Actions of Platelet Activating Factor (PAF) Homologues and Their Combinations on Neutrophil Chemokinesis and Cutaneous Inflammatory Responses in Man

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The inflammatory actions of synthetic C16:0 and C18:0 platelet activating factor (PAF) homologues, both alone and in combination, have been compared in an in vitro human neutrophil chemokinesis assay and by intradermal injection in human skin. In the chemokinesis assay, the maximum distance moved by neutrophils in the presence of C18:0 PAF was significantly greater than that seen with the C16:0 compound. A mixture of C16:0 and C18:0 PAFs in a ratio of 1:9 appeared to be more active than in a ratio of 3:1. Intradermal injection of the C16:0 and C18:0 PAF homologues induced dose-dependent increases in weal volume and flare area responses which were not significantly different. Combination of these phospholipids in a ratio of 3:1 or 1:9 of C16:0:C18:0 did not significantly alter the dose response curves. Thus, changes in the chain length of the alkyl substituent of synthetic PAF homologues and combination of these homologues, in ratios found in vivo or formed by leukocytes in vitro, did not alter the cutaneous inflammatory responses to PAF in man. The C18:0 homologue was, however, more active as a human neutrophil chemoattractant in vitro. *J Invest Dermatol 91:82–85, 1988*

There is now increasing evidence to suggest that the phospholipid platelet activating factor (PAF, Paf-acether, AGEPC), an ether-linked analogue of phosphatidyl choline (1-o-alkyl-2-o-acetyl-sn-glyceryl-3-phosphorylcholine) in which the length of the alkyl side chain can vary [1,2], is a mediator of inflammation. PAF, which is produced by many inflammatory cells [3], is a potent chemoattractant for polymorphonuclear neutrophils in vitro [4] and induces increased vascular permeability and inflammatory cell accumulation in the skin of experimental animals [5–7] and man [8–10]. A role for PAF in the pathogenesis of inflammatory dermatoses has been suggested [8–12], and its inflammatory actions in human skin have recently been shown to be inhibited by an orally administered PAF antagonist [13,14].

The biological activity of PAF has also been suggested to be a function of the length and degree of unsaturation of the alkyl chain [1,5,15]. The molecular forms that most commonly occur in vivo are the C16:0 and C18:0 PAFs, or mixtures of these homologues in varying amounts. Rabbit leukocytes were initially reported to release PAF, mainly as the C18:0 compound, in a ratio of 9:1 C18:0:C16:0 [16]. Studies using human neutrophils have demonstrated a predominance of the C16:0 homologue [18,19] and PAF has been isolated from psoriatic scale in a ratio of 3:1 C16:0:C18:0 [11].

The aim of this study was to compare the inflammatory actions of C16:0 and C18:0 PAF homologues, both alone and in combination, in two systems. The effects of PAF on cellular responses were examined using an agarose microdroplet chemokinesis assay for human neutrophils, and the effects on blood vessel reactivity in vivo in human skin were examined by measuring early weal and flare responses following intradermal injection.

MATERIALS AND METHODS

Drugs Synthetic C16:0 and C18:0 PAF homologues were obtained from Bachem AG (Switzerland). One milligram per milliliter stock solutions, prepared in absolute ethanol, were diluted in Eagle's minimal essential medium (MEM) (Wellcome Diagnostics, U.K.) buffered to pH 7.4 with N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid for in vitro studies, and in sterile 0.9% saline containing 0.25% human serum albumin (HSA) for intradermal injection.

