Acetyl-CoA:1-O-Alkyl-2-lyso-sn-glycero-3-phosphocholine
Acetyltransferase Is Directly Activated by p38 Kinase*

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Acetyl-CoA:1-O-Alkyl-2-lyso-sn-glycero-3-phosphocholine acetyltransferase, along with phospholipase A₂, is a key regulator of platelet-activating factor biosynthesis via the remodeling pathway. We have now obtained evidence in human neutrophils indicating that this enzyme is regulated by a specific member of the mitogen-activated protein kinases, namely the p38 kinase. We earlier demonstrated that tumor necrosis factor-α (TNF-α) as well as N-formyl-methionyl-leucyl-phenylalanine treatment leads to increased phosphorylation and activation of p38 kinase in human neutrophils. Strikingly, in the present study these stimuli increased the catalytic activity of acetyltransferase up to 3-fold, whereas 4-phorbol 12-myristate 13-acetate, which activates the extracellular-regulated kinases (ERKs) but not p38 kinase, had no effect. Furthermore, a selective inhibitor of p38 kinase, SB 203580, was able to abolish the TNF-α- and N-formyl-methionyl-leucyl-phenylalanine-induced activation of acetyltransferase. The same effect was not observed in the presence of an inhibitor that blocked ERK activation (PD 98059). Complementing the findings in intact cells, we have shown that recombinant, activated p38 kinase added to microsomes in the presence of Mg²⁺ and ATP increased acetyltransferase activity to the same degree as in microsomes obtained from TNF-α-stimulated cells. No activation of acetyltransferase occurred upon treatment of microsomes with either recombinant, activated ERK-1 or ERK-2. Finally, the increases in acetyltransferase activity induced by TNF-α could be ablated by treating the microsomes with alkaline phosphatase. Thus acetyltransferase appears to be a downstream target for p38 kinase but not ERKs. These data from whole cells as well as cell-free systems fit a model wherein stimulus-induced acetyltransferase activation is mediated by a phosphorylation event catalyzed directly by p38 kinase.

The platelet-activating factor (PAF), 1-O-alkyl-2-acyetyl-sn-glycero-3-phosphocholine, is a potent lipid mediator eliciting responses in a wide variety of cell types (1–3). It is a major mediator of inflammation often acting in coordination with arachidonic acid metabolites (4–9). Much work has been devoted to determine how inflammatory lipid mediators are formed and their production regulated. In human neutrophils and other inflammatory cells, the PAF is synthesized in response to a variety of stimuli by a remodeling pathway in which 1-O-alkyl-2-arachidonoyl-sn-glycero-3-phosphocholine (1-alkyl-2-AA-GPC) is converted to lyso-PAF (1-alkyl-2-lyso-GPC), which is then acetylated by acetyl-CoA:lyso-PAF acetyltransferase to form PAF. Physiologically, it is not clear whether the lyso-PAF is primarily generated by the direct action of PLA₂ on 1-alkyl-2-AA-GPC (1, 10) or whether PLA₂ first hydrolyzes AA from the ethanolamine plasmalogens, initiating a CoA-independent transacylase-catalyzed reaction that transfers AA from 1-alkyl-2-AA-GPC to the lyso-plasmalogen generating lyso-PAF by an indirect route (11). In either the direct or indirect routes, the actions of both PLA₂ and the acetyltransferase are required for PAF synthesis.

Acetyl-CoA:lyso-PAF acetyltransferase was initially demonstrated and partially characterized by Wykle et al. (12). The enzyme was found to be present in several rat tissues with the highest activity found in spleen. Furthermore, the reaction could be inhibited by divalent cations (Cu²⁺, Mn²⁺, and Mg²⁺). Both 1-O-alkyl- and 1-O-acyl-2-lyso-sn-glycero-3-phosphocholine could act as substrates, but 3-O-alkyl-2-lyso-sn-glycero-1-phosphocholine could not, indicating the stereospecificity of this enzyme. It was later shown that this activity could be increased in PMN stimulated by A23187 (13), opsinized zymosan (13, 14), and PAF itself (15). Increases in acetyltransferase activity have also been observed in eosinophils from patients with eosinophilia compared with cells isolated from those with normal eosinophil numbers (16). Subsequent to these findings, it was proposed by Lenihan and Lee (17) that the activation of acetyltransferase is regulated in a reversible activation/inactivation manner by phosphorylation. They showed that rat spleen microsomal acetyltransferase activity could be enhanced by the addition of the soluble fraction from rat spleen in the presence of ATP and Mg²⁺ (17). Moreover, the addition of phosphatidylerine, diolein, and Ca²⁺ further enhanced the activity of acetyltransferase, leading the authors to speculate that protein kinase C may be involved in regulation of the acetyltransferase. Gomez-Cambronero et al. (18) later showed that rat spleen microsomal acetyltransferase activity could be increased by the catalytic subunit of cyclic AMP-dependent protein kinase in the presence of Mg²⁺ and ATP; subsequent treatment of the microsomal preparations with alkaline phosphatase abolished the observed increase. It has also been shown that calcium/calmodulin-dependent protein kinases can lead to increased activation of acetyltransferase in vitro (19).

