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BIOSYNTHESIS OF PAF-ACETHER XIV. PAF-ACETHER OUTPUT IN MURINE PERITONEAL MACROPHAGES IS REGULATED BY THE LEVEL OF ACETYLHYDROLASE [1]

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SUMMARY Paf-acether (paf) synthesis was previously shown to be impaired in 24 hr-adherent and Bacillus Calmette-Guérin-activated murine peritoneal macrophages as compared to resident macrophages. We report here that the induction of acetylhydrolase was responsible for the decreased paf output in 24 hr-adherent macrophages. The kinetic analysis of the enzymes derived from 2 hr-, 24 hr- and BCG-activated adherent macrophages and from plasma revealed that the K_m for paf was similar whatever the source of the acetylhydrolase whereas the V_{max} was five-fold increased in 24 hr-cultured macrophages. The acetylhydrolase activity was Ca^{2+}-independent and was not inhibited by addition of alkyl-acyl(long chain)-glycero-phosphocholine suggesting that the enzyme was not a phospholipase A_2.

Paf-acether (paf) metabolic pathway via the deacylation/acetylation mechanism has been shown to be instrumental in several cell types (reviewed in 1 and 2). It involves phospholipase A_2 (PLA_2) hydrolysis of alkyl-acyl-glycero-phosphocholine (alkyl-acyl-GPC) from membrane phospholipids into alkyl-GPC (lyso paf) and its subsequent acetylation by an 1-O-alkyl-GPC, acetyl-CoA : acetyltransferase (EC 2.3.1.67). The acetyltransferase level is responsible for the regulation of paf biosynthesis in leukocytes and platelets, mouse bone marrow-derived mast cells, and murine macrophages (reviewed in 2). Paf degradation was first attributed to an Acid-Labile Factor described in human sera (3, 4), and later to an 1-O-alkyl-2-acetyl-GPC : acetylhydrolase (EC 3.1.1.48) present in cytosolic fractions of various rat tissues (5), in human sera (6), in plasma and platelets from several species, and in human neutrophils, eosinophils and endothelial cells (7-10). Human or rat plasma

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acetylhydrolase is bound to lipoproteins (4-12). Both of them are acid-labile, Ca^{2+}-independent and can hydrolyse only short chain fatty acids at sn-2 position of glycerol (6).

Acetylhydrolase acts as an important modulating factor in paf output and thus can control paf concentration in blood. Indeed, higher level of acetylhydrolase was found in plasma from hypertensive rats than from normotensive ones (13) and in plasma from patients with ischemic cerebrovascular disease as compared to control subjects (14).
Moreover, acetylhydrolase can be released from platelets during aggregation (15).

The low level of paf biosynthesis in murine BCG-activated macrophages (BCG-MO) or macrophages maintained 24 hours in culture (24 hr-MO) was not attributed to a defect in the anabolic pathway (16-18). Thus, it was postulated that in these cells, the regulation could be governed by the level of acetylhydrolase. We brink now evidence that in murine macrophages maintained in culture for 24 hr but not in BCG-activated macrophages, the enhanced catabolism of paf via acetylhydrolase induction is responsible for the low output of the mediator.

MATERIALS AND METHODS

Macrophages

Macrophages were obtained as described in (16) and plated for 2 or 24 hr. Two hour-adherent activated-MO were obtained from mice injected with Bacillus Calmette-Guérin (BCG) (Institut Pasteur Production, Marnes-La-Coquette, France) for a period of 1 to 2 weeks (BCG-MO, 16).

Identification and quantitation of paf metabolites

Paf metabolism by murine peritoneal macrophages was determined using [^{3}H]octadecyl-paf (1-O-[^{3}H]octadecyl-2-acetyl-GPC, 179 Ci/mmol, Amersham Int., Amersham, U.K.) or [^{3}H]acetyl paf (1-O-hexadecyl-2-[^{3}H]acetyl-GPC, 10 Ci/mmol, Amersham). Labeled paf from 15 to 500 nM, (0.01 to 0.05 μCi) was added to adherent cells in 500 μl culture medium and incubated for 5 to 120 min at 37°C. Reactions were stopped by adding ethanol (80% final concentration). Lipids were extracted from supernatants and cell monolayers by overnight incubation at 4°C. After centrifugation (1500 x g, 20 min, 4°C) the ethanolic supernatants were collected and dried under an air stream at 40°C and kept at -20°C prior to the high pressure liquid chromatography (HPLC) analysis. Unspecific paf metabolism (less than 5%) was determined using the same experimental conditions, except that the macrophages were lacking. In some experiments, macrophages were incubated for 1 hr at 37°C with zymosan (200 μg/ml, Sigma Chem. Co, St Louis, MO, USA) before addition of [^{3}H]octadecyl-paf. Lipid extracts were resolved by HPLC (Microporasil column, Waters, Milford, MA, USA), using dichloromethane/methanol/water (60:50:5, v/v) as mobile phase. Fractions were collected at 1 min intervals, dried, dissolved in ethanol and mixed with OCS (Amersham Int.) prior to scintillation counting. Sphingomyelin and phosphatidylcholine from Sigma and radiolabeled paf and lyso paf (Amersham Int.) were used as standards. When [^{3}H]acetyl-paf was used, the release of [^{3}H]acetate was determined as described below.