Human Neutrophil Chemokinesis Assay The chemokinetic activity of C16:0 and C18:0 PAF, alone and in combinations of 3:1 and 1:9 C16:0:C18:0, was examined in an agarose microdroplet assay as previously described [20]. Briefly, mixed peripheral blood leukocytes (predominantly neutrophils) were prepared from heparinized human blood by dextran sedimentation and hypotonic lysis of residual erythrocytes. The cells were washed and resus-

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Abbreviations:

HSA: human serum albumin

MEM; minimal essential medium

PAF: platelet activating factor

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pended in a small volume of MEM which was then mixed with an equal volume of agarose containing MEM and heat inactivated human serum. Two microliters of the mixture were then placed in each well of a microtitre plate and overlaid with 100 μl MEM, with or without PAF (10^{-10} to 10^{-5} M). After a 2-h incubation at 37°C, the distance that the neutrophils moved was measured by projection of a magnified image onto a digitizer pad interfaced to an Apple computer. Results have been expressed in millimeters as the mean (±sem) radial distance moved from the edge of the gel to the leading front of cells in the presence of PAF, after subtraction of random migration in the presence of MEM alone.

Human Skin Studies Twelve healthy, nonatopic volunteers were given intradermal injections (volume 50 μl) of 10, 30, and 100 ng C16:0 and C18:0 PAF homologues, and vehicle (0.25% HSA in 0.9% saline) alone. Solutions were coded and injected in random order on the volar aspect of the forearms.

Skin fold thickness was measured using a spring-loaded thickness gauge immediately prior to injection (To) and 15 min after injection, the time of maximal response (Tt). Weal area was determined by measurement of two perpendicular diameters (D1 and D2), and weal volume estimated (assuming the weal to be a cyclinder) as

$$\text{area} \times \text{depth} = \frac{\Pi}{4}\left(\frac{D1 + D2}{2}\right)^2 \times \frac{(Tt - To)}{2}\ \mu\text{l}.$$

Flare area was assessed by measuring two perpendicular diameters (d1 and d2) 5 min after injection, the time of maximal response:

$$\text{area} = \frac{\Pi}{4}\left(\frac{d1 + d2}{2}\right)^2\ \text{cm}^2.$$

Intradermal injections of combinations of C16:0 and C18:0 PAF solutions (1:9 and 3:1) were repeated in these subjects after a time interval of at least 1 wk.

Student's t test was employed for statistical analysis, using unpaired observations for between subject analysis (for neutrophil chemokinesis data) and paired observations for within subject analysis (for intracutaneous responses on the same occasion). Significance was taken to be at the $p < 0.05$ level.

RESULTS

The effects of the C16:0 and C18:0 PAF homologues in the neutrophil chemokinesis assay are shown in Fig 1*a*. Each compound produced a dose-related increase in neutrophil chemokinesis over the dose range of 10^{-10} to 10^{-7} M, higher concentrations being inhibitory. Although the maximal response occurred at a dose of 10^{-7} M for both C16:0 and C18:0 PAF, neutrophils moved significantly further in response to C18:0 PAF (0.73 ± 0.05 versus 0.36 ± 0.02 mm for C18:0 and C16:0 PAFs, respectively, $p < 0.05$, $n = 6$, two-tailed t test). In two further experiemnts, a mixture of 1:9 C16:0:C18:0 PAF appeared to be more active than a combination of 3:1 C16:0:C18:0 PAF (Fig 1*b*), each mixture producing dose-related increases in neutrophil movement over the range of 10^{-9} to 10^{-7} M.

In human skin, both the C16:0 and C18:0 PAF homologues caused dose-dependent increases in weal volume over the range 10 to 100 ng/site, but responses to the C16:0 and C18:0 compounds were not significantly different (Fig 2*a*). No significant differences in weal volume were observed following the injection of the C16:0 and C18:0 PAF combinations in the ratios of 1:9 and 3:1 (Fig 2*b*). Over the dose range of 10 to 100 ng/site, each PAF solution induced a dose-dependent flare response, but for a given dose of PAF, responses to the C18:0 and C16:0 homologues, and to the combinations thereof, were not significantly different (Table I).

