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Ligation of the α_2-Macroglobulin Signaling Receptor on Macrophages Induces Synthesis of Platelet Activating Factor

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Abstract The binding of receptor-recognized forms of α_2-macroglobulin (α_2M) to macrophage α_2M signaling receptors increases inositol-1,4,5-triphosphate synthesis and induces Ca^{2+} mobilization. In this report, we demonstrate that ligation of the macrophage α_2M signaling receptor is also associated with synthesis of platelet activating factor (PAF) by both the de novo and remodeling pathways. Both α_2M-methylamine and a cloned and expressed 20-kDa receptor binding fragment (RBF) from rat α_1M+, stimulated macrophage synthesis of PAF from [^{3}H]acetate, [^{3}H]methylcholine, and 1-O-[^{3}H]alkyl lyso-PAF by two- to threefold. PAF levels reached a peak in 20 min after the cells were exposed to α_2M-methylamine or RBF; they remained elevated for about 1 h after ligand addition to the cells. When [^{3}H]methylcholine was the substrate, pertussis toxin did not block PAF synthesis, but the protein kinase C inhibitor staurosporin reduced synthesis by 65–70%. Cycloheximide completely abolished the increase in synthesis of PAF by macrophages exposed to α_2M-methylamine. By contrast, when [^{3}H]acetate was employed as a precursor, staurosporin or cycloheximide did not abolish the increase in PAF synthesis. These studies suggest that protein kinase C is necessary for the induction of the de novo pathway by α_2M-methylamine. Both α_2M-methylamine and RBF stimulated the activity of lyso-PAF acetyltransferase by about fourfold. Both ligands also stimulated the activity of PAF acetylhydrolase by about six- to sevenfold, indicating that ligation of the α_2M signaling receptor also regulates the degradation of PAF. The ability of receptor-recognized forms of α_2M to regulate levels of PAF suggests that α_2M–proteinase complexes not only regulate macrophage function by activating intracellular signaling but also may indirectly regulate the function of other cells that cannot bind α_2M–proteinase complexes. © 1996 Wiley-Liss, Inc.

Key words: α_2M, PAF, RBF, PKC, lyso-PAF acetyltransferase

α_2M is a proteinase inhibitor that is part of a large superfamily, including both proteinase inhibitors and a number of complement components [see Sottrup-Jensen, 1987, for review]. Reaction of α_2M with members of all four mechanistic classes of proteinase induces a major conformational change in the inhibitor that exposes receptor recognition sites present on each of its four subunits [see Chu and Pizzo, 1994, for review]. Each α_2M subunit also contains an internal β-cysteinyl-γ-glutamyl thiolester whose attack by methylamine exposes the receptor recognition sites of the inhibitor. α_2M receptor-recognized forms (α_2M*) bind to receptors on a variety of cells, including macrophages. Two distinct types of α_2M receptors have been identified. The first of these is low-density lipoprotein receptor-related protein/α_2M receptor (LRP/α_2MR), a multiligand receptor related to the low-density lipoprotein (LDL) receptor [see Krieger and Herz, 1994, for review]. LRP/α_2MR is a typical scavenger receptor capable of binding and internalizing diverse ligands such as α_2M*, apolipoprotein E, *Pseudomonas* exotoxin A, plasminogen activators alone or in complex with plasminogen activator inhibitor, lactoferrin, and lipoprotein lipase. The second α_2M receptor is

Abbreviations used: α_2M, α_2-macroglobulin; α_2M*, receptor-recognized forms of α_2M derived by reacting the inhibitor with proteinases or methylamine; RBF, cloned and expressed receptor recognition fragment from rat α_1M; LRP/α_2MR, low-density lipoprotein receptor-related protein/α_2M receptor; α_2M*SR, the α_2M signaling receptor; RAP, the 39-kDa receptor-associated protein; PAF, platelet activating factor; IP_3, inositol 1,4,5-triphosphate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; PKC, protein kinase C; PGE_2, prostaglandin E_2.

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known to bind only α_2M^* and its homologues, including a cloned and expressed receptor recognition fragment (RBF) from rat α_1M [Misra et al., 1994a,b]. Binding of α_2M^* or RBF to this so-called α_2M signaling receptor (α_2M^*SR) activates an intracellular signaling cascade causing rapid production of IP_3 and an increase in $[Ca^{2+}]_i$ [Misra et al., 1993, 1994a,b]. Ligation of α_2MSR on macrophages also stimulates the activities of phospholipase C, phospholipase A_2, and protein kinase C [Misra and Pizzo, 1994].

