Presence of Lipid Hydroperoxide in Human Plasma

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Abstract

Using purified prostaglandin (PG) H synthase, which synthesizes PGG_2 and PGH_2 from arachidonic acid, we were able to assay for the presence of peroxide activators in biological tissues. This assay system, capable of detecting both hydrogen peroxide (H_2O_2) and lipid hydroperoxides, detected a significant amount of synthase activator in plasma. Treatment of the active preparations with catalase and glutathione peroxidase showed that the principal activator in normal human plasma was a lipid hydroperoxide rather than H_2O_2.

Introduction

Eicosanoid products of arachidonic acid metabolism are important in the regulation of numerous biological processes, ranging from acute inflammation and thrombosis to chronic cardiovascular disorders like atherosclerosis. Because hydroperoxide activator is required for the first committed step of eicosanoid biosynthesis (1–3), it is important to know what amounts of activator can occur in a tissue and whether any such activators are available under physiological conditions.

It is well established that prostaglandin H (PGH)[1] synthase requires the continued presence of a peroxide activator for catalytic activity (4), and that a wide variety of peroxides can stimulate in vitro systems (1). Both hydrogen peroxide (H_2O_2) and short chain hydroperoxides increase the cyclooxygenase velocity and shorten the time taken by PGH synthase to reach optimal velocity (1). In previous studies, PG production by intact cells was stimulated by the addition of 50–900 μM H_2O_2 to the medium (5, 6) and prevented by the addition of catalase (6). In other studies, however, straight chain lipid hydroperoxides and PGG_2 were shown to be much more potent than H_2O_2 with the purified synthase, able to achieve maximal activation with 100 to 1,000-fold lower levels (7, 8).

In using purified PGH synthase to assay for the presence of activating substances in biological samples, we observed significant levels of activator in human plasma (9). This report describes evidence that the major activator material in human plasma is lipid hydroperoxide, not H_2O_2.

Methods

Blood was obtained from healthy human volunteers, poured into citrated tubes (Vacutainer Systems, Rutherford, NJ), kept on ice for

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1. *Abbreviations used in this paper:* FA, fractional activation; GSP, glutathione peroxidase; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; NEM, *N*-ethylmaleimide; PG, prostaglandin; PGG_2, PGH, and PGH_2, prostaglandins G_2, H, and H_2.

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no more than 30 min, and centrifuged at 1,000 g for 15 min. The plasma was removed to a separate tube and kept on ice for analysis the same day.

H_2O_2 (30%) was purchased from Fisher Scientific Co., Allied Corp. (Pittsburgh, PA). A standard lipid hydroperoxide, 15-hydroperoxyeicosatetraenoic acid (15-HPETE), was prepared by the method of Graff (10). The purity of the 15-HPETE was checked by thin layer chromatography on Silica G (Analtech, Inc., Newark, DE) in a solvent system of petroleum ether/diethyl ether/acetic acid at 50:50:0.5 and was >90%.

Arachidonic acid was purchased from Nu-Chek Prep., Inc. (Elysian, MN). Before use it was rendered peroxide-free by treatment with sodium borohydride. The treated, washed arachidonate was stored at 4°C in toluene with butylated hydroxytoluene as antioxidant. Immediately before use the arachidonate solution was dried under a stream of nitrogen and resuspended in 0.1 M Tris buffer at pH 8.5.

PGH synthase was prepared using the method of Van der Ouderaa et al. (11) with slight modifications. Cyclooxygenase activity was assayed with a polarographic electrode as described previously (12). The cyclooxygenase specific activity ranged from 70 to 140 nmol O_2/min per mg protein. Catalase (155 U/mg sp act) was purchased from Sigma Chemical Co. (St. Louis, MO). Glutathione peroxidase (GSP) was prepared from rat liver as described by Nakamura et al. (13). Its catalytic activity was assayed by the method of Lawrence et al. (14) and found to be 69 Kimbel units (KU)/ml (16 KU/mg protein). The protein concentration, as assayed by the method of Peterson (15), was 4.3 mg/ml.

Sodium cyanide, phenol, GSH, and *N*-ethylmaleimide (NEM) were purchased from Sigma Chemical Co.

