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Biosynthesis of platelet-activating factor (PAF) in human polymorphonuclear leucocytes

The role of lyso-PAF disposal and free arachidonic acid

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Theophylline and 1-methyl-3-isobutylxanthine (MIX), compounds that block eicosanoid formation and modulate phospholipase A_2 activity, inhibited in a dose-dependent manner the formation of both leukotriene B_4 (LTB_4) and platelet-activating factor (PAF) by human polymorphonuclear leucocytes (PMN) in response to ionophore A23187. Theophylline and MIX lacked any inhibitory effect on acetyl-CoA: lyso-PAF acetyltransferase activity, which is the rate-limiting step for PAF biosynthesis in PMN. The effect of theophylline and MIX on PAF formation could be reversed by incubating the cells in the presence of 1–10 μM exogenous lyso-PAF. Incubation of PMN homogenates in the presence of unsaturated non-esterified fatty acids resulted in dose-dependent inhibition of the acetyltransferase. This effect was linked to the presence of a free carboxyl group, since both arachidonic acid methyl ester and palmitoyl-arachidonoyl phosphatidylcholine lacked inhibitory activity. This inhibitory effect was also dependent on the number of double bonds, since arachidonic acid ($C_{20:4}$) and eicosapentaenoic acid ($C_{20:5}$) displayed maximal effect. Kinetic analysis showed that the effect of arachidonic acid was consistent with competitive inhibition, with a K_i value of about 19 μM. Oxidative metabolites of arachidonic acid showed a lesser inhibitory effect with the following order of potency: arachidonic acid > 15-HETE (15-hydroxy-6,8,11,14-eicosatetraenoic acid) > LTB_4 > 5-HETE (5-hydroxy-6,8,11,14-eicosatetraenoic acid) > lipoxin A_4. Examination of enzymes involved in CoA-dependent acylation revealed a low activity of both arachidonoyl-CoA synthetase and arachidonoyl-CoA: lyso-PAF arachidonoyltransferase. These data indicate a strong influence on PAF biosynthesis of the products of the phospholipase A_2 reaction, with lyso-PAF disposal being a critical event for PAF formation, and unsaturated fatty acids acting as feed-back inhibitors. The conversion of arachidonic acid via oxidative metabolism into less active inhibitors of acetyl-CoA:lyso-PAF acetyltransferase seems to be an additional mechanism of modulation of this enzyme activity, linked to the function of lipoxygenases. Finally, the enzyme activities involved in arachidonoyl-CoA-dependent acylation of lyso-PAF show a low efficiency in capturing arachidonic acid.

INTRODUCTION

Activation of polymorphonuclear leucocytes (PMN) on interaction with secretagogues initiates the biosynthesis of eicosanoids, especially leukotriene B_4 (LTB_4) in intact cells [1], and platelet-activating factor (PAF) [2–4]. These lipid mediators show a number of similarities, e.g. biosynthesis from common precursors, induction or/and modulation of their biosynthesis by each other, and overlapping spectra of biological activities.

The compound 1-alkyl-2-arachidonoyl-*sn*-3-glycerophosphocholine is a common precursor for the biosynthesis of eicosanoids and of PAF in PMN [5,6]. Accordingly, a phospholipase A_2 (EC 3.1.1.4) acting on this compound [7] produces both arachidonic acid to be used for the synthesis of eicosanoids, and lyso-PAF to be utilized in the remodelling pathway for the synthesis of PAF by an acetyl-CoA:lyso-PAF acetyltransferase (1-alkylglycerophosphocholine acetyltransferase; EC 2.3.1.67). Current views stress the role of phospholipase A_2 and 5-lipoxygenase activities [8–10] as rate-limiting for the synthesis of LTB_4, whereas acetyl-CoA: lyso-PAF acetyltransferase [11] seems to be limiting step in the formation of PAF, since only those secretagogues that activate this enzyme via a phosphorylation–dephosphorylation mechanism initiate the biosynthesis of PAF in PMN, macrophages and exocrine secretory glands [12–19].

Moreover, some 5-lipoxygenase products have been reported to modulate PAF biosynthesis by acting at the phospholipase A_2 level [20,21], and unsaturated non-esterified fatty acids are either reversible and non-competitive inhibitors of phospholipase A_2 by a mechanism that is best explained by an interaction at an allosteric site(s) [22], or reversible and competitive inhibitors [23]. A study by Ramesha & Pickett [24] has shown a significant diminution of the generation of both PAF and LTB_4 in PMN from rats depleted of arachidonic acid and the almost complete recovery of the generation of these mediators on addition of exogenous arachidonic acid. In another study, the production of PAF by human monocytes was enhanced by exogenous arachidonic acid and diminished by eicosapentaenoic acid [25], a prominent component of diets enriched with fish oil, the metabolism of which is associated with inhibition of LTB_4 generation from membrane-derived arachidonic acid and with conversion to chemotactically inactive 5-hydroxyeicosapentanoic acid or attenuated LTB_5 [26]. So far, two explanations for this effect of arachidonic acid have been provided: either (1) a 1-*O*-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine precursor is required for PAF biosynthesis [24] or (2) lipoxygenase products of arachidonic acid modulate PAF biosynthesis by enhancing the expression of phospholipase A_2 [20,21]. This study is intended to characterize some factors that can influence PAF formation

Abbreviations used: IC_{50}, concentration causing half-maximal inhibition; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 5-HPETE, 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid; LTB_4, leukotriene B_4; MIX, 1-methyl-3-isobutylxanthine; PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); lyso-PAF, 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine; PMN, polymorphonuclear leucocytes.

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through the remodelling pathway acting at the acetyl-CoA: lyso-PAF acetyltransferase level, and that are independent of the already characterized mechanism of modulation of this enzyme activity linked to a phosphorylation–dephosphorylation reaction.

