Platelet activating factor, lyso-platelet activating factor and arachidonic acid release in normal human skin and the influence of topical steroid treatment

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- 1 Previous, *in vitro*, studies have established the synthesis of platelet activating factor (PAF) by the 're-modelling' pathways in which the activation of a phospholipase A₂ (PLA₂) enzyme catalyses the hydrolysis of an ether-acyl-phosphocholine to give concomitant release of lyso-PAF, the immediate precursor of PAF, and arachidonic acid, the precursor of the eicosanoids. The aim of this study was to investigate the relationship between PAF and eicosanoid release in human skin, and to study the effect of treatment of skin with a topical steroid, on the release of PAF, lyso-PAF and arachidonic acid.
- 2 A novel assay procedure was developed for the simultaneous assay of PAF and lyso-PAF in skin exudates from abrasions and suction blisters in normal human skin. In addition we assayed arachidonic acid and prostaglandin E₂ (PGE₂), a representative eicosanoid.
- 3 The mean amounts of mediator recovered in the first 30 min period following abrasion were PAF 0.43, lyso-PAF 11.9, PGE₂ 25.7 and arachidonic acid 760 pmol/sample. The molar ratio of PAF:lyso-PAF: arachidonic acid in skin exudates from abrasions was 1:30:1800 and in suction blister exudates was 1:90:3660.
- 4 Time course studies showed a decline in the recoveries of arachidonic acid and lyso-PAF, of about 50% in 2 h. In contrast, PAF was recovered in exudates at a constant rate over 2 h but PGE₂ release decreased by more than 90% after the initial 30 min period.
- 5 Topical application under occlusion, of 0.05% clobetasol propionate, a potent corticosteroid, significantly reduced lyso-PAF by 30% in suction blister exudates but did not significantly alter the concentrations of PAF or arachidonic acid.
- 6 These results show that, in normal human skin, the recovered quantities of PAF, lyso-PAF and the eicosanoids do not demonstrate a common metabolic pathway for their production. Although corticosteroid treatment reduced lyso-PAF the reduction was not sufficient to affect levels of PAF in skin.

Keywords PAF lyso-PAF corticosteroid inflammation skin

Introduction

PAF is an ether-phospholipid which has properties consistent with those of a potent mediator of inflammation (Camussi *et al.*, 1990). It occurs in psoriatic scale (Mallet & Cunningham, 1985) and can be released from human epidermal cell suspensions (Cunningham *et al.*, 1987; Michel *et al.*, 1990). PAF elicits an inflammatory response when injected intradermally (Archer *et al.*, 1985) and release of PAF has been associated with late

phase cutaneous reactions to antigen in skin (Michel *et al.*, 1988).

The mechanisms by which PAF is synthesized and released in human skin remain poorly defined. *In vitro* studies, mainly in neutrophils and in platelets, have established the synthesis of PAF via the 're-modelling' pathway (Chilton & Connell, 1988; Wykle *et al.*, 1980) in which PLA₂ catalyses the hydrolysis at the *sn*-2

carbon on the glycerol backbone of the phosphocholine, releasing lyso-PAF and, predominantly, arachidonic acid. PLA₂ activity *in vivo* is thought to be regulated by membrane associated lipocortins which on proteolytic cleavage can activate PLA₂ (Davidson *et al.*, 1987). This hypothesis is supported by the observation that PAF synthesis is inhibited by anti-proteinases and stimulated by proteinases (Camussi *et al.*, 1988). The newly released lyso-PAF is acetylated to PAF by an acetyl transferase enzyme; this step is rate limiting in inflammatory cells (Alam & Silver 1986). Inhibition of PLA₂ has been described as a mechanism of action of steroidal anti-inflammatory drugs (Flower, 1985).

PLA₂ activity in the re-modelling pathway has the potential to release arachidonic acid, the precursor of many inflammatory eicosanoids, in addition to lyso-PAF and PAF. The aim of this study was to investigate the relationship between PAF and eicosanoid release in human skin, PGE₂ being selected as a recognised inflammatory mediator product of the cyclo-oxygenase metabolism of arachidonic acid. In addition, the effect of treatment of skin with a topical steroid on the release of arachidonic acid, lyso-PAF and PAF was investigated.

