Lysophosphatidylcholine and lyso-PAF display PAF-like activity derived from contaminating phospholipids

Gopal K. Marathe,[††] Adriana Ribeiro Silva,[††] Hugo Caire de Castro Faria Neto,[††] Larry W. Tjoelker,[**] Stephen M. Prescott,[†,§] Guy A. Zimmerman,[†] and Thomas M. McIntyre[1,*,†]

Departments of Pathology* and Internal Medicine,[†] and the Huntsman Cancer Institute,[§] University of Utah, Salt Lake City, UT 84112; ICOS Corporation,** Bothell, WA 98021; and Departamento de Fisiologia e Farmacodinamica,[††] Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, CEP 21045-900, Brazil

Abstract **Lysophosphatidylcholine is an abundant component of plasma and oxidized LDL that displays several biological activities, some of which may occur through the platelet-activating factor (PAF) receptor. We find that commercial lysophosphatidylcholine, its alkyl homolog (lyso-PAF), and PAF all induce inflammation in a murine model of pleurisy. Hydrolysis of PAF to lyso-PAF by recombinant PAF acetylhydrolase abolished this eosinophilic infiltration, implying that lyso-PAF should not have displayed inflammatory activity. Saponification of lyso-PAF or PAF acetylhydrolase treatment of lyso-PAF or lysophosphatidylcholine abolished activity; neither lysolipid should contain susceptible *sn*-2 residues, suggesting contaminants account for the bioactivity. Lyso-PAF and to a lesser extent lysophosphatidylcholine stimulated Ca^{2+} accumulation in 293 cells stably transfected with the human PAF receptor, and this was inhibited by specific PAF receptor antagonists. Again, treatment of lyso-PAF or lysophosphatidylcholine with recombinant PAF acetylhydrolase, a nonselective phospholipase A_2, or saponification of lyso-PAF destroyed the PAF-like activity, a result incompatible with lyso-PAF or lysophosphatidylcholine being the actual agonist. We conclude that neither lyso-PAF nor lysophosphatidylcholine is a PAF receptor agonist, nor are they inflammatory by themselves. We suggest that PAF or a PAF-like mimetic accounts for inflammatory effects of lysophosphatidylcholine and lyso-PAF.**—Marathe, G. K., A. R. Silva, H. C. de Castro Faria Neto, L. W. Tjoelker, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. **Lysophosphatidylcholine and lyso-PAF display PAF-like activity derived from contaminating phospholipids.** ***J. Lipid Res.*** **2001.** 42: **1430–1437.**

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Lysophosphatidylcholine is an abundant plasma component. Independent methods of analysis place the value in normal blood between 140 to 150 μM (1, 2), and it accumulates to millimolar levels in hyperlipidemic subjects (3). Most of the circulating lysophosphatidylcholine is likely bound to albumin, but some is associated with lipoprotein particles, where it comprises 1% to 5% of the total phosphatidylcholine content of LDL particles. Oxidation and fragmentation of the polyunsaturated *sn*-2 fatty acyl residues of phosphatidylcholine, followed by the hydrolysis of the shortened fatty acyl residues by LDL-associated platelet-activating factor (PAF) acetylhydrolase (4, 5), also increase the content of lysophosphatidylcholine during the oxidative modification of LDL that accompanies their conversion to atherogenic particles (6).

Lysophosphatidylcholines activate a wide range of cell types and events in the vascular system, and therefore understanding its actions is relevant to a host of immunologic and inflammatory events. For instance, lysophosphatidylcholine induces cytokine synthesis and migration of lymphoid cells (7–9) and monocytes (10–12), and it is a chemoattractant for arterial smooth muscle cells (13). It stimulates growth factor expression by endothelial cells (14, 15) and it induces inflammation and leukocyte accumulation after intracutaneous injection (16). The mechanism by which any of these events occur is undiscovered as lysophosphatidylcholine does not act through the newly discovered lysophosphatidic acid or sphingosine 1-phosphate endothelial differentiation gene (*edg*) receptors (17). Also, lysophosphatidylcholine is a poor ligand (18) for the only other known receptor for phospholipids, the PAF receptor.

The single PAF receptor (19) recognizes PAF (1-*O*-alkyl-2-acetoyl-*sn*-glycero-3-phosphocholine) by the choline head group, the short *sn*-2 residue, and the *sn*-1 ether bond (20, 21). The potency of PAF differs markedly among cells that contain the PAF receptor, and indeed its discrimination among related phospholipids depends on the cell type (22, 23). This variation in sensitivity is potentially relevant as such cell-specific effects could alter the low affinity of the PAF receptor for lysophosphatidylcholine and thereby allow the PAF receptor to act as a lysophosphatidylcholine receptor in some cells. In fact, Ca^{2+} signaling (24) and phospholipase D activation (25) in mac-

Abbreviations: *edg*, endothelial differentiation gene; hPAFR293, 293 cells stably transfected with the human PAF receptor; PAF, platelet-activating factor.

[1] To whom correspondence should be addressed at 4130 EIHG, 2030 East 15 North, University of Utah, Salt Lake City, UT 84112.
e-mail: tom.mcintyre@hmbg.utah.edu

rophages, cytokine induction in monocytes (7), and monocyte chemoattractant protein 1 production by endothelial cells (26) are proposed to occur in just this way.

Recognition of lysophosphatidylcholine by the PAF receptor may underlie in vivo events as well, because the vasorelaxation induced by lysophosphatidylcholine in aortic rings (27) and the edema in the microvascular circulation of guinea pig lungs challenged with alkyl lysophosphatidylcholine (lyso-PAF) (28) are prevented with specific PAF receptor antagonists. In addition, the in vivo anti-inflammatory nature of the PAF acetylhydrolase (29) has been questioned on the basis of a proinflammatory role for the lysophosphatidylcholine that this enzyme generates (30) as it hydrolyzes PAF and oxidatively fragmented phospholipids.

One feature of lysophosphatidylcholine stimulation in many assay systems is the need for high concentrations compared with other lipid mediators where, typically, lysophosphatidylcholine at 10^{-5} to 10^{-4} M is required for a full effect. In the course of pursuing the identity of biologically active phospholipids derived from the oxidation of synthetic phosphatidylcholines (31, 32), we found that commercially available sources of lysophosphatidylcholine contained potent inflammatory mediators. Here we show that commercial preparations of lysophosphatidylcholine and lyso-PAF contain material that activates leukocytes through their PAF receptors and activates 293 cells transfected with the PAF receptor. However, this activity was susceptible to maneuvers that remove *sn*-2 residues—maneuvers that cannot by definition affect the structure of lysophosphatidylcholines. This information means that neither lysophosphatidylcholine nor lyso-PAF signals through the PAF receptor. It also means that some actions ascribed to lysophosphatidylcholines result from contaminants that do interact with the PAF receptor to initiate its Ca^{2+} signaling functions.

