Identification of platelet-activating factor as the inflammatory lipid mediator in CCl_4-metabolizing rat liver

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Abstract Unmitigated oxidative stress is deleterious, as epitomized by CCl_4 intoxication. In this well-characterized model of free radical-initiated damage, liver metabolism of CCl_4 to $CCl_3^{\cdot}$ causes lipid peroxidation, F-ring isoprostane formation, and pathologic leukocyte activation. The nature of the mediator that couples oxidation to the hepatotoxic inflammatory response is uncharacterized. We found that oxidatively modified phosphatidylcholines were present in the livers of CCl_4-exposed rats and not in livers from control animals, that CCl_4 metabolism generated lipids that activated 293 cells stably transfected with the human platelet-activating factor (PAF) receptor, and that this PAF-like activity was formed as rapidly as isoprostane-containing phosphatidylcholine (iPC) during oxidation. iPC and the PAF-like activity also had similar chromatographic properties. The potential for iPC activation of the PAF receptor has been unexplored, but we conclude that iPC themselves did not activate the PAF receptor, as phospholipase A_1 hydrolysis completely destroyed iPC, but none of the PAF-like bioactivity. Oxidatively fragmented phospholipids are potent agonists of the PAF receptor, but mass spectrometry characterized PAF as the major inflammatory component coeluting with iPC. Oxidatively fragmented phospholipids and iPC are markers of free radical generation in CCl_4-intoxicated liver, but PAF generation by activated hepatic cells generated the inflammatory agent.—Marathe, G. K., K. A. Harrison, L. J. Roberts II, J. D. Morrow, R. C. Murphy, L. W. Tjoelker, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. **Identification of platelet-activating factor as the inflammatory lipid mediator in CCl_4-metabolizing rat liver. *J. Lipid Res.* 2001.** 42: **587–596.**

Supplementary key words PAF • inflammation • liver • isoprostane • oxidation

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) activates inflammatory cells expressing the single known receptor for PAF (1) and does so at exceedingly low concentrations. Receptor specificity depends on the *sn*-1 ether bond, the choline head group, and, as a distinguishing feature, the short *sn*-2 acetyl residue (2). Biosynthesis by inflammatory cells occurs primarily by transacetylation from acetyl-CoA, and both this final enzyme and the phospholipase A_2 that generates the lysolipid acceptor are activated in appropriately stimulated cells.

We (3) and others (4, 5) find that a second way exists to create PAF receptor agonists: through the oxidative fragmentation of phosphatidylcholines containing a polyunsaturated fatty acyl residue in the *sn*-2 position (6). The esterified fatty acyl peroxides formed by such an oxidative attack can undergo β scission that, because of the varied location of the peroxide function along the fatty acyl residue, generates a large number of phospholipid products with short and/or functionalized *sn*-2 residues (7–9). Some of these directly bind and activate the PAF receptor, and do so with an affinity that is within an order of magnitude of that for PAF (10). These oxidatively generated PAF receptor agonists are products of an unregulated chemical reaction and are not under the stringent control accorded cellular enzymatic reactions. The structural diversity between PAF and its mimetics therefore represents mechanistic differences in the formation of potent PAF receptor agonists, and it implies that the unregulated formation of PAF-like mimetics may circumvent the tight control of a potent inflammatory system.

Oxidation of arachidonoyl-containing phosphatidylcholine has a second outcome, which is the formation of a

Abbreviations: iPC, isoprostane-containing phosphatidylcholine; PAF, platelet-activating factor; HPLC, high performance liquid chromatography

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series of isomers of prostaglandins. Again this occurs through unregulated chemical reactions that are not regulated or directed by the cyclo-oxygenases responsible for cellular prostanoid synthesis. The ability to detect these products of chemical oxidation with great specificity and sensitivity has made this class of lipid oxidation products premier markers of oxidative processes, particularly in vivo, where measures of oxidative stresses have been limited (11, 12). Isoprostanes are initially formed by oxidation of arachidonate esterified at the *sn*-2 position of cellular and lipoprotein phospholipids (13), and these arachidonoyl phospholipid peroxides may rearrange to a series of regio- and stereoisomers of eicosanoid mediators including the F_2-isoprostanes (12, 14). An E-ring isoprostane-containing phosphatidylcholine (iPC), generated in the absence of cellular reductants, has been implicated as an inflammatory agonist (15). The PAF receptor, ordinarily selective for the *sn*-2 acetyl residue of PAF, responds to phosphatidylcholines with longer, functionalized *sn*-2 residues (S. S. Davies, G. K. Marathe, G. A. Zimmerman, S. M. Prescott, and T. M. McIntyre, unpublished data), but whether an iPC might also interact with the PAF receptor is undetermined.

Oxidatively fragmented phospholipids with PAF-like activity are readily formed in vitro during the oxidation of lipoprotein particles (4, 5, 10) or cultured cells (16). These and other oxidatively fragmented phospholipids are found in vivo in atherosclerotic lesions (5) and in the circulation after exposure to cigarette smoke (17–19) or other oxidative stress (19). Isoprostanes are recovered under the same circumstances: F-ring isoprostanes mark the oxidative stress of smokers (20, 21), ex vivo lipoprotein oxidation (21, 22), and atherosclerotic plaques (23, 24). Whether isoprostanes and PAF-like lipids are formed in parallel or always coexist is unclear, but both are the products of phospholipid oxidation.

CCl_4 metabolism is an established model of liver necrosis and fibrosis, and the liver damage created by this metabolism is free radical dependent. CCl_4 is oxidized by cytochrome P-450 to the $CCl_3^{\cdot}$ radical, which generates oxygen radicals (25) and phospholipid peroxides (26) in abundance. These underlie liver damage in this model because either introduction of the protective enzyme superoxide dismutase (25) or interference with the inflammatory oxidative burst suppresses both peroxide formation and liver damage. In the context of CCl_4 poisoning, F-ring iPC increase dramatically, as much as 140-fold, in the liver (13, 27), confirming the oxidative nature of this insult. The PAF receptor in other models contributes to leukocyte influx in inflamed liver (28), and it appears to be responsible for the hemologic derangement subsequent to CCl_4-induced liver damage (29).

Receptor antagonists define a role for the PAF receptor in oxidative liver disease but do not distinguish between PAF, PAF-like lipids, or iPC as the relevant agonist. Here we show that the livers of rats metabolizing CCl_4 contain inflammatory phospholipids. These copurify with iPC, and they activate leukocytes via their PAF receptor. However, neither iPC nor oxidatively fragmented PAF-like lipids were the proinflammatory agonist(s) extracted from CCl_4-metabolizing liver. Instead we find that oxidants, which stimulate cellular PAF generation in vitro (30), lead to the accumulation of significant amounts of hepatic PAF. Thus in an established model where the proximal insult is free radical generation, oxidation activates PAF generation rather than increasing the formation of oxidatively fragmented phospholipids with PAF-like activity.

MATERIALS AND METHODS

Recombinant PAF acetylhydrolase (PAF-AH) was from ICOS (Bothell, WA). PAF, acyl PAF, and BN52021 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). FURA-2AM ester was from Molecular Probes (Eugene, OR). Pefabloc was from Pentapharm (Basel, Switzerland). Human albumin was from Baxter Health Care (Glendale, CA). WEB 2086 was a generous gift from Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT). The solvents (J. T. Baker, Phillipsburg, NJ) used were high performance liquid chromatography (HPLC) grade. Trypsin-ethylenediaminetetraacetic acid (EDTA) was from GIBCO (Grand Island, NY), and fetal bovine serum was from HyClone (Logan, UT). Lipase (with phospholipase A_1 activity) from *Rhizopus arrhizus* was from Boehringer Mannheim (Indianapolis, IN). In experiments where recombinant PAF-AH was used, PAF-like lipids or PAF was treated with 4 μg of this enzyme in Hanks' balanced salt solution containing 0.5% human serum albumin (HBSS-A) for 1 h.