In the present report, we demonstrate that ligation of the α_2MSR induces synthesis of platelet activating factor (PAF). PAF is a naturally occurring biologically active phosphoglyceride produced by most cells involved in inflammatory responses, including platelets, neutrophils, basophils, endothelial cells, monocytes, and tissue macrophages [see Prescott et al., 1990; Chao and Olson, 1993; Venable et al., 1993; Snyder, 1995, for review]. PAF production is involved in a variety of pathophysiological events, including shock, myocardial ischemia, and asthma [see McManus et al., 1993, for review].

PAF is synthesized by two different mechanisms; namely, a so-called remodeling pathway and a de novo pathway [see Chao and Olson, 1993; Venable et al., 1993; Snyder, 1995, for review]. The remodeling pathway is more important in various types of activated cells during responses to injury. The remodeling pathway is initiated by an arachidonate-specific phospholipase A_2 [Chao and Olson, 1993; Venable et al., 1993; Snyder, 1995]. The resultant lyso-PAF is then acetylated by an acetyl CoA-dependent acetyltransferase. By contrast, the de novo pathway has been viewed as a mechanism for constitutive production of PAF, which serves unknown physiological roles. Other studies however, suggest that the de novo pathway is also inducible in cells [Heller et al., 1991]. PAF made by either route is degraded to an inactive form by various PAF acetylhydrolases [Venable et al., 1993; Snyder, 1995].

Macrophage de novo PAF synthesis induced by α_2M-methylamine or RBF is not blocked by pertussis toxin, but both staurosporin and cycloheximide prevent the induction of PAF by these ligands. The ability of α_2M^* to induce macrophage PAF synthesis provides a mechanism by which α_2M–proteinase complexes produced during tissue injury alters not only macrophage function but also the function of other types of cells that may lack α_2M^*SR.

MATERIALS AND METHODS

Reagents

Sterile distilled water was obtained from Abbott Laboratories (Chicago, IL). Brewer's thioglycollate broth was purchased from Difco Laboratories (Baltimore, MD). Culture media were purchased from Life Technologies (Grand Island, NY). Bovine serum albumin (BSA), pertussis toxin, staurosporin, phosphatidylcholine, and lysophosphatidylcholine were purchased from Sigma Chemical Co. (St. Louis, MO). PAF-18 (1-O-stearyl-2-acetyl-sn-glycerol-3-phosphocholine) and lyso-PAF (1-O-hexadecyl-sn-glycero-3-phosphocholine) were purchased from Cal Biochem (La Jolla, CA). Sodium [^{3}H]acetate (spec act 100 mCi/mmol), [^{3}H]acetyl CoA (spec act 20 Ci/mmol), and [^{3}H]methylcholine chloride (spec act 60 Ci/mmol) were purchased from American RadioChemicals (St. Louis, MO). (1-O-[^{3}H])octadecyl lyso-PAF (spec act 151 Ci/mmol) and (1-O-[^{3}H])octadecyl PAF (spec act 185 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). All other chemicals used were of reagent grade. RAP was expressed and purified as described by Herz et al. [1991]. The pGEX-39-kDa expression construct was the kind gift of Dr. Joachim Herz (University of Texas, Southwestern, Dallas, TX).

Preparation of α_2M and RBF

Human α_2M was purified as previously described [Imber and Pizzo, 1981]. RBF was cloned and expressed in *Escherichia coli* and purified as reported [Salvesen et al., 1992]. Preparations were assayed for endotoxin and, where necessary, rendered free of endotoxin as previously reported [Chu and Pizzo, 1993]. Endotoxin levels were < 10 pg/ml in stock solutions of ≥ 1.0 μM α_2M-methylamine or RBF. The concentrations of α_2M (100 nM) and RBF (40 nM) chosen for comparative experiments were based on previous studies [Misra et al., 1993, 1994a,b] demonstrating a similar effect on macrophage signaling by α_2M-methylamine and RBF at these concentrations. The reason RBF is a more potent agonist is unknown.