MATERIALS AND METHODS

Materials

Tissue culture-grade chemicals were from Whittaker Bioproducts (Walkersville, MD) and tissue culture dishes were from Falcon Labware (Lincoln Park, NJ). Fetal bovine serum was from HyClone (Logan, UT), and human albumin was from Baxter Health Care (Glendale, CA). The PAF receptor antagonist BN52021 was purchased from Biomol (Plymouth Meeting, PA). A second PAF receptor antagonist, WEB 2086, was a generous gift from Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT). Phospholipase A_2 (bee venom) was from Sigma (St. Louis, MO). PAF was obtained from Biomol. 1-*O*-Hexadecyl-*sn*-glycero-3-phosphocholine (lyso-PAF) and its acyl analogs 1-palmitoyl-*sn*-glycero-3-phosphocholine and 1-stearoyl-*sn*-glycero-3-phosphocholine were from Sigma, Avanti Polar Lipids (Alabaster, AL), Biomol, or CalBiochem (San Diego, CA) as stated in the figures.

Lipid structural analysis

PAF, lyso-PAF, or lysophosphatidylcholine (5 to 10 μg) was treated with 8 μg of recombinant human PAF acetylhydrolase in a total volume of 0.1 ml of Hanks' balanced salt solution containing 0.5% human serum albumin (HBSS/A) for 1 h at 37°C. When the lipids were to be used ex vivo, no further purification steps were performed. For animal experiments, the remaining lipid was re-extracted into chloroform according to the procedure of Bligh and Dyer (33), the solvent was removed with a stream of N_2, and the material was dissolved in HBSS/A. Recovery is nearly quantitative as saponification of PAF, extraction, and reacetylation result in quantitative recovery of bioactivity (not shown). When the recovered material was to be used in the animal model described below, the recovered material was tested with 293 cells stably transfected with the human PAF receptor (hPAFR293 cells) for PAF-like activity to ensure complete loss of PAF-like activity. Treatment of these three lipids with bee venom phospholipase A_2 (10 μg) was carried out in a similar fashion before the resulting products were repurified by extraction into chloroform, except that this incubation also contained 10 mM Ca^{2+}. The presence of inappropriate ester residues in the preparations of lyso-PAF was investigated by a chemical procedure in which commercial lyso-PAF, or PAF as a positive control, was subjected to saponification with 0.5 N NaOH in methanol for 4 h at 24°C. This material was carefully brought to pH 7.4 with 0.5 M HCl before use. We have previously shown (31) that PAF (or its acyl analog) saponified in this way did not induce Ca^{2+} accumulation in hPAFR293 cells. Conversely, these experiments showed that reacetylation of saponified PAF resulted in complete recovery of activity in the hPAFR293 bioassay.

Measurement of intracellular Ca^{2+} fluxes

Human polymorphonuclear leukocytes were freshly isolated just before use as described (34). Subconfluent hPAFR293 cells were treated with Versene (ethylenediaminetetraacetic acid tetrasodium salt, Na_4EDTA; 0.2 g/liter) (GIBCO-BRL, Gaithersburg, MD) and resuspended in fresh culture medium ($1.0–1.2 \times 10^7$ cells/ml). The acetoxy methyl (AM) ester of fura-2 (fura-2 AM) was loaded into cells at 1 μM from a 1 mM DMSO stock, and after incubation in the dark for 45 min at 37°C the cells were washed with HBSS/A and resuspended in HBSS/A at a density of 2.25×10^6 cells/ml. Fluorescence of 1.5 ml of cells was measured at 37°C, with dual excitation at 340 and 380 nm and the emission recorded at 510 nm (35). The response of each batch of cells was tested with 0.1 and 1 nM authentic PAF to generate the maximal PAF response. Mock-transfected 293 cells were processed in the same way and their response was tested with PAF, or with thrombin or lysophosphatidic acid as positive controls. PAF receptor antagonists were used at a final concentration of 10 μM after preincubation of the cells with these competitive antagonists for 20 to 30 min. hPAFR293 cells stably expressing the human PAF receptor were isolated as described (31).

Murine pleurisy model

Swiss mice (25–30 g) from the Oswaldo Cruz breeding facility (Instituto Oswaldo Cruz, Rio de Janeiro, Brazil) received an intrathoracic injection of PAF (1 μg/0.1 ml per cavity), lysophosphatidylcholine from egg yolk (lysophosphatidylcholine, 25 μg/cavity), or lyso-PAF (5 or 25 μg/cavity). For this, the lipids were recovered from an organic phase by drying under nitrogen and then reconstituted in sterile saline containing 0.01% BSA. This material was sterilized (with a 0.2 μm pore size γ-irradiated filter unit), and the saline-BSA solution (0.1 ml/cavity) was administered intrathoracically into naive animals. The control group was injected with the saline-BSA vehicle alone. Lyso-PAF was subjected to alkaline hydrolysis, or PAF, lyso-PAF, and lysophosphatidylcholine were treated with recombinant PAF acetylhydrolase as described above. The recovered and resuspended material was sterilized and injected into the thoracic cavities of mice. The animals were killed 6 h after this challenge in a CO_2 gas chamber, and the thoracic cavity of each was opened and washed with 1 ml of HBSS. The pleural wash was recovered by syringe and

samples were diluted in Turk fluid (2% acetic acid) for total leukocyte counts in Neubauer chambers. Differential analysis was performed in cytosmears stained by the May-Grünwald-Giemsa method.

RESULTS

Commercial lysophosphatidylcholine and lyso-PAF are inflammatory

We tested the ability of lysophosphatidylcholine and lyso-PAF to induce an inflammatory reaction in a system where cellular infiltrates could be quantitated (36). We found that the intrathoracic injection of PAF, as a positive control, induced a strong inflammatory response within 6 h, as measured by an eosinophilic influx into this cavity (**Fig. 1A**). Treatment of this PAF with PAF acetylhydrolase, a phospholipase A_2 that specifically removes short or oxidized *sn*-2 residues from phospholipids (4, 5), before its injection significantly reduced the ability of PAF to elicit the inflammatory infiltrate. We then found that lysophosphatidylcholine, albeit at much higher concentrations, also induced an inflammatory infiltrate and that the extent of this reaction was equivalent to that of PAF. Unexpectedly, in what should have been a negative control, we found that PAF acetylhydrolase treatment of lysophosphatidylcholine also abolished the ability of lysophosphatidylcholine to induce an eosinophilic influx in this model.

The first issue to be resolved was whether lyso-PAF, the presumably inactive product generated by PAF acetylhydrolase digestion of PAF, behaved differently than the lysophosphatidylcholine that appeared to be proinflammatory. However, we found that lyso-PAF also induced an accumulation of eosinophils after intrathoracic injection (Fig. 1B), and was equally or slightly better than lysophosphatidylcholine as an agonist. But again, we found the unanticipated result that PAF acetylhydrolase treatment abolished this inflammatory response. Either the PAF acetylhydrolase was in some fashion responsible for this result (although it alone had no effect and it was not carried through the extraction of the reaction products and so was not injected into the cavity) or it was having an effect by removing *sn*-2 residues that should not have been present.

