Modulation of platelet-activating-factor production by incorporation of naturally occurring 1-*O*-alkylglycerols in phospholipids of human leukemic monocyte-like THP-1 cells

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1-*O*-Alkylglycerols (alkyl-Gro), naturally occurring compounds abundant in shark liver oil, protect patients from radiotherapy side-effects. However, the protection mechanism is not well understood. It might be mediated by alkyl-Gro incorporation into pools of platelet-activating factor (PAF) precursor and subsequent modification of PAF biosynthesis. Using a ^{3}H-labelled or unlabelled natural alkyl-Gro mixture, in which prominent alkyl chains were C18:1(9) (54−65%), C16:1(7) (5−15.5%), and C16:0 (5−10%), we investigated the incorporation of alkyl-Gro into phospholipids of human leukemic monocyte-like THP-1 cells. Incubation of cells for 24 h with [^{3}H]alkyl-Gro (10 μM) resulted in their incorporation into 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (1097 ± 25.1 pmol/2×10^6 cells) and into 1-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (640.4 ± 12.5 pmol/2×10^6 cells) with a total yield of 6.5%. Such incorporation induced production of 1-*O*-[^{3}H]alkyl-2-acetyl-*sn*-glycero-3-phosphocholine ([^{3}H]PAF), which was increased after stimulation by the calcium ionophore A23187. HPLC analysis of the [^{3}H]PAF molecular species indicated that the three major [^{3}H]alkyl-Gro were used for [^{3}H]PAF synthesis in ratios similar to that of the mixture. Total production of biologically active PAF, as measured by the platelet-aggregation bioassay, was also increased by alkyl-Gro incorporation in resting (+20%) and in A23187-stimulated (+59%) THP-1 cells. HPLC analysis of the [^{3}H]PAF produced in the presence of [^{3}H]acetate, confirmed that levels of PAF, but not of its 1-acyl analog, were increased by alkyl-Gro incorporation in resting and stimulated cells. However, the rise in [^{3}H]acetyl-PAF, which resulted mainly from C16:0 PAF, was reduced by about 50% in the presence of the PAF-receptor antagonist SR 27417, providing evidence that stimulation of total PAF synthesis was caused by the increase in the precursor pool and autocrine amplification of PAF-induced PAF production. Thus, the supplementation of THP-1 cells in culture with naturally occurring alkyl-Gro led to the incorporation of alkyl-Gro into ether-containing phospholipids, which were subsequently used for PAF synthesis. Furthermore, alkyl-Gro incorporation resulted in a significant rise in PAF production by THP-1 cells under resting and stimulated conditions. These results may be of importance for modulating PAF production in several pathophysiological conditions, such as peroxysome deficiencies, that are associated with a lack of ether lipid synthesis.

Keywords: alkylglycerol; platelet-activating factor; platelet aggregation; phospholipid.

1-*O*-Alkylglycerols (alkyl-Gro), naturally occurring lipids, are found in notable quantities in hematopoietic organs, such as bone marrow, and in milk. They are especially abundant in the liver of several species of sharks, whose liver oil may contain

as much as 50% alkyl-Gro [1, 2]. Shark liver oil has been traditionally used in Scandinavian medicine against debility and for wound healing. Studies have been performed to confirm and establish the therapeutic properties of these compounds. Beneficial effects in cancer treatment, such as preventive action of alkyl-Gro on radiotherapy side effects, including leukopenia and thrombocytopenia, have been reported [2−4]. Furthermore, synthetic ether lipids possess anti-cancer properties [5]. The molecular basis of such effects are poorly understood.

In mammals, alkyl-Gro from dietary sources are absorbed without cleavage of their ether bond, and are used as precursors of membrane phospholipids in different tissues [6]. Dietary alkyl-Gro are incorporated into 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (RAcylGro*P*Etn) and 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (RAcylGro*P*Cho) in rat intestinal mucosal cells [7], and in various other organs [6]. This incorporation into 1-alkyl-phospholipids is of particular interest because RAcylGro*P*Cho represent the pool of precursors for biosynthesis

of platelet-activating factor (PAF), a synthesized mediator with potent biological activities on various cell types and systems, including circulation, inflammation, reproduction and development [8−9]. 1-*O*-Alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine also serves as an important source of arachidonic acid, the major precursor of several families of mediators. The structure of PAF was identified as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phophocholine. Naturally occurring PAF includes several molecular species, differing by their hydrocarbon chain length and/or unsaturation at the *sn*-1 position of the glycerol. A 1-acyl analog of PAF with low biological activity may be produced simultaneously with PAF; however. The ratio between PAF and its acyl-analog may vary depending on the cell type [10].

The molecular basis of alkyl-Gro biological activities may include modifications of the pool of precursors for PAF, resulting in an alteration of its biosynthesis. To test this hypothesis, the human promonocyte leukemia cell line THP-1 [11] was used to study the incorporation of natural alkyl-Gro isolated and purified from shark liver oil into phospholipids, and its influence on PAF synthesis. Our results show that alkyl-Gro incorporated into RAcylGro*P*Etn and RAcylGro*P*Cho, and therefore may account for an increase in PAF precursor pools, resulting in increased production of PAF under resting and stimulated conditions.