DISCUSSION

The results of this study confirm previous findings regarding the inflammatory properties of synthetic PAF in human skin, obtained using a 1:9 ratio of C16:0:C18:0 PAF [8–10]. We have further shown that C16:0 and C18:0 PAFs, both alone and combined in ratios found in vivo and formed by inflammatory cells in vitro, produce dose-dependent weal and flare responses that do not differ statistically from each other. A difference in the chemokinetic activity of the two homologues was, however, found in vitro, as human neutrophils migrated significantly further after maximal stimulation with C18:0 PAF when compared to the maximal responses to C16:0 PAF. The late response to PAF, which occurs 3–6 h after intradermal injection of doses of 100 ng/site or above has not been measured in the present study. This late onset response is thought to be associated with cell accumulation [10]. In view of the difference in activity between the C18:0 and C16:0 PAF homologues in vitro on neutrophil migration it would be of interest to compare cellular accumulation in vivo 3–6 h after intradermal injection of the PAF homologues or by using a modified skin window technique [21].

Minor structural alterations of the PAF molecule have been shown to have marked effects on its biological properties [1,5,15]. The absence of the acetyl group renders the compound biologically inactive (lyso-PAF); alteration of the length and degree of unsaturation of the alkyl chain has less profound effects. Pinckard's group

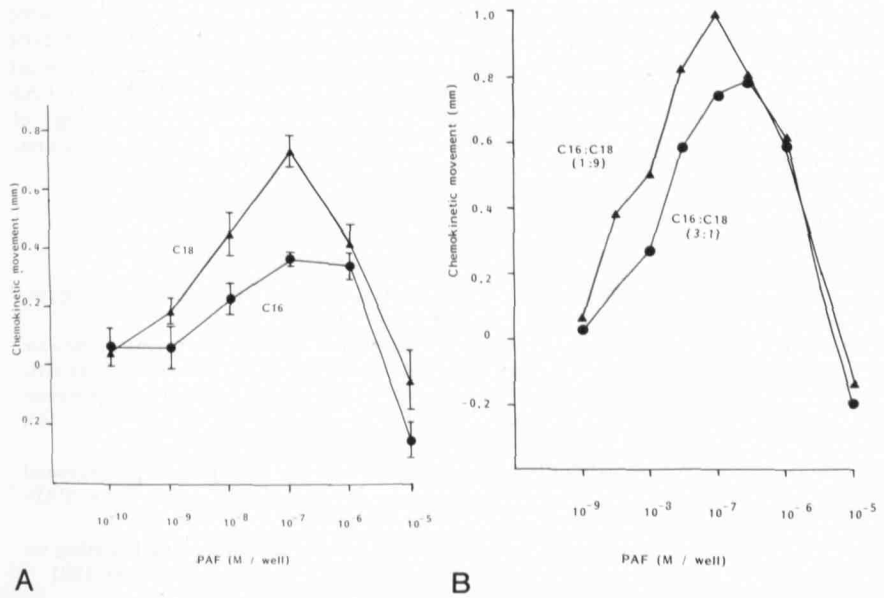

Figure 1. Chemokinetic movement of human neutrophils, after subtraction of random migration, in response to (*a*) C16:0 (●) and C18:0 (▲) PAF homologues (mean ±sem; n = 6), and (*b*) combinations of C16:0 and C18:0 PAF homologues in the ratios of 1:9 (▲) and 3:1 (●). Each point is the mean of duplicate experiments (4 wells per concentration in each). Neutrophils migrated significantly further after maximal stimulation with C18:0 PAF compared to the maximal response to C16:0 PAF ($p < 0.05$).