For the enzymatic identification of activator, GSH (to 2 mM), H_2O_2 (to 5.3 mM), catalase (1.55 U), or GSP (1.4 U) was added to 0.5 ml plasma. NEM (to 4 mM) was added after a 10-min incubation at room temperature to react with the GSH. To measure recovery of added material, either 2.65 μmol H_2O_2 or 600 pmol 15-HPETE was added to 0.5 ml plasma and allowed to equilibrate before the processing of the sample in the usual manner. Processing continued with the addition of 0.5 ml of 100% ethanol to each tube to precipitate proteins in the sample. The contents of the tubes were mixed, placed on ice for 10 min, and then centrifuged at 6,500 g for 15 min.

Aliquots of the clear supernatant were assayed for their ability to stimulate cyclooxygenase activity using an oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH). Samples were added to a 3-ml reaction cuvette containing 0.1 M potassium phosphate (pH 7.2), 1 mM phenol, 100 μM arachidonate, and 2.5 mM sodium cyanide. PGH synthase ($\sim$175 nmol O_2/min in the absence of cyanide) was added to begin the reaction, and the lag time (time required to reach maximal velocity) was determined from the chart record tracing of oxygen consumption versus time. The results were expressed as fractional activation (FA) based on the following relationship:

$$FA = lag_{(control)} - lag_{(sample)}/lag_{(control)} - lag_{(uninhibited)}.$$

Analysis of variance (16) was used to compare the FAs observed after treatment with catalase and GSP.

Results

Characteristics of the enzyme reaction. The basic form of the oxygen electrode tracing is shown in Fig. 1. After addition of cyclooxygenase to the reaction cuvette, there was a short period of no detectable oxygen consumption. This was followed by an acceleratory phase, which has been ascribed to the accu-
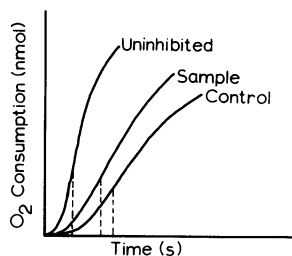
Figure 1. Reduction of cyclooxygenase lag times by added peroxide. A typical chart recording is shown of the oxygen consumption by PGH synthase as a function of time. The uninhibited system contained buffer, phenol, substrate arachidonate, and enzyme. The addition of cyanide resulted in a partially inhibited system (control). The peroxide-containing sample was able to partially overcome the inhibition caused by the cyanide.

Figure 3. Activation by hydroperoxide. Increasing amounts of 15-HPETE added to the reaction chamber before the PGH synthase resulted in a progressive stimulation of cyclooxygenase activity.

mulation of PGG_2 (7). Shortly after the maximal velocity was reached, the effect of enzyme self-inactivation was apparent as the oxygen consumption slowed. The lag times shown are the times taken for the reactions to reach their maximal velocities. The uninhibited system in the absence of cyanide exhibited lag times ($lag_{uninhibited}$) that ranged from 6.6 to 7.8 s. The variation appeared to be independent of any other factors. The mean value of $lag_{uninhibited}$ was determined and used when calculating the FA. The presence of 2.5 mM sodium cyanide increased the lag time ($lag_{control}$) 2–3 times. The length of the cyanide-induced lag time correlated inversely with the age of the stock arachidonic acid solution in toluene. Reproducible cyanide-induced lag times were, however, obtained from individual suspensions of arachidonic acid in buffer. Controls were run before and after unknown samples and the mean was used to determine the FA.

Activation by added peroxides. Additions of small amounts of standard peroxide to the cuvette before the addition of enzyme partly overcame the inhibition caused by the cyanide. The resultant lag time (lag_{sample}) was between that of the uninhibited system and the control. The concentration dependence of the activation by peroxides was studied using both H_2O_2 and 15-HPETE. Fig. 2 shows the effect on FA of increasing amounts of H_2O_2. Aliquots containing from 9 to 620 nmol H_2O_2 were added to the reaction cuvette just before the addition of cyclooxygenase. These amounts corresponded to final H_2O_2 concentrations of ~3–206 μM. There was a curvilinear relationship between the amount of H_2O_2 added and the FA observed. The greatest change in FA was seen with the lower levels of added H_2O_2. The FA observed with 206 μM H_2O_2 was 0.56 and was not saturated.