To resolve this issue we repeated this experiment, but this time we treated the lyso-PAF with a mild base. The mild saponification conditions we used would release any esterified residues, but would spare the alkyl lysophospholipid (not shown). We found that chemical saponification also destroyed the in vivo inflammatory potential of lyso-PAF (Fig. 1B). We therefore conclude that lyso-PAF itself was not the inflammatory principle, and that an alkyl phospholipid containing an *sn*-2 ester moiety was completely responsible for the apparent in vivo inflammatory activity of lyso-PAF. While we could not perform the same experiment with lysophosphatidylcholine, as it is susceptible to saponification, the results with PAF acetylhydrolase suggest that it too is contaminated with biologically active material whose effects were evident after intrathoracic injection.

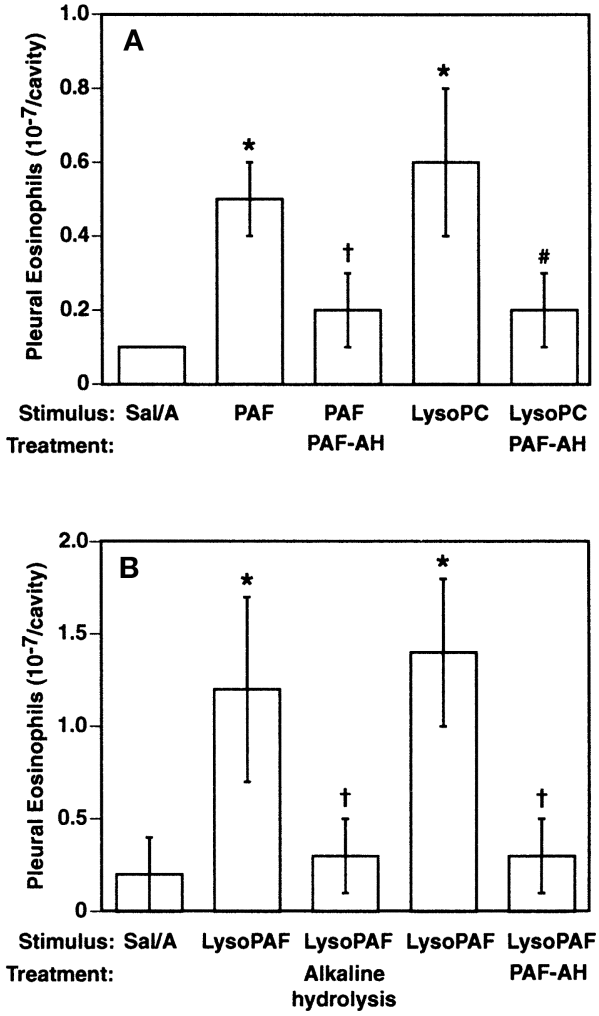

Fig. 1. Contaminants in lysophosphatidylcholine and lyso-PAF cause eosinophilia in a model of pleurisy. A: PAF acetylhydrolase (PAF-AH) treatment abolished pleural eosinophil infiltration induced by PAF (1 μg/cavity) or Avanti egg lysophosphatidylcholine (Lyso-PC, 25 μg/cavity). Sal/A, Saline-BSA. B: Inhibitory effect of alkaline hydrolysis and PAF acetylhydrolase treatment on eosinophil accumulation induced by lyso-PAF (5 μg/cavity). Each column represents the mean ± SEM of five to eight animals. * Significant difference ($P < 0.05$) compared with Sal/A group; † significant difference compared with lyso-PAF-stimulated group (B) or PAF-stimulated group (A); # significant difference compared with lysophosphatidylcholine-injected group.

Commercial lysophosphatidylcholines activate the PAF receptor

We anticipated that material sensitive to PAF acetylhydrolase digestion in preparations of lyso-PAF and lysophosphatidylcholine would be recognized by the PAF receptor, given the similarities between the substrate preferences of the enzyme and the ligands recognized by this receptor (21). That is, we anticipated that the proinflammatory effect of lyso-PAF and lysophosphatidylcholine preparations resulted from activation of the PAF receptor. We tested this postulate with hPAFR293 cells (31). In this assay, the cells are preloaded with the Ca^{2+}-sensitive dye fura-2 and changes in the fluorescence ratio sensitively probe for

activation of the PAF receptor. We found (**Fig. 2A**) that 293 cells expressing the PAF receptor, and not untransfected 293 cells (not shown), responded to lyso-PAF in a concentration-dependent fashion with a transient increase in fluorescence. These hPAFR293 cells also displayed a concentration-dependent increase in fluorescence in response to each of four supplier's preparations of lyso-PAF (Fig. 2A). It is also apparent that the concen-