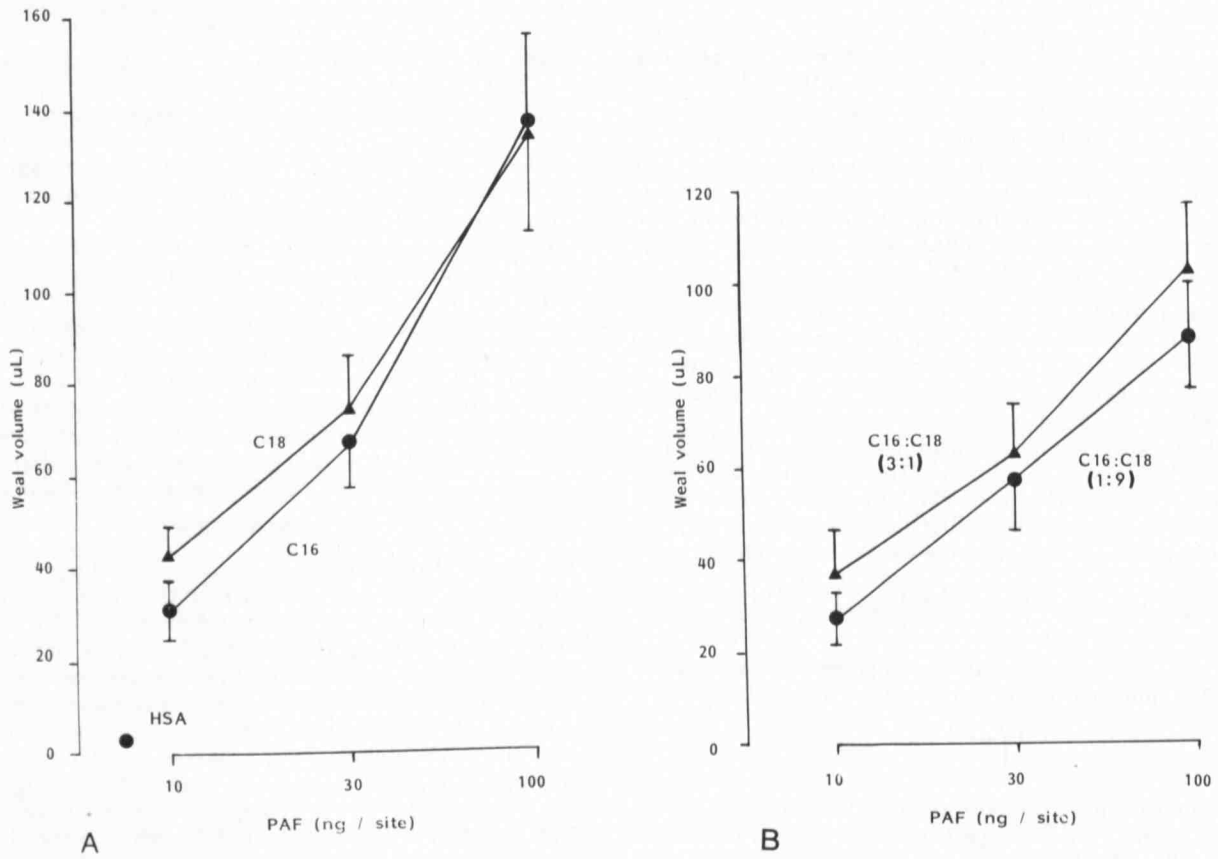

Figure 2. Weal volume (mean ± sem; n = 12) responses in human forearm skin to intradermal injections of (*a*) C16:0 (●) and C18:0 (▲) PAF homologues, and (*b*) combinations of C16:0 and C18:0 PAF homologues in the ratios of 1:9 (●) and 3:1 (▲). Measurements were made 15 min after injection, the time of maximal response. The response to vehicle (0.25% HSA in 0.9% saline) alone is also shown.

has reported a higher potency of hexadecyl PAF compared with octadecyl PAF by measuring effects on vascular permeability [5] and platelet secretion [15] in the rabbit, results which are in contrast to our in vitro data demonstrating greater activity of the C18:0 homologue for human neutrophils.