These earlier findings provided evidence that acetyltransferase activity is modulated by phosphorylation but have led to...
an unclear picture as to the specific kinase responsible for activation of the acetyltransferase in vivo. We now have evidence in human neutrophils that a member of the MAP kinase family, namely p38 kinase, is responsible for the phosphorylation, and hence, activation of acetyltransferase. The recent development of specific inhibitors of p38 kinase (20), as well as the availability of purified recombinant, activated p38 enzyme, has allowed us to demonstrate that this enzyme activates acetyltransferase in whole cells and in a cell-free system.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-O-[1,2-3H]Hexadecyl-2-lyso-GPC (56 Ci/mmol) was synthesized as described previously (21), except that 16:0 plasmeycholine isolated from beef heart by reverse phase high performance liquid chromatography was used instead of 1-choline isolated from beef heart by reverse phase high performance liquid chromatography was used instead of 1-choline synthesized as described previously (21), except that 16:0 plasmenyl-carrier was carried out at 37 °C for 15 min and terminated by extraction of the saline without CaCl2, and cells were pelleted at 300 g for 10 min at 4 °C. Pelleted cells were resuspended in 1 ml of protection buffer (0.2 M Tris-HCl (pH 9.3), 1 mM MgCl2, 100 μM ZnCl2, 1 mM spermadine) in a final volume of 100 μl. After 15 min, the entire 100 μl of kinase reaction mixture was added to a vessel containing acetyl-CoA (100 μM) and [3H]lyso-PAF (16 μM, 0.1 μCi/tube) in 900 μl of 0.2 M Tris-HCl (pH 7.5) and incubated at 37 °C. The reaction mixtures were extracted after 15 min and analyzed for [3H]PAF formation as described above. As a positive control, microsomes from TNF-α-treated cells were incubated in the same manner except no protein kinases were added.

**Acetyltransferase Activity Measurements**—PMN (1 × 107/ml, 3 ml total) in phosphate-buffered saline containing 1.4 mM CaCl2 was incubated for 30 min at 4 °C, warmed at 37 °C for 5 min, treated with or without SB 203580 and/or PD 98059 for 30 min at 37 °C, and stimulated with the various agonists as detailed in figure legends. Reactions were terminated by the addition of 30 ml of ice-cold phosphate-buffered saline without CaCl2, and cells were pelleted at 300 g for 10 min at 4 °C. Pelleted cells were resuspended in 1 ml of protease buffer (0.2 M Tris-HCl (pH 7.5), containing 50 μg/ml leupeptin, 50 μg/ml peptatin A, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 0.2 mM Na3VO4) and sonicated twice at a power setting of 2 and 10% output with a probe sonicator (Heat System Inc.). Sonicates were centrifuged for 10 min at 10,000 g for 10 min. Treatments were incubated at 37 °C with 2 μg of recombinant, activated p38, ERK-1, and ERK-2 were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Tumor necrosis factor-α was from PeproTech, Inc. (Rocky Hill, NJ). Phorbol 12-myristate 13-acetate (PMA) was purchased from LC Laboratories (Woburn, MA). Calf intestinal alkaline phosphatase was from Promega (Madison, WI). Dulbecco’s phosphate-buffered saline, essentially lipid-free bovine serum albumin, N-formyl-methionyl-leucyl-phenylalanine (fMLP), peptatin A, leupeptin, and phenylmethylsulfonyl fluoride were obtained from Sigma.

**Preparation of Neutrophils**—Neutrophils were prepared from heparinized, venous blood collected from healthy, medication-free donors using dextran sedimentation, isopych sedimentation, and brief hypotonic lysis to remove red blood cells as described previously (22). The resulting cell population consisted of ~95% PMN.