Lipid analysis

Human low density lipoprotein (LDL) was isolated by density flotation from normolipidic subjects, oxidized for the stated period of time, and quenched with 100 μM butylated hydroxytoluene and 200 μM EDTA, and the extracted lipids were separated on an aminopropyl column before being subjected to reversed-phase HPLC separation as described (10). An aliquot of the most active PAF-like fraction eluting at minute 6 was dried under nitrogen, resuspended in HBSS-A, and sonicated before being assayed for bioactivity as described below.

iPC was isolated and purified by HPLC from rat liver 2 h after administration of CCl_4 as described previously (13, 27) in accordance with National Institutes of Health (Bethesda, MD) guidelines. The amount of esterified F_2-isoprostane was determined by subjecting an aliquot to alkaline hydrolysis followed by quantitation of the free F_2-isoprostane by negative ion chemical ionization gas chromatography-mass spectrometry (GC-MS). This was accomplished by derivatization to the pentafluorobenzoyl ester, trimethylsilyl ether derivatives as previously described (14). A portion of the HPLC-purified iPC extracted from liver lipids (approximately 10 μg) was subjected to hydrolysis with 50 units of phospholipase A_1 (*R. arrhizus*) for 11 h at 37°C (10) and then injected into a 5-μm ODS reversed-phase HPLC column (2 × 150 mm; Phenomenex, Torrance, CA) and resolved with a gradient from 80% to 98% solvent B over a period of 10 min, where solvent A is H_2O-0.05% acetic acid (pH 5.7) and solvent B is CH_3CN–methanol 65:35. The fractions eluting from this column were dried under nitrogen and assayed for PAF-like bioactivity and for isoprostane content (14). In addition, PAF and certain PAF-like lipids were quantitated by negative ion GC-MS after hydrolysis with phospholipase C and derivatization of the liberated diacylglycerols with pentafluorobenzoyl chloride as described previously (10).

On-line HPLC mass spectral analysis was carried out with a Sciex API-III+ triple quadrupole mass spectrometer (PE-Sciex,

Thornhill, Ontario, Canada). For all electrospray ionization analyses, the curtain gas flow was 1.2 l/min nitrogen with a nebulizer pressure at 38 lb/in^2. The orifice potential was maintained at 75 V and the electrospray ionization potential was maintained at approximately +4,200 V. Precursor ion scanning MS/MS was carried out with collision energy E_{lab} = 20 eV and collision gas thickness 240 × 10^{13} argon atoms/cm^2.

PAF-like activity analysis

The human PAF receptor (hPAFR) cDNA was obtained by the polymerase chain reaction (PCR) (sense primer, 5′-ATAAGCGGC CGCGACCAGCTGATCATTCCAGCCCACAGCAATG; antisense primer, 5′-GAAGTCTAGAAAGACTTCAGGCCTGGAAGCAGG). The PCR product was cloned into the *Not*I and *Xba*I restriction sites of the mammalian expression plasmid pRc/CMV (InVitrogen, Carlsbad, CA), via the *Not*I and *Xba*I sites located within the PCR primers. The expression construct was introduced into HEK293 cells, using the 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) transfection reagent (Boehringer Mannheim, Indianapolis, IN) according to the supplier protocol. After 17 days of selection in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 1 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), 10% fetal calf serum, and Geneticin (0.8 mg/ml; GIBCO), clones were isolated with cloning rings. Levels of PAF receptor expression by individual clones were monitored by Northern blotting. Signaling via the overexpressed PAF receptor was confirmed by measuring calcium mobilization in response to 25 nM PAF.

Subconfluent hPAFR 293 cells were used to quantitate intracellular Ca^{2+} after they were suspended with Versene (GIBCO-BRL, Gaithersburg, MD). These cells were pelleted and resuspended in fresh culture medium containing FURA-2AM to a final concentration of 1 μM. After incubation in the dark for 45 min at 37°C, the cells were washed with HBSS-A and resuspended at a density of 2.25 × 10^6 cells/ml. Fluorescence was measured at 37°C with dual excitation at 340 and 380 nm and emission at 510 nm (10). In a few experiments, cells were treated with the PAF receptor antagonist BN52021 (10 μM) and then used in this assay. In experiments involving rPAF-AH, polar phosphatidylcholine preparations were dried under nitrogen, suspended in HBSS-A, sonicated, and then treated with recombinant PAF-AH. Human neutrophils were isolated by dextran sedimentation and centrifugation over Ficoll as described (31), labeled with FURA-2, and analyzed for intracellular Ca^{2+} mobilization as described previously (10).

RESULTS

CCl_4-exposed liver contains inflammatory phospholipids

CCl_4 metabolism produces a profound oxidative stress through its metabolism to $CCl_3^{\cdot}$ and other free radicals, and should produce both fragmented PAF-like phospholipids and iPC. The polar phospholipid fraction we extracted from CCl_4-exposed livers included iPC, based on the isoprostane content after phospholipid hydrolysis (see below). This material was then tested as an inflammatory, Ca^{2+}-mobilizing agonist for freshly isolated human polymorphonuclear leukocytes (PMN). PMN loaded with the Ca^{2+}-sensitive dye FURA-2 demonstrated a sharp and rapid increase in intracellular Ca^{2+} in response to the positive control 10^{-9} M PAF (**Fig. 1A**). We next exposed PMN to various concentrations of purified iPC, where the quantity of phospholipid was determined by phosphorus analysis, a value that agreed with the content of isoprostane determined after hydrolysis to free fatty acids and analysis by GC-MS. We found that this preparation caused transient increases in intracellular Ca^{2+} in PMN, and did so in a concentration-dependent fashion (Fig. 1B), where full activation was achieved at submicromolar concentrations. We determined whether oxidized phospholipids (or PAF) accounted for the biologic activity in CCl_4-exposed livers by enzymatically digesting the preparation with a nonspecific phospholipase A_2 (not shown), or with PAF-AH (Fig. 1C), an enzyme that selectively inactivates phospholipids with modified or short *sn*-2 residues, to find that this treatment abolished the inflammatory activity of iPC. Clearly the active species were intact phospholipids and could not be lysophosphatidylcholine, which was increased by this hydrolysis of oxidized phospholipids. These data show that the iPC preparations were inflammatory agonists for