In this paper we report the following: (1) the effect of methylxanthines, which modulate phospholipase A_2 activity, on the generation of both LTB_4 and PAF; (2) the partial reversal by exogenous lyso-PAF of the inhibition of PAF production caused by methylxanthines; (3) the effect of non-esterified fatty acids as feed-back inhibitors of PAF biosynthesis via the acetyl-CoA:lyso-PAF acetyltransferase reaction; and (4) the possible influence of arachidonoyl-CoA synthetase and acyl-CoA: lyso-phosphatide acyltransferase activities on PAF biosynthesis by diverting arachidonic acid into membrane phospholipids.

MATERIALS AND METHODS

Materials

1-Methyl-3-isobutylxanthine (MIX), theophylline, 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), methyl arachidonate, and palmitic, arachidonic, oleic, eicosadienoic, eicosatrienoic, eicosapentaenoic and docosahexaenoic acids were from Sigma Chemical Co., St. Louis, MO, U.S.A. Dextran T-500 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Lyso-PAF was from Bachem Feinchemikalien, Bubendorf, Switzerland. Calcium ionophore A23187 was from Calbiochem–Behring, La Jolla, CA, U.S.A. Acetyl-CoA was from Boehringer, Mannheim, Germany. [^{3}H]Acetyl-CoA (2 Ci/mmol), [^{14}C]acetate (55 mCi/mmol), [^{3}H]lyso-PAF (120 Ci/mmol), [1-^{14}C]arachidonoyl-CoA (55 mCi/mmol), [1-^{14}C]oleoyl-CoA (50 mCi/mmol) and [1-^{14}C]-arachidonic acid (58 mCi/mmol) were from Amersham International, Amersham, Bucks, U.K. LTB_4 (a gift from Dr. Joshua Rokach) was from Merck Frosst, Pointe Claire-Dorval, Quebec, Canada. *p*-Bromophenacyl bromide was from Aldrich-Europe, Beerse, Belgium. Lipoxin A_4 and 15-HETE were from Peninsula Laboratories Europe Ltd., Merseyside, U.K.

Isolation of human PMN

Human PMN were obtained from peripheral blood of normal volunteers which was anti-coagulated with citric acid/dextrose solution. Red cells were removed by Dextran T-500 sedimentation and PMN were separated from mononuclear cells by centrifugation on Ficoll–Hypaque cushions. PMN were washed twice in a Hepes-buffered medium in the absence of Ca^{2+} and resuspended at a concentration of 10^7 cells/ml in the same medium supplemented with 1 mM-$CaCl_2$ for the different experiments. Contamination of the PMN preparation by platelets was usually less than one platelet per two PMN, and in all cases less than two platelets per PMN. This allows us to rule out a significant synergism of platelets and neutrophils in PAF production (since this has been shown at a ratio of platelets/PMN of 100:1 [27]) as well as in the formation of 5,12-diHETE (see below).

Isolation of rat spleen microsomes

Spleens from normally fed Wistar rats of about 200 g body weight were homogenized in 12 vol. of ice-cold 0.1 M-Tris/HCl buffer, pH 7.4, containing 0.3 M-sucrose, 0.5 mg of soybean trypsin inhibitor/ml and 50 μg of leupeptin/ml. The microsomal fraction (referred to simply as microsomes) was isolated as previously described [28].

Assay of arachidonate metabolite production

PMN at a concentration of 10^7 cells/ml were incubated for 10 min in a Hepes-buffered medium containing 0.1 % BSA and 1 mM-$CaCl_2$, in the presence of either vehicle or 10 μM-ionophore A23187 previously dissolved in dimethyl sulphoxide. The final concentration of dimethyl sulphoxide never reached a value higher than 0.1 %. The supernatant buffer of stimulated and control PMN was removed by centrifugation and extracted with 2.5 vol. of chloroform/methanol/formic acid (12:12:1, by vol.). The lower phase was removed, the upper phase was washed with 1 ml of chloroform, and the resultant lower phase was pooled with the previous one. The chloroform extract was evaporated under a stream of N_2 and redissolved in the mobile phase for reverse-phase h.p.l.c. analysis [26,29]. This was carried out using a dual-pump Kontron system model 420 (Kontron Instruments, Zurich, Switzerland), fitted with a Spheri-5-RP 18 column (220 mm × 4.6 mm; pore size 5 μm), and a 15 mm × 4.6 mm precolumn, using as solvent methanol/water/acetic acid (750:250:0.1, by vol.) at 1 ml/min. The column was eluted for 60 min and 1 ml fractions were collected to characterize the u.v. absorbance spectrum of LTB_4. The system was equipped with a Uvikon 735LC variable wavelength detector which was operated at 270 mm. Characterization of the compound present as LTB_4 was carried out by comparison of the retention time with that of a pure standard of LTB_4 (12 ± 1 min), and by analysis of the u.v. absorbance spectrum of the appropriate fraction with a Cary model 219 spectrophotometer using methanol as solvent. The quantity of LTB_4 was estimated by integration of the peaks using a Spectra-Physics SP-4290 apparatus (Spectra-Physics, San Jose, CA, U.S.A.) and comparison with known amounts of exogenous LTB_4 used as an external standard.

Assay of 5-lipoxygenase activity

5-Lipoxygenase activity was measured in PMN homogenates in a medium containing 0.1 M-Tris/HCl, pH 7.4, 2 mM-ATP, 2 mM-$CaCl_2$, 1.6 mM-EDTA, 25 μM-5,8,14-eicosatrienoic acid and 24 μg of phosphatidylcholine in a volume of 1 ml in glass tubes [9,10,30]. The reaction was initiated by the addition of 0.2 μCi of [1-^{14}C]arachidonic acid and 100 μM unlabelled arachidonic acid dissolved in ethanol (0.5 %, v/v, final concentration), and allowed to proceed for up to 5 min. At the end of this period the reaction was terminated by adding 2.5 vol. of chloroform/methanol/formic acid (12:12:1, by vol.). After extraction, 5-HPETE and 5-HETE were identified by reverse-phase h.p.l.c. using as mobile phase methanol/water/acetic acid (750:250:0.1, by vol.) at 1 ml/min during 30 min followed by a step gradient of 100 % methanol. The effluent was monitored at 234 nm.