**Acetyltransferase Activation of Acetyltransferase**—PMN membranes (40 μl, ~5 μg of total protein) from untreated cells were immediately incubated at 37 °C with 2 μg of recombinant, activated p38, ERK-1, or ERK-2 in the presence of 500 μM Mg2+ and 40 μM ATP in a final volume of 50 μl. After 15 min, the entire 50 μl of kinase reaction mixture was added to a vessel containing acetyl-CoA (100 μM) and [3H]lyso-PAF (16 μM, 0.1 μCi/tube) in 950 μl of 0.2 M Tris-HCl (pH 7.5) and incubated at 37 °C. The reaction mixtures were extracted after 15 min and analyzed for [3H]PAF formation as described above. As a positive control, microsomes from TNF-α-treated cells were incubated in the same manner except no protein kinases were added.

**Alkaline Phosphatase Treatment of PMN Membranes**—Isolated membranes (15 μl, ~5 μg of total protein) from either control cells or cells treated with TNF-α (1 ng) were incubated at 25 °C with 5 units of calf intestinal alkaline phosphatase in the presence of assay buffer (50 mM Tris (pH 9.3), 1 mM MgCl2, 100 μM ZnCl2, 1 mM spermidine) in a final volume of 100 μl. After 15 min, the entire 100-μl reaction mixture was added to a reaction vessel containing acetyl-CoA (100 μM) and [3H]lyso-PAF (16 μM, 0.1 μCi/tube) in 900 μl of 0.2 M Tris-HCl (pH 7.5) and shaken at 37 °C. The reaction mixtures were extracted after 15 min and analyzed for [3H]PAF formation as described above.

**RESULTS**

**Activation of MAP Kinase(s) and Acetyltransferase by TNF-α, PMA, and Chemotactic Factors**—Previously, we investigated the abilities of selected agents (TNF-α, PMA, and chemotactic factors) to activate MAP kinases in neutrophils and evaluated their subsequent impact on cPLA2 activation. We found that although all the agents tested activated cPLA2, there were differences with regard to which MAP kinase cascade was utilized by the individual stimuli. TNF-α and PMA were shown to preferentially activate p38 kinase and ERKs, respectively. Further, it was shown that both pathways were activated by chemotactic factors (i.e. fMLP, C5a, and interleukin-8).

We have now extended our studies to focus on the activation of acetyl-CoA:lyso-PAF acetyltransferase using the three test stimuli previously examined. Human PMN were treated with TNF-α (1 ng, 10 min), PMA (10 ng, 10 min), or IMLP (100 ng, 1 min), sonicated, and assayed to determine acetyltransferase activity (Fig. 1). We observed that treatment with TNF-α induced a 2–3-fold increase in acetyltransferase activity. Activating concentrations were determined according to the method of Bradford (24) using bovine serum albumin as the standard.

**Isolated membranes (40 μl, ~5 μg of total protein) from untreated cells were immediately incubated at 37 °C with 2 μg of recombinant, activated p38, ERK-1, or ERK-2 in the presence of 500 μM Mg2+ and 40 μM ATP in a final volume of 50 μl. After 15 min, the entire 50 μl of kinase reaction mixture was added to a vessel containing acetyl-CoA (100 μM) and [3H]lyso-PAF (16 μM, 0.1 μCi/tube) in 950 μl of 0.2 M Tris-HCl (pH 7.5) and shaken at 37 °C. The reaction mixtures were extracted after 15 min and analyzed for [3H]PAF formation as described above.**

**Acetyltransferase Activity by various stimuli in intact human PMN.** PMN (3 × 106) were stimulated with 1 nm TNF-α, 10 nm PMA, or 100 nm IMLP for 10, 10, or 1 min, respectively, or untreated for 10 min (control). Cells were sonicated and subsequently assayed for acetyltransferase activity as described under “Experimental Procedures.” Data represent the mean ± S.E. of six separate experiments using different donors.

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tions were found to contain the highest specific activity of acetyltransferase activity.