Studies of the profiles of PAF homologues formed by cells in vitro or measured in vivo have been contradictory. Hanahan et al [16] found that the predominant PAFs synthesised by suspensions of rabbit leukocytes, containing 10% basophils, were the C18:0 and C16:0 compounds in a ratio of 9:1. The PAF released from isolated human neutrophils following stimulation with ionophore A 23187 has been variously reported to be a mixture of C16:0 and C18:0 homologues in proportions between 2.5 and 5:1 by Satouchi et al [17], whereas Clay et al [18] found evidence of the C16:0 homologue only, with no detectable C18:0 or any unsaturated ethers. Pinckard et al reported at least five distinct molecular forms of PAF in pooled extracts from neutrophils stimulated with N-formyl-methionyl-leucyl-phenylalanine, the major species present being the C16:0 alkyl ether [19]. Nanogram quantities of PAF have been recovered from 100 mg samples of lesional psoriatic scale, and both the C16:0 and C18:0 alkyl side chains were found to be present in a ratio of 3:1 [11]. The source of PAF derived from psoriatic scale may be the neutrophil, a neutrophilic infiltrate being found in lesional skin. Keratinocytes or Langerhans cells are other possible cellular sources, because mixed human epidermal cells have been shown to produce PAF in vitro [22], in addition to other putative mediators of inflammation, including epidermal cell derived thymocyte activating factor [23] and leukotriene B4 [24].

Although measurement of the ratios of the PAF homologues detected in vivo may provide some indication of the cellular source of the material, it appears that alterations in the relative amounts of C16:0 and C18:0 PAF, the predominant molecular species found to date, are unlikely to affect the early inflammatory effects of PAF in human skin, which may contribute to the pathophysiology of psoriasis and other inflammatory dermatoses [8,10,11], cold urticaria [12], and the late allergic response [8,9,25,26].

Table I. Flare area (cm²) (mean ± sem) responses in human forearm skin to intradermal injections of C18:0 and C16:0 PAF homologues and to the combinations of C16:0 and C18:0 PAF homologues in the ratios of 1:9 and 3:1. Flare area was measured 5 min after injection, the time of maximal response.

PAF (ng/site)	C18:0	C16:0	C16:0:C18:0 (1:9)	C16:0:C18:0 (3:1)
10	3.2 ± 1.7	2.7 ± 1.1	4.3 ± 1.1	4.1 ± 1.1
30	7.4 ± 1.0	5.0 ± 0.8	6.7 ± 1.2	5.7 ± 1.0
100	9.0 ± 1.0	9.3 ± 1.2	8.4 ± 1.2	9.8 ± 1.7

REFERENCES

1. Demopoulos CA, Pinckard RN, Hanahan DJ: Platelet-activating factor. J Biol Chem 254:9355–9358, 1979
2. Polonsky J, Tence M, Varenne P, Das B, Lunel J, Benveniste J: Release of 1-0-alkylglyceryl 3-phosphorylcholine, 0-deacetyl platelet-activating factor, from leukocytes: Chemical ionization mass spectrometry of phospholipids. Proc Natl Acad Sci USA 77:7019–7023, 1980
3. Vargaftig BB, Chignard M, Benveniste J, Lefor J, Wal F: Background and present status of research on platelet-activating factor (PAF-acether). Ann N Y Acad Sci 370:119–137, 1981
4. Czarnetzki BM, Benveniste J: Effect of synthetic PAF-acether on human neutrophil function. Agents Actions 11:549–550, 1981

