its biological action through the endoperoxide instead of the prostaglandins (3). The activation by hemoproteins of prostaglandin formation by vesicular gland microsomes (4,5) as well as the accompanying oxygen uptake (4) suggests that a hemoprotein may be involved in the initial cyclo-oxygenase step. Recently it has been shown that hemoproteins, particularly cytochrome P450, can readily oxygenate polyunsaturated fatty acids presumably by a peroxidase mechanism with the unsaturated fatty acid acting as a peroxidase hydrogen donor. The hemoprotein becomes inactivated during the reaction.

In the following, vesicular gland microsomes have been found to have a very active peroxidase activity with unsaturated fatty acid hydroperoxides. Hydrogen donors oxidized included cofactors required for prostaglandin biosynthesis. The properties of the peroxidase and prostaglandin synthetase have been compared.

MATERIALS AND METHODS:

Cytochrome c, catalase, hemin and indomethacin were purchased from Sigma Chemical Company. Epinephrine, N,N,N^1,N^1-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) and hydrogen peroxide were obtained from BDH Chemicals.

Abbreviations: SVG, sheep vesicular glands; TMPD, N,N,N^1,N-tetramethyl-p-phenylenediamine-dihydrochloride; NDGA, nordihydroguaiaretic acid.

Cumene hydroperoxide, p-menthane hydroperoxide and 2,5-dimethylhexane-2,5-dihydroperoxide were obtained from ICN pharmaceuticals. t-Butyl hydroperoxide and 1,3-diphenylisobenzofuran were purchased from Aldrich Chemical Co. Mefenemic acid was obtained from Parke Davis and Company and superoxide dismutase from Miles Laboratories. 8,11,14 eicosatrienoic acid was a gift of the Upjohn Company, Kalamazoo, Michigan. 2,7 naphthalenediol was purchased from Eastman Organic Chemicals. All other chemicals were of the highest grade commercially available.

Freshly frozen sheep vesicular glands were supplied by Dr. Hulan, NRC Laboratories, Ottawa. Microsomes from these frozen glands were prepared according to Miyamoto et al (5). The frozen glands were homogenized in a Waring blender for 90 seconds with three volumes of 50 mM potassium phosphate buffer (pH 7.0). The resulting homogenate was filtered through cheesecloth and the filtrate was centrifuged at 10,000 x g for 15 minutes. The supernatant obtained was further centrifuged at 105,000 x g for 75 minutes and the pellet obtained was resuspended in one third the volume of the original buffer and centrifuged at 105,000 x g for 60 minutes. The microsomal fraction that collected at the bottom of the centrifuge tube was suspended in 10 mM potassium phosphate buffer (pH 7.0) and adjusted to a protein concentration of 20 mg/ml.

TMPD PEROXIDASE: To a total volume of 6.0 ml of 0.1 M potassium phosphate buffer (pH 8.0, 37 C.) was added 0.2 mM TMPD, 1 mM EDTA, 74 μg SVG microsomes and the appropriate agent (when included). After mixing, the contents were divided between two 3 ml cuvettes and the reaction started by the addition of either 0.1 mM or 1.0 mM hydroperoxide to the sample cuvette. The rate of increase in O.D. at 610 nm due to formation of Wurster's blue (mM extinction coefficient =11.6) was taken as a measure of the peroxidase activity. The reaction was not linear possibly due to destruction of the enzyme system by the peroxide, hence only initial rates (first 30-60 seconds) were used in calculating the activity.

PROSTAGLANDIN SYNTHETASE AND PEROXIDASE ACTIVITY: Prostaglandin synthetase activity was measured spectrophotometrically according to the method of Takaguchi and Sih (6). The reaction was carried out in 1 ml cuvettes. Each 2.0 ml of the reaction medium contained 50 mM tris HCl - 0.04% cutscum (pH 8.3 at 25° C.), 1 mM epinephrine , 0.175 mg SVG microsomes and the appropriate agent (when included). Autoxidation of epinephrine was minimized by the inclusion of EDTA in the reaction medium. After mixing, the contents were divided equally between two 1.0 ml cuvettes. The reaction was initiated by the addition of either 8,11,14 eicosatrienoic acid (0.33 mM final concentration) for synthetase activity or H_2O_2 for peroxidase activity. Activity was measured by recording the increase in O.D. at 480 nm due to formation of adrenochrome (molar extinction coefficient at 480 nm = 4000). Only initial rates were measured. Synthetase activity of the microsomes 4-5 hours after isolation had declined 50-70%. Synthetase activity was therefore checked at regular intervals and the percentage stimulation or inhibition caused by the various agents was calculated accordingly.

