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Potentiating effects of clofibrate on prostaglandin-dependent and -independent pathways of human platelet activation: evidence for involvement of cyclic AMP

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Plasma lipids and blood platelets both play major roles in atherogenesis. A relationship between elevated plasma lipids and an increased platelet sensitivity to aggregatory agents in hyperlipidemic patients has been reported [1]. It has been shown that clofibrate, which lowers plasma lipids, also decreases the sensitivity of platelets to ADP, collagen and epinephrine in Type II_B hyperlipoproteinemic patients [1]. These dual hypolipidemic and antithrombotic actions make clofibrate a potentially effective and unique drug for use in the treatment of thromboembolic and coronary artery diseases.

Biochemical mechanisms of the hypolipidemic action of clofibrate have been linked to an inhibition of adenylate cyclase activity and a lowering of cAMP levels in various tissues [2, 3]. However, the antiaggregatory action of clofibrate appears to be unrelated to this biochemical effect since cAMP lowering in platelets is associated with an induction of aggregation, and not with an inhibition of platelet function [4, 5]. In earlier reports, we have examined the antiaggregatory effects of clofibrate on human platelets and have shown that this drug inhibits aggregation, secretion of serotonin and β-glucuronidase, platelet factor 3 activity, incorporation of [^{14}C]acetate into platelet phospholipids, release of arachidonic acid from platelet phospholipids, and prostaglandin biosynthesis [6–8]. Our recent findings suggest that clofibrate inhibits platelet activation by blocking prostaglandin biosynthesis in platelets [8].

By contrast, we have observed that the aggregation response to arachidonic acid in human platelets is potentiated in the presence of clofibrate [6, 8]. In another report, we have shown that phospholipase C (PLC) causes platelet aggregation and secretion of serotonin by a mechanism independent of prostaglandin biosynthesis and that clofibrate also enhances platelet activation by PLC [9]. These studies show that clofibrate exhibits divergent antiaggregatory and proaggregatory effects on human platelet function. Whereas the antiaggregatory action of clofibrate may be related to the inhibition of prostaglandin biosynthesis [8], the stimulatory effect of this drug remains to be explained. An elucidation of the mechanism of proaggregatory action by clofibrate may be helpful in assessing the overall effect of this drug on platelet function, *in vivo*.

These studies were initiated to better understand the mechanism of this proaggregatory action of clofibrate on arachidonic acid- and PLC-induced pathways of platelet activation. The latter pathway, using low concentrations of PLC as an inducer of platelet aggregation and serotonin secretion, is independent of prostaglandin biosynthesis [10, 11]. The concentration-dependent actions of clofibrate were examined on (a) arachidonic acid-induced aggregation and oxygen consumption in washed platelets, (b) platelet aggregation and secretion of [^{14}C]serotonin ([^{14}C]-5HT) by PLC, and (c) PGE_1-elevated cAMP levels in platelets. The data in this report suggest that clofibrate achieves its stimulatory effect on platelet function by lowering cAMP levels.

Methods and reagents used for collection of human blood, preparation of platelet-rich plasma, washed platelets, and platelet aggregation and secretion of serotonin ([^{14}C]-5HT) are identical to those published recently in this journal [8]. The sodium salt of clofibrate [2-(4′-chlorophenoxy)-2-methylpropionic acid] was provided by Ayerst Laboratories, Inc., New York, NY. Phospholipase C from *Clostridium perfringens* and arachidonic acid were obtained from the Sigma Chemical Co. (St. Louis, MO).

Oxygen consumption by platelets was measured by the method of Pickett and Cohen [12]. Incubations were done in a reaction vessel fitted with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH), in a total volume of 3 ml. The platelet count was adjusted to 5×10^8/ml. Arachidonic acid (final concentration, 200 μM) was added in a volume of 20 μl.