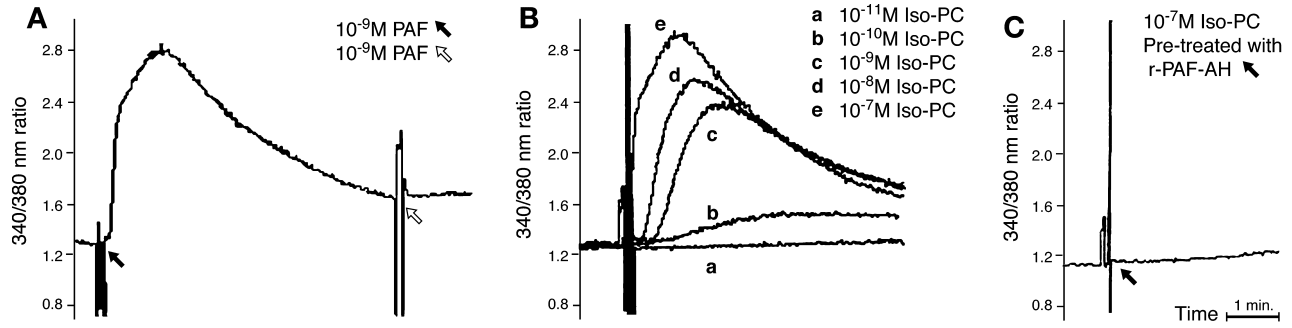

Fig. 1. Intact isoprostane-containing phosphatidylcholines (iPC) from CCl_4-treated rat liver are inflammatory. The effect of high performance liquid chromatography (HPLC)-purified iPC derived from CCl_4-treated rats on human polymorphonuclear leukocytes (PMN) was tested as follows. Freshly isolated PMN were loaded with the Ca^{2+}-sensitive dye FURA-2 and then treated with synthetic platelet-activating factor (PAF) (A) or iPC purified from the livers of CCl_4-treated rats (B) at the stated concentrations. A: Full activation of human PMN with 10^{-9} M PAF that completely desensitized these cells to a second exposure to the same concentration of PAF. B: Dose-dependent increase in calcium concentration, in response to iPC: line a = 10^{-11} M iPC; line b = 10^{-10} M iPC; line c = 10^{-9} M iPC; line d = 10^{-8} M iPC; line e = 10^{-7} M iPC. C: Stimulation by the maximally effective concentration of iPC is abolished by pretreatment with recombinant human PAF-AH (acetylhydrolase) (rPAF-AH) that inactivates PAF and oxidatively fragmented phospholipids.

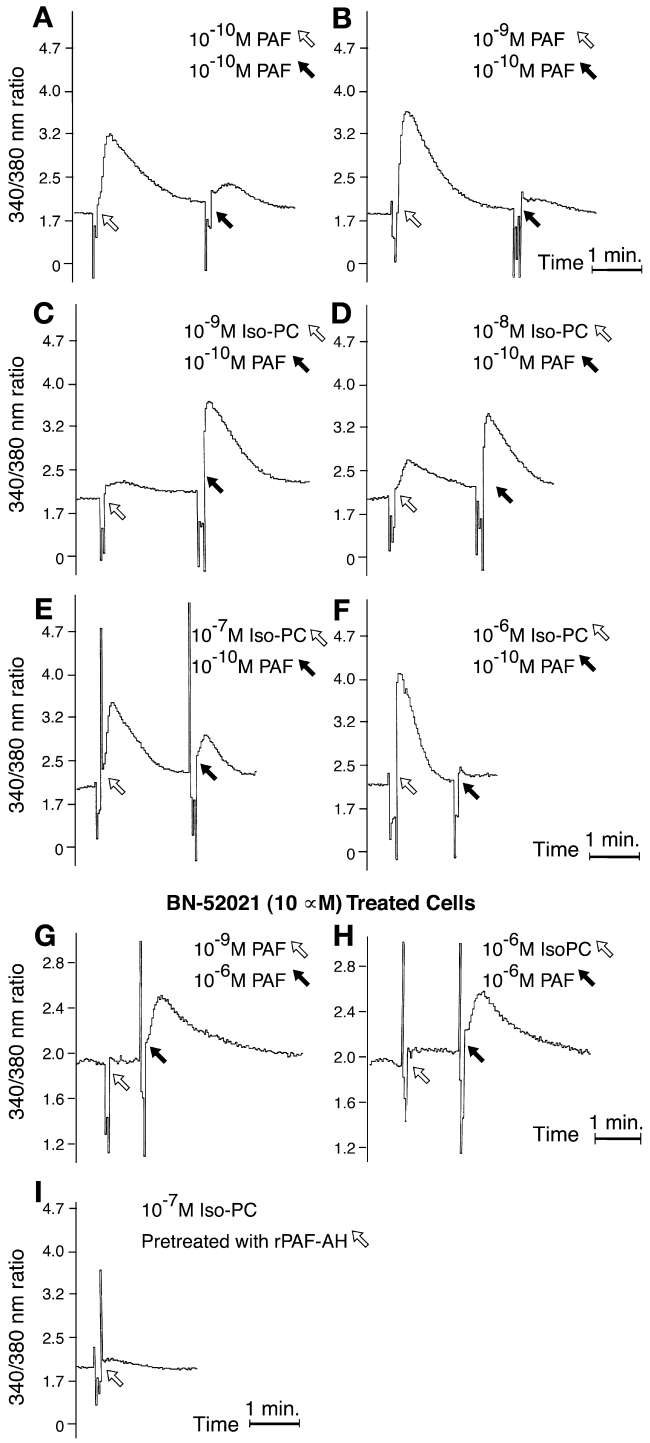

Fig. 2. Intact iPC from CCl_4-treated rat liver are PAF receptor agonists. iPC was purified from CCl_4-treated rat liver and the specified concentration was added to FURA-2AM-loaded hPAFR 293 cells. A: and B: establish the sensitivity of changes in the 340/380 nm ratio to PAF for these cells (open arrow), and PAF receptor desensitization was confirmed by a subsequent exposure to 10^{-10} M PAF (closed arrow). C–F: define the concentration-dependent increase in intracellular Ca^{2+} accumulation by iPC (open arrow) and PAF receptor desensitization (filled arrow) in the same batch of hPAFR 293 cells. G: and H: show inhibition of iPC- or PAF-induced increases in Ca^{2+} accumulation by BN-52021, a selective PAF receptor antagonist. I: demonstrates the loss of iPC PAF receptor agonistic activity after treatment with recombinant PAF-AH. IsoPC, Isoprostane-containing phosphatidylcholine; rPAF-AH, recombinant PAF-AH.

human leukocytes, but were less potent than PAF on a molar basis.

iPC preparations act through the PAF receptor

We questioned whether the polar phospholipids isolated from CCl_4-metabolizing liver activated leukocytes via their PAF receptors, or through some other phospholipid-recognizing entity. We therefore determined whether the active phospholipids extracted from liver were capable of activating 293 cells stably expressing the hPAFR, as this receptor recognizes intact phospholipids, albeit ones with an exceedingly short *sn*-2 residue. These cells specifically responded to PAF (**Fig. 2A** and **B**) and to oxidatively fragmented phospholipids with PAF-like activity (10). We examined Ca^{2+} transients in these hPAFR 293 cells in response to 10^{-9} and 10^{-10} M PAF and found (Fig. 2A and B) that both concentrations were effective in inducing a marked increase in intracellular Ca^{2+} levels. There was not a significant difference in fluorescence with this 10-fold change in concentration, but only the 10^{-9} M initial exposure completely desensitized the cells to a subsequent challenge with 10^{-10} M PAF.

With the sensitivity of the system established, we examined the effect of iPC to find a concentration-dependent increase in fluorescence (Fig. 2C–F). iPC at a concentration of 10^{-7} M was just submaximal, with an incomplete desensitization to rechallenge with 10^{-10} M PAF. However, iPC at 10^{-6} M induced the same level of fluorescence as 10^{-9} M PAF, and it completely desensitized the cells to a subsequent exposure to 10^{-10} M PAF. Equivalent fractions derived from a control rat liver lipid extract produced only a weak increase in intracellular Ca^{2+} (data not shown), showing that accumulation of the PAF-like bioactivity resulted from CCl_4 metabolism. To confirm that the change in Ca^{2+} levels was due to activation of the ectopic PAF receptor, we included the selective PAF receptor antagonist BN52021 in the assay. This abolished the Ca^{2+} flux induced by PAF and iPC, and this effect could be overcome, because this is a competitive inhibitor, by restimulation with a much higher concentration of PAF (Fig. 2G and H). In addition, this activity required the phospholipid esterified *sn*-2 residue because hydrolysis of this residue destroyed the ability of iPC preparations to interact with the PAF receptor (Fig. 2I). We conclude from this bioassay that the iPC fraction contains a PAF receptor agonist(s), but is approximately 1,000-fold less potent than the natural ligand PAF.

