ACCELERATIVE AUTOACTIVATION OF

PROSTAGLANDIN BIOSYNTHESIS BY PGG_2

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Summary: Cyclooxygenase catalysis is stimulated by its product, PGG_2, and by
other lipid hydroperoxides. The endoperoxide, PGH_2, was not stimulatory. The
results provide a direct demonstration of an essential role for lipid hydro-
peroxides in prostaglandin biosynthesis, and show how the biosynthetic inter-
mediate PGG_2 has a positive accelerative effect.

INTRODUCTION

A bis-dioxygenation of unsaturated fatty acids by cyclooxygenase produces

the endoperoxide which leads to the formation of all subsequent prostaglandins,

thromboxanes and prostacyclin. Recent reviews have noted a variety of physio-

logic and pharmacologic mechanisms for the regulation of cyclooxygenase acti-

vity (1-3). For example, reductions in the available levels of the required

heme cofactor and the substrates, oxygen and unesterified fatty acid, are

known to reduce prostaglandin formation in vitro. In addition, the extent of

product formation is limited by a self-inactivation of the cyclooxygenase (4,5)

which perhaps protects against overproduction of prostaglandins. Another

important feature of the reaction mechanism is the requirement for a minimal

level of peroxide to initiate and maintain cyclooxygenase catalysis.

This peroxide requirement is not readily apparent in the routine cyclo-

oxygenase assays and as a result, it can be easily overlooked. The require-

ment was clearly demonstrated when the removal of peroxide by additions of

glutathione peroxidase (GSP) inhibited oxygenation by crude (4,6) and purified

(5) cyclooxygenase and interrupted prostaglandin biosynthesis already in

Abbreviations: DDC, diethyldithiocarbamate; GSP, glutathione
peroxidase; GSH, glutathione; HPET, hydroperoxyeicosatetraenoic acid;
PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2.

progress (7). In later studies, an enhanced requirement for peroxide (lags

up to 50-60 seconds) was seen when crude cyclooxygenase preparations were

inhibited by sodium cyanide (8,9). Acting synergistically with cyanide, the

GSP present in bovine vesicular gland was inhibitory (8), supporting the concept

that the in vivo levels of GSP and its cosubstrate, glutathione (GSH), may

physiologically regulate prostaglandin biosynthesis by removal of essential

peroxide(s). After extraction of a reaction mixture with diethyl ether, the

resulting crude lipid peroxide fraction was stimulatory when added back to

lagging, accelerating reaction systems (9), but the self-generated, stimula-

tory peroxide responsible for accelerative oxygenation was not characterized.

To clearly identify the autoactivating peroxide factor(s), we secured

electrophoretically pure cyclooxygenase, isolated pure reaction products

PGG_2 and PGH_2, and established reaction conditions suitable for showing the

peroxide requirement with the pure enzyme.

MATERIALS AND METHODS

Hemin chloride (Calbiochem), manganese protoporphyrin IX (Porphyrin
Products, Logan, Utah), arachidonic acid (99% pure, Supelco), and sodium
diethyldithiocarbamate (Sigma) were used as supplied. Cyclooxygenase apo-
enzyme was obtained as previously described (5), and mangano-heme cyclo-
oxygenase was prepared by incubating apoenzyme with a 5-10-fold molar excess
of mangano-heme and then removing the unbound heme by chromatography on DEAE-
cellulose equilibrated with 20 mM sodium phosphate (pH 7.0) containing 20%
glycerol. PGG_2 was isolated and purified from cyclooxygenase reaction mix-
tures (10) and PGH_2 and 15-hydroperoxyeicosatetraenoic acid were prepared as
previously described (11). Enzyme activity was determined polarigraphically
(12) in reaction vessels with 50 uM arachidonate (20:4) in a total volume of
3 ml of Tris chloride 0.1 M (pH 8.5) at 30°C. Continuous analysis of oxy-
genation rates was facilitated by an electronic differentiator.

RESULTS AND DISCUSSION

Although previous studies using inhibited ferri-heme cyclooxygenase

preparations were adequate for establishing the existence of a peroxide

requirement, assays for peroxide stimulation were somewhat inconvenient

because rapid acceleration made the lag phase relatively short. We have

recently observed that mangano-heme cyclooxygenase has a much more obvious

lag phase (compare ferri-heme and mangano-heme in Fig. 2A) and provides a

more suitable assay system for screening potential peroxide activators.
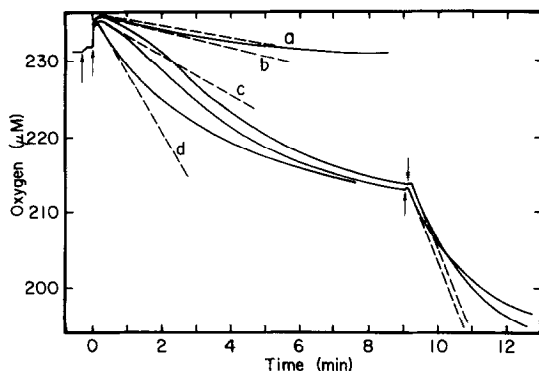
Figure 1: The accelerative effect of peroxide on mangano-heme cyclooxygenase
activity.

