THE POSSIBLE INVOLVEMENT OF SINGLET OXYGEN IN PROSTAGLANDIN BIOSYNTHESIS

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Summary: It has been found that both the peroxidase and synthetase activity of sheep vesicular gland microsomes catalyze the oxygenation of singlet oxygen trapping or quenching agents. Furthermore the synthetase was also readily inactivated by these agents, particularly bilirubin, and suggests that singlet oxygen formed by the peroxidase activity may initiate prostaglandin biosynthesis. The singlet oxygen agents also protected the synthetase from self-catalyzed destruction or inactivation by peroxides and suggests that singlet oxygen may also be responsible for the inactivation.

Hydroperoxides can be formed from unsaturated fatty acids by the action of singlet oxygen (1,2,3,4). The possibility that singlet oxygen may be involved in some biological oxidations is not proven but some investigators studying enzymic mechanisms of lipid peroxidation of liver microsomes believe the accompanying chemiluminescence is due to singlet oxygen decay (5,6,7) whilst others have demonstrated the co-oxygenation of some singlet oxygen scavengers; diphenylisobenzofuran (8,9), diphenylfuran (10) and 2,5 dimethylfuran (11) during lipid peroxidation. It has been suggested that the O_2^1 was formed during lipid peroxide decomposition (10,12). However other investigators have found no evidence of co-oxygenation of other singlet oxygen scavengers (13,14). The partial inhibition of lipid peroxidation by efficient singlet oxygen quenching or trapping compounds (8,9,10) suggests singlet oxygen may promote part of the lipid peroxidation. Evidence for singlet oxygen formation by lipoxygenase has also been presented (12,15) and may be responsible for the bleaching of cytochrome c if present but not for the self-catalyzed destruction of lipoxidase (15).

Recently Marnett et al (16) have demonstrated the co-oxygenation of DPBF during prostaglandin synthesis catalyzed by vesicular gland microsomes. In the

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Abbreviations: O_2^1, singlet oxygen; SVG, sheep vesicular gland; DPBF, diphenylisobenzofuran.

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following we present evidence that O_2^1 formed by a peroxidase mechanism may initiate prostaglandin synthesis.

MATERIALS AND METHODS:

Prostaglandin synthetase activity was measured spectrophotometrically according to the method of Takaguchi and Sih (17). The reaction was carried out in 1 ml cuvettes. Each 1 ml of the reaction medium contained 50 mM tris HCl - 0.04% cutscum (pH 8.3 at 25°C), 1 mM epinephrine and 175 μg SVG microsomal protein and the appropriate agent when included. The reaction was initiated by the addition of either H_2O_2 for the peroxidase activity or 8,11,14-eicosatrienoic acid for the synthetase activity. The reaction was monitored by recording the increase in O.D. at 480 nm due to adrenochrome formation (mM extinction coefficient = 4.00)

Bilirubin oxygenation: Bilirubin was made water soluble as its dianion by dissolving in water containing two equivalents of base per equivalent of bilirubin. The bilirubin reaction was studied by measurement of the pseudo-first order decay of bilirubin absorbance at the absorption maximum 435 nm. The reaction was carried out in 1 ml cuvettes. Each 1.0 ml of the reaction medium contained 50 mM tris HCl buffer (pH 8.3 at 25°C), 15 μM bilirubin, 50-100 μg SVG microsomes and the appropriate agent (when included). The reaction was initiated by the addition of either H_2O_2 or 8,11,14 eicosatrienoic acid (0.33 mM final concentration). Activity was measured as n moles of bilirubin disappearing initially.

1,3 diphenylisobenzofuran: The DPBF reaction was studied by measurement of the initial decay rate of DPBF absorbance at the absorption maxima at 420 nm. The reaction medium and method was similar to that described for bilirubin oxygenation using .05 mM DPBF (added in acetone). The product(s) of DPBF oxygenation were checked according to the method of Mayeda and Bard (18) using DPBF purified by column and thin layer chromatography.

Chemicals and Materials: These were supplied as described in the previous paper (19).

RESULTS:

In Figure 1 the ability of microsomes to catalyze the oxygenation of bilirubin by peroxides is demonstrated and found to be similar to the oxygenation by eicosatrienoic acid substrate. The microsomal peroxidase and synthetase also catalyzed the oxygenation of 1,3 diphenylisobenzofuran followed at 420 nm (18). The principal products identified by thin layer chromatography were similar to those expected from singlet oxygen attack. These products apparently derive from cleavage of enamine double bonds and by 1,4 addition of oxygen to pyrrole rings (20) or furan rings (18).