The shape of the plot of added 15-HPETE versus FA (Fig. 3) was basically the same as that of H_2O_2. However, 15-HPETE could activate the cyclooxygenase at much lower levels than H_2O_2. As the amount of added 15-HPETE increased
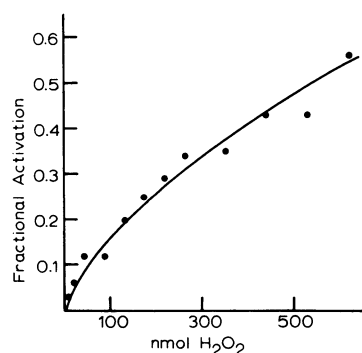

Figure 2. Activation by H_2O_2. The addition of H_2O_2 to the reaction chamber resulted in a stimulation of cyclooxygenase activity. The effect was nonlinear but directly correlated with the amount of peroxide added.
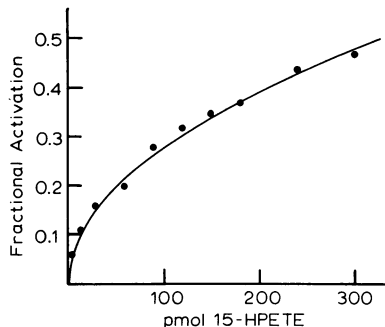
from 6 to 300 pmol (2–100 nM), the FA increased from 0.06 to 0.47. The relationship was again curvilinear, with the most sensitive increase in FA resulting from additions of small amounts of 15-HPETE (~6–30 pmol).

Activation by added plasma. The addition of the supernatant from ethanol-precipitated plasma resulted in a significant shortening of the lag time. Aliquots of 100 μl from two different plasma samples resulted in FAs of 0.185 and 0.13. The second sample of ethanol-precipitated plasma was examined in more detail. Aliquots from 40 to 120 μl were added to the reaction cuvette just before the addition of cyclooxygenase (Table I). There was a direct relationship between the size of the aliquot added and the FA. The amount of H_2O_2 or 15-HPETE equivalent to the observed FA was determined using the standard curves in Figs. 2 and 3, respectively.

Recovery of peroxides added to plasma. Considering the activating substance in plasma to be a peroxide, we checked to see that added peroxides were not affected by the ethanol precipitation and were detectable in plasma. The recoverability of H_2O_2 from plasma was studied first by adding 2.65 μmol of H_2O_2 to 0.5 ml plasma. The mixture was allowed to equilibrate for 2 min at room temperature before the addition of ethanol. Aliquots (40–120 μl) of these modified plasma samples after ethanol precipitation resulted in observed FAs of 0.13–0.40 (Table II). The observed FAs increased with increasing aliquot size and were consistently larger than those obtained with the same amount of supernatant from unmodified plasma. A comparison of the observed FA with that expected

Table I. Activation by Added Plasma

Sample	FA	H_2O_2 equivalents*	15-HPETE equivalents‡
μl		nmol	pmol
40	0.02	7	0
60	0.06	30	3
80	0.09	50	9
100	0.13	80	18
120	0.16	106	30

The addition of increasing aliquots of ethanol-precipitated plasma to the activator assay resulted in a linear increase in FA. The data also show a direct correlation between the amount of plasma added and the peroxide equivalents.
* From Fig. 2.
‡ From Fig. 3.

Table II. Recovery of Hydrogen Peroxide Added to Plasma

Sample aliquot	Endogenous H_2O_2*	Added H_2O_2	Expected H_2O_2‡	Expected activation	Observed FA
μl	nmol equivalents	nmol	nmol equivalents		
40	7	106	113	0.17	0.13
60	30	159	189	0.25	0.30
80	50	212	262	0.32	0.37
100	80	265	345	0.38	0.40
120	106	318	424	0.42	0.40

H_2O_2 (2.65 μmol) was added to 0.5 ml plasma. After a 2-min incubation at room temperature aliquots were assayed for their ability to initiate cyclooxygenase activity. Data show a comparison between the observed FA and that expected from the total of the added H_2O_2 plus endogenous H_2O_2 equivalents.
* From Table I.
‡ From Fig. 2.

from the total of the endogenous H_2O_2 equivalents and added H_2O_2 indicated that the recovery of added H_2O_2 was 118±17% (SD).

