The Inhibition of Platelet-activating Factor-induced Platelet Activation by Oleic Acid Is Associated with a Decrease in Polyphosphoinositide Metabolism*

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In an earlier study (Miwa, M., Hill, C., Kumar, R., Sugatani, J., Olson, M. S., and Hanahan, D. J. (1987) J. Biol. Chem. 262, 527–530) it was shown that an inhibitor of platelet-activating factor (PAF), a powerful endogenous mediator of platelet aggregation, was present in freeze-clamped perfused livers. Subsequently, we determined that this substance was a mixture of unsaturated free fatty acids (FFA). Among these FFA, oleic acid between 10 and 100 μM was found to be a potent inhibitor of PAF-induced platelet aggregation and serotonin secretion. Consequently, in order to understand the molecular mechanism of oleic acid action, we investigated the effects of this FFA on several biochemical events associated with platelet aggregation induced by PAF. The effect of oleic acid and/ or PAF on the level of [32P]phosphatidylinositol 4-phosphate (PIP) and [32P]phosphatidylinositol 4,5-bisphosphate (PIP2) was examined by using platelets labeled with [32P]phosphate. Oleic acid induced a dose-dependent decrease in the levels of [32P]PIP and [32P]PIP2, a maximal decrease in [32P]PIP and [32P]PIP2 of approximately 50 and 25%, respectively, was observed within seconds after the addition of 20 μM oleic acid and persisted for at least 15 min. Oleic acid did not induce the formation of [3H]inositol phosphates in platelets prelabeled with [3H]inositol, suggesting that the decrease in [32P]PIP and [32P]PIP2 was not due to a stimulation of phospholipase C. In contrast to oleic acid, PAF induced a dose-dependent increase in the [32P]PIP level, reaching a maximum of approximately 200% 3 min after the addition of 1 nM PAF to the platelets. This increase in [32P]PIP was accompanied by platelet aggregation and secretion, and a close correlation was established between the [32P]PIP level and the degree of aggregation. Oleic acid and PAF, when added together to the platelets, interacted by affecting the level of [32P]PIP and [32P]PIP2 in an opposite way since the decrease in the level of [32P]PIP and [32P]PIP2 induced by oleic acid was partially reversed by an excess of PAF. The decrease in the levels of [32P]PIP and [32P]PIP2 caused by oleic acid was associated with an inhibition of platelet aggregation induced by PAF. Interestingly, oleic acid did not block PIP2 binding to platelets but inhibited the PAF-induced phosphorylation of platelet proteins of 20 kDa and 40 kDa. These results suggest that inhibition of the PAF response by oleic acid may be at one of the steps in the signal transduction. The observed decrease in the level of [32P]PIP and [32P]PIP2 in response to oleic treatment was also associated with an inhibition of PAF-induced formation of [3H]inositol-1-P, [3H]inositol-1,4-P2, and [3H]inositol-1,4,5-P3, indicating an inhibition of the synthesis of PIP and PIP2 by oleic acid. The inhibition of PIP and PIP2 synthesis could explain the strong inhibitory effect of oleic acid upon platelet activation induced by PAF.

The potential thrombotic or antithrombotic effects of saturated and unsaturated free fatty acids (FFA) have been studied for several years. Studies in vivo and in vitro have indicated that long chain saturated FFA increase the platelet response (1) whereas unsaturated FFA inhibit platelet reactivity (2). Some polyunsaturated FFA, particularly arachidonic acid, are substrates for the platelet cyclooxygenase and are metabolized into eicosanoids that inhibit or induce platelet aggregation; the role of these products and their action have been well documented (3). FFA such as eicosapentaenoic acid or docosahexaenoic acid extracted from fish oil reduced platelet aggregation (4). Their mechanism of action was suggested to be due to a competition with arachidonic acid replacing the active thromboxane A2 by thromboxane A2, (5), to an inhibition of the cyclooxygenase (6, 7), or to a direct antagonistic effect on the thromboxane A2/prostaglandin H2 receptor in human platelets (8). Another group of cis-unsaturated FFA such as oleic acid or linoleic acid is not metabolized into eicosanoids and exhibits stimulating or inhibitory effects on platelet function (9–11, 52). The aggregating effect (52) is observed at high concentrations of FFA (above 40 μM). The inhibitory effect on platelet aggregation induced by ADP, thrombin, and U46619 is induced by lower concentrations of FFA (1–35 μM) and is not prevented by cyclooxygenase inhibitors such as indomethacin (12). The inhibition occurs within seconds after the addition of FFA and is selective since trans-unsaturated FFA exhibit a minor inhibitory effect compared with the corresponding cis-unsaturated FFA. The effects of

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unsaturated FFA have not been attributed only to an inhibition of phospholipase A₂, as suggested by others (13), or to an increase of the CAMP level of the cell (12). McIntyre et al. (12) proposed that oleic acid and linoleic acid could inhibit platelet aggregation by a structural perturbation of the lipid bilayer and "specific lipid domains" of the platelet. Surprisingly, the molecular events of certain aspects of platelet activation which may be inhibited following addition of oleic acid have not been examined, particularly when the platelets have been stimulated with PAF.