Assay of long-chain acyl-CoA synthetase activity

This assay was carried out with the membrane fraction according to the procedure described by Wilson *et al.* [30]. Briefly, the standard reaction mixture contained 0.1 M-Tris/HCl, 0.2 mM-$MgCl_2$, 6 mM-ATP, 1 mM-2-mercaptoethanol, 2 mM-Triton X-100, 40 μl of a 0.5 mM solution of radiolabelled arachidonic acid in 50 mM-$NaHCO_3$ and a source of enzyme from human PMN or platelet membrane preparations in a total volume of 0.15 ml, pH 8. After incubation at 37 °C for 10 min, the reaction was stopped by the addition of 2.25 ml of propan-2-ol/heptane/2 M-H_2SO_4 (40:10:1, by vol.). After addition of heptane and water, the mixture was vortex-mixed and the aqueous phase was taken and extracted with heptane containing palmitic acid as a carrier to remove unreacted radiolabelled fatty acid. The product of the reaction was identified by t.l.c. according to the procedure of Mishina *et al.* [31] in the system propan-2-ol/pyridine/acetic acid/water (60:15:1:25, by vol.). The R_F of authentic arachidonoyl-CoA in this system is 0.29.

Assay of PAF production

PMN at a concentration of 10^7/ml were incubated in the presence of 20 μCi of [^{14}C]acetate/ml for 10 min at 37 °C, followed by an additional period of 15 min after addition of stimuli or control solutions. At the end of this period, both cells and supernatants were extracted into chloroform/methanol/water according to the Bligh & Dyer procedure [32], and the lipid extract was evaporated to dryness under an N_2 stream and resuspended in 100 μl of propan-2-ol/hexane (1:1, v/v) for straight-phase h.p.l.c. as described by Blank & Snyder [33]. H.p.l.c. was carried out using the equipment mentioned for the LTB_4 assay fitted with a Spheri-5 silica column. The mobile phase was 96% B [propan-2-ol/hexane (1:1, v/v) supplemented with 0.005% acetic acid] and 4% A (water) which was linearly increased to 8% during a 15 min period. The column was eluted for 60 min at a flow rate of 2 ml/min, and 1 ml fractions were collected and used for liquid scintillation spectrometry after addition of a scintillation cocktail for non-aqueous samples. Absorbance at 206 nm was recorded continuously. Under these chromatographic conditions, the time of retention of a [^{3}H]hexadecyl-PAF standard was of 20±2 min.

Assay of lyso-PAF:acetyl-CoA acetyltransferase

The assay was performed as described in [12]. For this purpose, PMN at a concentration of 10^7 cells/ml in a Tris/HCl-buffered medium, pH 7.4, were sonicated for 15 s in a Branson B-12 sonifier equipped with microtip at position 3. The assay was carried out in the presence of 20 μM-lyso-PAF, 0.5 μCi of [^{3}H]acetyl-CoA and 100 μM unlabelled acetyl-CoA. The enzyme reaction was carried out for 15 min at 37 °C and stopped by the addition of 3.7 ml of chloroform/methanol (1:2, v/v) for lipid extraction. The product extracted into the chloroform layer was separated by t.l.c. on silica-gel plates using as developer the system propionic acid/propanol/chloroform/water (2:2:1:1, by vol.). The areas of the plates coincident with the R_F of a standard of synthetic PAF were scraped off and counted for radioactivity.

Assay of acyl-CoA: lysophosphatide acyltransferase (EC 2.3.1.23)

The assay was carried out in homogenates of PMN as described by Lands *et al.* [34]. The medium contained either oleoyl- or arachidonoyl-CoA, 0.2 μCi of [1-^{14}C]acyl-CoA and 20 μM-lyso-PAF in 1 ml of 0.1 M-Tris/HCl-buffered medium, pH 7.4. The reaction was carried out and stopped as described for acetyl-CoA: lyso-PAF acetyltransferase, except that the phosphatidylcholine zone was scraped for scintillation counting.

Data analysis

All data are expressed as mean values ± S.D. The significance of differences between group means was assessed by using the paired Student's *t* test, or by one-way analysis of variance, as appropriate; values of $P < 0.05$ were considered statistically significant. To study inhibition, simple IC_{50} values (concn. causing half-maximal inhibition) were used to screen fatty acids as inhibitors of acetyl-CoA:lyso-PAF acetyltransferase. A more detailed kinetic analysis was carried out with arachidonate to determine the nature of enzyme inhibition by using double-reciprocal representation and linear least-squares regression analysis. A replot of the slope versus inhibitor concentration was used to obtain the K_i value.

RESULTS

Effect of methylxanthines on LTB_4 production in intact cells

Initial attempts to ascertain the effect of phospholipase A_2 inhibition on PAF formation were planned with *p*-bromophenacyl bromide, an active-site-directed inhibitor, which has been used to characterize phospholipases. However, *p*-bromophenacyl bromide was also found to inhibit acetyl-CoA:lyso-PAF acetyltransferase activity in PMN homogenates in the range 1–100 μM (Table 1), i.e. similar to reports on phospholipase A_2 [35,36] and rat spleen acetyltransferase [11]. This finding discouraged the use of this compound and prompted us to utilize methylxanthines, which have a rapid inhibitory effect on phospholipase A_2 activity in intact cells [8,37] that seems to be independent from their effect on cyclic AMP-dependent phosphodiesterase [38,39] and which also lack a significant direct effect on acetyl-CoA:lyso-PAF acetyltransferase (see below). In addition, in a previous report we have observed that MIX is a potent inhibitor of the release of arachidonic acid from human PMN [40]. Preincubation of PMN with either MIX or theophylline for 10 min before the addition of 10 μM-ionophore

Table 1. Effect of different additions on acetyl-CoA: lyso-PAF acetyltransferase activity

Data represent mean values of two experiments done in duplicate. All compounds were tested in the 1–100 μM range and were added before starting the enzyme reaction by substrate addition.