Cell Culture

Pathogen-free C57BI/6 mice (6 weeks old) were obtained from Charles River Laboratories (Raleigh, NC). Thioglycollate-elicited macrophages were routinely obtained by peritoneal lavage with Hanks balanced salt solution con-

taining 10 mM Hepes, 3.5 mM $NaHCO_3$, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4 (HHBSS). The cells were washed once with HHBSS, suspended in RPMI 1645 medium containing 2 mM glutamine, 12.5 U/ml penicillin, 6.25 μg/ml streptomycin, and 0.25% fatty acid free BSA, and plated at 3.5–4 × 10^6 cells/4.5 cm^2. The cells were incubated for 2 h at 37°C in a humidified CO_2 (5%) incubator. The monolayers were washed with HHBSS three times to remove nonadherent cells, and monolayers were incubated in RPMI 1640 medium containing the additions listed above overnight at 37°C.

Synthesis and Secretions of PAF in Sodium [^{3}H]Acetate-Labeled Cells

Macrophage monolayers were washed twice with HHBSS; a volume of RPMI 1640 medium containing additives described under Cell Culture was added and incubated with the monolayers for 5 min as above. Sodium [^{3}H]acetate (20 μCi/ml) was added to the monolayers and cells labeled for 30 min at 37°C. The labeled monolayers were washed three times with HHBSS and a volume of RPMI 1640 medium with the above additions described under Cell Culture added and preincubated for 5 min at 37°C prior to the addition of buffer, native α_2M (100 nM), α_2M-methylamine (100 nM), or RBF (40 nM) to respective cells. The monolayers were incubated for the desired period of time as above. The reaction was terminated by quantitatively transferring the medium to clean glass tubes to assess the secretion of newly synthesized PAF and adding a volume of chilled methanol containing 50 mM acetic acid to the monolayers. The monolayers were scraped into glass tubes and the wells washed once with 1 ml of methanol and the wash combined with the monolayer suspensions. The lipids from the medium and cells were isolated by the Bligh and Dyer method [1959]. The two phases were separated by centrifugation at 2,000*g* at 4°C for 15 min, the chloroform layer was dried under N_2 at 40°C and dissolved in chloroform–methanol (9:1 v/v) and processed immediately for the fractionation of PAF by thin-layer chromatography (TLC) on silica gel G plates (Analtech, Dover, DE). The silica gel plates were activated for 3 h at 105°C prior to application of the samples, each of which was spiked with 4 μg of authentic standards of PAF and lyso-PAF. The plates were developed in chloroform–methanol–acetic acid–water at a ratio of 50:30:8:6 (v/v). The plates were dried at 60°C for 2 h and lipid spots on the gel visualized by exposure to I_2 vapors. PAF (Rf = 0.45) and lyso-PAF (Rf = 0.32) were well resolved in this solvent system, and their recoveries were about 90–93%. Gel areas corresponding in Rf values to authentic standards of PAF and lyso-PAF were scraped into a scintillation vial and their radioactivity determined by liquid scintillation counting.

The identity of PAF was further confirmed by its susceptibility to alkaline hydrolysis. For these studies, the silica gel plates with samples applied were exposed to NH_3 vapors for 5 min at room temperature. After air drying, the plates were developed as described above. Authentic [^{3}H]-PAF and lyso-PAF were spotted on these plates for reference. Alkaline hydrolysis demonstrated greater than 75% loss of radiolabel from spots identified as PAF in all studies in which the effect of α_2M-methylamine on PAF synthesis was studied. This compares well to the 80% loss in counts from authentic [^{3}H]-PAF spotted on the plates for reference.

Synthesis of PAF From (1-0-[^{3}H])Alkyl Lyso-PAF

Macrophage monolayers were washed twice with HHBSS and to the cells was added RPMI 1640 medium with the additions listed under Cell Culture. The cells were incubated for 5 min at 37°C and labeled with (1-0-[^{3}H]) Alkyl Lyso-PAF (1.0 μCi/ml medium) for 20 min at 37°C. The labeled monolayers were washed three times with HHBSS, a volume of RPMI 1640 medium added and cells stimulated with buffer, α_2M-methylamine (100 nM) or RBF (40 nM) for varying periods of time. The reaction was terminated by aspirating the medium and adding a volume of chilled methanol containing 50 mM acetic acid. The monolayers were scraped into glass tubes and processed for lipid extraction and fractionation of PAF by TLC and determination of radioactivity as described above.