Recovery of added lipid hydroperoxide from plasma was studied using 15-HPETE (Table III). 600 pmol of 15-HPETE was added to 0.5 ml plasma and allowed to equilibrate for 2 min before the addition of ethanol. Observed FA increased progressively from 0.06 to 0.32 with increasing aliquot size. A comparison of the observed FA with that expected from the total amount of added lipid hydroperoxide indicated that, as for H_2O_2, the recovery of added 15-HPETE appeared to be near 100% (85±48 [SD]).

Effect of catalase and GSP upon the cyclooxygenase activator in human plasma. To differentiate whether H_2O_2 or lipid hydroperoxide was the endogenous activating substance in human plasma, samples were incubated for 10 min with either catalase (1.55 U) or GSP (1.4 U) (Table IV). Before incubation, the FA resulting from the addition of 100 μl of a normal, untreated sample was 0.15. After the incubation with catalase, the FA of the plasma (sample B, 0.17) was unchanged; however, incubation with GSP resulted in the disappearance of the activator substance from the plasma (sample H, 0.02).

Table III. Recovery of 15-HPETE Added to Plasma

Sample aliquot	Endogenous ROOH*	Added 15-HPETE	Expected ROOH‡	Expected activation	Observed FA
μl	nmol equivalents	nmol	nmol equivalents		
40	0	24	24	0.14	0.06
60	3	36	39	0.18	0.16
80	9	48	57	0.21	0.19
100	18	60	78	0.25	0.29
120	30	72	102	0.28	0.32

15-HPETE (600 pmol) was added to 0.5 ml plasma and incubated at room temperature for 2 min. The observed FA yielded by increasing aliquots of the mixture was compared with the expected activation, the result of adding 15-HPETE and endogenous 15-HPETE equivalents.
* Radical hydroperoxide; from Table I.
‡ From Fig. 3.

Table IV. Effect of Catalase and GSP on the Cyclooxygenase Activator in Human Plasma

Sample (n)	Additions First	Additions Second	Fractional activations
A plasma (18)	—	—	0.15 (0.04)
B plasma (14)	Catalase	—	0.17 (0.05)
C plasma (12)	GSH	—	0.17 (0.07)
D plasma (8)	NEM	—	0.16 (0.03)
E plasma (4)	GSH + NEM	—	0.15 (0.06)
F plasma (4)	GSH + NEM	GSP	0.185 (0.05)
G buffer (6)	GSH + GSP	NEM	0.02 (0.02)
H plasma (12)	GSH + GSP	NEM	0.02 (0.03)
I plasma (10)	GSH + GSP + catalase	NEM	0.01 (0.02)
J plasma (6)	H_2O_2	—	0.25 (0.05)
K plasma (6)	H_2O_2	catalase	0.15 (0.03)

Additions of 10–20 μl of the indicated material were made to a 0.5-ml sample. After a 10-min incubation at room temperature the second addition was made and left to stand for 2 min. Then an aliquot (100 μl) was tested for its ability to stimulate cyclooxygenase activity. All values for fractional activation are expressed as the mean (SD) of n experiments.

The FA of 0.02 was statistically equivalent to zero ($P < 0.01$). Because the incubations with GSP also contained GSH, NEM was added before assay to combine with the GSH to stop the peroxidase activity. The addition of either or both of these materials to plasma did not cause a significant change in the amount of activator detected (sample C, 0.17; sample D, 0.16; and sample E, 0.15). An effect from the GSP on the PGH synthase was ruled out by adding the NEM to the sample before the GSP, resulting in an FA of 0.185 (sample F), not significantly different from samples A to E. The addition of this inactive peroxide scavenging system to the assay had no significant effect on the FA observed.

An additional set of experiments was performed to verify the ability of exogenous catalase to decompose H_2O_2 in plasma. Exogenous H_2O_2 (2.65 μmol) was added to 0.5 ml plasma (sample J). After 10 min of incubation, the addition of 10 μl of this modified sample to the assay gave a higher FA, 0.25 (a recovery of 70% of the H_2O_2 as determined from Fig. 2; the incomplete recovery is probably due to the small amount of peroxidase activity intrinsic to plasma). Addition of 1.5 U of catalase to H_2O_2 resulted in a loss of the added FA, originally caused by the exogenous H_2O_2, so that the resultant FA (sample K, 0.15) was the same as that in plasma before any treatment.