Formation of PAF-like activity and iPC are temporally correlated

We used a cell-free model system to determine whether fragmentation of fatty acyl peroxides to PAF-like lipids proceeds in parallel with nonenzymatic rearrangement of esterified arachidonoyl residues to esterified isoprostanes. We oxidized LDL with Cu^+ for various times, isolated the newly generated polar phospholipids by reversed-phase chromatography, and then assayed the fractions for activity, using 293 cells ectopically expressing the cloned hPAFR. We found (**Fig. 3**) that PAF-like activity could be detected by 30

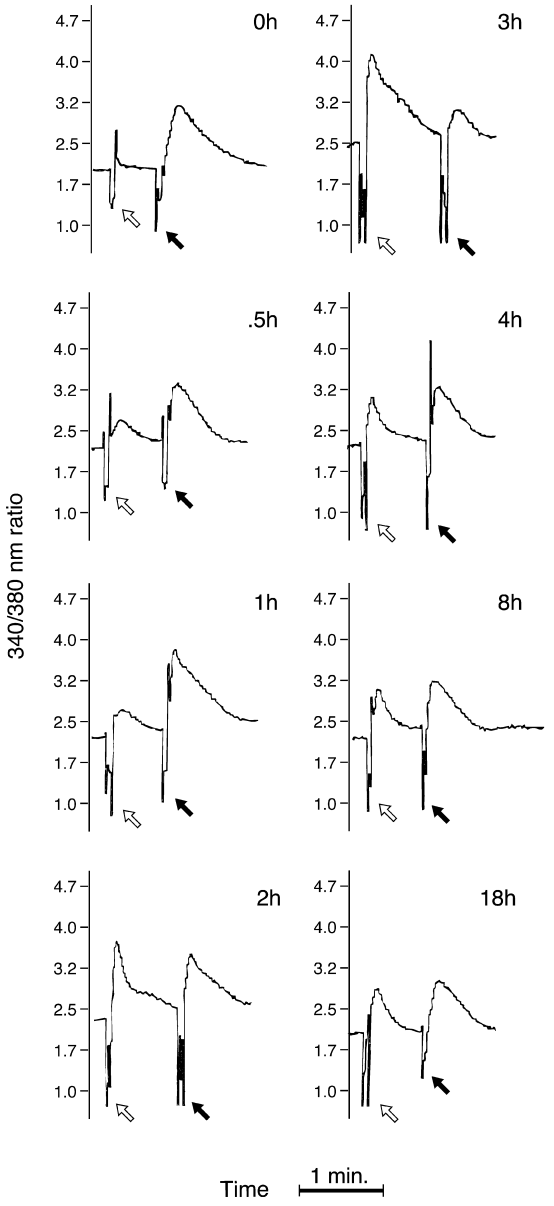

Fig. 3. Formation of PAF-like activity is an early event in acellular low density lipoprotein (LDL) oxidation. The rate of accumulation of PAF-like activity in oxidized LDL was measured by examining changes in intracellular calcium levels in Fura-2AM-loaded hPAFR 293 cells that ectopically express the hPAFR. LDL was isolated from human blood by density flotation and oxidized with copper for the stated periods of time before PAF-like phospholipids were isolated as described in Materials and Methods. An equal aliquot of this HPLC-purified material was added to the Ca^{2+}-reporting hPAFR 293 cells (open arrow), and the extent of PAF receptor desensitization was subsequently tested by adding $10^{-}10$ M PAF (filled arrow). There is a time-dependent accumulation of a PAF receptor agonist that accordingly induces homologous desensitization of the PAF receptor.

min, was maximal after 2 h of oxidation, remained at this level for 2 h, and then declined to a constant level that was maintained for at least 18 h. This parallels esterified isoprostane formation under these same conditions, where isoprostane accumulation is just detectable by 60 min of Cu^{+} oxidation and maximal by 90 min (22).

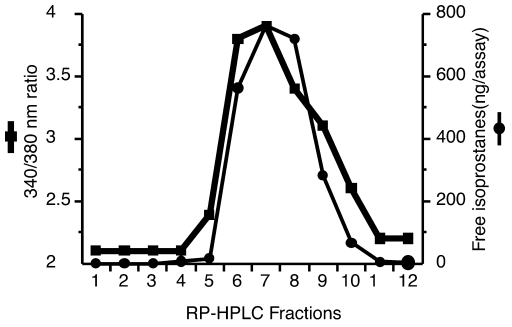

Fig. 4. PAF-like bioactivity and iPC coelute during RP-HPLC. Polar phospholipids from CCl_4-treated rat liver were purified by reversed-phase HPLC, and aliquots of the collected fractions were examined for their effect on Fura-2AM-loaded hPAFR 293 cells (squares) or subjected to alkaline hydrolysis to quantitate isoprostane content (circles) as described in Materials and Methods.

PAF-like activity and iPC cochromatograph during HPLC

The polar phospholipids derived from the extracts of CCl_4-exposed liver were isolated by reversed-phase HPLC and the elution profile of PAF-like activity was compared with that of esterified isoprostanes. This experiment showed that the major portion of the PAF-like bioactivity eluted 6, 7, and 8 min after injection (**Fig. 4**), which resembled the elution profile of the PAF-like lipids isolated from oxidized LDL (10, 32). Quantitation of the isoprostane content of these fractions by GC-MS showed that the elution profile of iPC mirrored PAF-like activity (Fig. 4).

Phosphatidylcholines with PAF-like activity and iPC differ at the *sn*-1 position

The results to this point do not define whether isoprostane-containing phospholipids themselves are PAF receptor agonists or whether PAF-like lipids copurify with these isoprostane reservoirs, an issue resolved as follows. The PAF-like lipids in oxidized LDL are all alkyl phosphatidylcholines (10), and therefore are resistant to phospholipase A_1 digestion. In contrast, rat liver phosphatidylcholines are virtually all diacyl phospholipids with only trace amounts of alkyl phosphatidylcholines (33). To explore whether the PAF-like activity found in CCl_4-poisoned livers was derived from this trace pool of alkyl phosphatidylcholines, we treated the individual fractions with phospholipase A_1. The controls show this protocol effectively distinguishes PAF (**Fig. 5A**) from the less potent acyl analogue (Fig. 5B). After hydrolysis of diacyl phosphatidylcholines, followed by chromatography on a normal-phase system to remove the reaction products, it was apparent that nearly all the biologic activity found in iPC preparations was recovered at the end of the procedure (Fig. 5C).

In sharp contrast to the results of the foregoing analysis of PAF-like activity, when we analyzed these phosphatidylcholines by electrospray liquid chromatography (LC)-MS, we found (**Fig. 6**) that virtually all the esterified isoprostanes were removed from the iPC preparation by phospholipase A_1 treatment. Before phospholipase A_1 digestion, the most abundant peaks in the analysis of total liver-derived iPC