Staurosporin and PAF Synthesis From [^{3}H]Methylcholine

Macrophages were adhered for 2 h, washed with HHBSS, and then labeled with [^{3}H]methylcholine (2 μCi/ml/16 h) in RPMI 1640 medium containing 0.25% fatty acid free BSA, 12.5 U/ml penicillin and 6.25 μg/ml streptomycin for 16 h at 37°C in a humidified CO_2 (5%) incubator. In cells in which the effect of staurosporin on PAF synthesis was to be examined, staurosporin (20 nM), a potent inhibitor of PKC, was added 12 h

before the end of the incubation. The labeled monolayers were preincubated for 5 min at 37°C prior to stimulation with buffer, α_2M-methylamine (100 nM) or RBF (40 nM) for 10 min at 37°C. The reaction was terminated by aspirating the medium and adding a volume of chilled methanol containing 50 mM acetic acid. The cells were scraped into tubes and processed for lipid extraction, fractionation of PAF by TLC and determination of radioactivity as described above.

Pertussis Toxin and PAF Synthesis From [^{3}H]Methylcoline

[^{3}H]methylcholine-labeled monolayers (see above) were incubated with pertussis toxin (1 μg/ml preactivated with 40 mM DTT for 1 h at 25°C) for 2 h at 37°C as above. The monolayers were washed twice with HHBSS, a volume of buffer added, and monolayers preincubated for 5 min at 37°C prior to stimulation with buffer, α_2M-methylamine (100 nM), or RBF (40 nM) for 10 min at 37°C. The reaction was terminated by aspirating the medium and adding a volume of chilled methanol containing 50 mM acetic acid. The cells were scraped into glass tubes and processed for lipid extraction, fractionation of PAF by TLC, and determination of radioactivity as described above.

Cycloheximide and PAF Synthesis From [^{3}H]Methylcholine

[^{3}H]methylcholine-preloaded macrophages were exposed to cycloheximide (10 μg/ml) at 37°C in HHBSS beginning 40 min before the addition of α_2M-methylamine (100 nM).

Effect of Staurosporin, Pertussis Toxin, or Cycloheximide on PAF Synthesis From [^{3}H]Acetate

For these studies, macrophages were adhered as described above and prelabeled with [^{3}H]acetate (10 μCi/ml/16 h). The effects of staurosporin, pertussis toxin, or cycloheximide were then studied as described above.

Assay of PAF Acetyl CoA Lyso-PAF Acetyltransferase Activity

The activity of PAF acetyl CoA lyso-PAF acetyltransferase in macrophages was assayed according to Billah et al. [1985]. Briefly macrophage monolayers in RPMI 1640 medium were incubated with buffer, α_2M (100 nM), α_2M-methylamine (100 nM) or RBF (40 nM) for varying periods of time at 37°C. The reaction was terminated by aspirating the medium and adding a volume of ice-cold sonication buffer containing 10 mM Hepes, 250 mM sucrose, 0.5 mM EGTA, and 1 mM DTT, pH 7.0. The cells were scraped into ice-cold tubes and sonicated (Vibra Cell VC50: Sonics & Materials, Danbury, CT) on ice (3 × 10-s bursts at the maximal setting with 30-s intervals). Sonicate (450 μl containing 180–200 μg protein) in glass tubes was used for the enzyme assay in the presence of 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 30 μM lyso-PAF. The reaction was initiated by adding 50 nM of [^{3}H]acetyl CoA to the sonicate preincubated for 5 min at 30°C. The reaction was stopped at the desired period of incubation at 37°C by adding a volume of chloroform:methanol–acetic acid (100:400:4 v/v). The lipids were extracted, fractionated by TLC, and radioactivity of PAF determined as described above.

Assay of PAF Acetylhydrolase Activity

Acetylhydrolase activity in macrophages was assayed by a slight modification of the method of Blank et al. [1981]. Briefly, macrophage monolayers in RPMI 1640 medium containing fatty acid free BSA (0.25%), penicillin (12.5 U/ml), and streptomycin (6.25 μg/ml) were incubated with buffer, α_2M-methylamine (100 nM), or RBF (40 nM) for 10 min at 37°C. The reaction was stopped by aspirating the medium. The monolayers were washed once with HHBSS buffer, a volume of ice-cold sonication buffer added, cells scraped into ice-cold tubes, and sonicated as above. Sonicate (450 μl containing 150–170 μg protein) in glass tubes was used for enzyme assay in the presence of 1.3 mM $CaCl_2$. The samples were preincubated for 5 min at 37°C prior to the initiation of the reaction with 10 μM of (1-0-[^{3}H])octadecyl-sn-2-acetyl-glycero-3-phosphocholine. The samples were incubated for 10 min at 37°C. The reaction was stopped with a volume of chloroform–methanol–acetic acid (100:200:4 v/v). The lipids were extracted, fractionated by TLC, and the radioactivity of lyso-PAF determined as described above.

