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Biosynthesis of paf-acether

XVII. Regulation by the CoA-independent transacylase in human neutrophils

Ewa Ninio[1], Michelyne Breton[2], Jocelyne Bidault[1] and Odile Colard[2]

[1]*INSERM U200, 32 rue des Carnets, 92140 Clamart, France* and [2]*URA 1283 CNRS, CHU Saint-Antoine, 27 rue Chaligny, 75571 Paris, France*

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Treatment of intact human polymorphonuclear neutrophils (PMN) with low concentrations of phorbol myristate acetate (PMA, 1–10 ng/ml) induced paf-acether (paf) and lyso paf formation, arachidonate release, and simultaneous inhibition of CoA-independent lyso paf : transacylase as assayed in a cell-free system. Inhibition of [^{3}H]lyso paf reacylation was also observed when it was exogenously added to the PMA-treated intact PMN. When higher concentrations of PMA (40–100 ng/ml) were used, paf biosynthesis was severely impaired and the level of the CoA-independent transacylase activity returned to basal level. Since lyso paf appears to be the substrate for PMA-activated paf formation (remodeling pathway), we showed that [^{14}C]acetate was incorporated into the paf molecule. By contrast, labeling with [^{3}H]choline was not appropriate in this model. The presented results are against the involvement of a de novo route in paf synthesis initiated by PMA and open a new possibility of an important role for the CoA-independent transacylase in controling the level of lyso paf availability for paf formation.

Polymorphonuclear neutrophil (human); Paf-acether; CoA-independent transacylase

1. INTRODUCTION

In human polymorphonuclear neutrophils (PMN) stimulated by a variety of agents, paf-acether (paf) is mainly synthesized via the remodeling pathway, i.e. deacylation/acetylation of pre-existing membrane phospholipid class of alkylacylglycerophosphocholine (AAGPC) (reviewed in [1]). However, conflicting results concerning the phorbol ester-activated de novo route for paf biosynthesis in PMN have also been published [2]. Two major factors limiting paf biosynthesis via the remodeling pathway were discovered: the level of activation of lyso paf (1-alkyl-GPC) : acetyl-CoA acetyltransferase (EC 2.3.1.67) and the avaibility of lyso paf [3–7]. The latter might be formed either directly by phospholipase A_2 action on AAGPC or by a CoA-independent transacylase inducing a transfer of arachidonic acid (AA) from arachidonyl-containing AAGPC [8]. In this context, phospholipase A_2 might generate lyso-derivative acceptor and thus participate indirectly in the deacylation of paf precursor. Under the conditions that are optimal for acetyltransferase activation, either the supply of exogenous lyso paf [6] or coincubation with thrombin-activated platelets, a source of abundant lyso paf [9], enhanced paf biosynthesis by activated PMN.

Correspondence address: E. Ninio, INSERM U200, 32 rue des Carnets, 92140 Clamart, France. Fax: (33) (1) 46310277.

Abbreviations: AA, arachidonic acid; AAGPC, alkylacylglycerophosphocholine; Io, ionophore A23187; paf, paf-acether; PMA, phorbol myristate acetate; PMN, polymorphonuclear neutrophils

When analyzing the time course of lyso paf formation in stimulated PMN we noticed that free lyso paf was only transiently present in these cells [6]. In addition, only concentrations of phorbol myristate acetate (PMA) between 1 and 10 ng/ml promoted lyso paf accumulation above the basal level [7]. In this report we address the question of the involvement of CoA-independent transacylase in the modulation of lyso paf availability by PMA. We also present new evidence suggesting that the phorbol esters activate the remodeling pathway rather than de novo one.

2. MATERIALS AND METHODS

Reagents were purchased as indicated. 4-α-phorbol-12-myristate-13-acetate (PMA), ionophore A23187 (Io; both prepared as 1 or 10 mg/ml stock solutions in dimethylsulfoxyde were kept at −20°C), fatty acid-free bovine serum albumin (BSA), creatine phosphate, creatine phosphokinase: Sigma Chemical Co., St. Louis, MO, USA. Aspegic: Laboratoire Egic, Amilly, France. Lipase from *Rhizopus arrhizus*: Boehringer, Mannheim, Germany. Paf C18:0 : Novabiochem, Cléry en Vexin, France. [^{3}H]lyso paf (1-*O*-[^{3}H]octadecyl-GPC, 163 Ci/mmol), [^{14}C]acetate (60 mCi/mmol), [^{3}H]choline (85 Ci/mmol) [^{3}H]arachidonic acid (2.9 Ci/mmol), [^{3}H]paf (1-*O*-[^{3}H]octadecyl-2-acetyl-GPC, 128 Ci/mmol), [^{3}H]phosphaditylcholine (62 Ci/mmol): Amersham Int., Amersham, UK.