EXPERIMENTAL PROCEDURES

Materials. RPMI medium, fetal calf serum, penicillin, kanamycin, Hepes and glutamine were obtained from Eurobio. Fatty-acid-free BSA, Phenylmethylsulfonyl fluoride (PhMeSO$_2$F) and 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) were purchased from Sigma Chemical Co. Deoxycholate and A23187 were obtained from Calbiochem. [^{3}H]acetate, sodium salt (100 mCi/mmol) was from Isotopchim. 1-Stearoyl-2-[^{14}C]-arachidonoyl-*sn*-glycero-3-phosphocholine (Ste[^{14}C]Δ_4AchGro*P*Cho; 55.6 mCi/mmol) and 1-octadecyl-2-acetyl-*sn*-glycero-3-phospho[^{14}C]choline (C18:0 [^{14}C]PAF; 55 mCi/mmol) were from Amersham International. Standards used in reverse-phase HPLC were 1-hexadecyl-2-[^{3}H]acetyl-PAF (10−30 Ci/mmol) from Du Pont-New England Nuclear, 1-[^{3}H]octadecyl-PAF (10−30 Ci/mmol) from Amersham International, and 1-[^{3}H]octadecenyl-PAF, which was prepared as described previously [12]. Lipase from *Rhizopus arrhizus* was obtained from Boehringer Mannheim. Silica gel 60 CF 254 plates were from Merck. All solvents were obtained from Prolabo. The specific PAF-receptor antagonist SR 27417 was kindly provided by Dr J. M. Herbert, Sanofi Research, Toulouse Cedex, France.

Preparation of unlabelled and tritiated alkyl-Gro. Alkyl-Gro from crude shark liver oil were prepared as follows. After separation of squalene (55% of total mass) by short path distillation, the residue was transesterified with methanol, then separated by chromatography on silica using a gradient of pentane/ethyl ether as solvent. The glycerol ether fraction was eluted with ethyl ether and analyzed by gas chromatography after silylation. Alkyl-chain composition depending on the batch varied as follows: 18:1(9) = 54−65%, 16:1(7) = 5−15.5%, 16:0 = 5−10%, 14:0 = 3%, 18:0 = 3%, and 17:1(9) = 1.5%. Tritiation was performed by ^{3}H-labeling on the *sn*-3 C position of glycerol. Specific radioactivity was established by measuring radioactivity of a weighed sample converted into moles using a molecular mass of = 344 (forthe C18:1 prominent alkyl-Gro).

Cell culture. THP-1 cells, a promonocyte leukemia cell line [11], were cultured at 37°C, in a humidified atmosphere of air + 5% CO$_2$, in RPMI 1640 medium supplemented with glutamine (2 mM), penicillin (50 UI/ml), streptomycin (50 μg/ml), kanamycin (50 μg/ml), 10% fetal calf serum and 2-mercaptoethanol

(20 μM). Cells were routinely cultured from 2×10^5 cells/ml to 10^6 cells/ml, then were centrifuged ($400 \times g$, 5 min) and suspended in fresh medium at 2×10^5 cells/ml. Cell viability was assessed by the trypan-blue-exclusion test. Untreated cells showed $94 \pm 0.9\%$ viability. After 24 h treatment with 20 μM and 100 μM alkyl-Gro, the cell viability was $94.3 \pm 0.9\%$ and $86 \pm 1.8\%$, respectively.

Incorporation of [^{3}H]alkyl-Gro into phospholipids. [^{3}H]alkyl-Gro were dissolved in medium supplemented with 10 mM Hepes and 0.2% BSA, pH 7.4. THP-1 cells (5×10^5 cells/ml) were seeded into Petri dishes and incubated with [^{3}H]alkyl-Gro (10 μM, 12.31 mCi/mmol) for the indicated periods of time. Cells were centrifuged, the supernatants were removed, and total lipids were extracted by the method of Bligh and Dyer [13]. Lipid extract was dried under a nitrogen stream and analyzed by TLC on silica-gel plates, using a mixture of chloroform/methanol/acetic acid (35:14:2.7, by vol.) as the mobile phase. Radioactive material was visualized on a radiochromatogram scanner (Bioscan), and the phospholipid classes were identified by their retention factor (R_f). The zones on silica-gel plates corresponding to radioactive phospholipids were scraped off, and radioactivity was measured in a liquid scintillation counter (Packard).

In selected experiments, THP-1 cells (5×10^5 cells/ml) were incubated for 48 h with [^{3}H]alkyl-Gro (10 μM, 24.82 mCi/mmol). The cells were centrifuged, washed three times with NaCl/P$_i$ (8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na$_2$HPO$_4$, 0.2 g/l KH$_2$PO$_4$, pH 7.4) + 0.2% BSA, suspended in fresh medium (5×10^5 cells/ml) and seeded into Petri dishes. At indicated times, the cells were centrifuged, the supernatants were removed, and the total lipids were extracted, analyzed by TLC, and quantified as described above.