Addition	IC_{50} (μM)	
	Spleen microsomes	A23187-stimulated PMN
p-Bromophenacyl bromide	22	23
Arachidonic acid	22	20
15-HETE	40	40
LTB_4	80	81
5-HETE	95	95
Lipoxin A_4	> 100	> 100
Arachidonic acid methyl ester	> 100	> 100

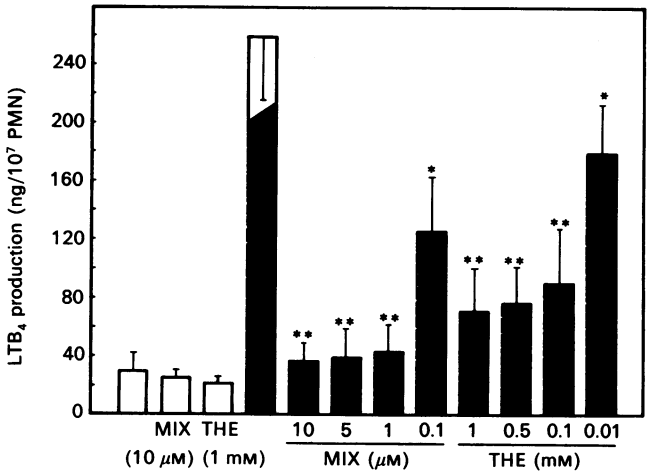

Fig. 1. Effect of methylxanthines on LTB_4 formation

PMN at a concentration of 10^7/ml were incubated for 10 min in the presence of different concentrations of MIX or theophylline (THE) before the addition of 10 μM-ionophore A23187 (■) or control solutions (□). After 10 min of incubation supernatants were taken for extraction and LTB_4 assay as described in the Materials and methods section. Data represent means ± S.D. of four (THE) or six (MIX) independent experiments done in duplicate. Significant differences from controls are indicated: * $P < 0.05$; ** $P < 0.01$.

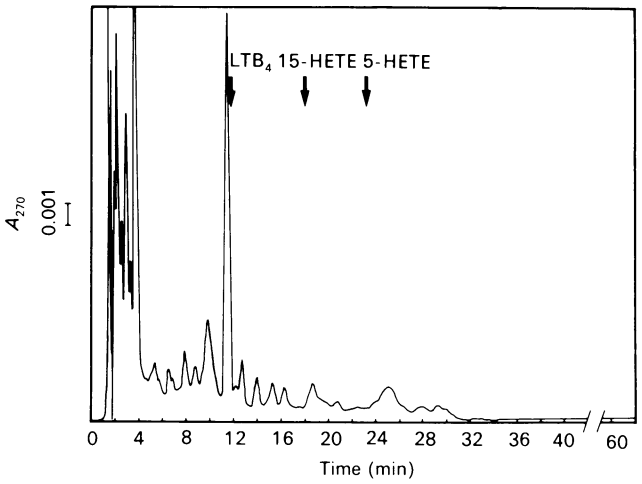

Fig. 2. Reverse-phase h.p.l.c. of supernatants from PMN activated with ionophore A23187

PMN at a concentration of 10^7/ml were kept at 37 °C for 10 min in the presence of 10 μM-ionophore A23187. At the end of this period, lipids were extracted and processed as described in the Materials and methods section. The arrows indicate the retention time of the standards.

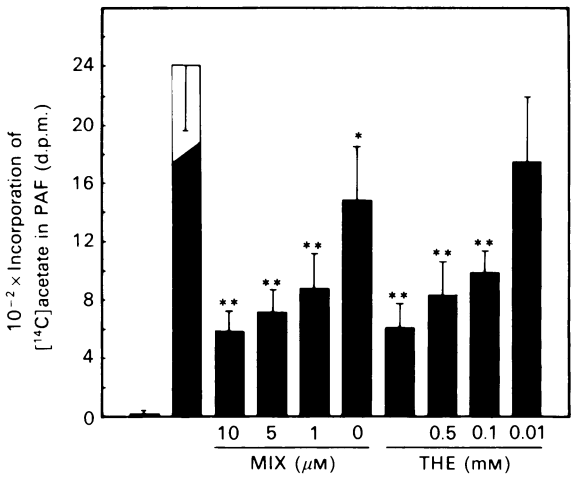

Fig. 3. Effects of MIX and theophylline on the incorporation of [^{14}C]acetate in PAF

PMN at a concentration of 10^7 cells/ml were incubated with [^{14}C]acetate in the presence of 10 μM-ionophore A23187 and various concentrations of MIX and theophylline (THE). After a period of 15 min of incubation in the presence of the agonist, pellets and supernatants were extracted, evaporated to dryness and analysed by straight-phase h.p.l.c. Fractions showing a retention time analogous to that of a [^{3}H]hexadecyl-PAF standard were collected and the associated radioactivity was quantified by scintillation spectrometry. ■, Incorporation of the label in the presence of ionophore A23187. Data represent means ± S.D. of four to six experiments. Significant differences from control are indicated: * $P < 0.05$; ** $P < 0.01$.