In an earlier study (22) it was shown that an endogenous inhibitor of platelet-activating factor (PAF) was present in freeze-clamped perfused rat livers, but its chemical structure was not established. Subsequently, Siafaka-Kapadai* showed that this substance was a mixture of PPA; among the PFA present, oleic acid was found to be a potent inhibitor of PAF-induced platelet aggregation.

PAF, as well as other platelet agonists, induces platelet shape change, aggregation, and release of serotonin. It has been suggested that PAF exerts these effects on platelets by interaction with a specific membrane receptor (14) that induces the degradation of platelet plasma membrane phosphatidylinositol (PI) (15, 16). In this pathway, PI is sequentially phosphorylated to PIP, phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP₂). The phosphodiesteric cleavage of PIP₂ by the action of phospholipase C leads to the generation of diacylglycerol, an endogenous activator of protein kinase C and isoinositol, 1,4,5-trisphosphate (Ins-P₃), which has been shown to induce the release of calcium from intracellular stores (17, 18). Diacylglycerol and calcium are considered second messenger molecules that are involved in the phosphorylation of platelet proteins of 40 kDa and 20 kDa; a major role in the platelet activation has been attributed to these two phosphopro teins (18).

The current study provides new data on the inhibitory effect of oleic acid, a cis-unsaturated PFA that is not metabolized into eicosanoids, on several aspects of PAF effects on rabbit platelets, i.e. aggregation and secretion, PAF binding to its receptor, phosphatidylinositol metabolism, and protein phosphorylation. It is proposed that oleic acid inhibits platelet activation induced by PAF by altering the metabolism of polyphosphoinositides.

**MATERIALS AND METHODS**

**Chemicals and Reagents—[³H]Serotonin (20 Ci/mmol) was purchased from Du Pont-New England Nuclear; [³H]Oleic acid (10 Ci/mmol) was from ICN; myo-[³H]inositol (80 Ci/mmol) and 1-O-[³H]octadecyl-2-acetyl-sn-glycerol-3-phosphocholine (179 Ci/mmol) were obtained from Amersham Corp. Unlabeled 1-O-hexadecyl-2-acetyl-sn-glycerol-3-phosphocholine was purchased from Bachem (Bubendorf, Switzerland) and solubilized in BSA (0.25%, w/v). Oleic acid was obtained from Nu Chek Prep, Inc. (Elysian, MN), dissolved in ethanol, and then resuspended in 0.1 M NaHCO₃.

Buffers—The washing buffer for rabbit platelets was composed of: (in m mol/l): KCl, 2.6; NaCl, 137; MgCl₂, 1.0; NaHCO₃, 12; EGTA, 0.2; glucose, 5.6; gelatin, 2.5% (w/v); pH 6.5 (Tyrode-gelatin-EGTA, pH 6.5). The same buffer was used for platelet aggregation except that 1 mM Ca²⁺ and 10 mM Li⁺ for 10 min. This concentration of Li⁺ inhibits the conversion of inositol phosphates to free inositol (28). The platelet suspension was stimulated with PAF in the presence of absence of oleic acid for 15 s; the reaction was stopped by the addition of 5% trichloroacetic acid (w/v), and the samples were centrifuged at 900 g for 5 min. The supernatant was removed, washed with water-saturated ether, lyophilized, and stored at −70 °C. Lyophilized extracts were dissolved in 200 μl of distilled water, filtered (0.45-μm Millipore type HV filter), and 200-μl aliquots were analyzed by HPLC for inositol phosphates essentially as described by Dean and Moyer (23).

Platelet Aggregation and [³H]Serotonin Secretion—Rabbit platelets (3 × 10⁸) were preincubated with [³H]serotonin as described previously (20); the platelet suspension, in 500 μl of Tyrode-gelatin, pH 7.2, containing 1 mM Ca²⁺ was preincubated in an aggregometer with oleic acid at 37 °C for 1 min and then exposed to PAF. Platelet aggregation was measured by the change in light transmission monitored by a Chrono-Log aggregometer (Hearne, PA). Each suspension of the cells was mixed with 25 μl of cold 1.5 M formaldehyde to stop the reaction and centrifuged at 900 × g for 3 min. The radioactivity in the supernatant was measured by liquid scintillation counting (Beckman LS 6800 liquid scintillation counter). Values for aggregation and secretion induced by PAF in the presence of oleic acid were calculated as percent inhibition of control (PAF-induced platelet aggregation and secretion in the absence of oleic acid).