Acetylhydrolase assay

The acetylhydrolase activity was measured in cell sonicates from 2 hr-, 24 hr- and BCG-macrophages and in murine plasma. Plasma was obtained after centrifugation of mouse blood (800 x g, 15 min) collected using EDTA (2.5 mM final) as anticoagulant. Then platelets were removed by additional centrifugation (1500 x g, 15 min, 4°C). Protein content was determined in cell lysates and in plasma using Lowry's method (19).

Macrophage lysates (50 µl, 5-15 µg protein) or plasma diluted 1:100 were added to 440 µl of a buffer pH 8.0 containing in mM: NaCl 137, KCl 2.6, HEPES 4.2, EDTA 2 or $CaCl_2$ 10. After 5 min preincubation at 37°C, the reaction was initiated with 10 µl of [^{3}H]acetyl-paf (0.01 µCi, 2.5 to 50 µM final concentration). Incubations were performed for 10 min at 37°C unless stated otherwise and stopped in an ice bath with simultaneous addition of 0.4 ml acetic acid (5%) solution containing [^{14}C]acetate (6000 dpm) as an internal standard. [^{3}H]acetyl-paf was extracted in organic phase by adding 2 ml of methanol/dichloromethane (1:1, v/v). Mixtures were centrifuged (3000 x g, 10 min) to facilitate phase separation. The amount of [^{3}H]acetate in the aqueous phase resulting from the acetylhydrolase assay was determined by liquid scintillation using ACS (Amersham Int.) as solvent. Blanks prepared using heat-denatured enzyme (100°C for 10 min) never exceeded 5% of tested samples and were subtracted prior to calculations of enzyme activities. The results are expressed in nmol x min^{-1} x mg^{-1} protein after correction for extraction losses calculated using the [^{14}C]acetate internal standard.

RESULTS

Metabolism of paf in intact macrophages

Incubation of [^{3}H]octadecyl-paf with intact macrophages for 1 hr at 37°C resulted in the formation of two products corresponding to lyso paf and alkyl-acyl-GPC (Table I). In 2 hr- and BCG-macrophages, only 18 to 38 % of paf was hydrolysed, whereas in 24 hr-macrophages, 69 to 90 % of

Table I. Paf metabolism in various macrophage populations

Exp. N°	Macrophages	Radioactivity distribution[a]		
		Paf	Lyso paf	AAGPC
I				
	2 hr-MO	82.0	18.0	0.0
	BCG-MO	55.5	37.5	7.0
	24 h-MO	27.0	69.0	4.0
II				
	2 hr-MO	70.0	27.0	3.0
	BCG-MO	61.0	32.0	7.0
	24 h-MO	6.0	90.0	4.0

2 hr-, BCG- and 24 hr-macrophages were incubated for 1 hr at 37°C with [^{3}H]octadecyl-paf (0.01 µCi, 100 nM). Lipids were extracted and separated using HPLC.

[a] Values are means of duplicate determinations with variations below 10 %.

the added [^{3}H]octadecyl-paf was recovered as lyso paf within 1 hr. In all types of macrophages, only a small part from 0 to 7 % of [^{3}H]octadecyl-paf was metabolised into alkyl-acyl-GPC. The initial velocity of paf degradation was further studied in 2 hr- and 24 hr-macrophages using [^{3}H]acetyl-paf. The release of [^{3}H]acetate was proportional with time up to 15 and 5 min for 2 hr- and 24 hr-macrophages respectively and reached the plateau at 15 min only for 24 hr macrophages. The hydrolysis of paf was still unsaturated at concentration 500 nM ; higher concentrations being stimulatory to intact macrophages (20). In two separate experiments the initial velocity calculated in the presence of 500 nM of paf and the linear portion of the time-course curve was : 13 vs 189 pmol x mg^{-1} prot. for 2 hr- and 24 hr-macrophages respectively.