Production of [^{3}H]PAF after [^{3}H]alkyl-Gro incorporation. THP-1 cells were incubated for 24 h with [^{3}H]alkyl-Gro (10 μM, 92.13 mCi/mmol), and were washed three times in buffer A [5 mM Hepes, 140 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 1.3 mM CaCl$_2$, 0.2% (mass/vol.) BSA, pH 7.4), and rsuspended in the same buffer supplemented with PhMeSO$_2$F (2 mM, 30 min). The cells were stimulated with the calcium ionophore A23187 (5 μM, 10 min) or with vehicle for controls, and the stimulation was stopped with methanol. C18:0 [^{14}C]PAF (10 000 dpm, 55 mCi/mmol) and 50 μg PAF were added as internal standard and carrier, respectively. Total lipids were extracted according to Bligh and Dyer [13] and [^{3}H]PAF samples were purified by using straight-phase HPLC on a 30-cm Intersil 10-μm Interchrom column (Interchim) with the following linear gradient solvent (1 ml/min): from 69.3:33.6:2.5 (by vol.) chloroform/methanol/water to 63.1:33.6:3.3 chloroform/methanol/water over 40 min and to 61.4:33.6:5 chloroform/methanol/water over 25 min. The retention time of PAF was determined using an authentic standard of C16:0 PAF. The peak corresponding to PAF was collected, dried under a nitrogen stream, and further analyzed by reverse-phase HPLC using a Spherisorb S5C6-30R HPLC column (Interchim) and the following solvent: 13.75% (by vol.) methanol, 41.25% 10 mM ammonium acetate, pH = 6.1, 45% acetonitrile (1 ml/min). Fractions of 1 ml were collected, dried, and radioactivity of ^{3}H and/or ^{14}C was measured in a liquid scintillation counter. The retention times of PAF molecular species were determined using ^{3}H-labelled C16:0, C18:1 and C18:0 PAF standards.

Bioassay estimation of PAF formation by THP-1 cells. THP-1 cells were incubated for 24 h in the presence or absence of alkyl-Gro (10 μM). They were washed three times and suspended (2×10^6 cells/ml) in buffer A. After a 30-min resting time, the cells were incubated with calcium ionophore A23187 (5 μM) or with vehicle for controls [ethanol concentration 0.2%

(by vol.)]. At indicated times, stimulation was stopped by adding 4 ml ethanol. After 1 h at room temperature, the tubes were centrifuged (200×*g*, 20 min) and the supernatant was dried under a nitrogen stream, and kept at −20°C until PAF titration. PAF extracted from unstimulated or A23187-stimulated THP-1 was dissolved in ethanol/water (60:40, by vol.) and biological activity was assayed by measuring its ability to aggregate washed rabbit platelets in an aggregometer as described previously [14]. PAF concentrations were calculated from a calibration curve constructed with C16:0 PAF standard.

[³H]acetate incorporation into PAF. THP-1 cells were incubated for 24 h with or without alkyl-Gro (10 µM), then the cells were harvested, washed three times, suspended in buffer A (5×10⁶ cells/ml), and incubated for 20 min with PhMeSO₂F (2 mM) and [³H]acetate (20 µCi, 100 mCi/mmol). In selected experiments the cells were treated for 30 min with the specific PAF receptor antagonist SR 27417 (0.1 µM). They were stimulated with calcium inophore A23187 (5 µM), or with vehicle for controls. At indicated times after stimulation, incubation was stopped with cold methanol, and [¹⁴C]octadecyl-PAF (10000 dpm, 55 mCi/mmol) and 50 µg PAF were added as internal standard and carrier, respectively. Total lipids were extracted according to Bligh and Dyer [13] and dried under a nitrogen stream, then 1-alkyl PAF and 1-acyl PAF were purified using straight-phase HPLC as described above. The peak of mixed radiolabelled 1-alkyl PAF and 1-acyl PAF was detected with a solid scintillation flow detector Radiomatic Flo-One (Packard), collected, dried under a nitrogen stream and dissolved in chloroform/methanol (60:40, by vol.). An aliquot was used for quantitative measurement of radioactivity. The *sn*-1-acyl analog of PAF was hydrolysed with lipase A1 (2000 U) from *Rhizopus arrhizus* [15] in the presence of 1-stearoyl-2-[¹⁴C]arachidonoyl-*sn*-3-glycerophosphocholine (Ste[¹⁴C]*Δ*₄AchGro*P*Cho) (5000 dpm, 55.6 mCi/mmol) as internal probe for lipase activity. The incubation with lipase A₁ was performed at room temperature in 1 ml buffer containing 0.1 M sodium borate, 10 mM CaCl₂, 1 mg/ml sodium deoxycholate, 0.04% BSA, pH 6.5, and 2 ml diethyl ether, for 30 h with constant stirring. Lipids were extracted according to Bligh and Dyer [13], and dried under a nitrogen stream. The extract was resolved on straight-phase HPLC as described above. The effluent was collected as 1-ml fractions, which were dried under vacuum/centrifuge (Jouan) and the ratio of ¹⁴C/³H radioactivity in the peak of PAF fraction was quantified in a liquid scintillation counter. The efficiency of lipase A1 (usually >80%) was estimated by the ratio of 1-lyso-[¹⁴C]PtdCho/Ste[¹⁴C]*Δ*₄AchGro*P*Cho (retention times of 52 min and 17 min, respectively), prior to the calculation of 1-alkyl-2-[³H]acetyl-PAF and 1-acyl-2-[³H]acetyl PAF analog formation. In selected experiments, performed in the absence of internal standard, the peak of PAF fraction obtained upon 10-min stimulation was further separated (before and after lipase A1) on reverse-phase HPLC as described above, and the distribution of 1-alkyl-2-[³H]acetyl-PAF species was analysed.