Methods

Clinical methods

Skin exudates were collected from normal healthy adult volunteers with no recent history of skin disease. The studies had approval from the West Lambeth Health Authority Ethics Committee and all volunteers gave written informed consent.

Nine subjects (six male, age range 21–46, mean 26 years) took part in a time course study. Exudates were collected from abraded skin into 1 ml phosphate buffered saline (PBS) contained in plastic chambers, as previously described (Barr *et al.*, 1988). A total of four sequential 30 min samples were collected over 2 h from each abrasion. Suction blisters were raised from the same groups of subjects, using established methods (Black *et al.*, 1977) and the exudates, approximately 0.2 ml, were collected for analysis.

The effect of topical corticosteroid treatment on PAF, lyso-PAF and arachidonic acid release in skin was studied in eight subjects (five male, age range 20–32, mean age 25 years). Clobetasol propionate, 0.05% and its base were applied in therapeutic amounts to square areas of skin (10 cm × 10 cm), on either anterior thigh in a double-blind design. Applications were made at 24 h intervals for 72 h and the sites were occluded with clingfilm. Suction blisters were then raised on each site and the exudates collected, as above. All exudates were analysed for PAF, lyso-PAF, arachidonic acid and, in the case of the time course studies, for PGE₂.

Mediator analysis

PGE₂ analysis was performed directly on an aliquot of skin chamber fluid by our published radio-immunoassay procedure (Barr *et al.*, 1988).

PAF, lyso-PAF and arachidonic acid were measured by gas chromatography-mass spectrometry (GC-MS). Deuterated internal standards (100 ng [²H₈]-arachidonic acid, 0.5 ng [7,7',8,8'-²H₄]-hexadecyl-PAF and 10 ng [7,7,8,8'-²H₄]-hexadecyl-lyso-PAF (Cascade Biochem Ltd, Reading, UK) were added to each sample. The volumes of suction blister exudates were made up to 0.9 ml with PBS. Each sample was mixed with 1 ml methanol containing 30 µl acetic acid and stored at –20 °C until analysed.

Lipids were extracted by the method of Folch *et al.* (1957); chloroform 1 ml was added to each sample and after vortexing and centrifugation the lower chloroform layer was removed. The remaining aqueous fraction was re-extracted with a further 1 ml chloroform and the pooled chloroform extracts were washed with 0.5 ml 0.17 M acetic acid and evaporated to dryness under nitrogen. The extracted lipids were partitioned between hexane and methanol (2:1) and the hexane layer collected for subsequent arachidonic acid analysis. The methanol layer was dried under nitrogen and this extract was purified on 1 ml amino-propyl extraction cartridges (BondElut, Jones Chromatography, Cardiff) (Kaluzny *et al.*, 1985). The columns were activated with 5 ml methanol and equilibrated with chloroform. The total lipid extracts were applied in chloroform:methanol (95:5) and the cartridge was eluted sequentially with 2 ml each of chloroform:methanol (95:5), chloroform:propan-2-ol (2:1), diethyl ether:acetic acid (98:2); the total phospholipids were recovered in 1.5 ml methanol:acetic acid (99.5:0.5). The solvent was removed from the latter fraction, under nitrogen, and the extract was dried under vacuum. Lyso-PAF in the extract was converted to the *sn*-2 propionyl homologue of PAF by dissolving the above phospholipid extract in a mixture of propionic acid:trifluoroacetic anhydride (1:2) (Dell & Tiller, 1986) at room temperature for 15 min. The reagents were removed under nitrogen. The combined PAF and propionyl PAF species were further purified by h.p.l.c. on a 4 mm × 250 mm LiChrosorb-DIOL column (Merck, Poole UK) eluted with methanol:propan-2-ol:trifluoroacetic acid (80:20:0.3) at 1 ml min⁻¹. A benzyl-PAF homologue was used to calibrate the column for the time of elution of PAF using u.v. detection at 244 nm and this was confirmed using 2500 d min⁻¹ [³H]-PAF (mixed hexadecyl and octadecyl forms, Amersham). PAF and its related derivative eluted at 5.5 to 6 min. The appropriate fraction was collected and taken to dryness under nitrogen.