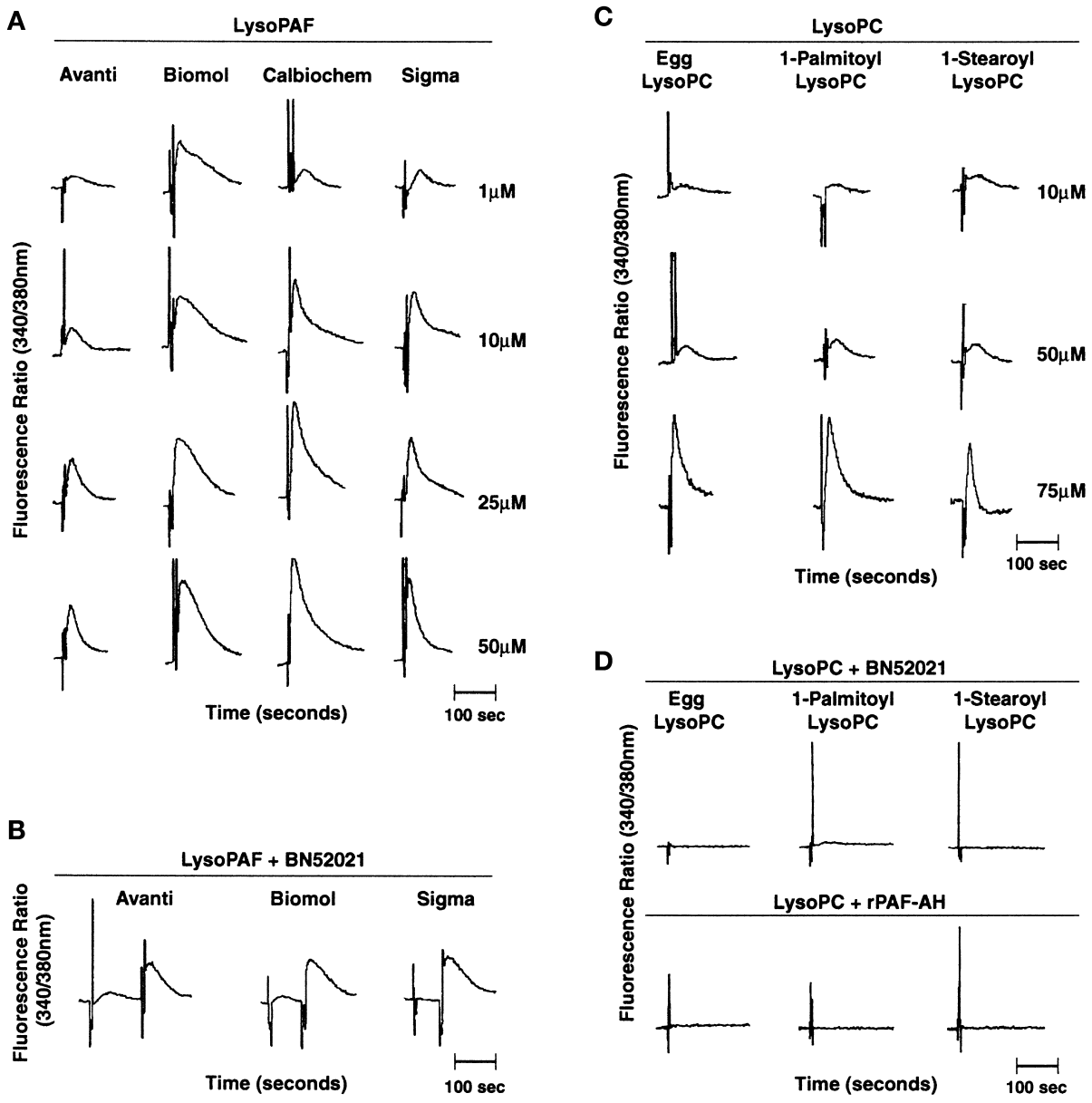

Fig. 2. Lysophosphatidylcholine (lyso-PC) preparations activate the PAF receptor, resulting in intracellular Ca^{2+} fluxes. A: Response of hPAFR293 cells to varying concentrations of lyso-PAF from various suppliers. A transfected cell line stably expressing the human PAF receptor (hPAFR293 cells) was loaded with the Ca^{2+} dye fura-2 AM and then exposed to the stated concentrations of alkyl lysophosphatidylcholine from the specified suppliers. Fluorescence was monitored by dual excitation at 340 and 380 nm with emission recorded at 510 nm as described in Materials and Methods. B: In this series of experiments, hPAFR293 cells were pre-exposed to the competitive PAF receptor antagonist BN52021 (10 μM) before addition of a 25 μM concentration of the various preparations of lyso-PAF (shown by the first injection). The PAF receptor antagonist has sharply reduced the Ca^{2+} fluxes caused by the stated lyso-PAF preparations. The effects of the competitive PAF receptor antagonist are reversible, as the addition of a saturating amount (1 μM) of PAF (shown by the pen deflection at the time of the second addition) overcame BN52021 inhibition of the receptor. C: Response of hPAFR293 cells to varying concentrations of acyl lysophosphatidylcholine. Cells were exposed to mixed acyl lysophosphatidylcholines derived from egg yolk or to lysophosphatidylcholine with a defined residue at the *sn*-1 position. The Ca^{2+} transients were recorded as in (A). D: Lysophosphatidylcholine is not a PAF receptor agonist. In this series of experiments, hPAFR293 cells were pre-exposed to the competitive PAF receptor antagonist BN52021 (10 μM) before addition of a 75 μM concentration of the various preparations of acyl lysophosphatidylcholines just as in (B). Alternatively, the lysophosphatidylcholine was digested with PAF acetylhydrolase as described in Material and Methods. Both maneuvers abolished the induction of a Ca^{2+} flux, yet the cells remained viable and responsive to an overwhelming amount of PAF added at the end of the trace (not shown).

tration of lyso-PAF required to produce a similar level of fluorescence differs among the various preparations despite the fact that the lyso-PAF concentration of each was standardized through phosphorus analysis (37).

We confirmed that lyso-PAF was signaling through the PAF receptor in hPAFR293 cells by including the specific PAF receptor antagonist BN52021 in the incubation. We found that this specific antagonist strongly suppressed induction of Ca^{2+} transients by each of the lyso-PAF preparations (compare Fig. 2A with 2B). BN52021 is a competitive antagonist, and we found the subsequent addition of excess PAF overcame its inhibitory effect on the PAF receptor. A similar result was obtained with a second competitive PAF receptor antagonist, WEB 2086 (not shown).

We determined whether lysophosphatidylcholine would also activate the PAF receptor, again using hPAFR293 cells. We found that lysophosphatidylcholine derived from egg induced a small Ca^{2+} transient in these cells, as did material with two different *sn*-1 fatty acyl residues (Fig. 2C). This response became apparent only by 50 μM and was significant by 75 μM. Comparison of these responses with those of lyso-PAF (Fig. 2A) suggests the preference for an *sn*-1 ether bond was about 50-fold. The response induced by acyl lysophosphatidylcholine was blocked by a PAF receptor antagonist or digestion with PAF acetylhydrolase (Fig. 2D). Such information normally would suggest that lyso-PAF and to some extent lysophosphatidylcholine are modestly successful PAF receptor agonists as this pair retains the selectivity for the *sn*-2 ether bond demonstrated by the PAF receptor (22).

Lysophosphatidylcholine activation of the PAF receptor displays inappropriate sensitivity to phospholipase hydrolysis

We determined whether lyso-PAF also activated human neutrophils, as a biological target, and found that lyso-PAF from several sources stimulated these leukocytes (**Fig. 3A**). The *sn*-2 position of lyso-PAF is a free hydroxyl function, and so is unaffected by phospholipase A_2 or PAF acetylhydrolase treatment. Despite this foregone result based on structure, we found that pretreating the various preparations of lyso-PAF with bee venom phospholipase A_2 abolished their activity (Fig. 3A). We also found that pretreatment with PAF acetylhydrolase also abolished Ca^{2+} transients in human neutrophils (Fig. 3B). This effect was also apparent when PAF acetylhydrolase-digested material was used to stimulate hPAFR293 cells (Fig. 3B). These data preclude lyso-PAF being a PAF receptor antagonist, and it shows that lyso-PAF is not the actual agonist in in vitro or in vivo assays of inflammation.

