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

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Daniele Nunez, Jacques Randon, Chandrashekhar Gandhi, Athanassia Siafaka-Kapadai, Merle S. Olson, and Donald J. Hanahan

From the Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78284-7760

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-biphosphate (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 [3H]PAF 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 also was associated with an inhibition of PAF-induced formation of [3H]inositol-P, [3H]inositol-P2, and [3H]inositol-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 aggregation induced by PAF.

The potential thrombotic or antithrombotic effects of saturated and unsaturated free fatty acids (FFA)1 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

1 The abbreviations used are: FFA, free fatty acids; PIP2, phosphatidylinositol 4,5-biphosphate; PIP, phosphatidylinositol 4-phosphate; PAF, platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine); HPLC, high performance liquid chromatography; [3H]Ins-P, [3H]inositol monophosphate; [3H]Ins-P2, [3H]inositol bisphosphate; [3H]Ins-P3, [3H]inositol trisphosphate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; BSA, bovine serum albumin.
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 FFA; among the FFA present, oleic acid was found to be a potent inhibitor of PAF-activated 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 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 inositol 1,4,5-trisphosphate (Ins-1,4,5-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 phosphoproteins (18).

The current study provides new data on the inhibitory effect of oleic acid, a cis-unsaturated FFA 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]PAF (10 μCi/ml) was from ICN, myo-[³H]inositol (80 Ci/mmol) and 1-O-[³H]lactadecyl-2-acetyl-sn-glycero-3-phosphocholine (179 Ci/mmol) were obtained from Amersham Corp. Unlabeled 1-O-hexadecyl-2-acetyl-sn-glycero-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 mm): 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-Evagreen, pH 6.5). The same buffer was used for platelet aggregation except that 1 mM Ca²⁺ was used instead of EGTA, and the pH was 7.2 (Tyrode-gelatin, pH 7.2).

**Isolation of Rabbit Platelets**—Platelets were prepared as described previously (19). Briefly, these cells were isolated from platelet-rich plasma by centrifugation at 100 × g for 15 min. The platelet pellet was resuspended in 5 ml of buffer (Tyrode-gelatin-EGTA, pH 6.5) and when necessary, labeled with either myo-[³H]inositol (50 μCi/ml) for 3 hr in [³H]phosphate (200 μCi/ml for 1 hr); the platelets were then washed twice by centrifugation (900 × g, 10 min) before the final resuspension in Tyrode-gelatin, pH 7.2.

² A. Siafaka-Kapadai, unpublished observations.

**Measurement of Platelet Aggregation and [³H]Serotonin Secretion**—The platelets (0.1 ml) 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 (Havertown, PA). Secretion was measured by the addition of the agonist to platelets preincubated with 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 5 × 10⁶ cells/ml. Since BSA avidly binds FFA and prevents the inhibition of platelet aggregation by oleic acid, the experiments were performed with a low concentration of BSA (0.095%). 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 for 10 min at room temperature, the platelet suspension was mixed with 5 ml of 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 ml 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 with a paired t test.

**Measurement of [³H]Polyphosphoinositide Levels**—Washed rabbit platelets (3 × 10⁴/ml) were incubated with [³H]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 [³H]-labeled phospholipids were separated on Silica Gel H plates (Analtech, Newark, DE) using a solvent system of chloroform/methanol/acetic acid/water (60:25:10:1). The standards used in the chromatography of lipids extracted from [³H]-labeled platelets were PIP₃, 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 (25). 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 13,000 × g for 10 min. The supernatants were resuspended in 5% trichloroacetic acid, lyophilized, and stored at −70°C. Lyophilized extracts were dissolved in 250 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 Scientific, Fair Lawn, NJ). Nonspecific binding was determined by the presence of a Milli-Q reverse osmosis device (Millipore Corporation, 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
(Phenomenex, Rancho Palos Verdes, CA). The A23 of the HPLC effluent was measured, and fractions were collected every 30 s. The radioactivity in various fractions was quantified by scintillation counting with a Beckman 5801 counter linked to a Beckman data transfer module. The results were analyzed by an unpaired t test.

Various inositol phosphates were identified by spiking the cell extracts with standard [3H]inositol phosphates obtained from Amer sham Corp.