Statistical analysis. Results are expressed as mean values ± SEM of the indicated number of observations. The computing program for statistics, STATPAK 4.1, was from Northwest Analytical, Inc. Significance of the difference between untreated and alkyl-Gro-treated cells was assessed by three-way analysis of variance (ANOVA). The three variables were (a) treatment by alkyl-Gro or not, (b) different times or chemical species of PAF, and (c) separate experiments performed in triplicate. ANOVA was followed in indicated cases by Student's paired *t*-tests. Significance of the treatment by SR 27417 on stimulated PAF release was tested by paired *t*-tests.
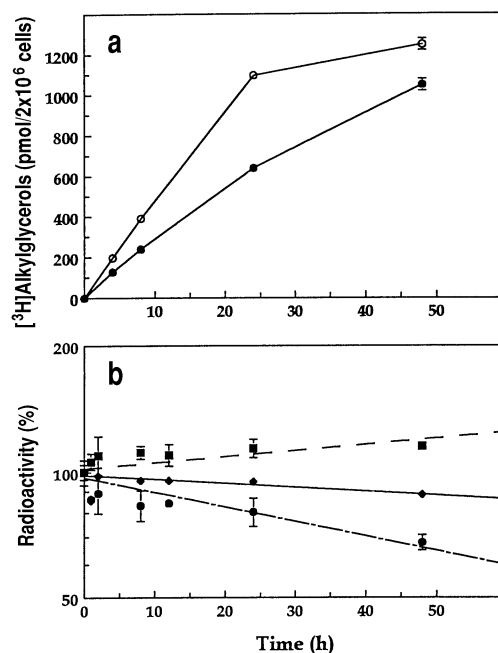

Fig. 1. Incorporation and persistence of alkylglycerols into phospholipids. (a) Incorporation. THP-1 cells were incubated with [³H]alkyl-Gro (10 µM, 12.31 mCi/mmol) for the indicated times. Cells were washed, total lipids were extracted, and radioactive phospholipids were separated by silica-gel TLC and visualized with a radiochromatogram scanner as described in Materials and Methods. Peaks migrating with standards of PtdEtn (●) and PtdCho (○) were scraped and quantified in a scintillation counter. Results are expressed as means ± SEM of 12 observations obtained from 3 experiments. (b) Persistence. THP-1 cells (5×10⁵ cells/ml) were incubated for 48 h with [³H]alkyl-Gro (10 µM, 24.82 mCi/mmol), washed three times with NaCl/Pᵢ containing 0.2% BSA, and suspended in alkyl-Gro-free medium (5×10⁵ cells/ml). At indicated times cells were centrifuged, the supernatant was removed, and total lipids were extracted, analyzed and quantified as described in Fig. 1. PtdCho (●), PtdEtn (■), and PtdCho+PtdEtn (◆). Results are expressed as means ± SEM of three experiments.

RESULTS

Alkyl-Gro incorporation and persistence in membrane phospholipids. When THP-1 cells were incubated with [³H]-alkyl-Gro (10 µM, 12.31 mCi/mmol), a significant amount was incorporated into RAcylGro*P*Etn) and RAcylGro*P*Cho). Alkyl-Gro incorporation into RAcylGro*P*Cho reached a plateau after 24 h, while incorporation into RAcylGro*P*Etn increased for another 24 h (Fig. 1a). After 24 h, alkyl-Gro were readily incorporated to a greater extent into RAcylGro*P*Cho than into RAcylGro*P*Etn, and incorporation into RAcylGro*P*Etn plus RAcylGro*P*Cho was 6.5% of the added radioactive alkyl-Gro. We did not detect any significant incorporation of alkyl-Gro into phosphatidylinositol. After incubation of THP-1 cells under similar conditions for 48 h, [³H]alkyl-Gro were removed by washing, and the time-dependent distribution of radioactivity into phospholipid classes was analyzed. We observed a substantial decrease in RAcylGro*P*Cho-associated radioactivity and conversely an increase in RAcylGro*P*Etn-associated radioactivity (Fig. 1b). After 48 h, the amount of [³H]RAcylGro*P*Cho decreased by 32%, whereas that of [³H]RAcylGro*P*Etn increased by 15%. This suggested a remodelling of 1-[³H]alkyl-PC to [³H]RAcylGro*P*Etn by phospholipases C and D and polar-head-group transferases. 48 h after the end of the incubation with [³H]alkyl-Gro, 88% of the initial radioactivity remained in cell phospholipids, indicating a slow turnover of alkyl-Gro in the membranes of the cells.
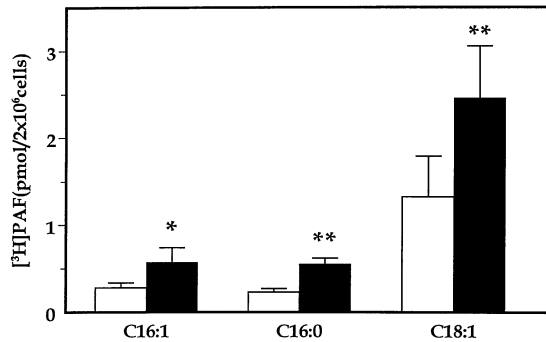
Fig. 2. Participation of incorporated alkylglycerols to PAF production. THP1 cells were incubated for 24 h in the presence of [³H]alkyl-Gro (10 µM, 92.13 mCi/mmol). The cells were centrifuged, washed and suspended in buffer A. Cells (2×10^7 cells/ml) were stimulated for 10 min with calcium ionophore A23187 (5 µM) (solid column) or with vehicle for controls (open column). Incubation was stopped with methanol, and [¹⁴C]octadecyl-PAF was added as an internal standard. Total lipids were extracted and PAF was purified on straight-phase HPLC. Molecular species were separated on reverse-phase HPLC and collected, and ³H/¹⁴C radioactivity was quantified in a liquid scintillation counter as described in Materials and Methods. Results are expressed as means ± SEM of five experiments. The significance of the difference between untreated and alkyl-Gro-treated cells was tested by three-way ANOVA ($P < 0.001$) followed by individual paired t-tests for each alkyl species: *, $P < 0.05$; **, $P < 0.01$.
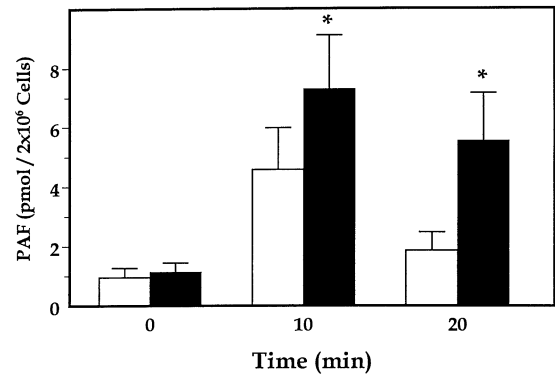