**Binding Studies with [³H]PAF—Washed rabbit platelets were prepared as described above and resuspended in 500 μl of Tyrode-gelatin buffer, pH 7.2, containing 1 mM Ca²⁺ in order to obtain a final platelet concentration of 3 × 10⁸ cells/ml. Since BSA avidly binds PFA and prevents the inhibition of platelet aggregation by oleic acid, the experiments were performed with a low concentration of BSA (0.05%). This concentration of BSA did not affect the inhibitory effect of oleic acid on platelet aggregation. [³H]PAF binding was performed as described previously (20, 21). Briefly, oleic acid or an excess of unlabeled PAF (1 μM) was added to 500 μl of platelet suspension 1 min before the addition of [³H]PAF. After incubation at room temperature, the platelet suspension was filtered through a 5 μl in cold Tyrode-gelatin buffer, pH 6.5, and immediately filtered through a Whatman type GF/C filter using a Millipore filtration manifold unit. The filters were washed twice with 5 μl of the same cold Tyrode-gelatin buffer, pH 6.5, and the radioactivity adsorbed to the filter was counted in 5 ml of Liquisint (National Diagnostic, Manville, NJ). Nonspecific binding was determined by including 1 μM unlabeled PAF in the assay mixture, and specific binding was defined as the difference between total and nonspecific binding. The significance of the data was assessed using a paired t test.

**Measurement of [³P]Polyphosphoinositide Levels—Washed rabbit platelets (3 × 10⁸/ml) were incubated with [³P]phosphate as described above for 1 h in order to study the labeling of polyphosphoinositides to isotopic equilibrium (40). Oleic acid, PAF, or PAF plus oleic acid was added to platelets resuspended in Tyrode-gelatin, pH 7.2, with 1 mM Ca²⁺. After incubation of the cells at 37 °C for the time indicated, with stirring in an aggregometer, the reaction was stopped with the addition of 3 ml of chloroform/methanol (1:2) and 1 ml of 2.4 M HCl. The phases were separated with the addition of 2 ml of chloroform, and the lower phase was collected and dried under nitrogen. The [³P]-labeled phospholipids were separated on Silica Gel H plates (Analtech, Inc., Newark, DE) using a solvent system of chloroform, methanol, 20% aqueous methylamine (30:18:5). After development in this solvent, plates were autoradiographed using Kodak XAR films. The standards used in the chromatography of lipids extracted from [³P]-labeled platelets were PIP, PIP₂, PI and PA (phosphatidic acid) and were purchased from Sigma.

**Measurement of [³H]Inositol Phosphates Formation—Inositol phosphates in platelets labeled with [³H]inositol were extracted with trichloroacetic acid and separated by HPLC. Briefly, the cells labeled with [³H]inositol were incubated in Tyrode-gelatin, pH 7.2, with 1 mM Ca²⁺ and 10 mM Li⁺ for 10 min. This concentration of Li⁺ inhibits the conversion of inositol phosphates to free inositol (28). The platelet suspension was stimulated with PAF in the presence or absence of oleic acid for 15 s; the reaction was stopped by the addition of 5% trichloroacetic acid (w/v), and the samples were centrifuged at 900 g for 5 min. The supernatant was removed, washed with water-saturated ether, lyophilized, and stored at −70 °C. Lyophilized extracts were dissolved in 200 μl of distilled water, filtered (0.45-μm Millipore type HV filter), and 200-μl aliquots were analyzed by HPLC for inositol phosphates essentially as described by Dean and Moyer (23).

Buffers were prepared using HPLC-grade ammonium phosphate (Fisher), and the water was purified using a Mini-Q system (Millipore type HV filter), and 200-μl aliquots were analyzed by HPLC for inositol phosphates essentially as described by Dean and Moyer (23). Buffers were prepared using HPLC-grade ammonium phosphate (Fisher), and the water was purified using a Mini-Q system (Millipore, Bedford, MA). Buffers were filtered (0.45-μm Millipore type HA filter) and degassed prior to use. A Waters HPLC system was used with a 25 × 0.4-cm Partisol 10 SAX column...
Platelet aggregation was dependent on the PAF concentration. However, when oleic acid at high concentrations was added to platelets together with PAF, aggregation was inhibited by oleic acid. The observed inhibition was dependent on the concentration of oleic acid (between 1 and 500 nM). In order to avoid the aggregating effect induced by high levels of PAF, platelets were preincubated for 1 min with stirring in an aggregometer at 37°C. The platelet pellets were dissolved in 50 μl of the following buffer: Tris-HCl (0.0625 M), 5% mercaptoethanol (v/v), 2.5% sodium dodecyl sulfate (w/v), 0.001% bromphenol blue (w/v), and 5% sucrose (w/v), pH 6.8. The samples were incubated for 3 min at 100°C. The platelet proteins were separated by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis under the conditions described previously (24). The dried gels were autoradiographed at -70°C using Kodak XAR films. The bands of proteins of 40 and 20 kDa were cut with scissors. The slices were placed in glass scintillation vials and reswollen into 0.5 ml of water. After 10 min, the remaining water was removed, and the slices were incubated overnight at 37°C with 1 ml of Protosol (Du Pont-New England Nuclear). The radioactivity in the samples was counted in 10 ml of Liquiscint, using a Beckman LS 6800 counter. The significance of the data was analyzed using a paired t test.