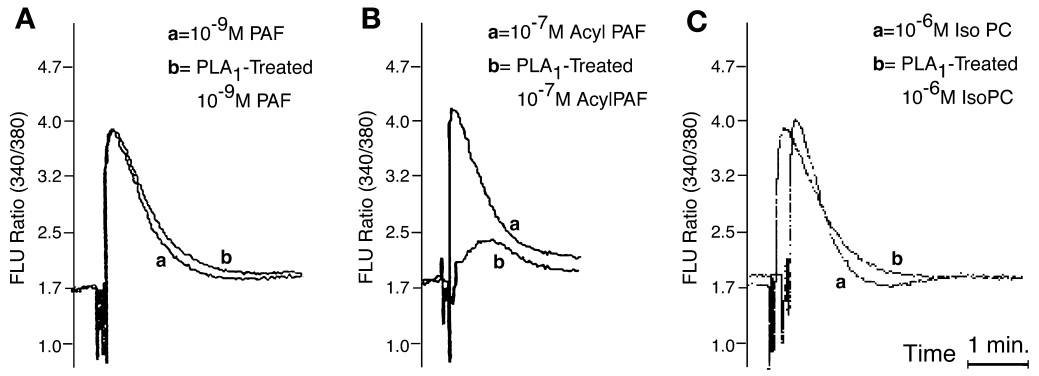

Fig. 5. PAF-like activity in CCl_4-exposed liver is resistant to phospholipase A_1 treatment. PAF as a negative control (A) or its acyl homolog (B) as a positive control were treated with phospholipase A_1 (PLA_1) and tested as Ca^{2+}-mobilizing agonists in hPAFR 293 cells. The iPC preparation from CCl_4-treated rat liver was similarly treated (or not treated) with phospholipase A_1 and tested (C) for its ability to stimulate intracellular Ca^{2+} accumulation in FURA-2AM-loaded hPAFR 293 cells. IsoPC, Isoprostane-containing phosphatidylcholine.

were positive ions at *m/z* 860.6 and 832.6, corresponding to 1-stearoyl-2-(F_2-isoprostanoyl)-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-(F_2-isoprostanoyl)-*sn*-glycero-3-phosphocholine, respectively. Other abundant oxidized components, quite possibly corresponding to the 1-palmitoyl 2-neuroprostane-*sn*-glycero-3-phosphocholine (34) and 1-palmitoyl hydroxylheptadecatrienoyl phosphatidylcholine, were detected at *m/z* 856.6 and 758.6, respectively. It is notable that all are polar phospholipids, and all were completely destroyed by phospholipase A_1 digestion (Fig. 6, inset). Thus, diacyl isoprostane-containing phosphatidylcholines or other diacyl polar phospholipids are not the PAF receptor agonists found in the livers of CCl_4-exposed animals.

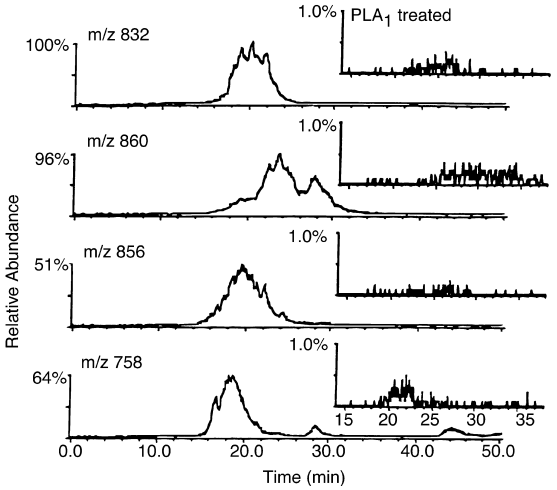

Fig. 6. iPC in CCl_4-exposed liver is sensitive to phospholipase A_1 (PLA_1) treatment. Reversed-phase liquid chromatography-mass spectrometry (LC-MS) of the iPC from CCl_4-treated rat liver was performed by electrospray ionization and monitoring of specific ions for phospholipid molecular species: *m/z* 832.6 corresponding to palmitoyl F_2-isoprostanoyl glycerophosphocholine; *m/z* 860.6 corresponding to stearoyl F_2-isoprostanoyl glycerophosphocholine; *m/z* 856.6 corresponding to palmitoyl docosapentenoyl (plus O_3) glycerophosphocholine; and *m/z* 758.6 corresponding to palmitoyl hydroxyheptadecatrienoyl glycerophosphocholine. Insets correspond to the identical ions monitored after PLA_1 treatment of the sample. These data show that all the detectable iPC is derived from diacyl phosphatidylcholines.

Identification of PAF in CCl_4-exposed liver

We searched for the identity of the inflammatory phospholipid(s) found in CCl_4-exposed livers by purifying the material remaining after phospholipase A_1 treatment, using reversed-phase HPLC and analysis by MS/MS. One abundant molecular ion in these polar liver phosphatidylcholines was observed at *m/z* 524 (**Fig. 7**), a mass-to-charge ratio that corresponds to $[M+H]^+$ for PAF, (1-*O*-hexadecyl-2-acetyl)phosphatidylcholine. This tentative identification was confirmed by derivatization and analysis by GC-MS (inset), and comparison of HPLC retention times with that of authentic PAF. This quantitative approach revealed the presence of nanogram amounts of PAF in the iPC preparation. A second peak of PAF-like activity eluting at 22.7 min (Fig. 7) in the polar phospholipids purified from liver lipids eluted after PAF, and analysis of this smaller amount of activity by electrospray MS/MS revealed the presence of a component that generated a protonated $[MH^+]$ ion at *m/z* 552. This corresponds to the previously identified (10) C_4 butanoyl analogue of PAF. The abundance of this C_4 PAF analogue, relative to the PAF in this sample as measured by GC-MS, could be readily assessed from the relative abundance of the corresponding $[M+H]^+$ precursor ions that formed *m/z* 184 after collision-induced dissociation (35) and the area of the corresponding LC-MS/MS response (Fig. 7A and D). The butanoyl analogue of PAF was estimated to be 24.3% as abundant, on a molar basis, as PAF present in this extract. This suggests the inflammatory activity in CCl_4-inflamed liver was predominantly the result of cellular PAF synthesis for two reasons. First, the C_4 butanoyl analogue of PAF is produced in far greater abundance than PAF during the oxidative fragmentation of phospholipid peroxides (10), so the ratio of their relative abundance is inverted in CCl_4-exposed liver. Second, the C_4 butanoyl analogue of PAF is only about 10% as po-

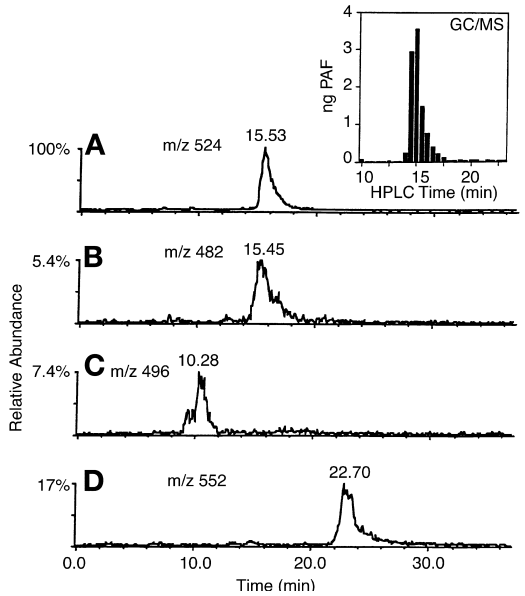

Fig. 7. PAF and butanoyl PAF are present in CCl_4-metabolizing liver. Reversed-phase LC-MS of the iPC preparations extracted from CCl_4-exposed rat liver after phospholipase A_1 treatment was performed, using electrospray ionization monitoring and MS/MS monitoring of those precursor ions of m/z 184 (phosphocholine) after collisional activation. A: m/z 524.5 corresponding to PAF. B: m/z 482.4 corresponding to the loss of ketene from m/z 524, a specific ion loss for PAF. C: m/z 496.4 corresponding to palmitoyl glycerophosphocholine. D: m/z 552.4 corresponding to butanoyl PAF. Inset: Histogram of quantitative gas chromatography-MS analysis of PAF after its hydrolysis with phospholipase C and derivatization with pentafluorobenzoyl chloride. The material analyzed was from 10% of the reversed-phase LC-MS fraction.

tent as PAF as an agonist for the PAF receptor (10), so only a few percent, at best, of the total biologic activity could come from this PAF-analogue.