Fig. 3. Influence of alkyl-Gro on the production of biologically active PAF. THP-1 cells were cultured for 24 h in the presence (solid column) or absence (open column) of alkyl-Gro (10 µM). The cells were washed and suspended in buffer A (2×10^6 cells/ml, 1 ml/tube) in glass tubes and incubated for 30 min at 37 °C prior to stimulation with calcium ionophore A23187 (5 µM) or with vehicle for controls. At indicated times, stimulation was stopped by adding 4 ml of ethanol. After 1 h at room temperature, the tubes were centrifuged ($200 \times g$, 20 min), the supernatant was dried under a nitrogen stream, and the remaining residue was resuspended in ethanol 60 % and tested for its ability to aggregate rabbit platelets. Results are expressed as means ± SEM of five experiments. The significance of the difference between untreated and alkyl-Gro-treated cells was tested by three-way ANOVA ($P < 0.01$) followed by individual paired t tests for each time-point: *, $P < 0.02$.

[³H]PAF formation after [³H]alkyl-Gro incorporation. To demonstrate that the alkyl-Gro incorporated into phospholipids was used for PAF synthesis, we measured [³H]PAF formation after [³H]alkyl-Gro incorporation into phospholipids of THP-1 cells. After [³H]alkyl-Gro incubation (10 µM, 24 h) and incorporation into phospholipids, the THP-1 cells produced 1.85 ± 0.54 pmol [³H]PAF/2×10^6 cells under resting conditions. After a 10-min stimulation by calcium ionophore A23187, [³H]PAF raised significantly ($P < 0.001$) to 3.58 ± 0.7 pmol [³H]PAF/2×10^6 cells. [³H]alkyl-Gro incorporation induced the formation of three distinct [³H]PAF molecular species, namely C16:0, 16:1, and C18:1 PAF. Their respective amounts were 15.2, 12.5 and 72.3 % in resting cells, and 16, 15.4 and 68.6 % in stimulated cells (Fig. 2).

Influence of alkyl-Gro incorporation on PAF production. To study the effect of alkyl-Gro incorporation into RAcylGro*P*Cho on PAF production, the production of PAF was measured by a bioassay based on aggregation of washed rabbit platelets, and by biochemical quantitation of [³H]PAF and the acyl analog of [³H]PAF produced during incubation with [³H]acetate.

PAF measured by platelet aggregation. THP-1 cells were incubated for 24 h with or without alkyl-Gro (10 µM), washed and stimulated with calcium ionophore A23187 (5 µM) for 10 min and 20 min, and PAF was measured. The A23187 addition strongly stimulated PAF production with a maximum at 10 min in treated or untreated cells (Fig. 3). The treatment with alkyl-Gro induced a significant increase in PAF production, compared withuntreated cells ($P < 0.01$). Furthermore, the rate of decrease in PAF after a 20-min stimulation dropped less in treated cells (59 % and 24 % decreases compared with that after a 10-min stimulation, in untreated and alkyl-Gro-treated cells, respectively). Alkyl-Gro treatment induced about 20 % increase in PAF production under resting conditions.