Platelet cyclic AMP levels were estimated by the radioimmunoassay of Brooker *et al.* [13]. At the end of the incubation of platelets with various agonists and antagonists, 1/10 volume of trichloroacetic acid (100%) was added to lyse platelets [14], and samples were centrifuged at 12,000 *g* for 1 min. Supernatant fractions (1 ml) were extracted three times with 3 ml of water-saturated diethyl ether. After evaporation of the ether phase, 100 μl of 1 M acetate buffer was added to bring the pH to 6.2. Aliquots (100 μl) of samples and standard solutions of cAMP (0.312 to 10,000 fmoles) were acetylated by mixing with 10 μl of triethylamine–acetic anhydride (5 : 2, v/v). All samples were incubated for 16 hr at 4° after addition of antiserum for cAMP and of [^{125}I]cAMP-tyrosine methyl ester containing bovine γ-globulin. After 16 hr, 2 ml of 60% ammonium sulfate was added to each sample, which was then centrifuged at 1500 *g* for 15 min at 4°. The supernatant fractions from sample tubes were discarded, and the tubes were counted in a Beckman model 8000 gamma scintillation counter (Irvine, CA).

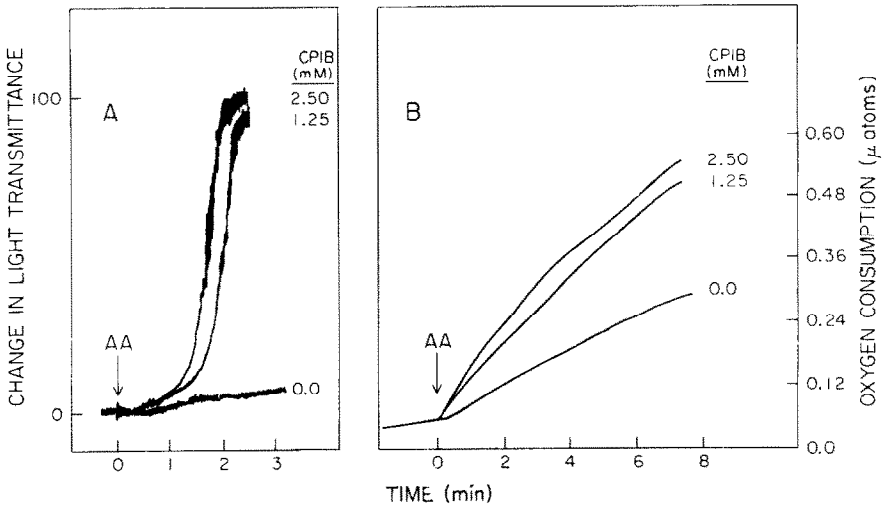

Fig. 1. Effects of clofibrate (CPIB) on platelet aggregation, measured as an increase in light transmittance (A), and on the rate of oxygen consumption (B) by human platelets. Diluent (0.0) or CPIB (concentration shown beside each tracing) was added to platelet-rich plasma for aggregation studies and to washed platelets for oxygen consumption studies 1 min before addition of arachidonic acid (200 μM). The tracings for aggregation (A) and oxygen consumption (B) are representative of at least four experiments.

The effects of clofibrate on arachidonic acid-induced platelet aggregation and on the rate of oxygen consumption by human platelets are shown in Fig. 1. Arachidonic acid in concentrations up to 200 μM did not cause platelet aggregation. However, addition of 1.25 mM and 2.5 mM clofibrate prior to the addition of arachidonic acid (200 μM) resulted in a maximum aggregation of platelets, as indicated by the maximum increase in light transmittance (Fig. 1A). Although the extent of change in light transmittance was maximum in the presence of either 1.25 or 2.5 mM clofibrate, it can be seen that an increase in the concentration of clofibrate reduced the time required for the onset of aggregation (Fig. 1A). Whereas clofibrate inhibits platelet aggregation induced by thrombin by blocking the release of arachidonic acid from platelet phospholipids [8], these experiments show that clofibrate enhanced arachidonic acid-induced aggregation. It is possible that this drug increases the enzymatic conversion of arachidonic acid into prostaglandin endoperoxides (PGG_2 and PGH_2) and thromboxane A_2 (TXA_2). A number of reports have shown that addition of arachidonic acid to platelets results in an increase in oxygen consumption which reflects an increased production of prostaglandin metabolites [12, 15]. Willem *et al.* [15] showed that 55 and 23% of the total increase in platelet oxygen consumption after arachidonic acid addition were due to increased production of prostaglandin metabolites as catalyzed by cyclooxygenase and lipoxygenase respectively. Therefore, we measured the effect of clofibrate on the arachidonic acid-induced burst in oxygen consumption in platelets as an index of rate of prostaglandin synthesis. Preincubation of platelets with clofibrate almost doubled the amount of oxygen that was consumed after arachidonic acid addition and, more importantly, the rate of oxygen consumption increased within a few seconds (Fig. 1B). A comparison of aggregation tracings (Fig. 1A) with those of oxygen consumption (Fig. 1B) shows that the clofibrate-induced increase in aggregation is related to an increase in oxygen consumption by platelets. These data suggest that clofibrate may enhance platelet aggregation induced by arachidonic acid by increasing the rate of oxygen consumption and, therefore, the rate of prostaglandin biosynthesis.