Measurement of Protein Phosphorylation—[32P]Phosphate-labeled platelets (3 x 10^9/ml) were resuspended in a final Tyrode-gelatin buffer, pH 7.2, with 1 mM Ca++ as described above. Oleic acid was added 1 min before PAF to the platelet suspension incubated at 22°C with external stirring. After incubation for an additional 2 min, the reaction was stopped by the addition of 0.1 M perchloric acid. The samples were washed twice with 0.1 M perchloric acid in order to remove [32P]phosphate that was not incorporated. 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 μM). 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 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 Kd 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 concentration (5-50 μM) that also inhibited platelet activation (51). 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.
Effect of Oleic Acid on PAF-induced Changes in $^{32}$P-Labeled Phosphoinositides and PA Levels in Rabbit Platelets

**Effect of Oleic Acid on PAF-induced Change of $^{32}$P|PIP Level**—After the addition of 1 nM PAF alone, the level of $^{32}$P|PIP increased gradually above the control value and at 3 min reached a maximum of 220 ± 20% of control (n = 5) (Fig. 1). The kinetics of the change in $^{32}$P|PIP level was also investigated following the addition of PAF and oleic acid to the platelets (Fig. 5A). A maximum decrease below the initial level was 15 s after the addition of 20 μM oleic acid and 1 nM PAF. This decrease in $^{32}$P|PIP level, which was similar to 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 $^{32}$P|PIP level was followed by a slow increase that reached a maximum above the basal value within 3 min. The maximum $^{32}$P|PIP level attained was proportional to the concentration of PAF added with 20 μM oleic acid (e.g. $^{32}$P|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 $^{32}$P|PIP and $^{32}$P|PIP$_2$ Levels in Rabbit Platelets**—Rabbit platelets prelabeled for 1 h at 20 °C with $^{32}$P|phosphate as described under "Materials and Methods" were washed twice and resuspended in Tyrode-gelatin, pH 7.2, with 1 mM Ca$^{2+}$, and 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. $^{32}$P|PIP (■) and $^{32}$P|PIP$_2$ (□) levels were determined as described under "Materials and Methods." Results are shown as the mean ± S.E. of nine separate experiments and are expressed as percent of control values of $^{32}$P|PIP and $^{32}$P|PIP$_2$ obtained in the absence of oleic acid and considered as being 100%. The control values of $^{32}$P|PIP and $^{32}$P|PIP$_2$ control were, respectively, 7,300 ± 1,900 and 19,000 ± 4,000 cpm/1.5 × 10$^9$ cells.

![Effect of oleic acid on $^{32}$P|PIP and $^{32}$P|PIP$_2$ levels](image-url)
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, compared with that for [32P]PIP in the presence of 1 nM PAF induced an initial decrease in [32P]PIP2, which within 15 s. This decrease as reported above for [32P]PIP was observed within 30 s of stimulation with PAF. The recovery of the [32P]PIP level using an excess of PAF was only partial since in the absence of oleic acid, [32P]PIP levels, 3 min after the addition of 0.1, 1, and 10 nM PAF, were 139 ± 6, 207 ± 11, 213 ± 7% of the control (n = 5) respectively.

Effect of Oleic Acid on PAF-induced Changes of [32P]PIP2 Level—In contrast to the effect of PAF on the [32P]PIP level described above, the [32P]PIP2 level was only slightly affected by this agonist. Furthermore, the kinetics of [32P]PIP2 changes compared with that for [32P]PIP was different. An initial but insignificant increase in the [32P]PIP2 level was observed with a peak of 137 ± 3% (n = 3) versus control 30 s after the addition of 1 nM PAF. This was followed by an insignificant decrease of the [32P]PIP2 level, which was 114 ± 8 and 117 ± 6% of the control value (n = 3) 3 min and 15 min after the addition of PAF, respectively (Fig. 5B).

The kinetics of the [32P]PIP2 change was also different as compared with that for [32P]PIP in the presence of 1 nM PAF and 20 μM oleic acid (Fig. 6A). Addition of 20 μM oleic acid with 1 nM PAF induced an initial decrease in [32P]PIP2 which reached a minimum value of 85 ± 6% of the control (n = 3) within 15 s. This decrease as reported above for [32P]PIP was similar to that obtained with platelets treated for 15 s with oleic acid alone (85 ± 6%) (n = 3). The decrease in [32P]PIP2 was followed by 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 in the absence of oleic acid were 102 ± 5, 107 ± 5, and 113 ± 7% of the control (n = 5) respectively. The difference between the values in the presence of oleic acid and PAF and those in the presence of PAF alone were significant.