RESULTS

Effect of Oleic Acid on Rabbit Platelet Aggregation and Secretion Induced by PAF

As shown in Fig. 1A, PAF-induced platelet aggregation was inhibited by oleic acid. The observed inhibition was dependent on the concentration of oleic acid (10-100 nM). When the concentration of PAF was 0.1 nM, the IC₅₀ for oleic acid was 12 μM. As the concentration of PAF increased, the inhibitory effect of oleic acid decreased, and the IC₅₀ values to inhibit the effects of 1 and 10 nM PAF were 25 and 40 μM, respectively. Platelet aggregation induced by 10 nM PAF was inhibited only by 25% in the presence of 100 μM oleic acid. These results indicated that the inhibitory effect of oleic acid on platelet aggregation was dependent on the PAF concentration. Addition of oleic acid alone at concentrations between 100 and 500 μM induced either a large shape change or a reversible aggregation, depending on the functional reactivity of the platelets. However, when oleic acid at high concentrations was added to platelets together with PAF, aggregation was less extensive than that induced by PAF alone, suggesting that even at concentrations capable of inducing platelet aggregation, oleic acid inhibited PAF responses (data not shown).

Lysis of platelets did not occur at any of the concentrations of oleic acid used in this study (between 1 and 500 μM). In order to avoid the aggregating effect induced by high levels of oleic acid, it was thought more suitable to dissect the mechanism of the inhibitory effect by using low concentrations of oleic acid.

The inhibition of PAF-induced secretion of serotonin by oleic acid was also dose dependent and decreased as the concentration of PAF increased. Serotonin secretion was affected more than aggregation since the IC₅₀ values against 0.1, 1, 10, and 100 nM PAF were 7, 18, 25, and 30 μM, respectively (Fig. 1B).

Effect of Oleic Acid on [3H]PAF Binding

Scatchard analysis demonstrated three types of [3H]PAF binding to rabbit platelets (Fig. 2, A and B), as described previously (51). One binding of high affinity and low capacity was linear, with a Kᵣ value of 0.16 nM and a calculated 1637 putative receptor site per platelet (n = 3). This binding was considered specific and associated to the biological response (Fig. 2, A and B). The second type of binding had a lower affinity and a higher capacity of sites and was shown previously to be associated with the internalization of PAF (51). The third type of binding was not saturable with an infinite number of binding sites and represented nonspecific binding. Twenty μM oleic acid did not inhibit the specific binding of [3H]PAF (Fig. 2B). A small insignificant increase in the binding of [3H]PAF was observed. Furthermore, oleic acid at a higher concentration (50 μM) did not affect the specific binding of 0.1 nM [3H]PAF to rabbit platelets. Since 19 ± 2 fmol of [3H]PAF could bind specifically to 1.5 × 10⁶ platelets (n = 3). This value was not significantly different from those obtained in the control (16 ± 2 fmol) (n = 3) (data not shown).

Effect of Oleic Acid on [32P]-Labeled Phosphoinositides and PA Levels in Rabbit Platelet

Oleic acid induced a decrease in the level of [32P]PIP and [32P]PIP2 associated with the platelets in the range of concentrations (5-50 μM) that also inhibited platelet activation induced by PAF. The effect of oleic acid on [32P]PIP and [32P]PIP2 levels was proportional to the concentration of oleic acid. The lowest values of [32P]PIP and [32P]PIP2 were obtained 3 min after treatment of platelets with 20 μM oleic acid and were 41 ± 5% and 71 ± 3% of control (n = 9), respectively (Fig. 3).

In contrast, the amount of [32P]PI and [32P]PA was not altered significantly by treatment of platelets with 20 μM oleic acid. These results suggested that the effect of oleic acid on the levels of [32P]PIP and [32P]PIP2 was not mediated by a decrease in the levels of [32P]PI and [32P]PA.
oleic acid is shown in Fig. 4. After the addition of 20 μM oleic acid to platelets, [32P]PIP and [32P]PIP2 levels decreased rapidly below the initial level to reach values of 53 ± 3 and 78 ± 3% of control, respectively, within 30 s (n = 5). The decrease of the [32P]PIP level persisted for at least 15 min. In contrast, the level of [32P]PIP increased slightly after 3 min of incubation with 20 μM oleic acid; at 15 min, the value of [32P]PIP was 68 ± 3% of the control (n = 5) (Fig. 4).