Mild saponification can be used to purify lyso-PAF

We determined whether mild saponification of carboxylate esters would reduce the PAF-like activity in a sensitive in vitro assay as it did in our in vivo assay. We found (**Fig. 4**) that saponification with dilute NaOH abolished leukocyte

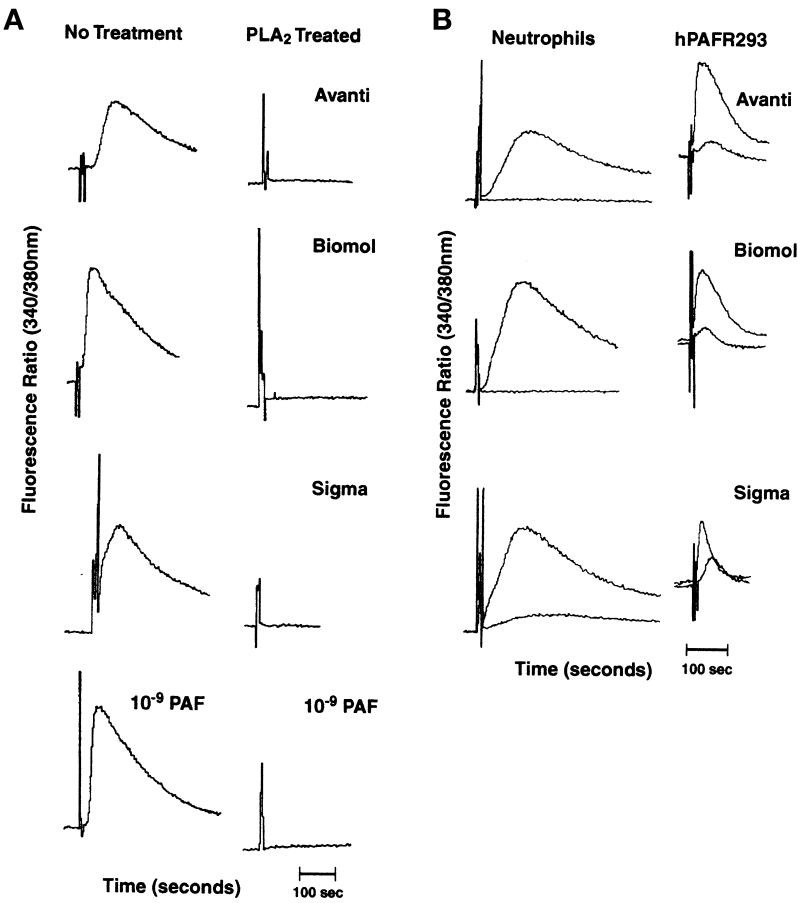

Fig. 3. Contaminants in lyso-PAF induce PAF receptor signaling. A: Phospholipase A_2 hydrolysis inappropriately abolishes lyso-PAF activation of the PAF receptor. PAF (1 nM) or alkyl lysophosphatidylcholine (25 μM) from the stated supplier was treated with bee venom phospholipase A_2 for 1 h before addition to fura-2-labeled leukocytes. The increase in fluorescence was determined as in Fig. 2. Left: No treatment. Right: Treated with phospholipase A_2. B: PAF acetylhydrolase digestion inappropriately inactivates lyso-PAF. Lyso-PAF (25 μM) was digested with recombinant human PAF acetylhydrolase for 1 h (lower tracing) or was subjected to a mock incubation (upper tracing) for an equal period of time. Ca^{2+} transients in freshly isolated human neutrophils (left) or hPAFR293 cells (right) in response to the stated source of lyso-PAF were then determined as described above.

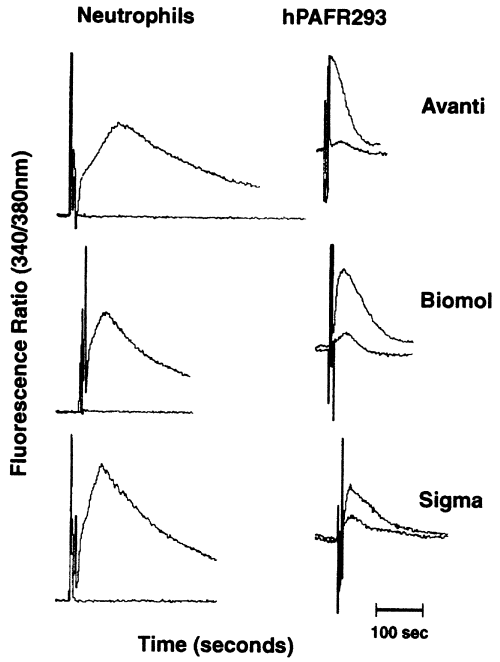

Fig. 4. Mild saponification removes PAF receptor agonists in lyso-PAF preparations. Lyso-PAF (25 μM) from the stated supplier was saponified with 0.5 M NaOH in methanol as described in Materials and Methods before analysis (lower tracings) or left untreated (upper tracings). Human leukocytes (left) or hPAFR293 cells (right) were loaded with fura-2 AM and intracellular Ca^{2+} levels were monitored as described above.

stimulation by lyso-PAF, and greatly reduced its effects in the more sensitive hPAFR293 cells. The conditions we used did not affect the phosphodiester bond of the *sn*-3 phosphocholine group, as thin-layer chromatography showed the migration of the lyso-PAF to be unchanged (data not shown). Saponification carried out under more basic conditions did generate lysosphosphatidic acid, which displays bioactivity in its own right through various Edg receptors. With care, a simple chemical method can be used to generate lyso-PAF preparations that are free of common bioactive contaminants.

DISCUSSION

We found that lyso-PAF and lysophosphatidylcholine from several sources appeared to stimulate leukocytes through their PAF receptors, an observation apparently confirmed when we found that each lysolipid also stimulated cells expressing the cloned human PAF receptor. In addition, we found in a quantifiable in vivo model that both lysolipids induced eosinophil infiltration in a murine model of pleurisy. As would be expected from the strong preference of the PAF receptor for an *sn*-1 ether bond (22), lyso-PAF was a better agonist than its acyl analog in each of these assays. In spite of this support for a role for the PAF receptor in responding to alkyl and acyl lysophosphatidylcholines in vivo and in vitro, it became apparent that this could not be true. By definition lysophosphatidylcholines, which lack an *sn*-2 residue, are not susceptible to attack by phospholipase A_2 or PAF acetylhydrolase. Furthermore, the alkyl isoform lyso-PAF, which lacks esterified fatty acyl functions, should not be susceptible to mild saponification. Yet each of these maneuvers abolished leukocyte stimulation, and severely attenuated the response in the more sensitive ectopic PAF receptor expression system. In a similar fashion, the in vivo biological activity of acyl lysophosphatidylcholine and its alkyl homolog, lyso-PAF, were abolished by such maneuvers. Thus our data clearly show *1*) neither lyso-PAF nor acyl lysophosphatidylcholine stimulates the PAF receptor; *2*) neither lysophosphatidylcholine nor lyso-PAF by itself is inflammatory in a murine model of pleurisy; and *3*) contaminating species account for the activity that we, and likely others, find when using unpurified lysophospholipid preparations.