Protein Assays

Protein concentrations were determined by the Bradford [1976] assay.

RESULTS

Synthesis of PAF in Macrophages Exposed to α_2M-Methylamine and RBF

Figure 1 shows the synthesis of PAF by macrophages preloaded with sodium [^{3}H]acetate, a precursor of both the de novo and remodeling pathways, for 30 min prior to addition of α_2M-methylamine (100 nM) or RBF (40 nM). Both ligands stimulated at least a 2.5-fold increase in PAF compared to buffer or native α_2M (data not shown) after 20 min of macrophage incubation with these ligands. RAP at a 100-fold molar excess did not block the induction of PAF synthesis by macrophages exposed to α_2M-methylamine or RBF, nor did it induce PAF synthesis on its own (data not shown). This observation is consistent with previous studies demonstrating that RAP prevents ligand binding to LRP/α_2MR, but not to α_2MSR [Misra et al., 1994a,b]. Thus RAP does not block signaling induced by ligation of α_2MSR. The level of PAF synthesis remained elevated as compared to the buffer control for at least 60 min. When 1-0-[^{3}H]alkyl lyso-PAF, a precursor of the remodeling pathway, was employed as a PAF precursor, the increase in PAF synthesis compared to buffer was 2.5-fold and 3.2-fold for macrophages exposed to α_2M-methylamine and RBF, respectively.

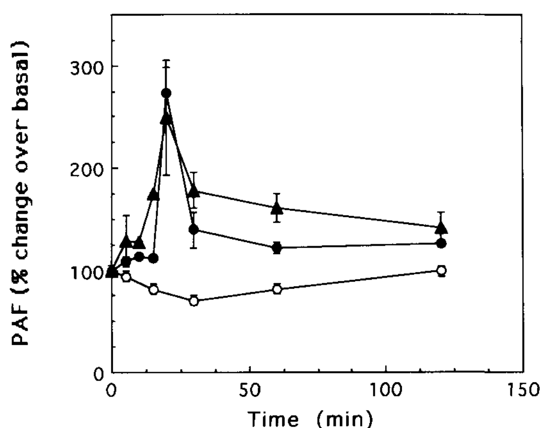

Fig. 1. Synthesis of PAF in macrophages stimulated with α_2M-methylamine and RBF. Details are given under Materials and Methods. Macrophage monolayers were labeled with sodium [^{3}H]acetate for 30 min at 37°C and the monolayers stimulated with buffer, α_2M-methylamine (100 nM), or RBF (40 nM) for varying periods of time at 37°C. After termination of the reaction, lipids were isolated from the cells and PAF separated and its radioactivity determined. The values are the mean ± SEM from four independent experiments for α_2M-methylamine (●), RBF (▲), and buffer (○).

Secretion of PAF by macrophages preloaded with sodium [^{3}H]acetate demonstrated a 20% increase in release compared to buffer over this time period when α_2M-methylamine (100 nM) was employed as the ligand (data not shown). Thus, most of the newly synthesized PAF were retained within the macrophages.

Synthesis of PAF from [^{3}H]Methylcholine and the Effects of Staurosporin, Pertussis Toxin, and Cycloheximide on Synthesis

[^{3}H]methylcholine can be used as a substrate to specifically probe the de novo pathway [see, e.g., Lee et al., 1986; Woodard et al., 1987]. Table I demonstrates the synthesis of PAF by macrophages preloaded with [^{3}H]methylcholine. Both α_2M-methylamine and RBF induce synthesis of PAF consistent with the data presented above. Staurosporin, a potent inhibitor of PKC activity, was associated with a 65–70% reduction in PAF synthesis by the de novo pathway in macrophages exposed to α_2M-methylamine and RBF. By contrast, pertussis toxin caused little or no effect.

The role of protein synthesis on the production of PAF was assessed by the use of cycloheximide. Figure 2 demonstrated complete suppression of the ability of α_2M-methylamine to stimulate PAF synthesis in macrophages.