2.1. *Preparation and stimulation of PMN*

Venous blood from healthy donors was collected and neutrophils were prepared as in [7]. The cell pellet was resuspended in HEPES buffer (pH 7.4) containing (mM) : KCl 2.6, NaCl 137, $CaCl_2$ 1.3, $MgCl_2$ 1.0, glucose 5.6, HEPES 4.2, and 0.25% BSA. Preparations were 98% PMN-positive (May Grunwald-Giemsa). Cells (10^6/ml) were stimulated for 60 min at 37°C with PMA (1 to 100 ng/ml) or 15 min with Io (2 μg/ml). When total formation of paf and lyso paf were to

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be assessed, the reaction was stopped by the addition of 4 vols. of ethanol and processed as in [6]. Paf and lyso paf, transformed into paf by chemical acetylation, were quantitated by aggregation of washed rabbit platelets and results expressed in pmol/10^6 PMN, as calculated over a calibration curve with C18:0 paf [10]. To assess the effect of PMA treatment on the CoA-independent transacylase, the stimulation of PMN was stopped by washing with NaCl 150 mM (4°C, 400 × *g* for 5 min). The cell pellets were resuspended in 0.2 ml of HEPES-EDTA (1 mM, pH 6.5) buffer, sonicated 15 s (Branson Sonic Power Co., Danbury, CT; microtype, position 3) and stored at −80°C until assay.

To assess the effect of PMA treatment on the lyso paf reacylation by intact PMN, cells were washed with NaCl as above, resuspended in 1 ml of HEPES-BSA containing [^{3}H]lyso paf (0.5 μCi) and reincubated for 15 min at 37°C. The reaction was stopped by adding 3 ml of chloroform/methanol (1:2, v/v) to achieve phase separation [11]. The organic phase was brought to dryness under an air flow at 40°C and the resulting residue was further analyzed by liquid chromatography (microporasil column, Waters, Milford, MA) using dichloromethane/methanol/water (60:50:5, v/v) as mobile phase at a flow rate of 1 ml/min [12]. [^{3}H]phosphatidylcholine (8 min), [^{3}H]paf (16 min) and [^{3}H]lyso paf (24 min) served as standards (retention time).

2.2. *Incorporation of [^{3}H]AA, [^{14}C]acetate and [^{3}H]choline by PMN*

PMN (3 × 10^7/ml) were preincubated for 10 min at 37°C in HEPES-BSA containing 30 μCi of [^{3}H]AA. The cells were centrifuged (20°C), then washed twice with 45 ml of HEPES-BSA buffer devoid of Ca^{2+} and Mg^{2+}. The cell pellet was resuspended in HEPES-BSA buffer. PMN were incubated at 37°C with defined concentrations of PMA or vehicle, the reaction was stopped by centrifugation (4°C) and the level of [^{3}H]AA release was assessed in the supernatants.

PMN (2 × 10^7/2 ml) were preincubated 10 min at 37°C with 20 μCi [^{14}C]acetate prior to stimulation as described above. Cells were also preincubated 1 or 2 h at 37°C with 20 μCi [^{3}H]choline alone, or 40 μCi [^{14}C]acetate was added 10 min before stimulation. All reactions were stopped by adding 6 ml of chloroform/methanol (1:2, v/v) as in [11]. Labeled phospholipids were treated overnight by lipase A_1 [13], reextracted and separated on TLC using chloroform/methanol/water/acetic acid (50:25:4.5:4.5, v/v) as mobile phase.

2.3. *Transacylase assay*

CoA-independent transacylase was assayed as in [14] using Tyrode buffer (pH 6.5) supplemented with 0.16 μM [^{3}H]lyso paf (0.05 μCi) and cell lysate prepared as described above. Incubations were carried on for 10 min at 37°C, samples were extracted [11] and analyzed by TLC as above.