Effect of Oleic Acid on PAF-induced Change in [32P]PA Level in Rabbit Platelets—As described previously (15, 16), PAF alone at a concentration between 0.1 and 10 nM induced an important increase in [32P]PA formation with values of 146 ± 43, 487 ± 86, and 1092 ± 300% of the control 3 min after the addition of 0.1, 1, and 10 nM PAF (n = 3), respectively. Twenty μM oleic acid did not induce any significant inhibition of the formation of PA induced by PAF since the values were 379 ± 86, 683 ± 170, and 896 ± 132% of the control (n = 3) in the presence of 0.1, 1, and 10 nM PAF, respectively.

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).

FIG. 7. Correlation between \(^{32P}\)PIP level and platelet aggregation induced by PAF. Platelets labeled with \(^{32P}\)phosphate as described in Fig. 1 were stimulated for 3 min by increasing the concentrations of PAF from 0.05 to 10 nM. \(^{32P}\)PIP level (□) was expressed as percent of the level of \(^{32P}\)PIP in the absence of PAF and considered as being 100\%. The results shown are the means \(\pm\) S.E. of 11 experiments. The control value of \(^{32P}\)PIP was 11,300 \(\pm\) 2,400 cpm/1.5 \(\times\) 10\(^7\) platelets. The percent of aggregation (○) was measured by the change of the light transmission in the aggregometer 3 min after the addition of PAF. The correlation between \(^{32P}\)PIP and platelet aggregation, analyzed by linear regression, is presented in the inset (\(r < 0.98; p < 0.001\)).

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 level induced by oleic acid alone was not produced by an accelerated degradation of PIP and PIP\(_2\) 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 \(\pm\) 53, 359 \(\pm\) 31, and 273 \(\pm\) 35\% of control (\(n = 3\)), respectively. The formation of \(^{3H}\)Ins-P, \(^{3H}\)Ins-P\(_3\), and \(^{3H}\)Ins-P\(_4\) 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 \(\pm\) 60, 150 \(\pm\) 22, and 156 \(\pm\) 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 \(\pm\) 15 and 50 \(\pm\) 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 \(\pm\) 8 and 45 \(\pm\) 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\(_2\) 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\(_2\) labeled with \(^{32P}\)phosphate reflect changes in the mass of these phospholipids in the cells since the labeling of these two
Oleic acid inhibition of PAF activation of platelets

Table II

| Effect of oleic acid on the formation of \[^{3}H\]inositol phosphates induced by PAF |
|-----------------|------------------|-----------------|-----------------|
| PAF (0.1 nM)    | Oleic acid (20 μM) | PAF (0.1 nM) + Oleic acid (20 μM) | % | % | % |
| \[^{3}H\]Ins-P | 441 ± 53            | 111 ± 7          | 211 ± 60*        |
| \[^{3}H\]Ins-P | 359 ± 31            | 82 ± 20          | 150 ± 22         |
| \[^{3}H\]Ins-P | 273 ± 35            | 103 ± 11         | 156 ± 98         |

\* p < 0.05.

Effect of oleic acid in the phosphorylation of proteins P40 kDa and P20 kDa.

Oleic acid stimulated phosphorylation of P40 kDa and P20 kDa. \[^{3}P\]Phosphate-labeled platelets preincubated for 1 min with 20 μM oleic acid were stimulated with PAF (0.1 nM) or oleic acid (20 μM). Phosphorylation of P40 kDa in the absence (■) or presence of 20 μM oleic acid (□) was measured. Phosphorylation of P20 kDa in the absence (■) or presence of 20 μM oleic acid (□) was also determined as described under "Materials and Methods." Results are expressed as percent of the control values of phosphorylation of P20 kDa and P40 kDa obtained in the absence of PAF and oleic acid and which were, respectively, 600 ± 300 and 800 ± 300 cpm/10^6 cells. The results are shown as the mean ± S.E. of three experiments. * p < 0.05; ** p < 0.01. Values in the presence of oleic plus PAF were compared with those in the presence of PAF alone.

polyphosphoinositides was studied under the conditions of isotopic equilibrium (30). Thus, the decrease of \[^{3}P\] labeling of PIP and PIPI was by oleic acid indicates a decrease in the amount of PIP and PIPI associated with the platelets.

The observed decrease of PIP and PIPI 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 μM oleic acid, when added to the platelets labeled with \[^{3}H\]inositol or by \[^{3}P\] phosphate, did not induce the formation of \[^{3}H\]inositol phosphates or \[^{3}P\] phosphatidic acid, it seems unlikely that the decrease of the amount of PIP and PIPI can be explained by an hydrolysis of these phospholipids by phospholipase C.