In contrast to the results described above when [^{3}H]methylcholine was used as a precur-

TABLE I. Effect of Staurosporin and Pertussis Toxin on PAF Synthesis by Macrophages

Treatment	PAF synthesis[a] (pmol/4 × 10^6 cells/10 min)
Buffer	0.206 ± 0.006
Buffer + staurosporin	0.04 ± 0.004*
Buffer + PTx[b]	0.165 ± 0.022
α_2M-methylamine (100 nM)	4.707 ± 0.316
α_2M-methylamine + staurosporin	1.670 ± 0.194*
α_2M-methylamine + PTx	5.339 ± 0.560
RBF (40 nM)	4.116 ± 0.326
RBF + staurosporin	1.262 ± 0.146*
RBF + PTx	4.457 ± 0.586

[a]PAF synthesis was determined in macrophages preloaded for 16 h with [^{3}H]methylcholine as described under Materials and Methods. The values reported are the mean ±SEM from two separate experiments. Asterisk (*) indicates a statistically significant difference within the experimental group ($P < 0.05$).
[b]PTx, pertussis toxin.

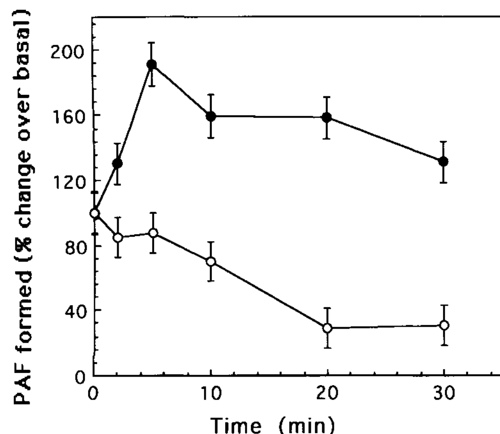

Fig. 2. Effect of cycloheximide on PAF synthesis by macrophages stimulated with α_2M-methylamine. Details are given under Materials and Methods. Cells preloaded with [^{3}H]methylcholine were exposed to cycloheximide (10 μg/ml) 40 min prior to adding α_2M-methylamine in the absence (●) or presence (○) of cycloheximide. The values are the mean ± SEM for two separate experiments.

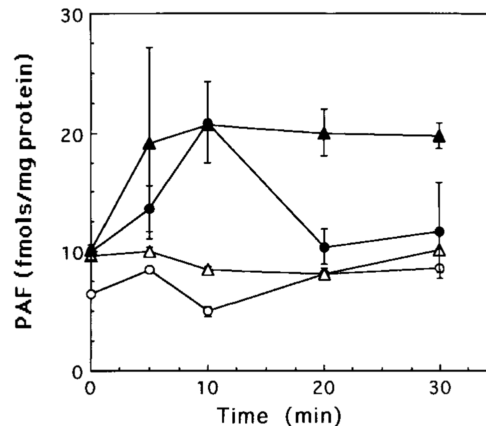

Fig. 3. Lyso-PAF acetyltransferase activity in macrophages stimulated with α_2M-methylamine and RBF. Details are given under Materials and Methods. The values are the mean ± SEM for four separate experiments. The symbols used are buffer (○), native α_2M (100 nM) (△), α_2M-methylamine (100 nM) (●), and RBF (40 nM) (▲).

sor, staurosporin, pertussis toxin, or cycloheximide caused little or no suppression of PAF synthesis when [^{3}H]acetate was employed as a precursor. Specifically, staurosporin caused only a 10% decrease, pertussis toxin a slight increase, and cycloheximide a 2% decrease in PAF synthesis induced by α_2M-methylamine. These studies suggest a role for PKC in the induction of PAF by the de novo pathway but not the remodeling pathway when α_2M-methylamine is employed as the ligand.

Effect of α_2M-Methylamine and RBF on Macrophage PAF Acetyl CoA Lyso-PAF Acetyltransferase and PAF Acetylhydrolase

The remodeling pathway uses lyso-PAF as a substrate for acetylation catalyzed by an acetyltransferase [Chao and Olson, 1993; Venable et al., 1993; Snyder, 1995]. Figure 3 and Table II show the effect of α_2M-methylamine and RBF on the activities of macrophage PAF acetyltransferase. Both α_2M-methylamine and RBF had a comparable stimulatory effect on PAF acetyltransferase, increasing the activity of the enzyme by more than fourfold. Peak stimulation occurred at about 10 min, but the effect of RBF was more prolonged. Native α_2M had no effect on PAF acetyl transferase activity (data not shown).