**Effect of Oleic Acid on PAF-induced Changes in 32P-Labeled Phosphoinositides and PA Levels in Rabbit Platelets**

**Effect of Oleic Acid on PAF-induced Change of [32P]PIP Level**—After the addition of 1 nM PAF alone, the level of [32P]PIP increased gradually above the control value and at 3 min reached a maximum of 220 ± 20% of the control (n = 3) (Fig. 5A). The kinetics of the change in [32P]PIP level was investigated following the addition of PAF and oleic acid to the platelets (Fig. 5A). A maximum decrease below the initial level of [32P]PIP with a value of 62 ± 6% of control (n = 3) was noted 15 s after the addition of 20 μM oleic acid and 1 nM PAF. This decrease in [32P]PIP level, which was similar to

![Image](http://www.jbc.org/content/252/42/18329/fig/3.expansion.png)

**Fig. 3. Effect of oleic acid on [32P]PIP and [32P]PIP2 levels in rabbit platelets.** Rabbit platelets prelabeled for 1 h at 20 °C with [3H]phosphate as described under "Material and Methods" were washed twice and resuspended in Tyrode-gelatin, pH 7.2, with 1 mM Ca2+. Then the cells were preincubated for 1 min with stirring in an aggregometer at 37 °C before the addition of increasing concentrations of oleic acid. Three min after the addition of oleic acid, the incubation was stopped, and the phospholipids were isolated and separated by thin layer chromatography. [32P]PIP (○) and [32P]PIP2 (○) levels determined as described under "Material and Methods." Results are shown as the mean ± S.E. of nine separate experiments and are expressed as percent of control values of [32P]PIP and [32P]PIP2 obtained in the absence of oleic acid and considered as being 100%. The control values of [32P]PIP and [32P]PIP2 control were, respectively, 7,300 ± 1,900 and 19,000 ± 4,000 cpm/1.5 x 10^8 cells.

![Image](http://www.jbc.org/content/252/42/18329/fig/4.expansion.png)

**Fig. 4. Kinetics of the decrease of [32P]PIP and [32P]PIP2 induced by oleic acid.** Platelets labeled with [32P]phosphate were incubated for the time indicated in an aggregometer with 20 μM oleic acid, as described in Fig. 1. [32P]-Labeled PIP (○) and PIP2 (○) levels were analyzed as described under "Material and Methods" and in Fig. 3. The results are shown as the mean ± S.E. of five separate experiments and are expressed as percent of control values of [32P]-polyphosphoinositides obtained in the absence of oleic acid and considered as being 100%. The control values of [32P]PIP and [32P]PIP2 control were, respectively, 11,100 ± 4,300 and 31,000 ± 9,500 cpm/1.5 x 10^8 cells.

that obtained with platelets treated for 15 s with oleic acid alone (60 ± 9% of the control (n = 3)), was different at longer incubation times. Thus, the decrease in the [32P]PIP level was followed by a slow increase that reached a maximum above the basal value within 3 min.

The maximum [32P]PIP level attained was proportional to the concentration of PAF added with 20 μM oleic acid (e.g. [32P]PIP levels were 65 ± 3, 114 ± 7, and 140 ± 12% of control values (n = 5), in the presence of PAF at 0.1, 1, and 10 nM.
Effect of Oleic Acid on PAF-induced Change in PIP

**FIG. 5.** Kinetics of the effect of oleic acid on PAF-induced change in [32P]PIP and [32P]PIP2 levels. Platelets labeled with [32P]phosphate for 1 h were stirred for the time indicated at 37 °C with 20 μM oleic acid (○), 1 nM PAF (●), or 1 nM PAF plus 20 μM oleic acid (■). [32P]PIP levels (A) and [32P]PIP2 (B) were expressed as percent of control values in the absence of oleic acid and PAF and considered as being 100%. The results are shown as the mean ± S.E. of five or six separate experiments. The control values of [32P]PIP and [32P]PIP2, obtained in the absence of oleic acid and PAF, were, respectively, 17,600 ± 6,800 and 46,900 ± 12,200 cpm/10^9 cells.

Effect of Oleic Acid on PAF-induced Changes of [32P]PIP2 Level—In contrast to the effect of PAF on the [32P]PIP level by this agonist. Furthermore, the kinetics of [32P]PIP, and compared with that for [32P]PIP2, was initiated a small increase in the [32P]PIP2 level, which rapidly reached a maximum close to the basal value within 30 s of stimulation with PAF.

[32P]PIP2 levels were 76 ± 2, 88 ± 3, and 93 ± 7% of the control 3 min after the addition of PAF at 0.1, 1, and 10 nM, with 20 μM oleic acid (n = 5–6), respectively (Fig. 6B). The decrease of the [32P]PIP2 level induced by 20 μM oleic acid was reversed partially by an excess of PAF since the values without oleic acid were 102 ± 5, 107 ± 5, and 113 ± 7% of the control in the presence of 0.1, 1, and 10 nM PAF (n = 5–6), respectively. The difference between the values in the presence of oleic acid and PAF and those in the presence of PAF alone were significant.

**FIG. 6.** Reversibility of the oleic acid-induced decrease of [32P]PIP and [32P]PIP2 by PAF. Platelets labeled with [32P]phosphate for 1 h and preincubated as described in Fig. 1 were stirred for 3 min at 37 °C with 20 μM oleic acid (○), PAF (■), PAF plus 20 μM oleic acid (●). [32P]PIP (A) and [32P]PIP2 (B) levels were expressed as percent of control values in the absence of oleic acid and PAF and considered as being 100%. The results shown are the means ± S.E. of five or six separate experiments. The control values of [32P]PIP and [32P]PIP2 were, respectively, 15,300 ± 3,200 and 37,400 ± 7,000 cpm/10^9 cells. * p < 0.05; ** p < 0.01; *** p < 0.001. Values in the presence of oleic acid plus PAF were compared with those in the presence of PAF alone.