Microsomes (~5 μg of total protein) were incubated with recombinant, constitutively activated ERK-1, ERK-2, or p38 kinase in the presence of ATP and Mg2+. It should be noted that Mg2+ dramatically inhibits acetyltransferase activity (12), and as a result, only 0.5 mM Mg2+ was used in the kinase assay. We have observed that this concentration of Mg2+ has little to no effect on acetyltransferase activity in our assay system (data not shown). Kinase reactions were allowed to incubate initially for 15 min, after which the entire mixture was added to a reaction vessel containing acetyl-CoA, [3H]lyso-PAF, and products assayed for acetyltransferase activity. We observed that acetyltransferase activity was increased 2–3-fold in microsomes isolated from neutrophils treated with TNF-α, confirming our earlier results with whole cell sonicates. Treatment with Mg2+ and ATP alone effected no change in acetyltransferase activity, but the addition of recombinant, constitutively activated p38 (2 μg) resulted in a dramatic increase in activity, reaching levels observed in microsomal membranes from TNF-α-treated cells (Fig. 3). This increase was not observed when equivalent amounts (2 μg) of ERK-1 or ERK-2 were added. 3-Fold more protein kinase was tested (6 μg), and no further increases in acetyltransferase activity were observed, indicating that 2 μg of p38 gave full activation whereas even at higher levels, ERKs were still inactive (data not shown). These data indicated that p38, not the ERKs, is responsible for the phosphorylation and activation of acetyltransferase in human neutrophils.

**Inactivation of Acetyltransferase in Microsomes by Treatment with Alkaline Phosphatase**—To further establish if the activation of acetyltransferase was because of a direct phosphorylation event, membranes from control or TNF-α-stimulated PMN were treated with alkaline phosphatase (Table I). Although the specific activity from unstimulated cells was higher than routinely observed, TNF-α still induced a 2–3-fold increase in acetyltransferase activity. Upon treatment with alkaline phosphatase, isolated membranes exhibited approximately 3-fold lower activity in control and 6-fold lower activity in stimulated preparations. In fact, phosphatase treatment reduced the activity of either treated or untreated membranes to the same level (0.234 nmol/μg/15 min). These results indicate that a phosphorylation event is crucial to not only the activation of acetyltransferase but also to its basal level of activation as assayed in stimulated or resting PMN, respectively.

**DISCUSSION**

Acetyl-CoA:lyso-PAF acetyltransferase has long been thought to be regulated by a phosphorylation event; however, the specific kinase(s) responsible for phosphorylation has yet to be unequivocally determined. It has been suggested that protein kinase C (17), cAMP-dependent protein kinase (18), or calcium/calmodulin-dependent protein kinases (19) could activate acetyltransferase in vitro, but evidence for any one of these kinases specifically activating the enzyme in whole cells is lacking. Because acetyltransferase plays a critical role in modulating the biosynthesis of PAF, understanding its regulation may lead to novel therapeutic approaches.

In the present studies, we investigated three agents (TNF-α, PMA, and fMLP) as potential activators of acetyltransferase in human neutrophils. We observed that the best activator of p38, TNF-α, was in fact the best activator of acetyltransferase. fMLP, which also activated p38, albeit to a lesser degree, correspondingly activated acetyltransferase to a lesser degree when compared with TNF-α. On the other hand, PMA had virtually no effect on p38 or acetyltransferase activity. The role of p38 in the activation of acetyltransferase was further sug-
effect of alkaline phosphatase treatment on acetyltransferase activity

TABLE I

| Treatment            | Alkaline phosphatase | Specific activity (nmol/µg/15 min) |
|----------------------|----------------------|----------------------------------|
| Control              | −                    | 0.625 ± 0.14                     |
| Control              | +                    | 0.234 ± 0.06                     |
| TNF-α                | −                    | 1.425 ± 0.11                     |
| TNF-α                | +                    | 0.234 ± 0.14                     |

FIG. 3. In vitro activation of microsomal acetyltransferase by recombinant, activated p38 kinase. Freshly isolated membranes (40 µl, ~5 µg of total protein) were incubated at 37 °C in the presence of 500 µM Mg²⁺ and 40 µM ATP in a final volume of 50 µl (complete system) with 2 µg of recombinant, activated p38, ERK-1, or ERK-2. Deletion of ATP/Mg²⁺ and additions of the kinases to the complete system are indicated. After 15 min, the entire 50-µl kinase reaction was added to a separate reaction vessel and subsequently assayed for acetyltransferase activity as described under “Experimental Procedures.” As a positive control, microsomes from TNF-α-treated neutrophils were incubated in the same manner except no protein kinases were added (TNF-α control). Data represent the mean ± S.E. of four separate experiments using different donors.