DISCUSSION

CCl_4 metabolism by hepatocytes, but also by endogenous macrophage-like Kupffer cells (36), results in severe hepatic necrosis and fibrosis. Liver injury in this established model derives from a mixture of free radicals and reactive oxygen species (25, 37), lipid peroxidation (26, 38), activated Kupffer cells (39), and infiltrating PMN (36, 40, 41), each of which has an underlying role in the resulting liver damage. For instance, selective blockade of selectin-mediated leukocyte influx (42), depletion of leukocytes with anti-PMN antibody (36), loss of the anti-inflammatory cytokine interleukin 10 (40, 41), and selective depletion of Kupffer cells with $GdCl_3$ (36, 39) all ameliorate hepatic damage in this model. Although activated leukocytes appear to be the direct cause of much of damage, the agonists for these effector cells in this established model of oxidative liver damage are unknown.

iPC are formed in abundance by liver metabolizing CCl_4 (13, 27). These are primarily of the F_2 class as endogenous substances reduce the isoprostane endoperoxide to F-ring compounds. D- and E-ring compounds are more abundant in the absence of reductants, as is the case for iPC isolated from LDL oxidized in the absence of reductants (15). This E-ring iPC preparation was inflammatory and stimulated monocyte-endothelial cell interactions through a G protein-linked receptor (15). A PAF receptor antagonist is effective in blocking bioactivity in this system, even though PAF itself is inactive (43). This information implicates the PAF receptor, but indicates that the relevant mediator was not PAF. The G protein-linked PAF receptor generally has a stringent requirement for the *sn*-2 acetyl residue of PAF, but we and others find that a modest increase in length to butanoyl residues or the presence of an ω-oxy function yields potent PAF receptor ligands and activators (6). The potential for phosphatidylcholines with longer, but highly modified, *sn*-2 residues, such as F_2 isoprostanes, to function as PAF receptor agonists is unknown. This became an important issue when we found that PAF receptor agonists and iPC were formed over the same time period, and that these activities comigrate.

We found that HPLC purification of iPC isolated from the livers of rats exposed to CCl_4 yields material that potently activated both human neutrophils and 293 cells stably expressing the hPAFR. Thus CCl_4 metabolism generates agents that attract and activate inflammatory cells that are critical in this model of hepatic injury. In light of the reported bioactivity of an E-ring iPC (15), we anticipated that the abundant F-ring iPC formed in livers metabolizing CCl_4 would be recognized by the PAF receptor and would define a new class of inflammatory mediators. Instead we found that virtually all the iPC were diacyl species that were sensitive to phospholipase A_1 digestion, but that none of the PAF receptor agonists were of this class. The latter point was expected as the PAF receptor has a several hundred-fold preference for *sn*-1 alkyl residues that are not substrates for phospholipase A_1 hydrolysis. Nevertheless, the disparity in lipase sensitivity of the iPC and PAF receptor agonists showed that iPC were not the active components in the livers of rats metabolizing CCl_4.

The paucity of alkyl iPC resistant to phospholipase A_1 digestion in the extracts of rat liver is not surprising in that their precursor, alkyl PC, is nearly undetectable in this tissue (33, 44). The situation in humans may differ from that of rats as, unlike the rat, the human liver pool of phosphatidylcholine contains 3.5% alkyl phosphatidylcholine (33). These less abundant phospholipids should oxidize in parallel with their diacyl analogues to yield alkyl iPC, which might possess biologic activity, and fragmented PAF-like phospholipids that will be biologically active. We expect this as even where alkyl phosphatidylcholines comprise just 0.5% of the total phosphatidylcholine pool, as they do in human LDL, we find that all the biologically active PAF-like lipids formed during oxidation are derived from these rare alkyl phosphatidylcholines (10). Thus, we anticipate that human liver subjected to severe oxidant stress should contain oxidatively modified phospholipids with PAF-like activity as well as the authentic PAF we find in the rat model. In fact, we did find small amounts of the C_4 analogue of PAF in the CCl_4-exposed rat liver, and both

this analogue and PAF can be generated from the oxidative fragmentation of alkyl arachidonoyl phosphatidylcholine (10, 45). However, the formation of PAF is not a preferred oxidative reaction and far more *sn*-2 butanoyl and butenoyl residues accumulate than acetyl residues (10, 46). The ratio of acetyl to butanoyl residues found in CCl_4-exposed liver was grossly inverted from this, so cellular generation of PAF is the major source of inflammatory lipids, at least in the rat model.

One might question how it is that PAF, a modified alkyl PC, is found in this rat model of liver injury when there is insufficient alkyl PC to produce alkyl iPC. The answer to this likely derives from the distribution of the limited amount of alkyl PC. Whereas rat hepatocytes may not contain this precursor for PAF synthesis, cells such as Kupffer cells, endothelial cells, platelets, and infiltrating monocytes that synthesize PAF do. For leukocytes, up to half of the phosphatidylcholine pool is alkyl phosphatidylcholine (47). Whether infiltrating leukocytes could be a significant source is currently unclear as the earliest reported (40, 41) time point for a CCl_4-induced neutrophilic influx was 12 h; nevertheless, these cells could aid in the further recruitment of an inflammatory response at later times.

The question then becomes how these cells were stimulated to produce PAF. CCl_4 metabolism by cytochrome P-450 generates the reactive trichloromethyl radical, and this event generates superoxide and hydrogen peroxide (25). Hydrogen peroxide stimulates the synthesis of PAF (30, 48, 49), and PAF is synthesized by stimulated macrophages (50) and Kupffer cells (51). The lyso-PAF acetyltransferase responsible for the bulk of PAF synthesis in stimulated cells remains unidentified, but it is known to be highly dependent on the rapid increase in intracellular Ca^{2+} in appropriately stimulated cells (52). Because CCl_4 metabolism elevates in vivo liver Ca^{2+} levels and Ca^{2+} channel blockers normalize markers of liver function in this hepatotoxic model (53), calcium-dependent activation of PAF synthesis may occur in Kupffer cells under these conditions.

Inappropriate or excessive PAF has the potential to modify liver function, and PAF is an established hepatotoxic agent (54, 55). PAF stimulates and primes liver Kupffer cells and neutrophils for an exaggerated production of superoxide (56), and it is a juxtacrine signaling molecule produced by activated endothelial cells in the recruitment of leukocytes to the extravascular compartment (57). Inappropriate production of PAF leads to pathologic inflammation, and PAF clearly is involved in liver damage as PAF receptor antagonists reduce hepatic damage after ischemia/reperfusion (28, 58), transplant (59, 60), and lipopolysaccharide-induced (61) injury. We conclude that the liver injury induced by CCl_4 metabolism is the inappropriate production of the powerful inflammatory and hepatotoxic lipid mediator PAF in common with these other routes to liver damage. We also conclude that even in a model where the precipitating insult is the abundant formation of free radicals that activated cellular synthesis of inflammatory mediators, rather than the uncontrolled oxidative fragmentation of phospholipids to PAF-like mimetics, is the main source of inflammatory mediators that promote and extend organ damage.