A23187 induced a dose-dependent inhibition of LTB_4 release with an IC_{50} of 0.13 μM for MIX and 60 μM for theophylline (Fig. 1). LTB_4 release was measured 10 min after the addition of the stimulus, since in preliminary experiments, maximal release of LTB_4 into the medium was observed after this period of incubation. Addition of either MIX or theophylline to PMN in the absence of 10 μM-ionophore A23187 slightly decreased basal production of LTB_4 (Fig. 1). Under the conditions of chromatography, the retention time of LTB_4 was 11.56 ± 0.3 min ($n = 15$) and the characterization of the material eluting at this time of retention as genuine LTB_4 stemmed also from its u.v. absorbance spectrum. Contamination of this fraction with 5,12-diHETE cannot be completely ruled out, since this product elutes at a similar time of retention and also shows a u.v. absorbance spectrum similar to that of LTB_4, but formation of 5,12-diHETE needs the concomitant presence of PMN and platelets at a ratio of about ten platelets per one PMN [41], i.e. far different from the contamination by platelets in our preparations, which never exceeded two platelets per one PMN. A typical reverse-phase h.p.l.c. chromatogram of supernatants from ionophore-A23187-stimulated PMN is shown in Fig. 2.

Effect of methylxanthines on PAF biosynthesis

Incubation of PMN with 10 μM-ionophore A23187 induced a time- and dose-dependent incorporation of [^{14}C]acetate in the PAF molecule as judged by the appearance of the label with a retention time of 18–22 min in straight-phase h.p.l.c., i.e. analogous to the retention time of a [^{3}H]hexadecyl-PAF standard. The label was lost after phospholipase A_2 treatment of some selected samples, which indicated that the label was incorporated at the *sn*-2 position and had not been added on to the alkyl chain. When PMN were preincubated in the presence of either theophylline or MIX, a dose-dependent inhibition of label incorporation was observed (Fig. 3). The IC_{50} was 0.53 μM for MIX and 81 μM for theophylline. Since these experiments in combination with those mentioned above on LTB_4 production suggested a possible effect of methylxanthines at the phospholipase A_2 level, additional experiments were planned to ascertain whether the effect of methylxanthines on [^{14}C]acetate incorporation could be due to the disposal of lyso-PAF, which is one of the products of the phospholipase A_2 reaction. Addition of lyso-PAF to the medium did not cause incorporation of [^{14}C]acetate in PAF in the absence of ionophore A23187 (results not shown); however, concentrations of lyso-PAF above 1 μM significantly reversed the inhibition of the incorporation of [^{14}C]acetate into the PAF molecule produced by either 10 μM-MIX or 1 mM-theophylline (Figs. 4*a* and 4*b*). This is in keeping with the hypothesis of a possible inhibitory effect of methylxanthines at the phospholipase A_2 level.

Effect of methylxanthines on acetyl-CoA: lyso-PAF acetyltransferase activity

Preincubation of PMN in the presence of 1 μM-MIX produced no significant effect on acetyl-CoA:lyso-PAF acetyltransferase activity, which increased from 122 ± 24 to 136 ± 21 pmol/min per 10^7 PMN ($n = 5$). Similar findings were observed when PMN were preincubated with 1 mM-theophylline, which also caused an increase in acetyl-CoA:lyso-PAF acetyltransferase activity to 148 ± 15 pmol/min per 10^7 PMN ($n = 4$). Similar non-inhibitory effects were also observed when methylxanthines were added to PMN stimulated with ionophore A23187 (Fig. 5). These findings indicate that methylxanthines diminish PAF formation by acting at a level other than the expression of acetyl-CoA:lyso-PAF acetyltransferase activity.

Effect of fatty acids on acetyl-CoA:lyso-PAF acetyltransferase activity

Since non-esterified fatty acids are products of the phospholipase A_2 reaction and have been demonstrated to influence the expression of many enzyme activities, including protein kinase C [42], phospholipase A_2 [22,23] and alkylacetylglycerol:CDP-choline phosphocholinetransferase activity [43], experiments were carried out to assess the effect of some fatty acids on acetyl-CoA:lyso-PAF acetyltransferase activity. As shown in Table 2, many fatty acids had an inhibitory

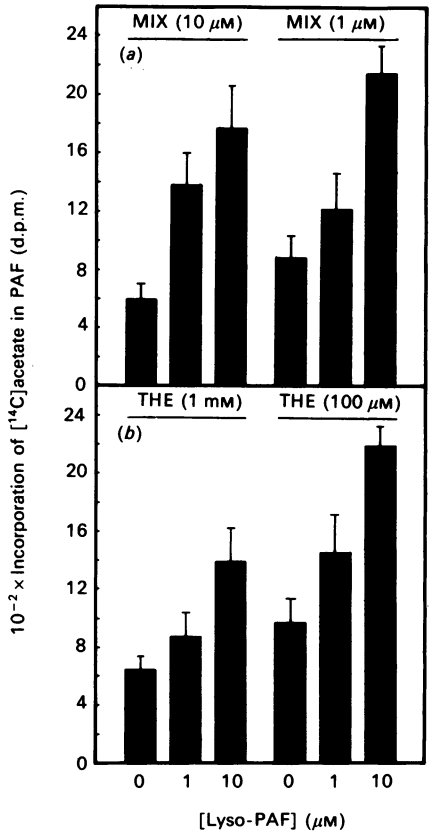

Fig. 4. Effect of lyso-PAF on the inhibition of the incorporation of [^{14}C]acetate in PAF produced by MIX and theophylline

PMN at a concentration of 10^7 cells/ml were incubated with [^{14}C]acetate in the presence of 10 μM-ionophore A23187 and various concentrations of MIX (*a*) or theophylline (THE) (*b*) and lyso-PAF for 15 min. At the end of the incubation period, cell pellets and supernatants were extracted and processed as described in the legend to Fig. 3. Fractions showing a retention time analogous to that of a [^{3}H]hexadecyl-PAF standard were collected and the associated radioactivity was quantified by scintillation spectrometry. Data represent means ± S.D. of three to six experiments with duplicate samples.