RESULTS:

The vesicular gland microsomes were found to have a very high peroxidase activity. Hydrogen donors oxidized included phenylenediamines, guaiacol, benzidine, diaminobenzidine and epinephrine. From Table I, it can be seen that the peroxide specificity was unusually wide. Linoleic acid hydroperoxide was particularly effective but the rate was too fast to measure. Hydrogen peroxide was more effective than organic hydroperoxides in spite of the presence of catalase in the microsomes. The peroxidase activity was unaffected by EDTA and was inactivated by heat treatment (80° C for 2 minutes) showing that the perox-

Table I: Efficiency of various hydroperoxides in supporting oxidation of TMPD by sheep vesicular gland microsomes[1]

Hydroperoxide used	0.1 mM Hydroperoxide		1.0 mM Hydroperoxide	
	Rate[2]	Efficiency[3]	Rate[2]	Efficiency[3]
Hydrogen peroxide	1.03	100	6.76	100
t-Butyl hydroperoxide	0.48	46	2.21	33
Cumene hydroperoxide[4]	0.25	24	0.50	7.4
p-Menthane hydroperoxide[4]	0.42	40	0.55	8.0
2,5-Dimethylhexane-2,5-dihydroperoxide	0.39	37	1.55	23

1. The assay was carried out as described in METHODS. The reaction mixture consisted of (final volume = 3.0 ml; 37° C): 0.1 M phosphate buffer (pH 8.0), 1 mM EDTA, 0.2 mM TMPD, 37 μg SVG microsomes. Hydroperoxide (final concentration 0.1 mM and 1.0 mM) was added to start the reaction. Due to nonlinearity only initial rates (first 30 seconds) were measured.
2. expressed as μmoles of TMPD oxidized/minute/mg microsomal protein
3. expressed relative to H_2O_2 as 100
4. causes rapid tailing off of the enzyme activity

idase was enzymic in nature. A subcellular distribution study showed that the peroxidase activity was located principally in the microsomal fraction with little activity in the cytosol fraction. If the peroxides were added before the hydrogen donor a much lower peroxidase activity was observed.

In Table II the effects of various inhibitors of prostaglandin biosynthesis were tested on the peroxidase activity. Prostaglandin synthetase activity was measured using the rapid, continuous, spectrophotometric assay for following the oxidation of epinephrine, a coenzyme providing reducing equivalents required for prostaglandin synthesis. In the peroxidase assay, H_2O_2 was substituted for the 8,11,14 eicosatrienoic acid. Rat liver microsomes, hemin (1 μM), methemoglobin (1 μM) or lipoxygenase did not have such a peroxidase or synthetase activity. However as can be seen from Table II hemin or methemoglobin markedly enhanced the activity of both the peroxidase and synthetase activity. Cytochrome c, however had little effect. In contrast cupric ions markedly inhibited these act-

Table II: Effects of various agents on 8,11,14 eicosatrienoic acid or hydrogen peroxide induced epinephrine oxidation by microsomes

Additions	% activity	
	Peroxidase	Synthetase
None	100	100
0.2 mM KCN	36	37
.15 mM aminotriazole	42	36
5 mM azide	34	80
20 mM azide	3	48
.1 μM hemin	210	205
1.0 μM hemin	280	285
1.0 μM methemoglobin	340	360
1.0 μM cytochrome c	102	104
.5 mM Cu^{2+}	10	43
5 μM linoleic acid hydroperoxide*	31	30
8 μM cumene hydroperoxide*	33	35
10 μM H_2O_2*	70	10

* preincubated with microsomes for 30 seconds

The assay was carried out as described in METHODS. The rates are expressed relative to "no addition" as 100. This corresponded to 1.2 μmoles adrenochrome formed/minute/mg protein for synthetase activity and 2.0 μmoles/minute/mg protein for peroxidase activity.

Table III: Effects of synthetase inhibitors on peroxidase activity

	Peroxidase	Synthetase
2,7-naphthalenediol (2 μM)	55	24
2,7-naphthalenediol (6 μM)	0	0
Indomethacin (2 μM)	95	50
Mefenemic acid (50 μM)	98	10
Flufenamic acid (50 μM)	92	48
Eicosatetraynoic acid (10 μM)	95	28
Eicosatetraynoic acid (50 μM)	85	6
Butylated hydroxyanisole (75 μM)*	136	47
Butylated hydroxyanisole (.75 mM)*	110	0
NDGA (.15 mM)	100	50
TMPD (.3 mM)	97	42

* increased the linearity of the peroxidase reaction

The assays were carried out as described in METHODS. The reaction conditions were identical to those in Table II. Rates are expressed relative to "no addition" as 100.