Lysophosphatidylcholine apparently is a multitalented agonist; included in its repertoire is the ability to stimulate lymphocytes (8, 9), monocytes (10–12), smooth muscle cells (13, 38), and endothelial cells (14, 15). A receptor that recognizes lysophosphatidylcholines with an appropriate distribution to account for its many effects has yet to be identified, although one abstract may shed light on such a receptor (39). Some of the effects of lysophosphatidylcholine and lyso-PAF might proceed through the molecularly characterized PAF receptor (21), and monocytes (40) and macrophages (41), but also smooth muscle cells (42), some lymphocytes (43, 44), and perhaps some endothelial cells (45, 46), express functional PAF receptors. Typically lysophosphatidylcholines are employed at high concentrations, ranging from ~3 to 100 μM, and this may allow the presence of low levels of potent contaminants in the preparation to become manifest. Our data show that stimulation of any cells that contain functional PAF receptors with unpurified lysophosphatidylcholine or especially lyso-PAF will yield erroneous results where the observed effect belongs to the PAF-like contaminants in the unpurified material.

One route to the preparation of alkyl lysophosphatidylcholine is saponification and hydrogenation of alkenyl lysophosphatidylcholine of bovine heart, a rich source of phosphatidylcholines with this structure (47). We find by mass spectrometry (not shown) that the lysolipid material as supplied has not undergone a saponification process sufficient to remove all the susceptible acyl residues because commercial alkyl lysophosphatidylcholine preparations contained residual stearoyl and palmitoyl fatty acyl residues at the *sn*-1 position. This would imply that *sn*-2 esters also would have been incompletely removed during preparation. We suggest that biological sources of the starting material either contain traces of biosynthetic PAF, or that the polyunsaturated residues that predominate at the *sn*-2 position of the intact phospholipids have undergone oxidation during their extraction and workup that leads to the formation of fragmented phosphatidylcholines with PAF-like biological activity (31, 48–50). These can migrate during chromatography much like lysophosphatidylcholine, can be isobaric contaminants, and—being quite potent—are exceedingly difficult to detect at low levels in the bulk lipid preparation.

Some of the activity ascribed to lysophosphatidylcholine appears to act through the PAF receptor because previous

work showed that in vivo vasodilatation (3, 27) and edema (28) induced by lysophosphatidylcholines are blocked by PAF receptor antagonists. A role for the PAF receptor in responding to lysophosphatidylcholine is directly addressed in isolated macrophages or Chinese hamster ovary cells ectopically expressing the human PAF receptor, where the Ca^{2+} increase induced in these cells by lysophosphatidylcholine is blocked by a PAF receptor antagonist (24). We now see from the results presented here that these events must result from contaminating PAF-like lipids because the PAF receptor does not respond to purified lysophosphatidylcholines.

Oxidation of LDL to modified particles is now a cornerstone of our understanding of the early steps in atherogenesis (51–53) and one result of the oxidation of LDL is an increase in the content of lysophosphatidylcholine from 1% to 5% of the total phosphatidylcholine content to 40% to 50% of this pool (3, 54, 55). Oxidatively fragmented phospholipids generated in this process are substrates (4, 5, 49, 56–58) for the LDL-associated (59) PAF acetylhydrolase, which is responsible for this increase in its hydrolysis product (30, 56, 58). Among the reported effects of lysophosphatidylcholine associated with oxidized LDL particles is an impairment of endothelial-dependent vasorelaxation in ex vivo arterial preparations (3, 60), as well as antibody and cytokine production by cells of the immune system (7). However, inhibition of vasodilation by PAF receptor antagonists in vivo (3, 27), coupled with the inability of lysophosphatidylcholine to activate the PAF receptor as shown here, suggests that LDL-associated lysophosphatidylcholine also is not vasoactive, at least through the PAF receptor. This also means that the PAF acetylhydrolase associated with LDL and HDL containing apolipoprotein E (59) inactivates the potent species PAF without generating an equivalent biologically active compound.

Lysophosphatidylcholines may have a role in homeostasis, but a potential role for contaminating PAF receptor agonists in unpurified preparations of lysophosphatidylcholine or lyso-PAF needs to be addressed. A key result of this work, beyond the identification of the problem, is that a simple chemical reaction, or in the case of lysophosphatidylcholine phospholipase A_2 treatment, can completely remove minor, but highly potent, contaminants that may lead to incorrect deductions in an important area of research.