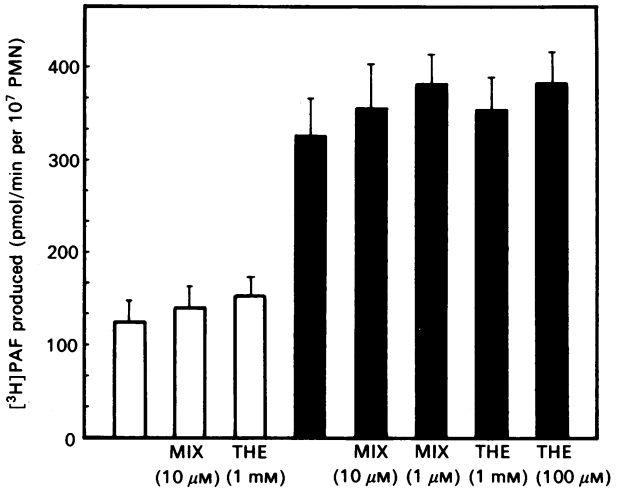

Fig. 5. Effects of methylxanthines on acetyl-CoA:lyso-PAF acetyltransferase activation

PMN were incubated in the presence of the additions indicated. After 10 min, 10 μM-ionophore A23187 (■) or control solutions (□) were added. After an additional period of incubation of 10 min, PMN were homogenized and acetyl-CoA:lyso-PAF acetyltransferase activity was assayed. Data represent means ± S.D. of four [theophylline (THE)] or five (MIX) experiments with duplicate samples.

Table 2. Effect of different fatty acids on acetyl-CoA:lyso-PAF acetyltransferase activity from spleen microsomes and PMN homogenates

Data represent mean values of two experiments done in duplicate. Fatty acids were tested in the range 1–100 μM.

Addition	IC_{50} (μM)	
	Spleen microsomes	A23187-stimulated PMN
Palmitic acid ($C_{16:0}$)	> 100	> 100
Oleic acid ($C_{18:1}$)	60	50
Linoleic acid ($C_{18:2}$)	51	43
Eicosadienoic acid ($C_{20:2}$)	42	37
Eicosatrienoic acid ($C_{20:3}$)	37	32
Eicosapentaenoic acid ($C_{20:5}$)	21	18
Docosahexaenoic acid ($C_{22:6}$)	48	43

effect on acetyl-CoA:lyso-PAF acetyltransferase activity in spleen microsomes and A23187-stimulated PMN. Structure–activity studies showed maximal inhibitory activity linked to arachidonic ($C_{20:4}$) and eicosapentaenoic ($C_{20:5}$) acids, whereas eicosadienoic ($C_{20:2}$), eicosatrienoic ($C_{20:3}$) and docosahexaenoic ($C_{22:6}$) acids were less active, and palmitic acid ($C_{16:0}$) lacked any significant inhibitory activity. The possible interference of arachidonic acid with the assay due to the concomitant occurrence of a transacylase reaction [44] was ruled out in parallel experiments on both spleen microsomes and A23187-stimulated PMN by using [^{3}H]lyso-PAF (hexadecyl) and unlabelled acetyl-CoA as substrates. Under these conditions, arachidonic acid also inhibited the acetyltransferase reaction, whereas no formation of [^{3}H]hexadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine could be demonstrated (results not shown). This was considered as evidence of a direct effect of fatty acids on the acetyl-CoA:lyso-PAF acetyltransferase reaction.

In order to look for an explanation for the outstanding inhibitory effect of arachidonic acid as compared with the effect of the other fatty acids, a possible effect of arachidonic acid linked to metabolic conversion into 5-lipoxygenase products was sought, since this is the central pathway for oxidative metabolism of arachidonate in PMN. For this purpose, the conversion of exogenously added [1-^{14}C]arachidonic acid into 5-lipoxygenase metabolites was monitored by extraction and analysis by reverse-phase h.p.l.c. of the metabolites formed after 10 min of incubation. Under these conditions, the label mostly appeared with a retention time of 34 ± 1 min, i.e. shortly after changing the mobile phase to pure methanol and analogous to that of the arachidonic acid standard, whereas no label appeared at the retention time of 15-HETE (18 ± 1 min) and only 0.2% of the label showed a retention time of 24 ± 1 min, i.e. analogous to that of 5-HETE, which is in keeping with the usual metabolism of arachidonate to 5-HETE and 5-HPETE in cell-free preparations, and with the low yield of the reaction in the absence of the stimulatory factors for the 5-lipoxygenase assay. In fact, in two parallel experiments with PMN homogenates supplemented with the stimulatory factors, the conversion of arachidonic acid into

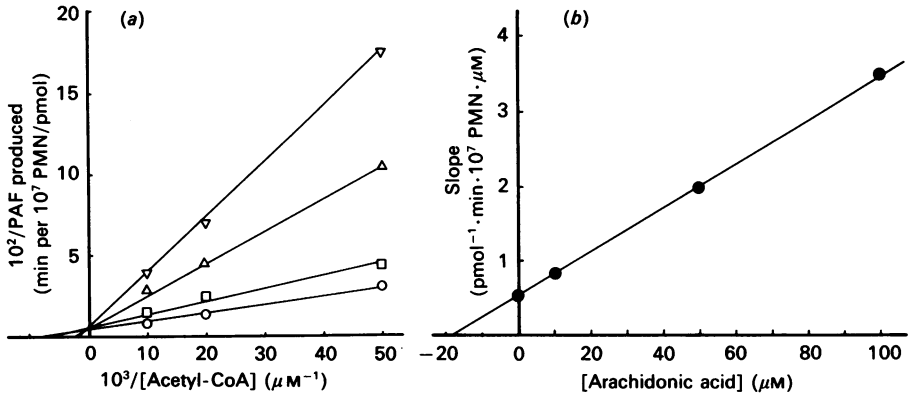

Fig. 6. Effects of arachidonic acid on acetyl-CoA:lyso-PAF acetyltransferase activity

(*a*) Double-reciprocal plot of substrate-dependence of acetyl-CoA:lyso-PAF acetyltransferase activity towards acetyl-CoA in the presence of 0 μM-(○), 10 μM- (□), 50 μM- (△) or 100 μM- (▽) arachidonate. PMN were incubated for 10 min at 37 °C in the presence of 10 μM-ionophore A23187 prior to homogenization, thereafter, 20 μM-lyso-PAF and various concentrations of acetyl-CoA were added for enzyme activity assay. Each point represents the average of triplicate samples. (*b*) Plot of the slopes as a function of arachidonate concentration.