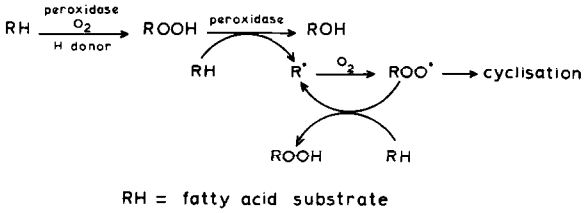
ivities. Inhibition was also observed for the heme ligands cyanide, aminotriazole and azide. The peroxidase and synthetase were also similar in their sensitivity to inactivation by different peroxides.

In Table III it can be seen that the most effective peroxidase inhibitor found was 2,7-naphthalenediol shown by others (6) to be the most effective inhibitor of prostaglandin synthetase. However, the inhibitors indomethacin, mefenamic acid, flufenamic acid, eicosatetraynoic acid believed to be specific inhibitors of the fatty acid binding site of the synthetase were ineffective in inhibiting peroxidase activity. The fatty acid site was probably inactivated by storage of the microsomes overnight at -20° C as the synthetase activity was inactivated more than the peroxidase activity. Most antioxidants also inhibited the synthetase activity without affecting the peroxidase activity as would be expected if they scavenge lipid free radicals.

DISCUSSION:

Most peroxidases eg. myeloperoxidase, lactoperoxidase and horseradish peroxidase utilize H_2O_2 but not organic hydroperoxides. Cytochrome P450 as a peroxidase utilizes organic hydroperoxides particularly cumene hydroperoxide (7) whereas cytochrome P420 can only utilize linoleic acid hydroperoxide (8). The peroxide specificity of vesicular gland microsomal peroxidase is unusually wide. Hydrogen donors oxidized by the peroxidase include coenzymes required for prostaglandin synthesis. Further evidence indicating that the peroxidase may be an integral part of the synthetase complex is their similar intracellular distribution, their similar activation by hematin or methemoglobin and their inhibition by heme ligands and destruction by peroxides.

Yoshimoto et al (4) found that hemin, myoglobin, hemoglobin greatly enhanced PGE_2 formation and accompanying O_2 uptake by bovine seminal vesicular microsomes and suggested that heme is involved in the oxygenation step. Other investigators (9,10) suggested that protein-bound copper ions play a role as diethyldithiocarbamate could reversibly inhibit oxygenation. However our finding that Cu ions inhibited synthetase and peroxidase activity and the reports that diethyldithiocarbamate and other thiol containing agents can trap peroxides (11) and inhibit non Cu-containing peroxidases (12) suggests that a case for Cu remains to be proven. Recently we have shown that a hemoprotein may be responsible for the



RH = fatty acid substrate

FIG 1. A PEROXIDASE MECHANISM FOR PROSTAGLANDIN SYNTHETASE

peroxidase and synthetase activity of vesicular gland microsomes(13). We have also recently discovered a hydroperoxide dependent oxygenase activity of cytochrome P450 preparations from liver microsomes which brought about hydroperoxide formation from polyunsaturated fatty acids (14) and aromatic hydroxylation of a variety of compounds (15,16). In the former reaction the cytochrome P450 underwent a self catalyzed destruction (7). Other hemoproteins also catalyzed lipid peroxidation (14) and were destroyed. Mameth et al (17) have also shown that the addition of endoperoxide or hydroperoxide intermediates of prostaglandin biosynthesis promote oxygen uptake by vesicular gland microsomes and may indicate a similar hydroperoxide dependent oxygenase medium. Furthermore Cook and Lands (18) have evidence that the oxygenation of 5,8,11,14 eicosatetraenoic acid is product activated.

The synthetase activity, unlike the peroxidase activity, was readily inhibited by substrate analogues and anti-inflammatory agents indicating that the hydrophobic substrate binding site is different from the peroxide reacting site. The inhibition of the synthetase activity by hydrophobic antioxidants is presumably due to free radical scavenging. However 2,7 naphthalenediol was very effective in inhibiting both peroxidase and synthetase activity indicating that it may act near the peroxide reacting site. Alternatively it may be a peroxide substrate and therefore a competitive inhibitor. However other investigators have shown that this inhibitor is apparently non-competetive with respect to arachidonate in PGE_2 formation (19).

In conclusion it is proposed that a peroxidase mechanism in the oxygenation

step(s) of prostaglandin biosynthesis as indicated in Figure 1. Hydrogen donors are probably involved in the initial hydroperoxide formation from substrate. Once formed the reaction may be propagated by the peroxidase with hydroperoxide intermediates catalyzing further oxygenation, utilizing the substrate as a hydrogen donor and resulting in a partial chain reaction (14)to form the 15 hydroperoxide or cyclization of the peroxy radical (20) to form the cyclic endoperoxide PGG (21).

ACKNOWLEDGEMENT:

The author (P.J.O'B) wishes to thank D.H. Nugteren for helpful advice and hospitality during part of a sabbatical leave in his laboratory at Unilever Research, Vlaardingen where this research was initiated.

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