The peroxidase catalyzed co-oxygenation of bilirubin showed the same peroxide specificity as the peroxidase (19). In Figure 1 and Table I, it can be seen that both peroxidase and synthetase catalyzed co-oxygenation were found

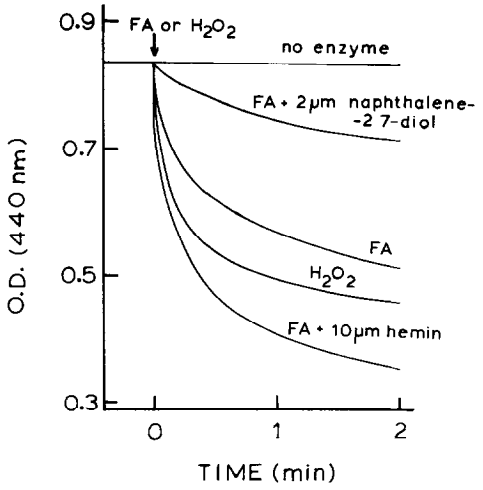

Figure 1:

The assay was carried out as described in METHODS. Each 1 ml of the reaction mixture contained 50 mM tris HCl buffer - 0.04% cutscum (pH 8.3 at 25°C), 52 μg SVG microsomes, 10 μM hemin or 2 μM 2,7-naphthalenediol (when added) and 15 μM bilirubin. The reaction was initiated by adding either H_2O_2 or 8,11,14 eicosatrienoic acid (final concentration 0.3 mM) and monitored by recording the disappearance of bilirubin at 440 nm. In case of H_2O_2 addition, 104 μg SVG microsomes were used.

Table I: Effects of various agents on bilirubin co-oxygenation

Additions	Peroxidase catalyzed	Synthetase catalyzed
None	100	100
Hemin (10 μM)	133*	183*
KCN (.2 mM)	74	90
2,7-naphthalenediol (2 μM)	27	20
Indomethacin (2 μM)	93	48
Butylated hydroxyanisole (.25 mM)	153*	65

* time curve more linear

Assays carried out as described in METHODS. To each 1 ml of the assay medium was added successively 52 μg SVG microsomes (for fatty acid dependent reaction) or 104 μg SVG microsomes (for H_2O_2 dependent reaction). The appropriate agent (when added) and 15 μM bilirubin. The reaction was initiated by the addition of either H_2O_2 or 8,11,14 eicosatrienoic acid (final concentration 0.3 mM). Rates are expressed relative to "no addition" as 100 and are based on the decrease in O.D. at 440 nm during the first six seconds of the reaction.

Table II: The involvement of activated oxygen species

	Synthetase	Peroxidase
Superoxydismutase (50 μg)	69	68
Tiron	90	95
Catalase (50 μg)	75	0
Mannitol (5 mM)	92	96
D_2O medium	46	46
Bilirubin (33 μM)	90	94
Bilirubin (100 μM)	2	70
Diphenylfuran (.4 mM)	36*	100
Diphenylisobenzofuran (.15 mM)	50	120+

* lag of 60 seconds + more linear time curve

Assays carried out as described in METHODS. To each 1 ml of the assay medium (final volume) was added successively 175 μg SVG microsomes, the appropriate agent (when added) and 1 mM epinephrine. The reaction was initiated by the addition of either H_2O_2 or 8,11,14 eicosatrienoic acid (final concentration 0.3 mM). Rates are expressed relative to "no addition" as 100 and are calculated on the basis of initial (maximum) rates only.

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to be highly sensitive to 2,7-naphthalenediol, an effective inhibitor of prostaglandin synthetase (17). The synthetase unlike the peroxidase reaction however was inhibited by the antioxidant, butylated hydroxyanisole or the anti-inflammatory drug, indomethacin. Both peroxidase and synthetase were activated by hemin and inhibited by cyanide.

The role of activated oxygen species in synthetase activity was investigated. In Table II, it can be seen that the superoxy radical quenchers, superoxy dismutase (50 μg) inhibited the synthetase activity by only 30-32% whereas Tiron (10 mM) was without effect. The hydroxyl radical scavenger mannitol (5 mM) was without effect. Catalase (50 μg) inhibited the synthetase activity by only 25%. However singlet oxygen quenchers markedly inhibit synthetase activity with only a small effect on peroxidase activity. The synthetase activity was inhibited by 54% in a deuterium oxide medium as was peroxidase activity.