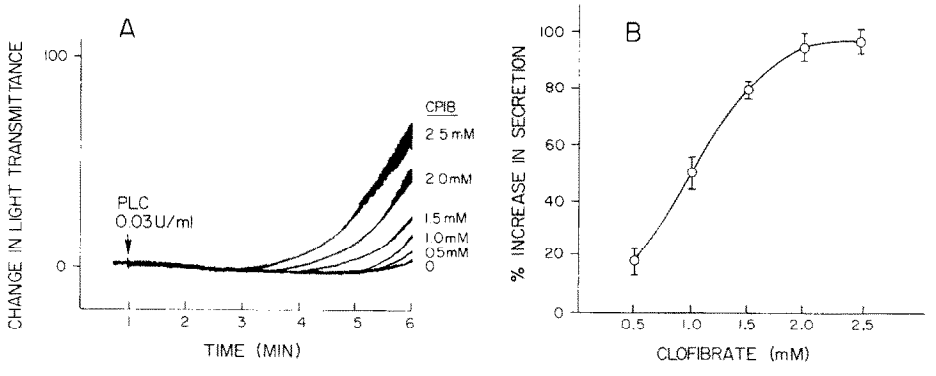

Fig. 2. Effects of clofibrate (CPIB) on PLC-induced platelet aggregation (A) and secretion of [^{14}C]-5HT (B) from washed human platelets. Diluent (0.0) or various concentrations of CPIB were added to platelets 1 min before addition of PLC (0.03 units/ml). Superimposed tracings of aggregation are representative of four experiments. Data on the secretion of [^{14}C]-5HT are expressed as percent and are the mean ± S.E.M. of four experiments.

The increase of arachidonic acid-dependent oxygen consumption by platelets may reflect an enhancement in the formation of prostaglandin metabolites by cyclooxygenase and lipoxygenase enzyme systems, as shown previously by Willem *et al.* [15]. Our studies do not indicate the relative role of the stimulation of the lipoxygenase enzyme system by clofibrate on platelet oxygen consumption. However, prostaglandin metabolites formed by the lipoxygenase system do not modify platelet function, whereas those of the cyclooxygenase pathway are known to be proaggregatory (e.g. $PGG_2/PGH_2/TXA_2$) [16]. Since clofibrate increases platelet aggregation in the presence of arachidonic acid, the enhancement of oxygen consumption by clofibrate should, in part, be related to a stimulation of platelet cyclooxygenase activity.

PLC has been shown recently to mediate platelet aggregation by a mechanism which is independent of prostaglandins [10, 11]. To further evaluate the proaggregatory action of clofibrate, the effects of various concentrations of this drug on PLC-induced aggregation and [^{14}C]-5HT secretion were examined. Prior addition of clofibrate to aspirin-treated (200 μM) platelets not only increased the extent of platelet aggregation, measured as an increase in light transmittance, by PLC but also shortened the time required for the onset of aggregation in a concentration-dependent manner (Fig. 2A). In a similar fashion, increasing concentrations of clofibrate increased the secretion of [^{14}C]-5HT from platelet dense granules (Fig. 2B).