Characteristics of paf acetylhydrolase activity in murine macrophage lysates and plasma

In the following experiments, acetylhydrolase activity was determined in suspension of sonicated unstimulated macrophages by the measurement of free [^{3}H]acetate released from 10 μM [^{3}H]acetyl-paf. Acetylhydrolase activity in all types of macrophages was linear as compared to the time of incubation through at least 10 min (Fig. 1) and to protein concentration at least up to 15 μg x ml^{-1} (data not shown). Paf hydrolysis was then studied as a function of paf concentration. [^{3}H]acetate release from [^{3}H]acetyl-paf reached a plateau in the presence of 25 μM paf in 2 hr-,

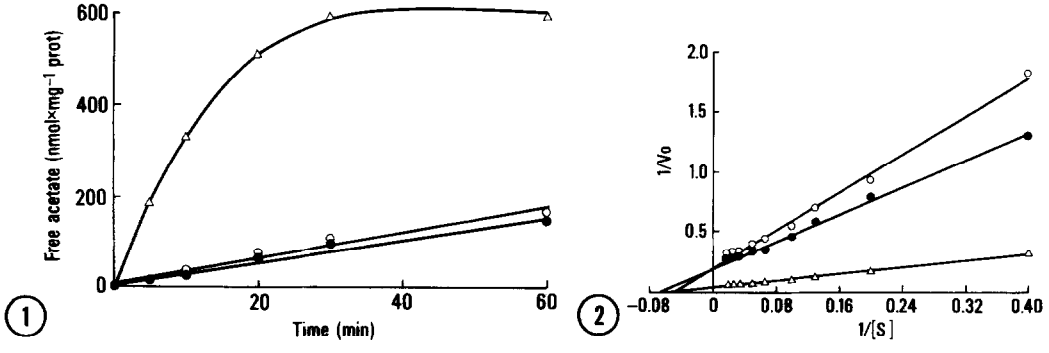

Figure 1. Time-course of [^{3}H]acetyl-paf hydrolysis by murine macrophages. Lysates from 2 hr- (●), 24 hr- (△) and BCG- (○) macrophages were incubated at 37°C with [^{3}H]acetyl-paf (0.01 μCI, 10 μM). At the indicated times, the amount of [^{3}H]acetate released was quantified as described in Materials and Methods.

Figure 2. Release of [^{3}H]acetate from 2 hr- (●), 24 hr- (△) and BCG- (○) macrophage lysates incubated for 10 min with defined concentrations of [^{3}H]acetyl-paf (0.01 μCi, 2.5 to 50 μM) as described in Materials and Methods. Lineweaver-Burk plot of the data. Results are means of duplicate determinations (with variations below 10%) and are representative of 3 to 6 experiments (statistical analysis is given in Table II).

Table II. Kinetic constants of acetylhydrolase in murine macrophages and in plasma

Enzyme source	K_m (μM)	V_{max} (nmol×min^{-1}×mg^{-1})
2 hr-MO (6)	14.8 ± 3.2	3.8
BCG-MO (3)	17.8 ± 2.2	5.1
24 hr-MO (3)	13.6 ± 1.4	21.9
Plasma (3)	19.3 ± 3.8	17.6

K_m and V_{max} for acetylhydrolase were calculated from the double reciprocal plots shown in Fig. 2 and 4 and represents means ± SEM. In parentheses, number of experiments.

24 hr- and BCG-macrophages. For all types of macrophages, the Lineweaver-Burk plot of the data gave apparent similar K_m values for paf (Fig. 2 and Table II).

Furthermore acetylhydrolase from 2 hr- and BCG-macrophages exhibited a comparable level of activity. In contrast, a markedly increased V_{max} was observed in 24 hr-macrophage lysates as compared to 2 hr- and BCG-macrophage lysates (Table II). After zymosan stimulation the acetylhydrolase kinetic constants remained unchanged for all macrophage populations studied as compared to unstimulated ones (data not shown).

Next, we characterised the deacetylation activity in murine plasma. The acetylhydrolase activity was linear with the time of incubation up to 15 min, reaching a plateau at 60 min and with protein concentration at least up to 50 $\mu g \times ml^{-1}$ (data not shown). K_m and V_{max} were determined as described for macrophage lysates (Fig. 3). We found that K_m values obtained using macrophage lysates and plasma were similar.

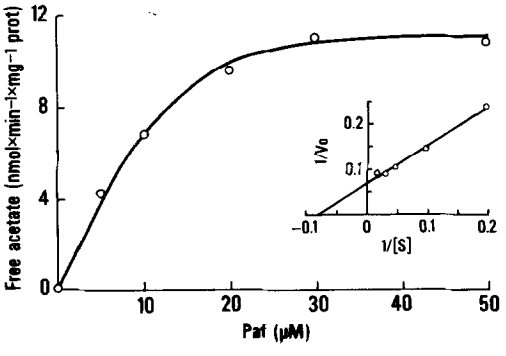

Figure 3. [^{3}H]acetate release in the presence of murine plasma incubated for 10 min with defined concentrations of [^{3}H]acetyl-paf (0.01 μCi, 5 to 50 μM). Inset : Lineweaver-Burk plot of the data. Results are means of duplicate determinations with variations below 10% and are representative of 3 separate experiments (statistical analysis is given in Table II).