The Relationship between [32P]PIP and [32P]PIP2 Levels and PAF-induced Platelet Aggregation

PAF concentrations between 0.02 and 0.5 nM initiated a dose-dependent increase in the amount of [32P]PIP after 3 min of platelet stimulation (Fig. 7). The maximum of [32P]PIP level was reached at 0.5 nM PAF. Concomitantly, PAF between 0.02 and 0.5 nM also induced an increase in platelet aggregation with the same maximum response at 0.5 nM PAF. When analyzed by linear regression analysis, the level of [32P]PIP and the amplitude of platelet aggregation were closely correlated (r > 0.98, p < 0.001).
Table I shows that when the PAF-induced effect on the \(^{[32P]}\)PIP level was blocked by 20 \(\mu\)M oleic acid, PAF-induced platelet aggregation also was inhibited. The increase in \(^{[32P]}\)PIP induced by 0.1, 1, and 10 \(nM\) PAF was inhibited by 70, 49, and 31\% in the presence of 20 \(\mu\)M oleic acid. Concomitantly, aggregation induced by 0.1, 1, and 10 \(nM\) PAF was blocked by 89, 30, and 8\%, respectively.

A similar relationship was established between the decrease in the \(^{[32P]}\)PIP level and inhibition of PAF-induced platelet aggregation by oleic acid (data not shown).

Effect of Oleic Acid on the Formation of Inositol Phosphates

Twenty \(\mu\)M oleic acid did not alter the levels of \(^{[3H]}\)inositol phosphates, as compared with the control (Table II). These results indicated that the decrease in \(^{[32P]}\)PIP and \(^{[32P]}\)PIP levels induced by oleic acid alone was not produced by an accelerated degradation of PIP and PIP, to inositol phosphates following stimulation of phospholipase C. In contrast, the formation of \(^{[3H]}\)Ins-P, \(^{[3H]}\)Ins-P\(_2\), and \(^{[3H]}\)Ins-P\(_3\) was stimulated by PAF since the values for the three species of inositol phosphates were 441 ± 53, 359 ± 31, and 273 ± 35\% of control (\(n = 3\)), respectively. The formation of \(^{[3H]}\)Ins-P, \(^{[3H]}\)Ins-P\(_2\), and \(^{[3H]}\)Ins-P\(_3\) induced by 0.1 \(nM\) PAF was partially blocked by the addition of 20 \(\mu\)M oleic acid since the values of these species decreased to 211 ± 60, 150 ± 22, and 156 ± 28\% of the control (\(n = 3\)), respectively.

Effect of Oleic Acid on the Phosphorylation of 20-kDa and 40-kDa Proteins

Oleic acid alone at 20 \(\mu\)M did not affect the phosphorylation of the platelet proteins P40 kDa and P20 kDa. However, 20 \(\mu\)M oleic acid inhibited partially but significantly the phosphorylation of P40 kDa induced by 0.1 and 1 \(nM\) PAF to 61 ± 15 and 50 ± 4\% of the control, respectively (\(n = 3\)). The phosphorylation of P20 kDa induced by 0.1 and 1 \(nM\) PAF was inhibited in a similar way by 20 \(\mu\)M oleic acid to 64 ± 8 and 46 ± 4\% of the control (\(n = 3\)), respectively (Fig. 8).

DISCUSSION

The present study demonstrates that oleic acid between 10 and 100 \(\mu\)M is a potent inhibitor of PAF-induced platelet aggregation and serotonin secretion. However, oleic acid did not inhibit \(^{[3H]}\)PAF binding to platelets. This contrasts with the activity of all reported antagonists of PAF which inhibit PAF effects by affecting agonist binding (14, 25). These results suggest that the inhibitory effect of oleic acid on the platelet response is distal to the site of binding of PAF to its platelet receptor.

The treatment of \(^{32P}\)-labeled platelets with oleic acid induced a rapid and dose-dependent decrease in the amount of \(^{[32P]}\)PIP and \(^{[32P]}\)PIP associated with the cells. The maximum decrease of \(^{[32P]}\)PIP and \(^{[32P]}\)PIP\(_2\) was approximately 50 and 25\%, respectively and was observed 30 s following the addition of 20 \(\mu\)M oleic acid. This decrease in \(^{[32P]}\)PIP and \(^{[32P]}\)PIP\(_2\) persisted for at least 15 min.

