Stimulation of human platelet guanylate cyclase by unsaturated fatty acid peroxides

(arachidonic acid peroxide/oxidation of sulphydryl residue/hemoglobin/lipoxidase)

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ABSTRACT Guanylate cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2] activity of human platelet homogenates was stimulated by the addition of phospholipase A2 or unsaturated fatty acids such as oleic, vaccenic, linoleic, linolenic, eicosenic, eicosadienic, and arachidonic acids. The addition of lipoxidase potentiated the fatty acid-induced stimulation of guanylate cyclase purified by DEAE-cellulose column chromatography. The extent of the stimulation was dependent on the concentration of the oxidized form of these fatty acids (peroxides). Saturated fatty acids such as stearic and arachidic acids had no effect on the guanylate cyclase activity in the presence or absence of lipoxidase, indicating that human platelet guanylate cyclase is stimulated by unsaturated fatty acid peroxides rather than by fatty acids. Hemoglobin prevented the enzyme stimulation produced by low concentrations of fatty acid peroxides, but enhanced stimulation of the enzyme activity with high concentrations of fatty acid peroxides. 2-Mercaptoethanol, dithiothreitol, and N-ethylmaleimide inhibited the guanylate cyclase activities both in the presence and absence of unsaturated fatty acid peroxide. The stimulation of guanylate cyclase activity by unsaturated fatty acid peroxides is attributed to oxidation of sulphydryl residues of the enzyme protein.

The role of cyclic nucleotides in platelet function has been the subject of considerable study since the report by Marcus and Zucker (1) on inhibition of platelet aggregation by adenosine 3',5'-cyclic monophosphate (cyclic AMP). Subsequent investigations from several laboratories revealed that platelet aggregation mediated by various substances such as ADP, collagen, and epinephrine is facilitated by a decrease in the platelet cyclic AMP concentration (2, 3), and that many compounds that inhibit platelet aggregation act by increasing the cyclic AMP concentration in the platelets (3, 4). The effect on the platelet guanosine 3',5'-cyclic monophosphate (cyclic GMP) level of various aggregating agents, however, appears to be the opposite of that seen with cyclic AMP in that aggregating agents such as collagen (5, 6), thrombin, and epinephrine (6, 7) produce an increase in the cyclic GMP concentration in the platelets.

Guanylate cyclase [GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2] activity has been detected in many types of cells and the enzyme is present in both membrane-bound and soluble forms. Several hormones increase cyclic GMP levels in cells (8-14); however, it has not been demonstrated in vitro that there is a direct effect of any of these hormones on guanylate cyclase activity (12, 15-17). Guanylate cyclase is stimulated by nonionic detergents (18, 19), sodium azide (20), lysolecithin, phospholipase A2 (21-23), sodium nitroprusside, nitroglycerin, and nitric oxide (24). Recently, it was demonstrated that fatty acids stimulate guanylate cyclase activities from mouse BALB 3T3 fibroblasts (25) and human platelets (26, 27), but a detailed description of the mechanism in fatty acid-induced stimulation of guanylate cyclase has apparently not been provided. In an attempt at elucidation of the mechanisms involved, we carried out experiments in which we found that the guanylate cyclase activity is stimulated by unsaturated fatty acid peroxides, not fatty acids, and the stimulation is apparently due to a peroxide-induced oxidation of —SH groups of the enzyme protein.

MATERIALS AND METHODS

Chemicals and Reagents. [8-3H]GTP and cyclic [3H]GTP were purchased from the Radiochemical Centre; cyclic GMP, human hemoglobin, and phospholipases were from Sigma Chemical Co.; GTP and phospholipase A2 (porcine pancreas) were obtained from Boehringer Mannheim Co.; and fatty acids and lipoxidase (soybean) were purchased from P-L Biochemicals, Inc. Neutral aluminum oxide was a product of M. Woelm Co. All other chemicals were of reagent grade or the best commercially available and were not purified further. Fatty acids and phospholipids were dispersed in 50 mM Tris-HCl (pH 7.5) by sonication and freshly prepared emulsions were used in all experiments.