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REFERENCES

1. Shimizu, T., Z. Honda, M. Nakamura, H. Bito, and T. Izumi. 1992. Platelet-activating factor receptor and signal transduction. *Biochem. Pharmacol.* **44:** 1001–1008.
2. Prescott, S. M., G. A. Zimmerman, D. M. Stafforini, and T. M. McIntyre. 2000. Platlet-activating factor and related lipid mediators. *Annu. Rev. Biochem.* **69:** 419–445.
3. Zimmerman, G. A., S. M. Prescott, and T. M. McIntyre. 1995. Oxidatively fragmented phospholipids as inflammatory mediators: the dark side of polyunsaturated lipids. *J. Nutr.* **125:** S1661–S1665.
4. 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.
5. Watson, A. D., N. Leitinger, M. Navab, K. F. Faull, S. Horkko, J. L. Witztum, W. Palinski, D. Schwenke, R. G. Salomon, W. Sha, G. Subbanagounder, A. M. Fogelman, and J. A. Berliner. 1997. Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. *J. Biol. Chem.* **272:** 13597–13607.
6. McIntyre, T. M., G. A. Zimmerman, and S. M. Prescott. 1999. Biologically active oxidized phospholipids. *J. Biol. Chem.* **274:** 25189–25192.
7. Tokumura, A., K. Kamiyasu, K. Takauchi, and H. Tsukatani. 1987. Evidence for existence of various homologues and analogues of platelet activating factor in a lipid extract of bovine brain. *Biochem. Biophys. Res. Commun.* **145:** 415–425.
8. 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.
9. 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.
10. 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.
11. Morrow, J. D., Y. Chen, C. J. Brame, J. Yang, S. C. Sanchez, J. Xu, W. E. Zackert, J. A. Awad, and L. J. Roberts. 1999. The isoprostanes: unique prostaglandin-like products of free-radical-initiated lipid peroxidation. *Drug Metab. Rev.* **31:** 117–139.
12. Lawson, J. A., J. Rokach, and G. A. FitzGerald. 1999. Isoprostanes: formation, analysis and use as indices of lipid peroxidation in vivo. *J. Biol. Chem.* **274:** 24441–24444.
13. Morrow, J. D., J. A. Awad, H. J. Boss, I. A. Blair, and L. J. Roberts. 1992. Non-cyclooxygenase-derived prostanoids (F2-isoprostanes) are formed in situ on phospholipids. *Proc. Natl. Acad. Sci. USA.* **89:** 10721–10725.
14. Morrow, J. D., K. E. Hill, R. F. Burk, T. M. Nammour, K. F. Badr, and L. J. Roberts. 1990. A series of prostaglandin F2-like compounds are produced *in vivo* in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. *Proc. Natl. Acad. Sci. USA.* **87:** 9383–9387.
15. Watson, A. D., G. Subbanagounder, D. S. Welsbie, K. F. Faull, M. Navab, M. E. Jung, A. M. Fogelman, and J. A. Berliner. 1999. Struc-