[³H]PAF and acyl analog of [³H]PAF production. Using unlabelled alkyl-Gro under the same conditions of incubation and

A23187 stimulation, we measured the production of [³H]PAF after incubation with [³H]acetate. Stimulation of untreated cells with the calcium ionophore induced increases in the [³H]acetate incorporation into PAF of 130 % and 134 % after 10 min and 20 min stimulation, respectively, as shown after separation on straight-phase HPLC (Fig. 4a). Alkyl-Gro treatment enhanced significantly ($P < 0.001$) 1-alkyl-2-[³H]acetyl-PAF production, which was raised by 64.3 % in resting cells, and by 24.5 % and 36.4 % after 10 min and 20 min stimulation, respectively, compared with untreated cells. By contrast, alkyl-Gro had no effect on 1-acyl-2-[³H]acetyl PAF analog production in resting or in A23187-stimulated THP-1 cells (Fig. 4b).

Analysis of PAF on reverse-phase HPLC allowed separation of 1-acyl-2-[³H]acetyl PAF analog from different species of 1-alkyl-2-[³H]acetyl-PAF. Untreated THP-1 cells produced mainly C16:0 1-alkyl-PAF (97.12 %) after a 10-min stimulation. When the cells were incubated with alkyl-Gro containing prominently the C18:1 hydrocarbon chain, a 15-fold increase in C18:1 PAF occurred, representing 9.07 % of total PAF, and the fraction of C16:0 PAF dropped to 88.3 % (Table 1).

Effect of PAF receptor antagonist on [³H]PAF production. After supplementation with alkyl-Gro, which contained predominantly the C18:1 alkyl chain, the THP-1 cells produced mainly C16:0 PAF. Therefore we proposed that endogenously produced PAF may increase its own production, as suggested by our earlier study [16]. After treatment with alkyl-Gro, THP-1 cells were incubated with the specific PAF-receptor antagonist SR 27417, before measurement of PAF in resting and ionophore-stimulated cells. In stimulated THP-1 cells, the A23187-induced increase in PAF production was significantly reduced, by about 50 %, in the presence of SR 27417, whereas this PAF-receptor inhibitor did not change PAF production in unstimulated THP-1 cells (Fig. 5).

DISCUSSION

In this study, we report that THP-1 cells incubated with naturally occurring alkyl-Gro incorporated such ether lipids into al-
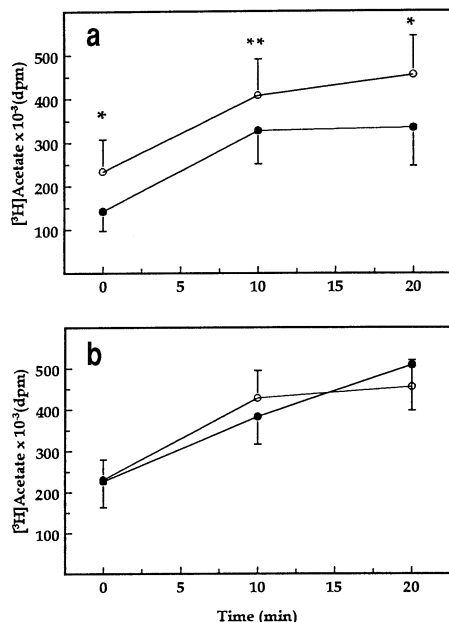
Fig. 4. Influence of alkyl-Gro on PAF (a) and 1-acyl-PAF (b) produc-
tion. THP-1 cells were cultured for 24 h without (●) or with (○) alkyl-
Gro (10 μM). Cells were washed and incubated with [³H]acetate (20 μCi/
assay) for 20 min, then stimulated with calcium ionophore A23187
(5 μM) or with vehicle for controls. At indicated times, cell stimulation
was stopped with methanol and [¹⁴C]octadecyl-PAF was added as in-
ternal standard. Total lipids were extracted, total (alkyl + acyl analog)
PAF was separated using straight-phase HPLC, and radioactivity was
counted in an aliquot. The acyl analog of PAF was hydrolyzed with
lipase A1 (from *R. arrhizus*) in the presence of Ste[¹⁴C]Δ_4AchGro*P*Cho
as probe for lipase activity. Non-hydrolyzed PAF was extracted, purified
by straight-phase HPLC, and its radioactivity measured in a liquid scin-
tillation counter (a). The yield of lipase activity was established from
the ratio lyso[¹⁴C]PtdCho/Ste[¹⁴C]Δ_4AchGro*P*Cho radioactivity, and the
initial quantity of the acyl analog of PAF was calculated (b). Results
are expressed as means*SEM of six experiments. Significance of the
difference between untreated and alkyl-Gro-treated cells in (a) was tested
by three-way ANOVA ($P < 0.001$) followed by individual paired *t*-tests
for each time-point: *, $P < 0.05$; **, $P < 0.001$.

kyl-phospholipid precursors of PAF, contributing to an increased
PAF production in resting and stimulated cells. This incorpora-
tion into alkyl-phospholipids is in agreement with previous data
showing that dietary alkyl-Gro or diacetyl derivatives may par-
ticipate in the elevation of alkylacylglycerophospholipid content
in several rat tissues, without modifying the amount of total
phospholipids in the cells, or the ratio of phospholipid classes
[6, 7].