Although we consistently observed no activation of acetyltransferase in response to PMA, various reports have shown that PMA can either activate or have no effect on acetyltransferase. Early reports by both Albert and Snyder (13) in rat alveolar macrophages and by Domenech et al. (19) in guinea pig exocrine cells clearly showed that PMA treatment did not significantly activate acetyltransferase. Conversely, Lenihan and Lee (17) found that acetyltransferase activity increased upon treatment of rat spleen microsomes with the soluble fraction from rat spleen along with Mg²⁺ and ATP, and because the activity could be further enhanced by the addition of phosphatidyserine, diolein, and calcium, the authors speculated that protein kinase C, the direct target of PMA, was playing a role (17). In the neutrophil, no clear consensus has been reached as to whether acetyltransferase is indeed activated by PMA. Our findings here and a number of earlier findings (35, 36) indicate that PMA has little measurable impact on acetyltransferase activity. These observations disagree with those of Leyravaud et al. (37), who showed that stimulation with PMA not only increased neutrophil acetyltransferase activity but also raised PAF production. These differences may reflect varying assay conditions including different concentrations and times of incubations with PMA. Also, under certain conditions, protein kinase C activation may be able to differentially link to p38 activation.

For the purpose of our analyses, we selected relatively short incubations of cells with high PMA concentrations. Under these conditions the phorbol ester achieved optimal activation of ERKs. We were unable to find any concentration of PMA that appreciably activated p38. In any event, other studies provided alternative explanations for some of the observed differences. Nieto et al. (36) found that PMA could in fact stimulate PAF production in PMN; however, the PAF appeared to be synthesized de novo via the dithiothreitol-insensitive cholinephosphotransferase pathway. Because PMA caused no activation of acetyltransferase, Nieto et al. (36) accordingly concluded that PMA could only initiate PAF biosynthesis through the de novo pathway. Similar results were later obtained in human umbilical vein endothelial cells where PMA induced PAF formation solely through the de novo pathway (38). Although dithiothreitol-insensitive cholinephosphotransferase activity is high in PMN, the remodeling pathway appears to be the more significant route by which PAF is synthesized in human PMN (39).

The formation of PAF is dependent upon at least two enzyme activities, PLA₂ and acetyltransferase. We have shown PMA can stimulate PMN to activate cPLA₂ as well as to induce AA release when measured by mass² so it appears that within the context of the remodeling pathway, PMA can induce the generation of the obligate PAF precursor, lyso-PAF, by activating PLA₂, but the acetylation of lyso-PAF does not occur at appreciable levels in response to the phorbol ester. Our results suggest one possible explanation for this relationship; acetyltransferase tightly controls the formation of PAF. Alternatively, the
lyso-PAF precursor could be generated at a distinct site within the cell not accessible to acetyltransferase.

The p38 kinase has largely been considered to be a stress-activated protein kinase in mammalian cells, as initial reports indicated that it was activated in cells exposed to hyperosmolarity, UV radiation, and endotoxin (40, 41). More recently, other activators of p38 that act through heterotrimeric G-protein-coupled receptors have been identified that include interleukin-8 (42), FMLP, and PAF (43). PAF itself is known to stimulate PAF biosynthesis in neutrophils (44) and other cell types (15), possibly through the activation of acetyltransferase directly. These observations agree with our findings, and we further conclude that the regulation of acetyltransferase occurs via a direct phosphorylation by p38. Although a complete definition of all the signaling components activated in response to PAF has not been elucidated, stimulation of neutrophils by PAF appears to trigger MAP kinase kinase-3 activation, and evidence suggests that this occurs through a pertussis toxin-insensitive pathway, possibly coupling to Gαq or Gα11 (43).

In conclusion, our results strongly indicate that p38 activates the acetyltransferase responsible for PAF biosynthesis both in intact neutrophils and cell-free preparations. The precise effect of the phosphorylation event on subsequent PAF formation is still unclear, because the activation of p38 impacts other enzymes involved in the biosynthesis of PAF, i.e. cPLA2 (29, 31). The determination of the specific enzyme, which may control the overall flux through the PAF biosynthetic pathway, remains to be determined. Our findings lead us to conclude that protein kinase C does not activate the acetyltransferase in intact neutrophils. The findings fit a model in which both protein kinase C and p38 activate the acetyltransferase in concert by the p38 kinase cascade in response to TNF-α and other cytokines.