tural identification of a novel pro-inflammatory epoxyisoprostane phospholipid in mildly oxidized low density lipoprotein. *J. Biol. Chem.* **274:** 24787–24798.
16. Patel, K. D., G. A. Zimmerman, S. M. Prescott, and T. M. McIntyre. 1992. Novel leukocyte agonists are released by endothelial cells exposed to peroxide. *J. Biol. Chem.* **267:** 15168–15175.
17. Imaizumi, T., K. Satoh, H. Yoshida, H. Kawamura, M. Hiramoto, and S. Takamatsu. 1991. Effect of cigarette smoking on the levels of platelet-activating factor-like lipid(s) in plasma lipoproteins. *Atherosclerosis.* **87:** 47–55.
18. Lehr, H. A., A. S. Weyrich, R. K. Saetzler, A. Jurek, K. E. Arfors, G. A. Zimmerman, S. M. Prescott, and T. M. McIntyre. 1997. Vitamin C blocks inflammatory PAF mimetics created by cigarette smoking. *J. Clin. Invest.* **99:** 2358–2364.
19. Frey, B., R. Haupt, S. Alms, G. Holzmann, T. Konig, H. Kern, W. Kox, B. Rustow, and M. Schlame. 2000. Increase in fragmented phosphatidylcholine in blood plasma by oxidative stress. *J. Lipid Res.* **41:** 1145–1153.
20. Morrow, J. D., B. Frei, A. W. Longmire, J. M. Gaziano, S. M. Lynch, Y. Shyr, W. E. Strauss, J. A. Oates, and L. J. Roberts. 1995. Increase in circulating products of lipid peroxidation (F_2-isoprostanes) in smokers. *N. Engl. J. Med.* **332:** 1198–1203.
21. Pratico, D., O. P. Barry, J. A. Lawson, M. Adiyaman, S. W. Hwang, S. P. Khanapure, L. Iuliano, J. Rokach, and G. A. FitzGerald. 1998. IPF2alpha-I: an index of lipid peroxidation in humans. *Proc. Natl. Acad. Sci. USA.* **95:** 3449–3454.
22. Lynch, S. M., J. D. Morrow, L. J. Roberts II, and B. Frei. 1994. Formation of non-cyclooxygenase-derived prostanoids (F_2-isoprostanes) in plasma and low density lipoprotein exposed to oxidative stress in vitro. *J. Clin. Invest.* **93:** 998–1004.
23. Mallat, Z., T. Nakamura, J. Ohan, G. Leseche, A. Tedgui, J. Maclouf, and R. C. Murphy. 1999. The relationship of hydroxyeicosatetraenoic acids and F2-isoprostanes to plaque instability in human carotid atherosclerosis. *J. Clin. Invest.* **103:** 421–427.
24. Pratico, D., L. Iuliano, A. Mauriello, L. Spagnoli, J. A. Lawson, J. Rokach, J. Maclouf, F. Violi, and G. A. FitzGerald. 1997. Localization of distinct F2-isoprostanes in human atherosclerotic lesions. *J. Clin. Invest.* **100:** 2028–2034.
25. el Sisi, A. E., D. L. Earnest, and I. G. Sipes. 1993. Vitamin A potentiation of carbon tetrachloride hepatotoxicity: role of liver macrophages and active oxygen species. *Toxicol. Appl. Pharmacol.* **119:** 295–301.
26. Terao, J., I. Asano, and S. Matsushita. 1984. High-performance liquid chromatographic determination of phospholipid peroxidation products of rat liver after carbon tetrachloride administration. *Arch. Biochem. Biophys.* **235:** 326–333.
27. Morrow, J. D., J. A. Awad, T. Kato, K. Takahashi, K. F. Badr, L. J. Roberts, and R. F. Burk. 1992. Formation of novel non-cyclooxygenase-derived prostanoids (F2-isoprostanes) in carbon tetrachloride hepatotoxicity. An animal model of lipid peroxidation. *J. Clin. Invest.* **90:** 2502–2507.
28. Horie, Y., R. Wolf, J. Russell, T. P. Shanley, and D. N. Granger. 1997. Role of Kupffer cells in gut ischemia/reperfusion-induced hepatic microvascular dysfunction in mice. *Hepatology.* **26:** 1499–1505.
29. Villamediana, L. M., E. Sanz, S. Fernandez-Gallardo, C. Caramelo, M. Sanchez Crespo, P. Braquet, and J. M. Lopez-Novoa. 1986. Effects of the platelet-activating factor antagonist BN 52021 on the hemodynamics of rats with experimental cirrhosis of the liver. *Life Sci.* **39:** 201–205.
30. Lewis, M. S., R. E. Whatley, P. Cain, T. M. McIntyre, S. M. Prescott, and G. A. Zimmerman. 1988. Hydrogen peroxide stimulates the synthesis of platelet-activating factor by endothelium and induces endothelial cell-dependent neutrophil adhesion. *J. Clin. Invest.* **82:** 2045–2055.
31. 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.
32. 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.
33. Diagne, A., J. Fauvel, M. Record, H. Chap, and L. Douste-Blazy. 1984. Studies on ether phospholipids: Comparative composition of various tissues from human, rat and guinea pig. *Biochim. Biophys. Acta.* **793:** 221–231.
34. Roberts II, L. J., T. J. Montine, W. R. Markesbery, A. R. Tapper, P. Hardy, S. Chemtob, W. D. Dettbarn, and J. D. Morrow. 1998. Formation of isoprostane-like compounds (neuroprostanes) in vivo from docosahexaenoic acid. *J. Biol. Chem.* **273:** 13605–13612.
35. Brugger, B., G. Erben, R. Sandhoff, F. T. Wieland, and W. D. Lehmann. 1997. Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry [published erratum appears in *Proc. Natl. Acad. Sci. USA.* 1999. **96:** 10943]. *Proc. Natl. Acad. Sci. USA.* **94:** 2339–2344.
36. Badger, D. A., J. M. Sauer, N. C. Hoglen, C. S. Jolley, and I. G. Sipes. 1996. The role of inflammatory cells and cytochrome P450 in the potentiation of CCl4-induced liver injury by a single dose of retinol. *Toxicol. Appl. Pharmacol.* **141:** 507–519.
37. Sipes, I. G., A. E. el Sisi, W. W. Sim, S. A. Mobley, and D. L. Earnest. 1991. Reactive oxygen species in the progression of CCl_4-induced liver injury. *Adv. Exp. Med. Biol.* **283:** 489–497.
38. el Sisi, A. E., D. L. Earnest, and I. G. Sipes. 1993. Vitamin A potentiation of carbon tetrachloride hepatotoxicity: enhanced lipid peroxidation without enhanced biotransformation. *Toxicol. Appl. Pharmacol.* **119:** 289–294.
39. Edwards, M. J., B. J. Keller, F. C. Kauffman, and R. G. Thurman. 1993. The involvement of Kupffer cells in carbon tetrachloride toxicity. *Toxicol. Appl. Pharmacol.* **119:** 275–279.
40. Louis, H., J. L. Van Laethem, W. Wu, E. Quertinmont, C. Degraef, K. Van den Berg, A. Demols, M. Goldman, O. Le Moine, A. Geerts, and J. Deviere. 1998. Interleukin-10 controls neutrophilic infiltration, hepatocyte proliferation, and liver fibrosis induced by carbon tetrachloride in mice. *Hepatology.* **28:** 1607–1615.
41. Thompson, K., J. Maltby, J. Fallowfield, M. McAulay, H. Millward-Sadler, and N. Sheron. 1998. Interleukin-10 expression and function in experimental murine liver inflammation and fibrosis. *Hepatology.* **28:** 1597–1606.
42. Kajihara, J., Y. Guoji, K. Kato, and Y. Suzuki. 1995. Sulfatide, a specific sugar ligand for L-selectin, blocks CCl_4-induced liver inflammation in rats. *Biosci. Biotechnol. Biochem.* **59:** 155–157.
43. Leitinger, N., A. D. Watson, K. F. Faull, A. M. Fogelman, and J. A. Berliner. 1997. Monocyte binding to endothelial cells induced by oxidized phospholipids present in minimally oxidized low density lipoprotein is inhibited by a platelet activating factor receptor antagonist. *Adv. Exp. Med. Biol.* **433:** 379–382.
44. Curstedt, T. 1977. Analysis of molecular species of ether analogues of phosphatidylcholines from biological samples. *Biochim. Biophys. Acta.* **489:** 79–88.
45. 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.
46. Tanaka, T., H. Minamino, S. Unezaki, H. Tsukatani, and A. Tokumura. 1993. Formation of platelet-activating factor-like phospholipids by Fe^{2+}/ascorbate/EDTA-induced lipid peroxidation. *Biochim. Biophys. Acta.* **1166:** 264–274.
47. Mueller, H. W., J. T. O'Flaherty, D. G. Greene, M. P. Samuel, and R. L. Wykle. 1984. 1-*O*-alkyl-linked glycerophospholipids of human neutrophils: Distribution of arachidonate and other acyl residues in the ether-linked and diacyl species. *J. Lipid Res.* **25:** 383–388.
48. Gasic, A. C., G. McGuire, S. Krater, A. I. Farhood, M. A. Goldstein, C. W. Smith, M. L. Entman, and A. A. Taylor. 1991. Hydrogen peroxide pretreatment of perfused canine vessels induces ICAM-1 and CD18-dependent neutrophil adherence. *Circulation.* **84:** 2154–2166.
49. Hughes, H., M. A. Sands, G. M. McGuire, and A. A. Taylor. 1994. PAF formation by H2O2-stimulated perfused canine carotid arteries. *Prostaglandins Leukot. Essent. Fatty Acids.* **51:** 323–328.
50. Elstad, M. R., S. M. Prescott, T. M. McIntyre, and G. A. Zimmerman. 1988. Synthesis and release of platelet-activating factor by stimulated human mononuclear phagocytes. *J. Immunol.* **140:** 1618–1624.
51. Chao, W., A. Siafaka-Kapadai, M. S. Olson, and D. J. Hanahan. 1989. Biosynthesis of platelet-activating factor by cultured rat Kupffer cells stimulated with calcium ionophore A23187. *Biochem. J.* **257:** 823–829.
52. Whatley, R. E., P. Nelson, G. A. Zimmerman, D. L. Stevens, C. J. Parker, T. M. McIntyre, and S. M. Prescott. 1989. The regulation of platelet-activating factor production in endothelial cells. The role of calcium and protein kinase C. *J. Biol. Chem.* **264:** 6325–6333.
53. Romero, G., B. Lasheras, L. Sainz Suberviola, and E. Cenarruzabe-

itia. 1994. Protective effects of calcium channel blockers in carbon tetrachloride-induced liver toxicity. *Life Sci.* **55:** 981–990.
54. Zhou, W., W. Chao, B. A. Levine, and M. S. Olson. 1992. Role of platelet-activating factor in hepatic responses after bile duct ligation in rats. *Am. J. Physiol.* **263:** G587–G592.
55. Zhou, W., M. O. McCollum, B. A. Levine, and M. S. Olson. 1992. Inflammation and platelet-activating factor production during hepatic ischemia/reperfusion. *Hepatology.* **16:** 1236–1240.
56. Bautista, A. P., and J. J. Spitzer. 1992. Platelet activating factor stimulates and primes the liver, Kupffer cells and neutrophils to release superoxide anion. *Free Radic. Res. Commun.* **17:** 195–209.
57. Zimmerman, G. A., D. E. Lorant, T. M. McIntyre, and S. M. Prescott. 1993. Juxtacrine intercellular signaling: another way to do it. *Am. J. Respir. Cell Mol. Biol.* **9:** 573–577.
58. Yamakawa, Y., M. Takano, M. Patel, N. Tien, T. Takada, and G. B. Bulkley. 2000. Interaction of platelet activating factor, reactive oxygen species generated by xanthine oxidase, and leukocytes in the generation of hepatic injury after shock/resuscitation. *Ann. Surg.* **231:** 387–398.
59. Walcher, F., I. Marzi, R. Fischer, M. Bauer, and R. Larsen. 1996. Platelet-activating factor is involved in the regulation of pathological leukocyte adhesion after liver transplantation. *J. Surg. Res.* **61:** 244–249.
60. Fukunaga, K., Y. Takada, H. Taniguchi, K. Yuzawa, M. Otsuka, T. Todoroki, K. Goto, and K. Fukao. 1998. Protecting the viability of hepatic allografts procured from non-heart-beating donors by blockade of endothelin and platelet activating factor in porcine liver transplantation. *Int. Surg.* **83:** 226–231.
61. Sakaguchi, T., S. Nakamura, S. Suzuki, T. Oda, A. Ichiyama, S. Baba, and T. Okamoto. 1999. Participation of platelet-activating factor in the lipopolysaccharide-induced liver injury in partially hepatectomized rats. *Hepatology.* **30:** 959–967.