Alkyl-Gro are phosphorylated by an ATP:alkylglycerol
phosphotransferase to form 1-*O*-alkyl-2-lyso-*sn*-glycerol-3-
phosphate. Via this pathway, the alkyl-Gro from dietary intake
or resulting from metabolism enter the biosynthetic pathways
responsible for the production of structural lipids that are com-
ponents of membrane bilayers, and precursors of lipid mediators
including PAF [17]. In THP-1 cells, the incorporation of alkyl-
Gro occurred predominantly into PtdCho, and to a lesser extent
and at a slower rate, into PtdEtn. The study of the turnover of
[³H]alkyl-Gro after pulse labelling showed that after 48 h, only
12% of the initial labelled phospholipids were lost, suggesting
a slow turnover of 1-alkyl-phospholipids in which [³H]alkyl-
Gro were incorporated. We noted a substantial decrease in
[³H]RAcylGro*P*Cho which paralleled the increase in [³H]-
RAcylGro*P*Etn, which suggests a conversion of RAcylGro*P*Cho
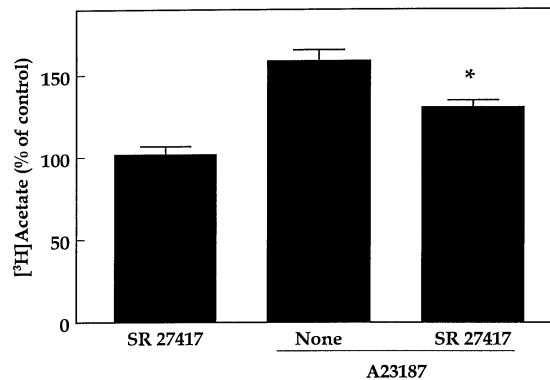into RAcylGro*P*Etn. Such a reaction involving the transfer of

Fig. 5. Effect of PAF-receptor antagonist on PAF production. THP-1
cells were cultured for 24 h with alkyl-Gro (10 μM). Cells were washed
and incubated with [³H]acetate (20 μCi/assay), and with or without PAF-
receptor antagonist SR 27417 (0.1 μM) for 30 min. Cells were stimulated
with calcium ionophore A23187 (5 μM) or with vehicle for controls.
After 10 min, cell stimulation was stopped with methanol and [¹⁴C]octa-
decyl-PAF was added as internal standard. Total lipids were extracted,
total (alkyl + acyl analog) PAF was separated using straight-phase
HPLC, and radioactivity was measured. Results are expressed as
means ± SEM of five experiments. The significance of the difference
between SR-27417-treated and untreated cells was assessed by paired
t-tests: *, $P = 0.025$.

Table 1. Influence of alkyl-Grp on PAF species production. THP-1
cells were cultured for 24 h with or without alkyl-Gro (10 μM). Cells
were washed and suspended in buffer A, then 5 ml cell suspension
(5×10^6 cells/ml) were incubated for 20 min with [³H]acetate (20 μCi/
tubeThe cells were stimulated with calcium inophore A23187 (5 μM)
for 10 min. Total lipids were extracted, and total PAF was separated as
in Fig. 6. Molecular species of 1-[³H]alkyl-PAF were separated using
a reverse-phase HPLC, quantified in a liquid scintillation counter,
and their distributions were calculated. Results are expressed as
means ± SEM of data from three experiments performed in duplicate.
The significance of the difference between untreated and treated cells
was tested by three-way ANOVA followed by paired *t*-tests. n.d., not
detected.

PAF species	Radioactivity incorporated into			
	control cells		alkyl-Gro−treated cells	
	dpm	%	dpm	%
C16:1	n.d.	n.d.	n.d.	n.d.
C16:0	7111 ± 1381	97.12	11184 ± 2718	88.3 [a]
C18:1	76 ± 35	1.04	1149 ± 338	9.07 [b]
C18:0	135 ± 20	1.84	333 ± 83	2.63 [a]
Total	7322 ± 1408	100	12666 ± 3118	100 [c]

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P = 0.026$.

polar groups such as choline and ethanolamine in ether phospho-
lipids has been reported [18].

PAF is a potent mediator involved in human pathophysiol-
ogy including septic shock, asthma and allergy. PAF plays a role
in neuronal functions, reproduction and fetal development [8, 9,
19]. Many PAF activities are mediated through transmembrane
receptors; however, PAF has been described recently as an intra-
cellular messenger with PAF-binding sites in subcellular frac-
tions of rat cerebral cortex [20]. PAF induces early-gene expres-
sion, such as c-*fos* and *zif-268* in rat hippocampus [21] or c-*fos*
and c-*jun* in human neuroblastoma cells [22]. This makes PAF

an almost unique type of molecule with autacoid and second-messenger properties. Previous reports have indicated that the exogenous PAF precursor 1-*O*-alkyl-*sn*-glycero-3-phosphocholine increases PAF production in human neutrophils [23] and macrophages [12]. As alkyl-Gro was incorporated predominantly into the PAF precursor RAcylGro*P*Cho, it was of interest to establish whether the incorporated alkyl-Gro could account for and/or modify PAF production in resting or stimulated THP-1 cells.