α_2M-Methylamine and RBF also increase the activity of PAF acetylhydrolase by about six- to sevenfold (Fig. 4; Table II). Thus, ligation of the α_2MSR activates both the enzymes that produce and degrade PAF, effectively regulating the ability of macrophages to produce PAF.

TABLE II. Lyso-PAF Acetyltransferase and PAF Acetylhydrolase Activities in Macrophages

Treatment	Lyso-PAF acetyltransferase (PAF formed, fmol/min/mg protein)	PAF acetylhydrolase (Lyso-PAF formed, fmol/min/mg protein)
Buffer	0.499 ± 0.042[a]	0.241 ± 0.011
α_2M-Methylamine (100 nM)	2.082 ± 0.340	1.603 ± 0.066
RBF (40 nM)	2.076 ± 0.020	1.469 ± 0.129

[a]Values are mean ±SEM from two to four independent experiments. See Materials and Methods for details.

DISCUSSION

α_2M is capable of binding proteinases of every known class; historically, it has been viewed as a proteinase inhibitor. However, there are several difficulties with this concept. The proteinase becomes trapped within the α_2M molecule once it has attacked the so-called "bait region" present in each α_2M subunit; however, proteolytic activity remains preserved, since the active site is not involved in the complex with the inhibitor [see Sottrup-Jensen, 1987; Salvesen and Pizzo, 1994, for review]. Generally, only small substrates can be cleaved by the trapped proteinase,

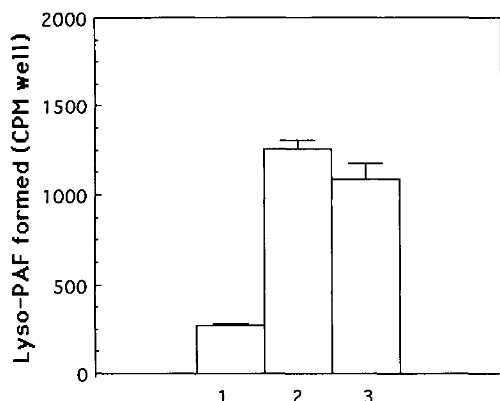

Fig. 4. PAF acetylhydrolase activity in macrophages stimulated with α_2M-methylamine and RBF. Details are given under Materials and Methods. The values given are the mean ± SEM for two separate experiments. *Bar 1* is the buffer control, *Bar 2* is α_2M-methylamine (100 nM), and *Bar 3* is RBF (40 nM).

but there are significant exceptions where the complex can cleave large macromolecular substrates, including fibrinogen. Moreover, during the course of evolution, many much more specific proteinase inhibitors, such as the serpin class, have evolved. The continued presence of α-macroglobulins throughout species, ranging from *Limulus* to humans, suggests other potential roles for α-macroglobulins.

It has been known for many years that α_2M–proteinase complexes bind to receptors on a variety of cells. Only more recently has it been shown that there exist specific receptors whose ligation by activated forms of α_2M trigger classical signaling cascades [see Chu and Pizzo, 1994, for review]. Binding of these receptor-recognized forms of α_2M (α_2M*) to α_2M*SR causes a rapid increase in IP_3 with mobilization of Ca^{2+} from internal stores, as well as entry of Ca^{2+} from the external milieu [Misra et al., 1993, 1994a,b]. Ligation of the signaling receptor by α_2M* also stimulates the activities of various phospholipases, including phospholipase A_2, and PKC [Misra and Pizzo, 1994]. While RAP blocks binding of all known ligands, including α_2M* to LRP/α_2M, it does not inhibit the interaction of α_2M-methylamine or RBF with α_2M*SR [Misra et al., 1994a,b].