3. RESULTS AND DISCUSSION

PMN were stimulated for 60 min with graded concentrations of PMA. As we have shown earlier [7], lyso paf and paf formation increased with PMA concentrations between 1 and 10 ng/ml, whereas at higher concentrations lyso paf remained at the basal level (Fig. 1A). Since the lyso paf formed during stimulation is not only the substrate for acetyltransferase, but also might be acylated back into AAGPC by the CoA-independent : lyso paf transacylase [15], we measured activity of this enzyme (Fig. 1B). We observed that the rate of transacylation, when assayed in a cell-free system supplemented with [^{3}H]lyso paf, was modulated by the treatment of intact the PMN by PMA. The enzyme activity was decreased by 15 to 38% in cells treated with low concentrations of PMA (1 to 25 ng/ml) and almost returned to the

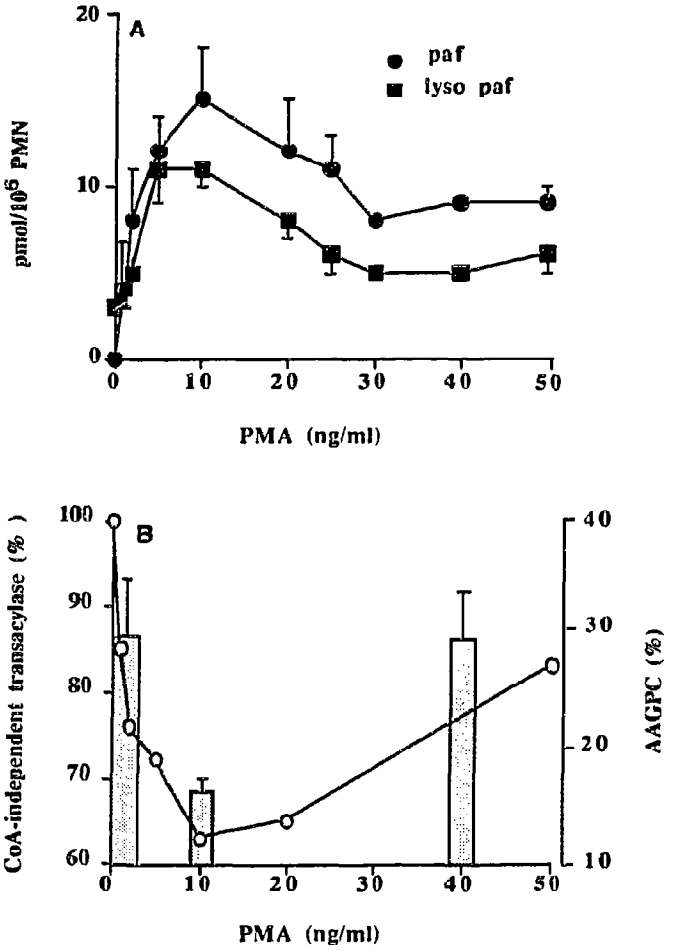

Fig. 1. Paf biosynthesis and activity of the CoA-independent transacylase. A. Paf (●) and lyso paf (■) formation by PMA-treated (60 min at 37°C) PMN. Results in pmol/10^6 cells are means ± SE of 3 to 5 experiments, except at 30 and 40 ng/ml PMA (n=2). B. Activity of [^{3}H]lyso paf : CoA-independent transacylase in sonicates of PMA-treated (60 min at 37°C) PMN. Results are the percentages of transacylase activity as compared to untreated PMN and are means of 2 separate experiments, where 100% of activity was 6.6 and 8.8 pmol/min per 10^6 cells. Bars: reacylation of [^{3}H]lyso paf into AAGPC by PMA-treated intact PMN. PMN were treated with PMA (60 min at 37°C), washed and reincubated with [^{3}H]lyso paf for 15 min. Results as percentage of the total radioactivity (259,738 ± 8,511 dpm, SE of 8 determinations) recovered after liquid chromatography are means of 4 separate experiments. When 100 ng/ml PMA was used 29 ± 7% of [^{3}H]lyso paf was reacylated into AAGPC.

basal level when higher concentrations were used. This biphasic effect of PMA might be due to selective activation of protein kinase C isoforms as shown in human platelets [16] and with purified rat brain isozymes [17]. A modulation of CoA-independent transacylase by protein kinase C activators has also been found in rat platelets [18]. In the next set of experiments, PMN were triggered with PMA at 10, 40 and 100 ng/ml, for 60 min at 37°C. After washing, the intact cells were reincubated for 15 min at 37°C with [^{3}H]lyso paf (Fig. 1B). The reacylation rate, i.e. the percentage of AAGPC formed with respect to the total radioactivity recovered after

Table I

Labeling of paf and its precursors with [^{14}C]acetate and [^{3}H]choline and paf quantitation.