To demonstrate that alkyl-Gro incorporated into cellular phospholipids acted as precursors for PAF synthesis, we measured [³H]PAF formation after [³H]alkyl-Gro incorporation into phospholipids of THP-1. The THP-1 cells that incorporated [³H]alkyl-Gro produced three major [³H]PAF molecular species, C18:1, C16:0 and C16:1 PAF under resting and stimulated conditions. The distribution of the three major molecular species of labelled PAF produced by stimulated cells was 68.7, 15.4 and 15.9% for C18:1, C16:0 and C16:1 PAF, respectively. As this distribution fits approximately the ratio of corresponding precursors contained in alkyl-Gro, it indicates that different alkyl-Gro were used as PAF precursors without species selectivity. Resting THP-1 cells produced [³H]PAF, and such formation was enhanced in A23187-stimulated cells. Thus, we demonstrated that alkyl-Gro were incorporated into phospholipid pools involved in PAF synthesis under resting and stimulated conditions. Dual PAF-biosynthetic pathways have been proposed [24]. One pathway, referred to as the remodeling pathway, involves the hydrolysis of pre-existing 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine by phospholipase A$_2$ and/or by transacylase, and acetylation of 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lyso-PAF) by acetyl-CoA:lyso-PAF acetyl transferase. The second pathway, or *de novo* route, involves acetylation of 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphate with a subsequent removal of the phosphate by a 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphate phosphohydrolase and the transfer of phosphocholine from CDP-choline to 1-alkyl-2-acetyl-*sn*-glycerol by a CDP-choline phosphotransferase. PAF formation induced by various stimuli is considered to occur mainly via the remodeling pathway, whereas in resting cells, the *de novo* synthetic pathway is predominantly involved in PAF production. Our data suggest that alkyl-Gro may participate in PAF synthesis via both pathways, since it increases PAF production in resting and stimulated cells. However, our unpublished data show that the basal activity of acetyltransferase of the remodelling pathway is high in THP-1 cells, and thus may be involved in PAF formation in resting cells.

The next step was to study the influence of alkyl-Gro incorporation on the quantity of PAF produced by the cells. PAF titration by platelet-aggregation assays showed that alkyl-Gro increased the amount of biologically active PAF produced by THP-1 cells under resting and stimulated conditions. The maximum increase, corresponding to a threefold stimulation, was reached after a 10-min stimulation. However, the PAF bioassay is not entirely specific to 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine. Some related compounds, such as 1-acyl and 1-alkenyl analogs of PAF, aggregate platelets, but with a lower potency than PAF [25]. Therefore the HPLC procedure was used to measure the amount of 2-[³H]acetyl-PAF before and after incubation with phospholipase A$_1$, which hydrolyses the 1-acyl analog of PAF. Our results confirmed that PAF, but not its 1-acyl analog, was increased after alkyl-Gro incorporation in resting and stimulated cells. However, analysis of PAF species in stimulated cells demonstrated that the ratio between the PAF species produced upon stimulation was different from the ratio of C16:0 alkyl-Gro /C18:1 alkyl-Gro in the mixture. The increase in PAF level resulted mainly from the increase in the C16:0 PAF molecular species. Upon stimulation with calcium ionophore, this species, which represented initially 97.1% of PAF in alkyl-Gro-free cells, rose by 57.3% after alkyl-Gro incorporation. On the other hand, C18:1 PAF represented only 1% of PAF in alkyl-Gro-free cells and was increased 15-fold after alkyl-Gro incorporation, rising to 9.1% its level produced by stimulated cells. Thus, after alkyl-Gro incorporation followed by stimulation, the ratio of C16:0/C18:1 was 9.7 in PAF, whereas it was only around 0.12 in the alkyl-Gro mixture. Since THP-1 cells possess PAF receptors, we propose that the rise in PAF production after alkyl-Gro incorporation results primarily from the increase in the pool of PAF precursors. This rise is amplified by PAF, which mobilizes mainly a C16:0 precursor, thus producing prominently C16:0 PAF. The highly specific PAF-receptor antagonist SR 27417 reduced the ionophore-stimulated PAF production by about 50%, indicating that such a mechanism could be operational in these cells. This proposed mechanism is supported by recent data suggesting autocrine amplification of PAF biosynthesis [16]. Previous data have shown that in isolated rat, mouse and guinea pig neutrophils, the composition of PAF molecular species produced by the unstimulated or A23187-stimulated cells had a profile that was different from potential precursors, indicating a high selectivity for utilization of precursor substrate [26]. Our data indicate that the PAF species profile could be significantly altered by the addition of an exogenous precursor, such as C18:1 alkyl-Gro. This modulation could occur under physiological situations since the molecular species distribution of PAF is independent of the stimulus used to elicit its synthesis [27]. Since various molecular species possess distinct biological activities [28], this represents an interesting way to modulate such activities. Such a modulation of PAF synthesis might represent benefits in various pathophysiological situations in which PAF may have an important role, i.e. stimulation of cells involved in immunological responses, such as lymphoid cells [29].

Our results suggest a possibility of dietary supplementation with alkyl-Gro to prevent the consequences of a genetic deficit in ether-lipid synthesis, such as in Zellweger syndrome. This disease is characterized by the absence of ultrastructurally detectable peroxisomes in patients' tissues, associated with the absence of ether-lipid synthesis [30−32].

In conclusion, our study demonstrates that exogenous alkyl-Gro may participate in PAF synthesis and increase its production. This could be of importance in several physiological and pathological conditions.

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