PAF is a phospholipid autocoid with a wide spectrum of diverse and potent proinflammatory effects [see Prescott et al., 1990; Venable et al., 1993; Chao and Olson, 1993; McManus et al., 1993, for review]. In the present study, we demonstrate that binding of α_2M-methylamine to α_2M*SR regulates the synthesis of PAF. This effect appears to involve both the de novo and remodeling pathways based on the substrate studies employed in our experiments. During the 120-min time course of our studies with macrophages, most of the PAF synthesized as a result of cell stimulation with α_2M-methylamine was retained; however, a fraction of this mediator was also secreted. Secretion of newly synthesized PAF in stimulated cells varies depending on a number of factors [see, e.g., Cluzel et al., 1989]. A large proportion of newly synthesized PAF is secreted by some cells while other cells retain a significant fraction of this mediator [see Prescott et al., 1990; Chao and Olson, 1993; McManus et al., 1993; Venable et al., 1993, for review]. While only a fraction of the newly synthesized PAF is secreted by α_2M-methylamine-treated macrophages, the amount released is consistent with effects of ligands on other cells that produce PAF, suggesting that the production of α_2M–proteinase complexes during inflammation may trigger a variety of proinflammatory events associated with release of PAF from cells.

It is known that both lyso-PAF and arachidonic acid can be released from a common precursor 1-O-alkyl-2-arachidonyl-sn-glycero-3-phosphocholine, thereby providing the intermediates for the synthesis of both PAF and prostaglandins [see Prescott et al., 1990; Chao and Olson, 1993; Venable et al., 1993; Snyder, 1995, for review]. PAF itself can stimulate the release of arachidonic acid and synthesis of eicosanoids [Chao and Olson, 1993]. Cultured rat mesangial cells stimulated with PAF, for example, mobilize arachidonic acid and secrete prostaglandin E_2 (PGE_2) [Schlondorff et al., 1984]. Previous studies from this laboratory have shown that exposure of macrophages to α_2M-methylamine triggers rapid release of PGE_2 within 2–10 min of ligand exposure [Uhing et al., 1991], somewhat faster than the time when we now demonstrate significant increases in PAF synthesis. However, there was also a sustained production of PGE_2 that may be related to effects induced by the increased synthesis of PAF for more than 1 h.

In these studies, we employed cycloheximide, staurosporin, and pertussis toxin to explore further the regulation of PAF synthesis by the de novo and remodeling pathways in macrophages treated with α_2M-methylamine. Cycloheximide completely blocked the effects of α_2M-methylamine, indicating that induction of the de novo

PAF synthetic pathway enzymes is required for α_2M-methylamine to exert its effects. Based on the data presented in this paper, this includes at least lyso-PAF acetyltransferase. Since α_2M-methylamine also promoted the activity of the PAF acetylhydrolase, the effects of α_2M-methylamine binding to macrophage α_2MSR are regulated with respect to both synthesis and degradation of PAF.

The effect of staurosporin, a PKC inhibitor, on α_2M-methylamine-induced PAF synthesis by macrophages is consistent with a number of other studies [see Venable et al., 1993; Chao and Olson, 1993; Snyder, 1995, for review]. The effect of PKC may be to regulate phospholipases involved in PAF synthesis, but PKC also may modulate PAF receptor activity in some types of cells, including neutrophils [O'Flaherty et al., 1990; Yamazaki et al., 1989] and Kupffer cells [Chao et al., 1989]. While the latter effects are not addressed in this study, the data obtained in this study suggest that when α_2M-methylamine is the ligand, the role of PKC is restricted to effects in the de novo, but not the remodeling, pathway.

Several reports have implicated G proteins in PAF receptor-induced transmembrane signaling [Chao and Olson, 1993]. The current study did not seek to address this issue but rather to use pertussis toxin to probe the role of G proteins in the induction of PAF synthesis after ligation of macrophage α_2M*SR by α_2M-methylamine. Pertussis toxin had no effect on the ability of α_2M-methylamine to induce PAF synthesis. This observation is consistent with our previous studies demonstrating that the α_2MSR is coupled to a heterotrimeric G protein which is pertussis toxin insensitive [Misra et al., 1994a,b].

In summary, the present work demonstrates that ligation of α_2M*SR on macrophages induces synthesis of PAF, a potent proinflammatory mediator. Production of PAF by stimulated macrophages offers a means by which proteinase generation and formation of α_2M–proteinase complexes can regulate activities by a variety of cells other than macrophages. While macrophages possess α_2M* receptors, many cells such as endothelial cells, neutrophils, and T and B lymphocytes lack α_2M* receptors [Salvesen and Pizzo, 1994]. Therefore, these cells do not have a direct capability of responding to the generation of α_2M–proteinase complexes during tissue injury. However, by stimulating macrophage production of PAF and prostaglandins, the binding of α_2M–proteinase complexes to macrophages offers the potential to regulate a variety of cells important during host responses to tissue injury.

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