Preparation of Human Platelets. Platelet-rich plasma was prepared by low-speed centrifugation of freshly obtained citrated whole blood (28). Platelets were isolated from the platelet-rich plasma by centrifugation and were stored at ~70° until use. Platelet homogenates were prepared from freeze-thaw platelets in 50 mM Tris-HCl buffer (pH 7.4) by using a homogenized tube with a tight Teflon pestle. The homogenates were centrifuged for 60 min at 105,000 X g. The homogenate or 105,000 X g supernatant was employed as the crude enzyme preparation.

Guanylate Cyclase Assay. Activity of guanylate cyclase was determined by the method of Nakazawa et al. (29). The standard assay mixture contained 1 mM [3H]GTP (5 Ci/mole), 2.5 mM cyclic GMP, 15 mM creatine phosphate, 40 µg of creatine kinase, 3 mM MnCl2, 50 mM Tris-HCl (pH 7.7), and an appropriate amount of the enzyme in a total volume of 0.2 ml. The reaction was started by the addition of enzyme unless otherwise indicated. After the mixture was incubated for 10 min at 30°, the reaction was stopped by heating for 2 min in a boiling-water bath, following the addition of 1 M HCl (40 µl). The radioactive cyclic GMP produced was isolated by the serial use of a neutral aluminum oxide/AG1-X2 (Bio-Rad Co.) column. The assay mixture for guanylate cyclase was adjusted to neutral pH with 1 M Tris solution and sodium pyrophosphate was added to make a final concentration of 7 mM. Each cyclase mixture was then applied onto a neutral aluminum oxide column (0.5 g, 0.6 X 2.0 cm). When this column was washed with 5 ml of 0.05 M Tris-HCl buffer (pH 7.4), cyclic GMP passed through the column
while other nucleotides remained adsorbed. The washings containing the cyclic GMP passed directly onto a AG-1-X2 column (chloride form, 0.7 × 3 cm). The neutral aluminum oxide column was removed and after the resin column was washed with 10 ml of 0.05 M HCl, cyclic GMP was eluted from this column with 2 ml of 0.5 M HCl. Over 90% of cyclic GMP was always recovered from the columns.

**Phosphodiesterase Assay.** Activity of phosphodiesterase was determined by the method of Hidaka and Shibuya (50).

**Preparation of Peroxide and Determination of Its Content in Emulsions.** One millimolar arachidonic or linoleic acid emulsion was incubated with various concentrations of lipoxidase solution for 10 min at 30°C. An aliquot of the incubated emulsion was diluted with 50 mM Tris-HCl (pH 7.5) and 10–30 μl of the diluted solution was immediately added to the assay mixture of guanylate cyclase prior to initiating the reaction. On the other hand, the final concentration (50%) was added to the remaining fatty acid emulsion treated with lipoxidase. This method solution was utilized for determination of the peroxide value (peroxide content of emulsions) by the ferrous thiocyanate method as described by Wagner et al. (31). Reagent (3 ml) was added to the sample (20 ml) and the absorption of 500 nm was measured and compared with standard ferric chloride that had been similarly treated. In all experiments, the oxidation of the fatty acid was also measured by determining the absorption at 235 nm. The results obtained by these two methods agreed.

**DEAE-Cellulose Column Chromatography.** DEAE-cellulose chromatography was carried out as described previously (28) on columns (1.5 × 20 cm) with bed volumes of 35 ml. The buffer was 50 mM Tris/acetate (pH 6.0) including 3.75 mM 2-mercaptoethanol and 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA). The enzyme preparation (105,000 × g supernatant) was applied to the column and then eluted with several column volumes of buffer. The initial wash contained no detectable guanylate cyclase activity. A linear gradient from 0 to 0.7 M sodium acetate was then applied with a flow rate of 0.5 ml/min and a total gradient volume of 300 ml. The fractions containing guanylate cyclase activity, which were free from cyclic GMP phosphodiesterase, were pooled and used for experiments. This preparation was referred to as the purified guanylate cyclase.

**RESULTS**

**Stimulation of a Crude Guanylate Cyclase Preparation by Unsaturated Fatty Acids.** As shown in Table 1, phospholipase A₂, phosphatidylcholine, and arachidonic acids stimulated guanylate cyclase activity in a human platelet homogenate. The effect of lyso phosphatidylcholine on guanylate cyclase activity was slight. All of the unsaturated fatty acids—in contrast to the saturated ones such as myristic, palmitic, stearic, and arachidic acids—stimulated guanylate cyclase activity significantly up to the concentration of 0.1 mM (Table 2). Polyunsaturated fatty acids such as linoleic, linolenic, and arachidonic acids were much more effective. These results suggest that unsaturated fatty acids, when released from phospholipids by phospholipase A₂, stimulate guanylate cyclase activity. The concentration of each fatty acid was 0.1 mM.