Discussion

The PGH synthase/oxygen electrode used to assay for the presence of cyclooxygenase activator is very sensitive. It can consistently detect about 45 nmol of H_2O_2 in the 3-ml reaction cuvette (15 μM). Its sensitivity for lipid hydroperoxides is about three orders of magnitude greater, with $\sim$6 pmol/3 ml reaction cuvette (2 nM) providing a significant response.

The use of the enzymatic assay for hydroperoxides with plasma samples was initially somewhat difficult due to the

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high amount of protein, which probably interfered with the assay, probably by nonspecific binding of the fatty acid substrate and activator. Addition of an equal volume of 100% ethanol reduced the interference considerably by precipitating ~90% of the protein (as determined by absorbance at 280 nm) while leaving most lipids in solution. The addition of the supernatant from the plasma-ethanol mixture to the cyclooxygenase system resulted in a measurable and reproducible response. These data alone, however, do not indicate whether the activation was due to H_2O_2 or lipid hydroperoxide in the sample because both can activate the cyclooxygenase (1). The observed FA might be attributed either to 2.0 mM H_2O_2, 0.5 μM lipid hydroperoxide, or some combination of the two. The preliminary evidence (9) that plasma and serum contain similar amounts of activator indicates that the platelets did not contribute enough peroxide spontaneously to interfere with the assay results, thus making it unlikely that the plasma activator was 12-HPETE formed while collecting the samples.

The quantitative recovery of both types of added peroxides indicated that the presence of plasma had no direct effect on the measurement of peroxide activator when ethanol was included to free the activator from lipid-protein interactions. After 10 min of equilibration, however, the recovery of added H_2O_2 dropped to ~70%. This difference in recovery due to the amount of time spent at room temperature can be explained by the small amount of GSP normally present in human plasma (17). The amounts of added lipid hydroperoxide in this study (0.6 μM) were too low to be effectively acted upon by the endogenous GSP, which has a K_m value for lipid hydroperoxide of ~5 μM (18). In contrast, the added H_2O_2 was at a concentration (5 mM) that permitted maximal peroxidase action.

The final identification of the activating substance in plasma as a lipid hydroperoxide was established by the effects of added catalase and GSP. The activator in plasma was abolished by preincubation with added GSP. The hydroxy fatty acid (15-hydroxyeicosatetraenoic acid) was not an effective activator of oxygenase activity (result not shown). The loss of activator does not indicate clearly which was the activating substance, because GSP is equally reactive with both H_2O_2 and lipid hydroperoxide (19, 20). On the other hand, incubation with catalase left the activator activity intact. Added catalase was indeed active in this system, as shown by its ability to abolish the additional activation caused by added H_2O_2 to plasma. Thus, the lack of effect of catalase on the endogenous activator in human plasma made unlikely the possibility that much of the endogenous activator in plasma was H_2O_2. This evidence, together with that of the effectiveness of GSP in diminishing the activation by plasma, led us to designate the activator in human plasma as a lipid hydroperoxide.

The hydroperoxide concentration of 0.5 μM found for normal plasma is close to that reported to be injurious to endothelial cells (Moncada et al. [21] reported that 1 μM lipid hydroperoxide was capable of causing a 50% reduction in prostacyclin production). Thus, its presence at ~0.5-μM levels in the plasma in both free and protein-bound forms may represent a previously unrecognized state of chronic oxidant stress in normal humans. Such levels of lipid hydroperoxide in plasma might also penetrate cells sufficiently to activate the cyclooxygenase function of PGH synthase, causing it to be "primed" for more rapid reaction upon the appearance of any

amount of the nonesterified substrate arachidonate. The cyclooxygenase reaction would cause a further elevation of the intracellular levels of lipid peroxides because of the accompanying increase in PG formation, which may occur in inflammation. One consequence of adding local inflammatory conditions to the apparent chronic hydroperoxide stress of normal plasma may be the reported decrease in prostacyclin generation by atherosclerotic arteries (22). The resultant decrease in the ratio of prostacyclin to thromboxane may then lead to the thrombotic tendencies associated with atherosclerosis, and the elevation in lipid peroxides reported to occur in atherosclerosis (23) may be the basis for continued pathophysiology.

Acknowledgments

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