It is widely accepted that the variations in PIP and PIP\(_2\) reflect changes in the mass of these phospholipids in the cells since the labeling of these two

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**Table I**

Relation between the decrease of \(^{[32P]}\)PIP level and the inhibition of platelet aggregation

\(^{[32P]}\)-Labeled platelets were stimulated for the time indicated at 37°C with PAF or PAF plus oleic acid. The increase of \(^{[32P]}\)PIP was calculated between 0 and 3 min after the addition of PAF alone or between 15 s and 3 min after the addition of oleic acid plus PAF and expressed in percent control in the absence of PAF and oleic acid, considered as being 100\%. The percent of aggregation was measured by the change of the light transmission in the aggregometer 3 min after the addition of PAF. The results shown are the means of two to three experiments.

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<th>Total increase in PIP</th>
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<td>3%</td>
</tr>
<tr>
<td>PAF, 1 nm</td>
<td>100</td>
<td>100</td>
<td>114</td>
<td>137</td>
<td>120%</td>
<td>49%</td>
</tr>
<tr>
<td>PAF, 1 nm + oleic acid, 20 (\mu)M</td>
<td>100</td>
<td>100</td>
<td>75</td>
<td>70</td>
<td>120%</td>
<td>34%</td>
</tr>
<tr>
<td>PAF, 10 nm</td>
<td>100</td>
<td>100</td>
<td>102</td>
<td>125</td>
<td>118%</td>
<td>56%</td>
</tr>
<tr>
<td>PAF, 10 nm + oleic acid, 20 (\mu)M</td>
<td>100</td>
<td>100</td>
<td>52</td>
<td>92</td>
<td>81%</td>
<td>51%</td>
</tr>
</tbody>
</table>

* PIP increase between 0 and 3 min.

\(^{32P}\)PIP increase between 15 s and 3 min.
polyphosphoinositides was studied under the conditions of isotopic equilibrium (30). Thus, the decrease of \(^{32}P\) labeling of PIP and PIP\(_2\) by oleic acid indicates a decrease in the amount of PIP and PIP\(_2\) associated with the platelets.

The observed decrease of PIP and PIP\(_2\) could be produced by an accelerated degradation of these phosphoinositides by a specific phospholipase C as described previously in the brain (29). The hydrolysis of polyphosphoinositides by phospholipase C generates inositol phosphates and 1,2-diacylglycerol in platelets; 1,2-diacylglycerol is phosphorylated rapidly to phosphatidic acid by a diacylglycerol kinase. Since 20 \(\mu\)M oleic acid, when added to the platelets labeled with myo-\(^{32}P\)inositol or by \(^{32}P\)phosphate, did not induce the formation of \(^{32}P\)inositol phosphates or \(^{32}P\)PA, it seems unlikely that the decrease of the amount of PIP and PIP\(_2\) can be explained by an hydrolysis of these phospholipids by phospholipase C.

The decrease in \(^{32}P\)PIP and \(^{32}P\)PIP\(_2\) could be due to a decrease in synthesis of these phospholipids which are formed from PIP by specific kinases that phosphorylate the 4 and 5 positions of PIP and PIP\(_2\), respectively (30). PIP and PIP\(_2\) kinases have been characterized in different cells and tissues (31–33). The concentrations of PIP and PIP\(_2\) in the cells are determined by the balance between the activity of kinases and phosphatases (34). Furthermore, Pike and DeMeester (37) have shown that competitive inhibition of PI kinase by Sadenosylhomocysteine could induce a decrease of PIP level associated with human leukocytes. Other adenine-containing compounds also have been shown to be competitive inhibitors of PI kinase in vascular smooth muscle membranes (38).

A similar effect on PI kinase could explain the decrease in the levels of \(^{32}P\)PIP and \(^{32}P\)PIP\(_2\) induced by oleic acid.

In contrast to the effect of oleic acid, PAF induced a dose-dependent increase in the \(^{32}P\)PIP level. However, PAF did not induce a significant change in the level of PIP\(_2\). During cell stimulation, there is some evidence that PI and PIP kinases are activated to replenish pools of PIP and PIP\(_2\) hydrolyzed by phospholipase C. Several investigators have reported an increase of the PIP level surpassing the control value of PIP after treatment of platelets with thrombin (40), ADP and adrenaline (41). The mechanism for regulating the increase of synthesis of PIP during cell stimulation remains inadequately defined. A number of reports of product inhibition of the PI and PIP kinases have been published (30). Recently it has been suggested that PIP and PIP\(_2\) kinases in the brain can be stimulated by GTP (42), indicating that the activation of these lipid kinases may be the result of the activation of G proteins. Also, it has been shown in platelets that the synthesis of PIP and PIP\(_2\) is stimulated by an increase in the cAMP level or by 12-O-tetradecanoylphorbol-13-acetate, suggesting that protein kinase C and/or the cAMP-dependent protein kinase may be involved in the activation of PI and PIP kinases (39,43).