Stimulation	Control	Io: (2 μg/ml)	PMA (20 ng/ml)	PMA (100 ng/ml)
Lyso paf [^{3}H]choline	76.0±22.5	58.5±9.1	265.3±6.7	301.8±106.1
AAGPC [^{3}H]choline	80.4±13.4	146.9±41.3	674.0±223.2	456.1±106.1
paf				
[^{3}H]choline[a]	ND	11.2[b]	<1.0[b]	<1.0[b]
[^{14}C]acetate	ND	31.3±4.7	8.3±2.1	6.7±2.1
Bioassay	ND	548±122	66±12	46±10
Specific activity	NA	70±30	128±34	147±16

PMN (2×10^7/2 ml) were preincubated at 37°C for 1 or 2 h with 20 μCi [^{3}H]choline, next 40 μCi [^{14}C]acetate was added for additional 10 min and cells were stimulated with Io for 15 min or PMA for 60 min. After extraction, phospholipids were analyzed on TLC or TLC and subsequent liquid chromatography[a]. Results of [^{3}H]choline and [^{14}C]acetate incorporation are expressed in dpm $\times 10^{-3}$, bioassay in pmol and specific activity of [^{14}C]paf in dpm/pmol. They are means±SEM of 3 or 4 independent experiments. ND, not detected; NA, not applicable; one positive result out of 4 independent experiments[b].

liquid chromatography separation, was lower in cells stimulated with 10 ng/ml PMA than in those exposed to 40 and 100 ng/ml.

Since the modulation of the CoA-independent transacylase might govern availability of lyso paf for paf synthesis, it was important to show that labeled acetate was incorporated into paf. We showed that both Io and PMA induced incorporation of the label into paf (Table I). In addition, mass of paf measured by the bioassay revealed that the specific activities of paf formed were not substantially different in Io- versus PMA-stimulated cells. In contrast, both agonists promoted only weak incorporation of labeled choline into paf (one positive experiment out of 4) that was only detectable after liquid chromatography of TLC eluates. We found that labeled choline was mainly incorporated, after lipase A_1 hydrolysis of the *sn*-1 acyl bond, into lyso paf and AAGPC (Table I). Io-stimulation slightly enhanced AAGPC labelling without any change in lyso paf, whereas PMA induced a several-fold increase in [^{3}H]choline incorporation into both. Fractions corresponding to lyso paf did not contain platelet aggregating activity, but when chemically acetylated, they became indistinguishable from our paf standard (data not shown). Thus the radioactivity comigrating with the lyso paf standard did not contain paf, which could be masked by such a substantial incorporation of [^{3}H]choline into lyso paf. The low amount of paf formed, together with the high [^{3}H]choline incorporation into paf precursors, might account for the discrepancies between our own and the former study [2].

Since labeled acetate but not choline is incorporated into paf, the remodeling pathway is most probably operational in paf biosynthesis initiated by PMA. We also checked that PMN prelabeled with arachidonate released this fatty acid after incubation with 10 ng/ml PMA: at 30 min, 15.7 ± 5.2% net release, n=3; at 120 min 24.4 ± 3.6%, n=5. This time-course is similar to that of lyso paf and paf biosynthesis [7] and is consistent with a close relationship between arachidonate release and paf biosynthesis [19]. The latter finding, together with our earlier description of acetyltransferase activation in PMN stimulated by PMA [7], indicate that paf is formed by these cells rather as a result of activation of the remodeling pathway than the de novo route [2]. In the former pathway, the CoA-independent transacylase might directly control lyso paf supply [8] or modulate lyso paf availability by changing the rate of its reacylation with long chain fatty acids.

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