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REFERENCES

1. Subbaiah, P. V., C. H. Chen, J. D. Bagdade, and J. J. Albers. 1985. Substrate specificity of plasma lysolecithin acyltransferase and the molecular species of lecithin formed by the reaction. *J. Biol. Chem.* **260:** 5308–5314.
2. Rabini, R. A., R. Galassi, P. Fumelli, N. Dousset, M. L. Solera, P. Valdiguie, G. Curatola, G. Ferretti, M. Taus, and L. Mazzanti. 1994. Reduced Na(+)-K(+)-ATPase activity and plasma lysophosphatidylcholine concentrations in diabetic patients. *Diabetes.* **43:** 915–919.
3. Chen, L., B. Liang, D. E. Froese, S. Liu, J. T. Wong, K. Tran, G. M. Hatch, D. Mymin, E. A. Kroeger, R. Y. Man, and P. C. Choy. 1997. Oxidative modification of low density lipoprotein in normal and hyperlipidemic patients: effect of lysophosphatidylcholine composition on vascular relaxation. *J. Lipid Res.* **38:** 546–553.
4. Stremler, K. E., D. M. Stafforini, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 1989. An oxidized derivative of phosphatidylcholine is a substrate for the platelet-activating factor acetylhydrolase from human plasma. *J. Biol. Chem.* **264:** 5331–5334.
5. Stremler, K. E., D. M. Stafforini, S. M. Prescott, and T. M. McIntyre. 1991. Human plasma platelet-activating factor acetylhydrolase: oxidatively-fragmented phospholipids as substrates. *J. Biol. Chem.* **266:** 11095–11103.
6. Chisolm, G. M., and Y. Chai. 2000. Regulation of cell growth by oxidized LDL. *Free Radic. Biol. Med.* **28:** 1697–1707.
7. Huang, Y. H., L. Schafer-Elinder, R. Wu, H. E. Claesson, and J. Frostegard. 1999. Lysophosphatidylcholine (LPC) induces proinflammatory cytokines by a platelet-activating factor (PAF) receptor-dependent mechanism. *Clin. Exp. Immunol.* **116:** 326–331.
8. Nishi, E., N. Kume, H. Ochi, H. Moriwaki, S. Higashiyama, N. Taniguchi, and T. Kita. 1997. Lysophosphatidylcholine induces heparin-binding epidermal growth factor-like growth factor and interferon-gamma in human T-lymphocytes. *Ann. N.Y. Acad. Sci.* **811:** 519–524.
9. Hoffman, R. D., M. Kligerman, T. M. Sundt, N. D. Anderson, and H. S. Shin. 1982. Stereospecific chemoattraction of lymphoblastic cells by gradients of lysophosphatidylcholine. *Proc. Natl. Acad. Sci. USA.* **79:** 3285–3289.
10. Ramos, M. A., M. Kuzuya, T. Esaki, S. Miura, S. Satake, T. Asai, S. Kanda, T. Hayashi, and A. Iguchi. 1998. Induction of macrophage VEGF in response to oxidized LDL and VEGF accumulation in human atherosclerotic lesions. *Arterioscler. Thromb. Vasc. Biol.* **18:** 1188–1196.
11. Liu-Wu, Y., E. Hurt-Camejo, and O. Wiklund. 1998. Lysophosphatidylcholine induces the production of IL-1beta by human monocytes. *Atherosclerosis.* **137:** 351–357.
12. Quinn, M. T., S. Parthasarathy, and D. Steinberg. 1988. Lysophosphatidylcholine: a chemotactic factor for human monocytes and its potential role in atherogenesis. *Proc. Natl. Acad. Sci. USA.* **85:** 2805–2809.
13. Kohno, M., K. Yokokawa, K. Yasunari, M. Minami, H. Kano, T. Hanehira, and J. Yoshikawa. 1998. Induction by lysophosphatidylcholine, a major phospholipid component of atherogenic lipoproteins, of human coronary artery smooth muscle cell migration. *Circulation.* **98:** 353–359.
14. Kume, N., M. I. Cybulsky, and M. A. Gimbrone, Jr. 1992. Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. *J. Clin. Invest.* **90:** 1138–1144.
15. Kume, N., and M. A. Gimbrone, Jr. 1994. Lysophosphatidylcholine transcriptionally induces growth factor gene expression in cultured human endothelial cells. *J. Clin. Invest.* **93:** 907–911.
16. Ryborg, A. K., B. Deleuran, H. Sogaard, and K. Kragballe. 2000. Intracutaneous injection of lysophosphatidylcholine induces skin inflammation and accumulation of leukocytes. *Acta Derm. Venereol.* **80:** 242–246.
17. Goetzl, E. J., and S. An. 1998. Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. *FASEB J.* **12:** 1589–1598.
18. Aoki, Y., M. Nakamura, H. Kodama, T. Matsumoto, T. Shimizu, and M. Noma. 1995. A radioreceptor binding assay for platelet-activating factor (PAF) using membranes from CHO cells expressing human PAF receptor. *J. Immunol. Methods.* **186:** 225–231.
19. Shimizu, T., Z. Honda, M. Nakamura, H. Bito, and T. Izumi. 1992. Platelet-activating factor receptor and signal transduction. *Biochem. Pharmacol.* **44:** 1001–1008.
20. Shen, T. Y., S-B. Hwang, T. W. Doebber, and J. C. Robbins. 1987. The chemical and biological properties of PAF agonists, antagonists, and biosynthetic inhibitors. *In* Platelet-Activating Factor and Related Lipid Mediators. F. Snyder, editor. Plenum Press, New York. 153–190.
21. Prescott, S. M., G. A. Zimmerman, D. M. Stafforini, and T. M.