**Table 1. Stimulation of guanylate cyclase activity in human platelet homogenates by lipids and phospholipase A₂**

| Addition                  | Guanylate cyclase activity pmol/min per mg protein | %
|---------------------------|-----------------------------------------------|
| None                      | 48                                            | 100%
| Phospholipase A₂, 25 μg/ml| 139                                           | 290%
| Phosphatidylcholine, 0.5 mg/ml | 196                             | 408%
| Lyso phosphatidylcholine, 0.5 mg/ml | 73                        | 152%
| Arachidonic acid, 0.1 mM | 346                                           | 721%

**Table 2. Effect of various fatty acids on guanylate cyclase activity in human platelet 105,000 × g supernatant**

| Addition                 | Guanylate cyclase activity pmol/min per mg protein | %
|--------------------------|---------------------------------------------------|
| None                     | 56                                               | 100%
| Saturated fatty acid     |                                                  |        |
| Stearic (C₁₈₂₀)          | 55                                               | 98%
| Arachidic (C₂₀₄)         | 70                                               | 125%
| Unsaturated fatty acid   |                                                  |        |
| Oleic (C₁₈₁₈)            | 235                                              | 420%
| Linoleic (C₁₈₂₀)         | 420                                              | 750%
| Linolenic (C₁₈₃₃)        | 393                                              | 702%
| Linolenic (C₁₈₂₁)        | 143                                              | 255%
| Eicosadienonic (C₂₀₂₃)   | 269                                              | 480%
| Arachidonic (C₂₀₄)       | 404                                              | 721%

The concentration of each fatty acid was 0.1 mM.

the stimulation of human platelet guanylate cyclase by the fatty acid peroxide may be due to the oxidation of a certain residue, probably sulphydryl, in the enzyme protein. If the —SH groups of the enzyme protein are actually oxidized to —S—S— for enzymatic activity by peroxides, this reaction is likely to be blocked and the stimulation of guanylate cyclase activity possibly will be prevented by the addition of sulfhydryl compounds or N-ethylmaleimide. Dithiothreitol, 2-mercaptoethanol, and N-ethylmaleimide inhibited the purified guanylate cyclase activities both in the presence and in the absence of unsaturated fatty acid peroxide. Dithiothreitol at 1 mM or 2-mercaptoethanol at 10 mM inhibited stimulation of the purified guanylate cyclase by arachidonic acid peroxide by 50% and N-ethylmaleimide at 5 mM inhibited the stimulation by 100%. While inhibition of the guanylate cyclase activity by dithiothreitol and 2-mercaptoethanol may be due to protection of —SH groups in enzyme protein as well as to reduction of fatty acid peroxide, the inhibition by N-ethylmaleimide is attributed exclusively to its effect on —SH groups in enzyme protein.

**Effect of Hemoglobin on Guanylate Cyclase.** The effect of hemoglobin on the stimulation of platelet guanylate cyclase induced by unsaturated fatty acid peroxide was also investigated (Table 4). Hemoglobin (15 μg/ml) prevented the enzyme stimulation produced by low concentrations of the unsaturated fatty acid peroxides. Although the effect of hemoglobin itself on guanylate cyclase was negligible at this concentration, this same concentration of hemoglobin enhanced the stimulation of the enzyme activity by high concentrations of these peroxides. In crude preparations containing various amounts of hemoglobin, the effect of fatty acid peroxide may vary with the ratio of peroxide and hemoglobin. The potentiation of this peroxide-induced stimulation of guanylate cyclase by hemoglobin has not yet been explained.