        Mangano-heme cyclooxygenase [0.062 μM subunit, based on 70,000-
72,000 daltons per subunit (17,25)] was added (first arrow) to standard reac-
tion mixtures and after 20 seconds, reactions were initiated (second arrow)
by the addition of 10 μl of ethanol which contained: no 20:4 (curve a),
SnCl$_2$-pretreated 20:4 (curve b), untreated 20:4 (curve c), and SnCl$_2$-pre-
treated 20:4 plus 15-hydroperoxy eicosatetraenoic acid (15-HPET) (curve d).
Final concentrations were 20:4 (50 μM) and 15-HPET (1 μM). After ∿ 9 minutes
(arrows), fresh enzyme (0.062 μM) was added to reactions b and c. The dashed
lines are tangents representing the velocities at 15 seconds.
        For pretreatment, 20:4 in toluene was made 4 mM with ethanolic
SnCl$_2$ and mixed vigorously. After several hours, and acidification with
aqueous citric acid, aliquots of the extracted organic layer were dried under
N$_2$, taken up in ethanol, and used immediately.

Also, the mangano-enzyme is free of the peroxidase activity (13) that is

associated with ferri-heme cyclooxygenase. Thus the kinetic results with

this enzyme can be attributed to cyclooxygenase activity that is uncomplicated

by the peroxidase mechanism.

    The importance of peroxide stimulation is clearly evident in Fig. 1.

The Mn-catalyzed oxygenation reaction needed more than a minute to reach its

optimum rate even in the absence of any added inhibitors (curve c). This lag

was accentuated when the substrate arachidonate (20:4) was treated with SnCl$_2$

to remove some of the contaminating peroxides which had spontaneously formed

(curve b). On the other hand, addition of 1 μM 15-hydroperoxy eicosatetrae-

noic acid practically eliminated the lag (15-HPET, curve d). These dif-

ferences in early reaction rates are particularly obvious when the velocities

at 15 seconds (represented by the dashed line tangents) are compared. Evi-

dence for a self-generated stimulating factor is also seen in the rapid

response to second additions of fresh enzyme (at 9 minutes for curves b and

c). The diminished lags (after the second enzyme additions) support the

concept that a stimulatory peroxide accumulated during the first reactions

and remained for several minutes. Since a second addition of enzyme provided

further uninhibited reaction (Fig. I), the recognized self-inactivating

properties of the crude (5) and purified (6) ferri-heme cyclooxygenase are

also characteristic of the pure mangano-heme cyclooxygenase.