McIntyre. 2000. Platelet-activating factor and related lipid mediators. *Annu. Rev. Biochem.* **69:** 419–445.
22. O'Flaherty, J. T., W. L. Salzer, S. Cousart, C. E. McCall, C. Piantadosi, J. R. Surles, M. J. Hammett, and R. L. Wykle. 1983. Platelet-activating factor and analogues: comparative studies with human neutrophils and rabbit platelets. *Res. Commun. Chem. Pathol. Pharmacol.* **39:** 291–309.
23. Kern, H., T. Volk, S. Knaure-Sciefer, T. Mieth, B. Rustow, W. J. Kox, and M. Schlame. 1998. Stimulation of monocytes and platelets by short-chain phosphatidylcholines with and without terminal carboxy groups. *Biochim. Biophys. Acta.* **1394:** 33–42.
24. Ogita, T., Y. Tanaka, T. Nakaoka, R. Matsuoka, Y. Kira, M. Nakamura, T. Shimizu, and T. Fujita. 1997. Lysophosphatidylcholine transduces Ca^{2+} signaling via the platelet-activating factor receptor in macrophages. *Am. J. Physiol.* **272:** H17–H24.
25. Gomez-Munoz, A., L. O'Brien, R. Hundal, and U. P. Steinbrecher. 1999. Lysophosphatidylcholine stimulates phospholipase D activity in mouse peritoneal macrophages. *J. Lipid Res.* **40:** 988–993.
26. Takahara, N., A. Kashiwagi, H. Maegawa, and Y. Shigeta. 1996. Lysophosphatidylcholine stimulates the expression and production of MCP-1 by human vascular endothelial cells. *Metabolism.* **45:** 559–564.
27. Hirayama, T., Y. Ogawa, K. Tobise, and K. Kikuchi. 1998. Mechanism of endothelium-dependent vasorelaxation evoked by lysophosphatidylcholine. *Hypertens. Res.* **21:** 137–145.
28. Sakamoto, T., W. Elwood, P. J. Barnes, and K. F. Chung. 1993. Effect of inhaled lyso-platelet-activating factor on airway microvascular leakage in the guinea pig. *J. Appl. Physiol.* **74:** 1117–1122.
29. Tjoelker, L. W., C. Wilder, C. Eberhardt, D. M. Stafforini, G. Dietsch, B. Schimpf, S. Hooper, H. Le Trong, L. S. Cousens, G. A. Zimmerman, Y. Yamada, T. M. McIntyre, S. M. Prescott, and P. W. Gray. 1995. Anti-inflammatory properties of a platelet-activating factor acetylhydrolase. *Nature.* **374:** 549–553.
30. Tew, D. G., C. Southan, S. Q. Rice, M. P. Lawrence, H. Li, H. F. Boyd, K. Moores, I. S. Gloger, and C. H. Macphee. 1996. Purification, properties, sequencing, and cloning of a lipoprotein-associated, serine-dependent phospholipase involved in the oxidative modification of low-density lipoproteins. *Arterioscler. Thromb. Vasc. Biol.* **16:** 591–599.
31. Marathe, G. K., S. S. Davies, K. A. Harrison, A. R. Silva, R. C. Murphy, H. Castro-Faria Neto, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 1999. Inflammatory PAF-like lipids in oxidized low density lipoproteins are fragmented alkyl phosphatidylcholines. *J. Biol. Chem.* **274:** 28395–28405.
32. Davies, S. S., A. V. Pontsler, G. K. Marathe, K. A. Harrison, R. C. Murphy, J. C. Hinshaw, G. D. Prestwich, A. St. Hilaire, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 2001. Oxidized phospholipids are specific, high affinity PPARγ ligands and agonists. *J. Biol. Chem.* **276:** 16015–16023.
33. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
34. Zimmerman, G. A., T. M. McIntyre, and S. M. Prescott. 1985. Thrombin stimulates the adherence of neutrophils to human endothelial cells *in vitro*. *J. Clin. Invest.* **76:** 2235–2246.
35. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260:** 3440–3450.
36. Pires, A. L. A., P. M. R. e Silva, C. Pasquale, H. C. Castro-Faria-Neto, P. T. Bozza, R. S. B. Cordeiro, G. A. Rae, P. Braquet, V. Lagente, and M. A. Martins. 1994. Long-lasting inhibitory activity of the hetrazepinic BN 50730 on exudation and cellular alterations evoked by PAF and LPS. *Br. J. Pharmacol.* **113:** 994–1000.
37. Ames, B. N., and D. T. Dubin. 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. *J. Biol. Chem.* **235:** 769–775.
38. Chai, Y. C., P. H. Howe, P. E. DiCorleto, and G. M. Chisolm. 1996. Oxidized low density lipoprotein and lysophosphatidylcholine stimulate cell cycle entry in vascular smooth muscle cells. Evidence for release of fibroblast growth factor-2. *J. Biol. Chem.* **271:** 17791–17797.
39. Zhu, K. 2001. Identification of the first two high affinity receptors for Sphingosylphosphorylcholine (SPC) and the first receptor for Lysophosphatidylcholine (LPC). *FASEB J.* **15:** A932.
40. Ouellet, S., E. Muller, and M. Rola-Pleszczynski. 1994. IFN-g upregulates platelet-activating factor receptor gene expression in human monocytes. *J. Immunol.* **152:** 5092–5099.
41. Pendino, K. J., C. R. Gardner, J. D. Laskin, and D. L. Laskin. 1993. Induction of functionally active platelet-activating factor receptors in rat alveolar macrophages. *J. Biol. Chem.* **268:** 19165–19168.
42. Heery, J. M., M. Kozak, D. M. Stafforini, D. A. Jones, G. A. Zimmerman, T. M. McIntyre, and S. M. Prescott. 1995. Oxidatively modified LDL contains phospholipids with PAF-like activity and stimulates the growth of smooth muscle cells. *J. Clin. Invest.* **96:** 2322–2330.
43. Muller, E., P. Dagenais, N. Alami, and M. Rola-Pleszczynski. 1993. Identification and functional characterization of platelet-activating receptors in human leukocyte populations using polyclonal anti-peptide antibody. *Proc. Natl. Acad. Sci. USA.* **90:** 5818–5822.
44. Calabresse, C., M. C. Nguer, O. Pellegrini, J. Benveniste, R. Yolande, and Y. Thomas. 1992. Induction of high-affinity PAF receptor expression during T cell activation. *Eur. J. Immunol.* **22:** 1349–1355.
45. Hirafuji, M., J. M. Mencia-Huerta, and J. Benveniste. 1987. Regulation of PAF-acether (platelet-activating factor) biosynthesis in cultured human vascular endothelial cells stimulated with thrombin. *Biochim. Biophys. Acta.* **930:** 359–369.
46. Predescu, D., K. Ihida, S. Predescu, and G. E. Palade. 1996. The vascular distribution of the platelet-activating factor receptor. *Eur. J. Cell Biol.* **69:** 86–98.
47. Pugh, E. L., M. Kates, and D. J. Hanahan. 1977. Characterization of the alkyl ether species of phosphatidylcholine in bovine heart. *J. Lipid Res.* **18:** 710–716.
48. Smiley, P. L., K. E. Stremler, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 1991. Oxidatively fragmented phosphatidylcholines activate human neutrophils through the receptor for platelet-activating factor. *J. Biol. Chem.* **266:** 11104–11110.
49. Tokumura, A., M. Toujima, Y. Yoshioka, and K. Fukuzawa. 1996. Lipid peroxidation in low density lipoproteins from human plasma and egg yolk promotes accumulation of 1-acyl analogues of platelet-activating factor-like lipids. *Lipids.* **31:** 1251–1258.
50. Schlame, M., R. Haupt, I. Wiswedel, W. J. Kox, and B. Rustow. 1996. Identification of short-chain oxidized phosphatidylcholine in human plasma. *J. Lipid Res.* **37:** 2608–2615.
51. Brown, M. S., and J. L. Goldstein. 1983. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* **52:** 223–261.
52. Steinberg, D. 1997. Low density lipoprotein oxidation and its pathobiological significance. *J. Biol. Chem.* **272:** 20963–20966.
53. Berliner, J. A., and J. W. Heinecke. 1996. The role of oxidized lipoproteins in atherogenesis. *Free Radic. Biol. Med.* **20:** 707–727.
54. Kugiyama, K., S. A. Kerns, J. D. Morrisett, R. Roberts, and P. D. Henry. 1990. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature.* **344:** 160–162.
55. Yokoyama, M., K-I. Hirata, R. Miyake, H. Akita, Y. Ishikawa, and H. Fukuzaki. 1990. Lysophosphatidylcholine: essential role in the inhibition of endothelium-dependent vasorelaxation by oxidized low density lipoprotein. *Biochem. Biophys. Res. Commun.* **168:** 301–308.
56. Steinbrecher, U. P., and P. H. Pritchard. 1989. Hydrolysis of phosphatidylcholine during LDL oxidation is mediated by platelet-activating factor acetylhydrolase. *J. Lipid Res.* **30:** 305–315.
57. Watson, A. D., M. Navab, S. Y. Hama, A. Sevanian, S. M. Prescott, D. M. Stafforini, T. M. McIntyre, B. N. La Du, A. M. Fogelman, and J. A. Berliner. 1995. Effect of platelet activating factor-acetylhydrolase on the formation and action of minimally oxidized low density lipoprotein. *J. Clin. Invest.* **95:** 774–782.
58. Tsoukatos, D. C., M. Arborati, T. Liapikos, K. L. Clay, R. C. Murphy, M. J. Chapman, and E. Ninio. 1997. Copper-catalyzed oxidation mediates PAF formation in human LDL subspecies. Protective role of PAF:acetylhydrolase in dense LDL. *Arterioscler. Thromb. Vasc. Biol.* **17:** 3505–3512.
59. Stafforini, D. M., T. M. McIntyre, M. E. Carter, and S. M. Prescott. 1987. Human plasma platelet-activating factor acetylhydrolase: association with lipoprotein particles and role in the degradation of platelet-activating factor. *J. Biol. Chem.* **262:** 4215–4222.
60. Mangin, E. L., Jr., K. Kugiyama, J. H. Nguy, S. A. Kerns, and P. D. Henry. 1993. Effects of lysolipids and oxidatively modified low density lipoprotein on endothelium-dependent relaxation of rabbit aorta. *Circ. Res.* **72:** 161–166.