**DISCUSSION**

Phospholipase A₂, which degrades phospholipids to lysophospholipids and unsaturated fatty acids, has been detected in virtually every organism, tissue, and subcellular fraction that has been investigated (34). Gullis and Rowe (35) reported that net phospholipase A₂ activity of synaptic membranes of guinea pig brain cortex is stimulated by Ca²⁺, noradrenaline, acetylcholine, carbamoylcholine, and serotonin. The effects on phospholipase A₂ parallel the effects of these agents on intracellular cyclic GMP levels in other systems (36). The induction of platelet aggregation by agents such as thrombin or collagen is potentiated by the release of arachidonic acid from phosphatidylcholine (37). It has recently been reported that the crude soluble guanylate cyclase of human platelets is stimulated by unsaturated fatty acids such as arachidonic acid (26, 27). Böhme et al. (32) found that human platelet guanylate cyclase activity is stimulated when crude preparations are used for incubation. The stimulation of guanylate cyclase by incubation is attributed to the effect of unsaturated fatty acids produced from endogenous phospholipids by endogenous phospholipase A₂.

Our results suggest that the stimulation of the crude guanylate cyclase preparation induced by unsaturated fatty acids was due to the unsaturated fatty acid peroxides produced during the incubation.

Unsaturated fatty acids readily take up oxygen to give peroxides. These are probably hydroperoxides (—OOH) formed on a carbon atom adjacent to the double bond (38) and this reaction is catalyzed by lipoxidase. Nugteren (39) reported that...
lipoxidase activity was detected in platelets from various species and arachidonic acid proved to be the best and most abundant natural substrate. Our Fig. 1 shows that the addition of lipoxidase enhanced this fatty acid-induced stimulation of guanylate cyclase and the extent of the stimulation was dependent on the peroxide value of fatty acid. A small amount of the peroxide (below 20 microequivalents/liter) was detected even in 1 mM fresh arachidonic acid emulsion and 1 microequivalent/liter of the peroxide was sufficient to stimulate the guanylate cyclase (Fig. 1). These results indicate that the guanylate cyclase activity was stimulated by unsaturated fatty acid peroxides rather than fatty acids. The inhibition of —SH enzymes is reportedly produced by unsaturated fatty acid peroxides (40, 41) and oxidation of —SH groups of proteins or amino acids is catalyzed by peroxides of unsaturated fatty acids (42, 43). Human platelet guanylate cyclase is thought to possess —SH groups (52) and our present data suggest that the oxidation is promoted by incubation with unsaturated fatty acid peroxides, which increases enzyme activity. N-ethylmaleimide prevents this stimulation by covalent binding to —SH residues in the protein. The concept is supported by the evidence that guanylate cyclase from rat lung is stimulated by hydrogen peroxide (44) and guanylate cyclase is inhibited upon application of thiols (27, 44).

Heme proteins are most effective catalysts for oxidation of unsaturated fatty acids (38, 45). In concentrated solutions, however, heme proteins inhibit oxidation (46, 47) because high concentrations apparently destroy the peroxide. The effect of hemoglobin is dependent on the ratio of molar concentration of fatty acid to molar concentration of hemoglobin (47). These findings support our present results that hemoglobin (3 μg per assay) prevents the enzyme stimulation produced by low concentrations of the oxidized fatty acids but enhances the stimulation of the enzyme activity by high concentrations of oxidized fatty acids. Little and O’Brien (41, 43) reported that heme protein accelerated oxidation of thiols by the fatty acid peroxide. Small amounts of hemoglobin derived from contaminating erythrocytes found in human platelet homogenates or 105,000 X g supernatant can be removed by DEAE-cellulose column chromatography. The fact that the degree of stimulation of guanylate cyclase by arachidonic acid peroxide was less with purified than with crude preparations was probably due to the removal of the hemoglobin.

Mickel and Horbar (48) reported that oxidized rather than nonoxidized arachidonic acid caused a much more rapid and marked aggregation. Glass et al. (49) indicated that prostaglandin endoperoxide, a potent inducer of platelet aggregation (50), produced an increase in cyclic GMP levels in human platelets. It is possible that prostaglandin endoperoxide may also have the same effect as unsaturated fatty acid peroxides on guanylate cyclase. Stimulation of guanylate cyclase activity in the homogenate by arachidonic acid was not inhibited by the addition of aspirin or indomethacin, which are inhibitors of prostaglandin endoperoxide synthesis (data not included); however, the possibility remains that, under the proper conditions, prostaglandin endoperoxide may stimulate the cyclase. If cyclic GMP acts as a "second messenger" for platelet aggregation, then it would be physiologically relevant for the guanylate cyclase to be activated by this process. The aggregation-induced activation of phospholipase A2 would liberate arachidonic acid and other unsaturated fatty acids and subsequent peroxidation would activate the guanylate cyclase.

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