5-lipoxygenase metabolites reached 4 % of the total arachidonate added. Structure–activity experiments on acetyl-CoA:lyso-PAF acetyltransferase activity using different eicosanoids showed that all of the compounds tested had a less marked effect than arachidonic acid, having an order of inhibitory potency of: arachidonic acid > 15-HETE > LTB_4 > 5-HETE > lipoxin A_4 (Table 1). A free carboxylic group was found to be an absolute requirement for the expression of inhibition of acetyl-CoA:lyso-PAF acetyltransferase, since methylarachidonate lacked an inhibitory effect and 100 μM-phosphatidylcholine (palmitoyl-arachidonoyl) increased the enzyme activity by 35 %. A set of drugs displaying interference with arachidonic acid metabolism showed, in general, inhibitory actions on acetyl-CoA:lyso-PAF acetyltransferase when added to PMN homogenates. Indomethacin was inhibitory at concentrations above 5 μM and nordihydroguaiaretic acid produced 20 % inhibition at 10 μM.

Kinetic analysis of arachidonic acid effect

In the absence of exogenous arachidonic acid, the apparent K_i for acetyl-CoA was about 147 μM and the $V_{max.}$ was 270 pmol/min per 10^7 PMN. The action of arachidonic acid was most consistent with competitive inhibition (Fig. 6*a*), with a K_i value of about 19 μM obtained from plotting arachidonate concentration versus the slope of double-reciprocal plots (Fig. 6*b*). Studies on the effect of arachidonic acid on the reaction kinetics compared with the other substrate were not carried out, since lyso-PAF causes an inhibition of the reaction at concentrations above 20 μM [11]. When the experiment was carried out with homogenates of resting PMN (results not shown), the inhibitory effect was similar to that observed on either A23187-stimulated PMN or spleen microsomes. The present studies have been carried without taking into account the endogenous free arachidonic acid, but its possible contribution seems unlikely in view of the very low concentrations reported in human PMN [45].

Acyl-CoA: lysophosphatide acyltransferase activity in human PMN

Incubation of homogenates of resting PMN with [1-^{14}C]arachidonoyl-CoA and lyso-PAF resulted in an enzyme activity of 11 pmol/min per 10^7 PMN, whereas the activity assayed in homogenates from A23187-stimulated PMN was 29 pmol/min per 10^7 PMN (Fig. 7*a*). When the assay was carried out with [1-^{14}C]oleoyl-CoA (Fig. 7*b*), the enzyme activity was 470 pmol/min per 10^7 PMN, which was similar to the activity in

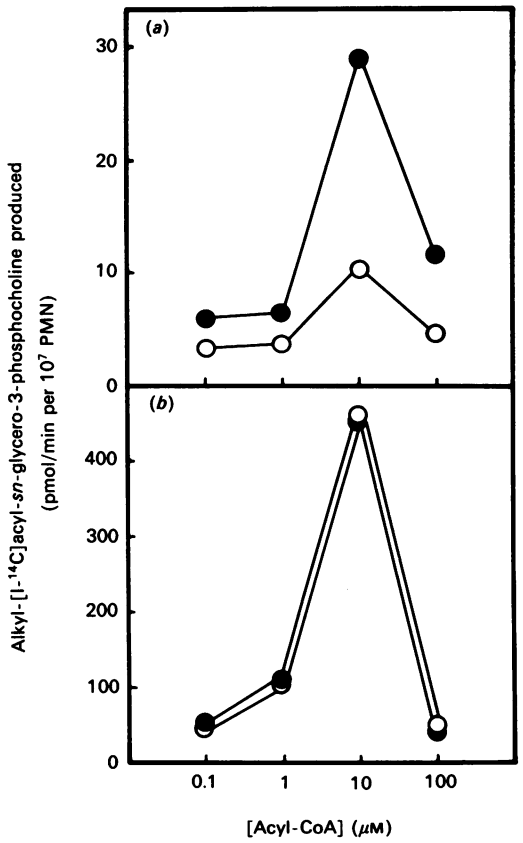

Fig. 7. Assay of acyl-CoA:lysophosphatide acyltransferase activity in homogenates from human PMN

The assay was carried out in homogenates of both resting (○) and ionophore-A23187-treated (●) PMN in the presence of 20 μM-lyso-PAF and various concentrations of [1-^{14}C]arachidonoyl-CoA (*a*) or [1-^{14}C]oleoyl-CoA (*b*). The results shown are representative of two experiments done in duplicate.

A23187-stimulated PMN (460 pmol/min per 10^7 PMN). These values are those obtained when the assays were carried out with 10 μM-acyl-CoA, since higher concentrations resulted in inhibition (Fig. 7). This suggests a low efficiency of the CoA-dependent reacylation reaction for arachidonic acid in PMN and

Table 3. Arachidonoyl-CoA synthetase activity in membrane fractions from human platelets and PMN

Platelets and PMN from the same individuals were disrupted by sonication and the membrane fractions were isolated by ultracentrifugation. In the case of PMN, a low-speed centrifugation step was included to remove nuclei and unbroken cells. The assay was carried out as described in the Materials and methods section. The data represent means ± S.D. of three independent experiments with duplicate samples.