The decrease in \[^{3}P\]PIP and \[^{3}P\]PIPI could be due to a decrease in synthesis of these phospholipids which are formed from PI by specific kinases that phosphorylate the 4 and 5 positions of PI and PIPI, respectively (30). PIP and PI PI kinases have been characterized in different cells and tissues (31-33). The concentrations of PIP and PIPI in the cells were 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 S-adenosylhomocysteine could induce a decrease of PIP level associated with human leukocytes. Other adenosine-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 \[^{32}P\]PIPI induced by oleic acid.

In contrast to the effect of oleic acid, PIPI induced a dose-dependent increase in the \[^{32}P\]PIP level. However, PAF did not induce a significant change in the level of PIPI. During cell stimulation, there is some evidence that PI and PIPI kinases are activated to replenish pools of PIP and PIPI hydrolyzed by phospholipase C. Several investigators have reported an increase of the PPI 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 PIPI kinases have been published (30). Recently it has been suggested that PI and PIPI 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 PIPI 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 PIPI kinases (39, 43).

Furthermore, oleic acid and PAF, when added together to platelets, interacted by affecting the level of PIP and PIPI in a noncompetitive manner since the decrease in the level of PIP and PIPI could be overcome only partially by an excess of PAF. Oleic acid could antagonize PAF effects by acting directly on a kinase/phosphatase of PI, or PIPI; also, oleic acid may affect the synthesis of PIP and PIPI indirectly by inhibiting the PAF-induced activation of protein kinase C or 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 \[^{32}P\]PIPI 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 \[^{3}H\]Ins-P, \[^{3}H\]Ins-P, and \[^{3}H\]Ins-P in platelets labeled with myo-\[^{3}H\]inositol, as described previously (44, 45). It is widely accepted that inositol phosphates are formed by hydrolysis of PIP and PIPI after activation by phospholipase C; a depletion of PIP and PIPI 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 \[^{3}P\]PA was not inhibited by oleic acid, suggesting that a phospholipase C, for which one of the major substrates could be PI, was not inhibited (46). Interestingly, it has been observed recently in pancreatic acinar cells prelabeled with \[^{3}P\]PA.
beneficial effects of oleic acid and other unsaturated FFA in preventing thrombotic diseases.

Of particular importance, the increase in [32P]PIP caused by PAF was always accompanied by an inhibition of PAF-induced formation of PIP and PIP,. This decrease in synthesis of PIP and PIP2 phosphorylation after treatment by oleic acid could explain the inhibition of platelet aggregation and secretion.

The inhibition of platelet aggregation and secretion can be explained by the decrease in synthesis of PIP and PIP2 caused by oleic acid. Oleic acid inhibited the phosphorylation of the P20 kDa, which is involved in the inhibition of platelet aggregation and secretion.

These results indicate that the level of PIP and PIP2 associated with the membrane is crucial for PAF-induced platelet activation. The decrease in the level of PIP and PIP2 may limit the formation of Ins-P2, which causes the release of calcium from intracellular stores. Recently it has been demonstrated that oleic acid inhibits the concanavalin A-mediated rise in cytoplasmic free Ca2+ in T lymphocytes. Furthermore, it has been observed that PIP2 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 PIP2 levels caused by oleic acid would limit the release of cytoplasmic free Ca2+ 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 is stimulated directly by protein kinase C, and phosphorylation of the P20 kDa, which is the light chain of myosin, occurs via the myosin light chain Ca2+- 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 [32P]PIP and [32P]PIP2 levels to a value above the control, platelet aggregation could occur.

The results presented in this study show that oleic acid, between 5 and 50 µM, is a potent inhibitor of PAF-induced rabbit platelet activation. Oleic acid did not seem to block the binding of PAF to platelets but inhibited the phosphorylation of P20 kDa and P40 kDa induced by PAF. These findings suggested that the mechanism of action of oleic acid was distal to the site of binding of PAF, at one of the steps in transmembrane signal transduction. The decrease in the level of [32P]PIP and [32P]PIP2 associated with an inhibition of PAF-induced formation of [3H]Ins-P, [3H]Ins-P2, and [3H]Ins-P3 indicated that oleic acid induced an inhibition of the synthesis of PIP and PIP2. This decrease in synthesis of PIP and PIP2 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.
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