These studies with arachidonic acid and PLC show that clofibrate is capable of potentiating platelet aggregation that is mediated by both prostaglandin-dependent and -independent mechanisms. Thus, clofibrate may affect a modulator of platelet function which is common to both the prostaglandin-dependent and -independent pathways of platelet activation. Cyclic AMP is a well-known modulator of platelet function. Agents which elevate platelet cAMP, e.g. PGE_1 and PGI_2, inhibit platelet aggregation whereas agents which induce aggregation lower cAMP in platelets [4, 5, 16]. Since clofibrate has been shown to lower cAMP in a number of tissues by inhibiting adenylate cyclase [2, 3], we examined the effect of clofibrate on PGE_1-mediated cAMP elevation in platelets. In preliminary experiments, clofibrate lowered PGE_1 (1 μM)-induced elevations in cAMP by 46 and 85% at 1.2 and 2.5 mM respectively (N = 3). As shown in Fig. 3, the inhibitory effects of PGE_1 on PLC-induced [^{14}C]-5HT secretion and cAMP levels were examined simultaneously in the presence or absence of clofibrate. PGE_1 increased platelet cAMP levels by 5-fold and consequently completely inhibited PLC-mediated secretion of [^{14}C]-5HT. In addition, preincubation of various concentrations of clofibrate with platelets before PGE_1 addition lowered the PGE_1-induced elevation of cAMP and also reversed the inhibitory effect of PGE_1 on [^{14}C]-5HT secretion by PLC in a concentration-dependent manner (Fig. 3). Lapetina and Cuatrecasas [17] have shown that PGE_1 inhibits the PLC-like activity in platelets, which may be a result of its ability to elevate cAMP in platelets. These findings are in agreement with our observation that PGE_1 elevated cAMP concentrations and blocked secretion of [^{14}C]-5HT by PLC (Fig. 3). Since clofibrate reversed the effects of PGE_1 on both cAMP concentration and the PLC-induced secretion of [^{14}C]-5HT, we suggest that clofibrate achieves its stimulating effects on PLC-induced secretion and aggregation by lowering cAMP concentration in platelets. The same mechanism that lowers cAMP may also be responsible for the converse ability of clofibrate to potentiate arachidonic acid-induced aggregation, that is, an increase in cAMP has been shown to inhibit cyclooxygenase, the enzyme responsible for oxygenation of arachidonic acid to PGG_2 and PGH_2 [16].

In summary, although clofibrate has been shown to inhibit platelet aggregation that is caused by thrombin, ADP and epinephrine, by blocking the release of arachidonic acid from platelet phospholipids [8], here we have demonstrated that clofibrate enhanced platelet aggregation by arachidonic acid and PLC and reversed the effects of PGE_1 on platelet cAMP concentration and on PLC-induced secretion of [^{14}C]-5HT in similar, concentration-dependent manners. Taken together, these findings strongly suggest that the proaggregatory effect of clofibrate is mediated by a lowering of cAMP in platelets.

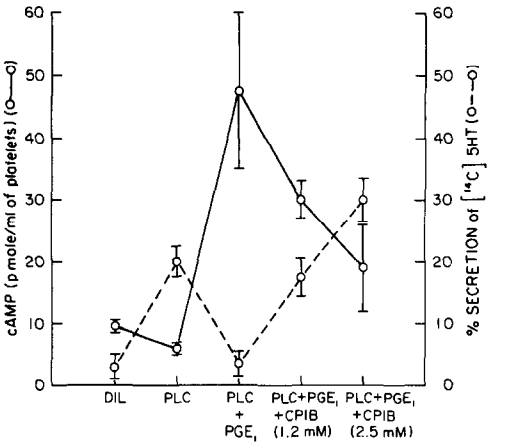

Fig. 3. Effects of clofibrate (CPIB) on PGE_1-elevated levels of cAMP (O——O) and on the inhibitory effect of PGE_1 on PLC-induced secretion of [^{14}C]-5HT (O- - -O) from washed human platelets. Diluent or CPIB was added to platelets 1 min before addition of PGE_1 (1 μM). PLC (0.03 units/ml) was added to platelets 1 min after addition of diluent or PGE_1. The samples for cAMP and percent secretion of [^{14}C]-5HT were collected after 3-min incubations. The data are expressed as the mean ± S.E.M. of duplicate observations from three experiments.

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