Table III. Acetylhydrolase inhibition and specificity

Additions[a]	Enzyme source		
	2 hr-MO	24 hr-MO	plasma
EDTA (2 mM)	2.1 ± 0.8	8.8 ± 2.7	6.9 ± 0.1
EDTA (2 mM) + PMSF (2 mM)	1.9	7.4	5.5 ± 0.6
Ca^{2+} (10 mM)	2.2 ± 0.9	7.2 ± 2.2	6.5
Ca^{2+} (10 mM) + PMSF (2 mM)	1.4	7.3	5.5
Alkyl-acyl-GPC (10 μM)	2.1 ± 0.8	7.7 ± 1.8	6.7
Alkyl-acyl-GPC (20 μM)	2.0 ± 0.8	7.5 ± 2.4	6.6
Alkyl-acyl-GPC (40 μM)	1.9 ± 0.9	7.0 ± 2.1	6.3

[a] Added compounds were dissolved in the acetylhydrolase buffer and preincubated 5 min at 37°C with the enzyme prior to [^{3}H]acetyl-paf supplementation for 10 min. See Materials and Methods section for details. Starting solution of PMSF (1 M in dimethylsulfoxyde) was dissolved by sonication in the buffer; alkyl-acyl-GPC (2 mM in 80 % ethanol) was directly added to the assay.
Results are expressed in nmol acetate released x min^{-1} x mg^{-1} prot. and are means ± SEM of 3 experiments, except where SEM are omitted, the results being means of 2 experiments.

In order to determine whether the acetylhydrolase activities observed in murine macrophages and plasma were different from that of typical PLA_2, various compounds known to influence the activity of PLA_2 and/or of acetylhydrolase, were added to the incubation medium. Results obtained after addition of EDTA, Ca^{2+}, phenylmethylsulfonyl fluoride (PMSF) and alkyl-acyl-GPC are shown in Table III. The presence of Ca^{2+} and PMSF did not modify the acetylhydrolase activity. Addition of alkyl-acyl-GPC (10 to 40 μM) to the assay did not diminish significantly the yield of acetylhydrolase reaction, supporting the specificity of the latter enzyme for short chain fatty acid esterified to the sn-2 position of glycerophospholipids. These results also suggest that PLA_2 did not contribute to paf hydrolysis in macrophages and in plasma.

DISCUSSION

Paf is degraded to various degrees by different cell types (5-10). Here we show that while intact 2 hr-adherent resident or BCG-activated macrophages degraded paf to some extent, this degradation was greatly enhanced when cells were cultured for 24 hr. Interestingly, only a small portion of lyso paf was reacylated with long chain fatty acid into alkyl-acyl-GPC by macrophages as opposed to human (21) and rabbit neutrophils (22) but in good agreement with the results obtained with

human endothelial cells (9). However a Ca^{2+}-independent PLA_2 was described in macrophages (23) it seems unlikely that the increased paf hydrolysis in 24 hr-macrophages was due to that particular enzyme. The induction of an acetylhydrolase that is Ca^{2+}-independent and not inhibited by alkyl-acyl(long chain)-GPC explains certainly better the observed phenomenon. The kinetic analysis of acetylhydrolase derived from 2 hr-, 24 hr-, and BCG-macrophages indicated that in 24 hr-macrophages, the V_{max} of the reaction was increased about 5 times as compared to 2 hr-adherent macrophages and BCG-macrophages. However, the specificity of the acetylhydrolase remained similar as judged by similar K_m values for paf. These data imply that most probably the level of acetylhydrolase is increased during 24 hr adherence but not its affinity for paf. This increase is likely due to the de novo synthesis of the enzyme because of the relatively long period of induction.

In contrast to acetyltransferase that is increased after zymosan challenge (16), we found no variation in acetylhydrolase activity after zymosan-induced macrophage stimulation. The latter result and the fact that acetylhydrolase activity was not increased in BCG-activated macrophages indicate that induction of acetylhydrolase is not a direct consequence of macrophage activation by phagocytic stimulus. The impaired paf formation by BCG-activated macrophages remains still an open question (16). In light of our results, this defect can not be readily explained by the enhanced catabolism of paf by these cells. However, we observed that the deacetylation/reacylation reaction leading to alkyl-acyl-GPC formation from exogenously added [^{3}H]octadecyl-paf was slighty increased in BCG-macrophages as compared to 2 hr-adherent macrophages.

Finally, it seems likely that acetylhydrolase activity in macrophages may represent one of the mechanisms that controls paf output. Such a self-controling mechanism could operate in vivo. Indeed, prolonged adherence of macrophages to biological surfaces such as adjacent cell membranes (e.g. in inflammatory lesions) and to blood vessels, could contribute to a reduction of paf availability and thus of its effects.

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