Source	Activity (pmol/min per mg)
PMN	4.5±0.7
A23187-treated PMN	12±2.3
Platelets	173±32

indicates that other pathways should be responsible for the enrichment of alkyl-ether lipids in arachidonate molecular species, as had been shown in rabbit alveolar macrophages [44] and platelets [46]. A further consequence of these findings is that changes in the rate of reacylation of lyso-PAF seem to play a limited role in the modulation of PAF formation.

Arachidonoyl-CoA synthetase activity in platelets and PMN

Experiments were carried out by processing in parallel platelet and PMN membrane fractions obtained from the same donor; for this purpose, blood was always centrifuged at low speed to remove platelet-rich plasma before PMN isolation. Under these conditions, there was a recognized source of enzyme in each experiment and the possible contribution by contaminating platelets to the activity assayed in PMN membranes could be assessed. As shown in Table 3, PMN contained much less enzyme activity than platelets, even when the membrane fraction had been obtained from cells stimulated previously with ionophore A23187, which has been shown to enhance this enzyme activity in human platelets [47].

DISCUSSION

The data herein presented indicate an important role for the products of the phospholipase A_2 reaction in the modulation of PAF biosynthesis. A consequence of these findings with potential therapeutic value is that pharmacological agents acting at this enzyme step can influence formation of both PAF and LTB_4. The first pharmacological tool we used to block phospholipase A_2 was *p*-bromophenacyl bromide, an active-site-directed inhibitor (although this concept has been challenged by Kyger & Franson [48] who found that *p*-bromophenacyl bromide can also react with thiol groups), but this was found not to be suitable.

Previous studies have shown the inhibition of the generation of LTB_4 [49,50] and PAF [51] in response to both complement-coated zymosan particles and calcium ionophore A23187 by increasing intracellular levels of cyclic AMP with pharmacological tools, methylxanthines being the most effective agents. However, few studies are available concerning the enzyme on which cyclic AMP and methylxanthines seem to exert this inhibitory role. Moreover, some discrepancies are raised by previous findings on the inhibition of PAF generation by methylxanthines and the description of the activation of acetyl-CoA: lyso-PAF acetyltransferase activity by the catalytic subunit of cyclic AMP-dependent protein kinase in rat spleen microsomes [17], PMN homogenates [19] and guinea-pig parotid gland microsomes [15]. The present results indicate a dual effect of methylxanthines on PAF formation. On the one hand, MIX and theophylline block the generation of both PAF and LTB_4, and this agrees with an inhibitory effect at the phospholipase A_2 level. On the other hand, they do not inhibit (and even slightly enhance) the passage of acetyl-CoA: lyso-PAF acetyltransferase from a low-activity form to a high-activity form, even though the incorporation of [^{14}C]acetate in [^{14}C]PAF is diminished. Since the effect of both theophylline and MIX could be partially reversed by the addition of lyso-PAF to the PMN, the most relevant inhibitory effect of these compounds on PAF biosynthesis can be related to an effect on lyso-PAF disposal.

Both phospholipase A_2 and acetyl-CoA: lyso-PAF acetyltransferase are inhibited by unsaturated fatty acids by a competitive mechanism. According to this finding, non-esterified unsaturated fatty acids seem to play a modulatory role in PAF biosynthesis which is linked to phospholipase A_2 activity, since their intracellular level depends on the deacylation/reacylation rate, and detectable levels of free arachidonic acid are only found after phospholipase activation in many cell types, e.g. human platelets [52]. Interestingly, the effect of arachidonic acid herein described is observed at concentrations below its critical micellar concentration. Those concentrations are equal to or lower than the concentrations required to display other pharmacological properties of unsaturated fatty acids, e.g. stimulation of glycolysis in intact lymphocytes [53] and inhibition of 5-lipoxygenase in alveolar macrophages [54]. The structure–activity study indicates a more potent effect of fatty acids containing double bonds, and the need for a free carboxylic group for the expression of the effect. This would suggest that the action of arachidonic acid may in turn be modulated by either oxidation or reacylation via formation of an arachidonoyl-CoA intermediate [30]. The comparable effects of unsaturated fatty acids on spleen microsomes and A23187-stimulated PMN indicate that the effect of arachidonic acid cannot be related to oxidative metabolism. This agrees with recent reports on 5-lipoxygenase, a soluble hydrophobic enzyme that rapidly loses activity and undergoes translocation to the membrane [10] when it catalyses ionophore-induced arachidonic acid metabolism. The low conversion rate of arachidonate into 5-lipoxygenase-produced metabolites found in our experiments, even in the resting homogenates, can be explained by the requirements of this enzyme for multiple stimulatory factors including ATP and hydroperoxy derivatives of polyenoic fatty acids [9,10].

Another factor that could influence PAF formation is the activity of the enzymes involved in the CoA-dependent reacylation of lyso-PAF. We have detected a low arachidonoyl-CoA synthetase activity in human PMN, which is at variance with reports on platelets [30,47], and a preference for oleoyl-CoA as a substrate of acyl-CoA: lyso-phosphatide acyltransferase. This activity was not influenced by previous activation of the PMN, whereas some differences in enzyme activity between resting and ionophore-A23187-activated PMN were detected with arachidonoyl-CoA as substrate. This suggests the existence of two enzymes with different affinities for the two substrates, and indicates that accumulation of arachidonoyl species in the alkyl subclass of choline-containing phosphoacylglycerols cannot be explained by an arachidonoyl-CoA: lysophosphatide acyltransferase reaction, but most probably by a CoA-independent transacylation such as that described in rabbit alveolar macrophages [44].

In summary, our data enlarge the list of factors implicated in the modulation of acetyl-CoA: lyso-PAF acetyltransferase. Lyso-PAF disposal, and the balance between unsaturated non-esterified fatty acids and arachidonate oxidative metabolites, should be added to the requirement for Ca^{2+} and a phosphorylation–dephosphorylation mechanism. In contrast, variations in the activity of acyl-CoA: lysophosphatide

acyltransferase appear to have a marginal effect on short-term variations in the rate of PAF biosynthesis.

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