The cyclooxygenase can produce an endoperoxide (PGH_2) or an endoperox-

ide-hydroperoxide (PGG_2), either or both of which might have caused the

accelerative autoactivation. A good system for testing the stimulatory

effects of the products PGG_2 and PGH_2 could be obtained by including di-

ethyldithiocarbamate, which caused more pronounced lags, in the reaction

mixtures [diethyldithiocarbamate reacts with peroxide (14)]. Using these

conditions, the mangano-heme reaction was preceded by a lag of at least

240-300 seconds (Figure 2A). When PGG_2 was then added directly to the

lagging system it caused a concentration-dependent rapid acceleration of

the early rates of oxygen consumption and gave a greatly diminished lag

period (Fig. 2A). To simplify comparison of reactions in which the veloci-

ties were continuously changing, only the velocity 60 seconds after peroxide

addition was graphed in Figure 2B. PGH_2 had little effect on reaction veloci-

ty at 60 seconds compared to PGG_2, which gave maximum stimulation at a con-

centration of 1 μM. The lipoxygenase product, 15-hydroperoxy eicosatetra-

enoic acid (15-HPET), was also stimulatory at 1 μM. Apparently it is the

hydroperoxide group rather than the endoperoxide group which is needed to

promote cyclooxygenase catalysis. This conclusion agrees well with the

earlier report that GSP (which can remove the activator peroxide) does not

catalyze reduction of the endoperoxide moeity of PGH_2, but it does reduce

the hydroperoxide of PGG_2 (15). To confirm that the mangano-heme cyclo-

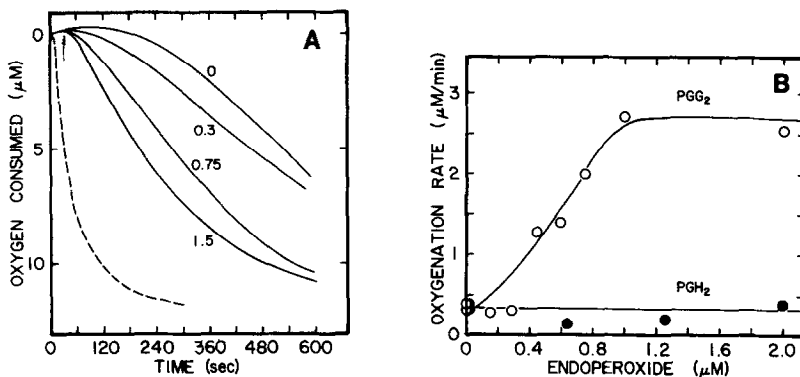oxygenase was free of peroxidase, a reaction was run in the absence of

Figure 2A: The accelerative effect of PGG_2 on cyclooxygenase action.

     Oxygenation of 20:4 (50 µM, standard reaction conditions), which had been preincubated with DDC (4 mM) for at least 5 minutes, was initiated at zero time by the addition of mangano-heme cyclooxygenase (0.031 µM), or cyclooxygenase (0.041 µM) in the presence of 0.9 µM ferri-heme (dashed line). At 30 seconds (arrow), various concentrations of PGG_2 were added, and reaction velocities were determined.

Figure 2B: Stimulation of cyclooxygenase; comparison of the endoperoxide products, PGG_2 and PGH_2.

     Reactions were carried out as in Fig. 2A using 0.041 µM mangano-heme cyclooxygenase. Various concentrations of PGG_2 or PGH_2 were added as shown and reaction velocities at 90 seconds (60 seconds after the addition of endoperoxide) were determined tangentially from the polarograph tracings.

diethyldithiocarbamate and with 0.67 mM phenol (potentially a peroxidase

cosubstrate). The ratio of O_2/20:4 consumed was near 2.0. Analysis showed

that 120 nmoles of 20:4 were converted in 3 minutes to products that co-

migrated on TLC (using ethyl acetate:isooctane:acetic acid (50:50:0.5)

without a tank liner) with PGG_2 (76%), PGH_2 (4%), and combined PGD, PGE, PGF

(20%). Confirmation that PGG_2 was the major product was obtained by co-

migration with PGG_2 standard in another system (isopentane:butanone:acetic

acid (50:40:1) with a tank liner). Also, the material was reducible to $PGF_{2\alpha}$

by triphenylphosphine. Since very little PGH_2 was formed by the mangano-

heme enzyme even in the presence of the peroxidase cosubstrate phenol, the

mangano-heme enzyme appears to have no peroxidase activity of the type

normally associated with the ferri-heme activity (16,17). Thus, our

results show that the stimulatory action of hydroperoxide is clearly dis-

tinct from its action as a substrate for the peroxidase activity of the

ferri-heme enzyme. In addition we find the characteristic self-inactivation

that occurs with both types of cyclooxygenase does not require a peroxidase-

catalyzed breakdown of PGG_2 to PGH_2 as suggested earlier (18).

Our present in vitro findings suggest that even in the presence of O_2

and 20:4, cyclooxygenase in vivo also may not catalyze prostaglandin formation

efficiently unless sufficient amounts of a hydroperoxide are also available.

For example, trace amounts of HPET produced by lipoxygenase action may trigger

cyclooxygenase activity which then accelerates as its product (PGG_2) contri-

butes to further catalysis. Thus, tissue hydroperoxides may have an important

role as proinflammatory agents, and significantly, lipoxygenase activity has

been found in tissues also known to produce prostaglandins (19-23). The

requirement for trace levels of an activator peroxide also means that condi-

tions which facilitate hydroperoxide removal are potentially inhibitory for

prostaglandin biosynthesis. This insight may be significant in terms of the

hypothesis that peroxide removal may be antiinflammatory (24).

In summary, our results have clearly shown that the accelerative early

phases of cyclooxygenase action can be due to accumulation of small amounts

of the product hydroperoxy endoperoxide, PGG_2, which enhances cyclooxygenase

activity. This positive feedback aspect and the negative feedback property

of self-catalyzed inactivation are characteristic of the cyclooxygenase

reaction and not the associated peroxidase reaction.

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