5. Humphrey DM, McManus LM, Satouchi K, Hanahan DJ, Pinckard RN: Vasoactive properties of acetyl glyceryl ether phosphorylcholine and analogues. Lab Invest 49:422–427, 1982
6. Morley J, Page CP, Paul W: Inflammatory actions of platelet activating factor (PAF-acether) in guinea-pig skin. Br J Pharmacol 80:503–509, 1983
7. Dewar A, Archer CB, Page CP, Paul W, Morley J, MacDonald DM: Cutaneous and pulmonary histopathological responses to platelet activating factor (PAF-acether) in the guinea pig. J Pathol 144:25–34, 1984
8. Archer CB, Page CP, Paul W, Morley J, MacDonald DM: Inflammatory characteristics of platelet activating factor (PAF-acether) in human skin. Br J Dermatol 110:45–50, 1984
9. Basran GS, Page CP, Paul W, Morley J: Platelet activating factor: a possible mediator of the dual response to allergen. Clin Allergy 14:75–79, 1984
10. Archer CB, Page CP, Morley J, MacDonald DM: Accumulation of inflammatory cells in response to intracutaneous platelet activating factor (PAF-acether) in man. Br J Dermatol 112:285–290, 1985
11. Mallet AI, Cunningham FM: Structural identification of platelet activating factor in psoriatic scale. Biochem Biophys Res Commun 126:192–198, 1985
12. Grandel KE, Farr RS, Wanderer AA, Eisenstadt TC, Wasserman SI: Association of platelet activating factor with primary acquired cold urticaria. N Engl J Med 313:405–409, 1985
13. Guinot P, Braquet P, Duchier J, Cournot A: Inhibition of PAF-acether induced weal and flare reaction in man by a specific PAF antagonist. Prostaglandins 32:160–163, 1986
14. Chung KF, Dent G, McCusker M, Guinot PH, Page CP, Barnes PJ: Effect of a ginkgolide mixture (BN 52063) in antagonising skin and platelet responses to platelet activating factor in man. Lancet i:248–251, 1987
15. Hanahan DJ, Munder PG, Satouchi K, McManus L, Pinckard RN: Potent platelet stimulating activity of enantiomers of acetyl glyceryl ether phosphorylcholine and its methoxy analogues. Biochem Biophys Res Commun 99:183–188, 1981
16. Hanahan DJ, Demopoulos CA, Liehr J, Pinckard RN: Identification of platelet activating factor isolated from rabbit basophils as acetyl glyceryl ether phosphorylcholine. J Biol Chem 255:5514–5516, 1980
17. Satouchi K, Oda M, Yasunaga K, Saito K: Application of selected ion monitoring to determination of platelet-activating factor. J Biochem 94:2067–2070, 1983
18. Clay KL, Murphy RC, Andres JL, Lynch J, Henson PM: Structure elucidation of platelet activating factor derived from human neutrophils. Biochem Biophys Res Commun 121:815–825, 1984
19. Pinckard RN, Jackson EM, Hoppens C, Weintraub ST, Ludwig JC, McManus LM, Mott GE: Molecular heterogeneity of platelet-activating factor produced by stimulated human polymorphonuclear leukocytes. Biochem Biophys Res Commun 122:325–332, 1984
20. Cunningham FM, Wong E, Woollard PM, Greaves MW: The chemokinetic response of psoriatic and normal polymorphonuclear leukocytes to arachidonic acid lipoxygenase products. Arch Dermatol Res 278:270–273, 1986
21. Michel L, Mencia-Huerta JM, Benveniste J, Dubertret L: Biologic properties of LTB4 and Paf-acether in vivo in human skin. J Invest Dermatol 88:675–681, 1987
22. Cunningham FM, Leigh I, Mallet AI: The production of platelet activating factor (PAF) by human epidermal cells. Br J Pharmacol 90:117P, 1987
23. Sauder DN, Carter CS, Katz SI, Oppenheim JJ: Epidermal cell production of thymocyte activating factor (ETAF). J Invest Dermatol 79:34–39, 1982
24. Grabbe J, Rosenbach T, Czarnetzki BM: Production of LTB4-like chemotactic arachidonate metabolites from human keratinocytes. J Invest Dermatol 85:527–530, 1985
25. Valone F, Shalit M, Atkins P, Goetzel E, Zweiman B: Platelet activating factor release in allergic skin sites in humans. J Allergy Clin Immunol 79:248, 1987
26. Henocq E, Vargaftig BB: Accumulation of eosinophils in response to intracutaneous PAF-acether and allergens in man. Lancet i:1378–1379, 1986