Prostaglandin synthetase is known to undergo a self-catalyzed destruction during the reaction. Addition of eicosatrienoic acid 30 seconds before the

Table III: Inactivation of synthetase-or peroxidase-catalyzed bilirubin oxygenation

Inhibitor	Synthetase	Peroxidase
Linoleic acid hydroperoxide (.8 μM)	12	14
Cumene hydroperoxide (5 μM)	12	17
Cumene hydroperoxide + bilirubin (15 μM)	53	64
LAHPO (.8 μM) + bilirubin (15 μM)	51	60

The assays were carried out as described in METHODS. Each 1 ml of the assay medium containing 110 μg SVG microsomes was preincubated with the appropriate hydroperoxide for 30 seconds either in the presence or absence of 15 μM bilirubin. When bilirubin was excluded during the preincubation it was added subsequently followed by addition of H_2O_2 or 8,11,14 eicosatrienoic acid. All activity is expressed relative to "no addition" as 100.

bilirubin in the synthetase catalyzed bilirubin oxygenation reaction resulted in 90% inactivation. Bilirubin therefore very effectively protects the synthetase from self-catalyzed destruction. Bilirubin also protected peroxidase and synthetase from inactivation by peroxides. In Table III, it can be seen that the singlet oxygen quencher, bilirubin, protected both peroxidase and synthetase catalyzed oxygenation of bilirubin from inactivation by various hydroperoxides. Tryptophan, another O_2^1 quencher (21) was also effective in protecting the synthetase.

DISCUSSION:

Recently the co-oxygenation of 1,3 diphenylisobenzofuran (DPBF) during prostaglandin biosynthesis in vesicular gland microsomes has been demonstrated (16). Our demonstration that these microsomes also catalyze the oxygenation of DPBF and bilirubin by peroxides indicates that a peroxidase is involved in O_2^1 formation. The synthetase inhibitor 2,7-naphthalenediol also inactivated the peroxidase. Chan also found a peroxidase and lipoxygenase could co-oxygenate DPBF (15) but tetracyclone, a less reactive quencher, was not oxidized by lipoxygenase via a singlet oxygen mechanism (23).

It is likely that the O_2^1 formed by the peroxidase is not generated into the medium as deuterium oxide, a solvent in which O_2^1 has a ten fold longer

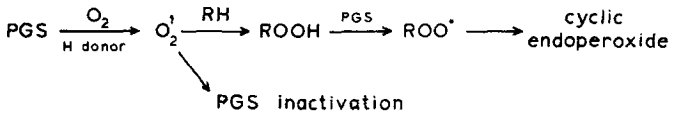
half-life than in water (22), inhibits the synthetase. Presumably the O_2^1 exists as a complex of the metal prosthetic group of the synthetase, in the lipid phase of the membrane, and able to oxygenate reactive quenchers.

Several investigators have proposed that singlet oxygen formation accompanying lipid peroxidation is the result of lipid peroxide decomposition (6,7,10) or the dismutation of peroxy radicals (12). Although the peroxidase seems to be responsible for O_2^1 formation in prostaglandin synthesis, whether additional O_2^1 formation may originate from the decomposition of the hydroperoxide intermediates of prostaglandin biosynthesis is doubtful. The inhibition of synthetase-catalyzed bilirubin co-oxygenation by antioxidants or indomethacin probably indicates that antioxidants or indomethacin prevent the formation of the hydroperoxide intermediates which are also substrates of the peroxidase.

Singlet oxygen quenchers inhibited the synthetase reaction with only a slight effect on peroxidase activity. The degree of inhibition was directly related to their ability to quench or react with singlet oxygen (24). Bilirubin was particularly effective probably because 83% of the singlet oxygen reaction is due to physical quenching (25). The furans used however quench singlet oxygen by chemical reaction and are therefore not as effective (25). Panganamala et al (26) showed that DPBF inhibited PGE and PGF formation from 8,11,14-eicosatrienoic acid catalyzed by lyophilized bovine vesicular gland microsomes.

It was also found that singlet oxygen quenchers at concentrations low enough not to inhibit the synthetase reaction protected prostaglandin synthetase from inactivation by peroxides as well as self catalyzed destruction. Tryptophan, another O_2^1 quencher (21), was also effective in protecting and may help explain the ability of tryptophan to act as a cofactor for prostaglandin synthetase (27).

In Figure 2 it is suggested that the cyclo-oxygenase step in prostaglandin biosynthesis involves a peroxidase mechanism in which a singlet oxygen metal complex reacts with the unsaturated fatty acid substrate to form an unconjugated hydroperoxide (2). The hydroperoxide reacts further with the peroxidase part of



PGS = prostaglandin synthetase peroxidase

RH = fatty acid substrate

Fig. 2. Possible Mechanism of Cyclooxygenase.

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the synthetase complex to form a peroxy radical which could form the endoperoxide by a two step consecutive radical cyclization reaction (28).

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