Furthermore, oleic acid and PAF, when added together to platelets, interacted by affecting the level of PIP and PIP\(_2\). In a noncompetitive manner since the decrease in the level of PIP and PIP\(_2\) could be overcome only partially by an excess of PAF. Oleic acid could antagonize PAF effects by acting directly on a kinase/phosphatase of PIP, or PIP\(_2\) hydrolyzed by phospholipase C. Several investigators have reported an increase of the PIP level surpassing the control value of PIP after treatment of platelets with thrombin (40), ADP and adrenaline (41). The mechanism for regulating the increase of synthesis of PIP during cell stimulation remains inadequately defined. A number of reports of product inhibition of the PI and PIP kinases have been published (30). Recently it has been suggested that PIP and PIP\(_2\) kinases in the brain can be stimulated by GTP (42), indicating that the activation of these lipid kinases may be the result of the activation of G proteins. However, it is unlikely that oleic acid could inhibit protein kinase C since in our hands, staurosporine, an inhibitor of protein kinase C, did not induce a decrease in \(^{32}P\)PIP and \(^{32}P\)PIP\(_2\) levels as observed with oleic acid (data not shown). Oleic acid stimulated in vitro protein kinase C (53). Under our experimental conditions (low concentrations of oleic acid, using whole platelets), oleic acid did not stimulate the phosphorylation of P40 kDa, suggesting that protein kinase C was not activated by this FFA.

PAF stimulated the formation of \([\text{H}]\text{Ins-P}, [\text{H}]\text{Ins-P}_2, \text{and} [\text{H}]\text{Ins-P}_3\) in platelets labeled with myo-[\text{H}]inositol, as described previously (44, 45). It is widely accepted that inositol phosphates are formed by hydrolysis of PIP and PIP\(_2\) after activation by phospholipase C; a depletion of PIP and PIP\(_2\) levels could explain the decrease of PAF-induced formation of inositol phosphates observed in this study after treatment with oleic acid. Surprisingly, the PAF-stimulated formation of \([\text{P}]\text{PA} was not inhibited by oleic acid, suggesting that a phospholipase C, for which one of the major substrates could be P1, was not inhibited (46).

Interestingly, it has been observed recently in pancreatic acinar cells prelabeled with \(^{32}P\)
or myo-[\(^{3}\)H]inositol that arachidonic acid also could rapidly decrease the steady-state levels of [\(^{32}\)P]PIP, and inhibit the formation of [\(^{3}\)H]Ins-P, induced by carbachol. This effect on the [\(^{32}\)P]PIP, level was due to arachidonic acid and not to its metabolites and, as suggested by the authors, could also involve the inhibition of the synthesis of PIP, (26, 27).

Of particular importance, the increase in [\(^{32}\)P]PIP caused by PAF was always accompanied by an activation of cell function. The [\(^{32}\)P]PIP level increased with the concentration of PAF and reached a plateau at a PAF concentration of 0.5 nM, which was also the concentration needed to effect maximum aggregation. A close relationship seemed evident between the extent of aggregation and the [\(^{32}\)P]PIP level. In contrast, the decrease in [\(^{32}\)P]PIP and [\(^{32}\)P]PIP, levels caused by oleic acid was always associated with an inhibition of cell function; when PAF reversed the decrease in [\(^{32}\)P]PIP and [\(^{32}\)P]PIP, levels to a value above the control, platelet aggregation could occur.

These results indicate that the level of PIP and PIP, associated with the membrane is crucial for PAF-induced platelet activation. The decrease in the level of PIP and PIP, may limit the formation of Ins-P, which causes the release of calcium from intracellular stores (17, 18). Recently it has been demonstrated that oleic acid inhibits the concanavalin A-mediated rise in cytoplasmic free Ca\(^{2+}\) in T lymphocytes (50). Furthermore, it has been observed that PIP, can enhance cytoplasmic free Ca\(^{2+}\) release from skeletal muscle sarcoplasmic reticulum (49). PIP, in vitro activates protein kinase C directly and as effectively as 12-O-tetradecanoylphorbol-13-acetate or diacylglycerol (47, 48). From these findings, it appears that the decrease of PIP and PIP, levels caused by oleic acid would limit the release of cytoplasmic free Ca\(^{2+}\) and the activation of protein kinase C. These observations could explain the inhibition of the phosphorylation of proteins P40 kDa and P20 kDa by oleic acid observed in this study since the phosphorylation of P40 kDa and P20 kDa, which is the light chain of myosin, occurs via the myosin light chain Ca\(^{2+}\)- and calmodulin-dependent kinase or by protein kinase C. The major role of P40 kDa and P20 kDa in platelet function; when PAF reversed the decrease in [\(^{32}\)P]PIP and [\(^{32}\)P]PIP, levels caused by oleic acid, which was partially reversed by an excess of PAF, could be the mechanism inducing the inhibition of the platelet response induced by PAF. This effect of oleic acid on platelet activation also could explain the well known beneficial effects of oleic acid and other unsaturated FFA in preventing thrombotic diseases.

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Oleic Acid Inhibition of PAF Activation of Platelets

The inhibition of platelet-activating factor-induced platelet activation by oleic acid is associated with a decrease in polyphosphoinositide metabolism.

D Nunez, J Randon, C Gandhi, A Siafaka-Kapadai, M S Olson and D J Hanahan


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