GC-MS analysis

PAF and propionyl PAF were derivatised directly (Weintraub *et al.*, 1990) by treatment with 30 µl pentafluorobenzoyl chloride (Sigma Chemical Co, Poole) at 60 °C for 12 h. The reagent was removed under vacuum and the residue dissolved in *n*-octane. The arachidonic acid extracts were esterified by treatment with pentafluorobenzyl chloride:di-isopropyl-ethylamine:acetonitrile (10:1:100) for 30 min at room temperature. After removing the reagents under vacuum the residue was dissolved in *n*-octane.

GC-MS was performed using electron capture negative ion mass spectrometry with ammonia as the moderating

gas. A VG Analytical VSEQ mass spectrometer (Fisons Instruments Ltd, Manchester) was used and chromatography was performed on a Restek Rtx-1 15 m $\times$ 0.33 mm column (Jones Chromatography, Cardiff). Samples were injected on a Gerstel cooled on-column injector (Thames Chromatography, Maidenhead). Only the signals corresponding to the hexadecyl PAF and lyso-PAF species were monitored, these species having been shown previously to comprise over 80% of the total PAF in human skin (Mallet & Cunningham, 1985).

Statistical analysis

Statistical analysis was by two way analysis of variance. Time course data were assessed using Tukey's procedure for multiple comparisons. The significance of the steroid treatment data was determined by paired *t*-test. All results are expressed as mean $\pm$ s.e. mean.

Results

Mediator concentrations in the suction blister exudates, are expressed in molar terms but those in the chamber fluids collected in PBS from abraded skin, are expressed as absolute amount per sample.

Mediator release from abraded skin

The time course of PAF, lyso-PAF, PGE₂ and arachidonic acid release into skin chambers on abraded normal skin is shown in Figure 1. The mean ($\pm$ s.e. mean) amounts of mediator recovered in the first 30 min

period were, PAF 0.43 ± 0.12 ($n = 9$); lyso-PAF 11.9 ± 2.9 ($n = 8$); PGE₂ 25.7 ± 4.5 ($n = 8$); arachidonic acid 760 ± 70 ($n = 4$) pmol/sample. The molar ratio relative to PAF was 1:30:60:1800 respectively. PAF was released at a constant rate throughout the 2 h sampling period; in contrast the mean PGE₂ release had declined by >90% for the second and subsequent samples collected. The amounts of lyso-PAF and arachidonic acid also showed significant decrease over the collection period.

Mediator release into suction blister exudates

The mean PAF, lyso-PAF and arachidonic acid concentrations in suction blister exudates from untreated normal skin were 1.86 ± 0.92 ($n = 7$), 160 ± 21 ($n = 8$) and 6820 ± 280 ($n = 4$) nmol l⁻¹ respectively a molar ratio relative to PAF of 1:90:3660.

Effect of corticosteroid treatment

The effect of topical clobetasol propionate treatment on PAF, lyso-PAF and arachidonic acid release into suction blister exudates for each of the eight subjects is shown in Figure 2. The steroid treatment decreased lyso-PAF concentration in 8/8 subjects. The difference in the mean between steroid and base treated sites was significant. PAF concentrations were decreased in 6/8 subjects but the difference in the means of clobetasol propionate and the base treatment did not achieve significance. Steroid treatment had no overall effect on the mean recovery of arachidonic acid from the eight subjects.

The mean ($\pm$ s.e. mean) concentrations for exudates from steroid and base treatment skin sites were re-

Figure 1 Time course of release of arachidonic acid, lyso-PAF, PGE₂ and PAF into skin chambers. Mean ($\pm$ s.e. mean) values expressed as pmol (PAF, lyso-PAF and PGE₂) or nmol (arachidonic acid). Significantly different from the value at 0-30 min, **P* < 0.05, ***P* < 0.01.

Figure 2 Effect of topical steroid treatment on mediator release into suction blister exudates. Mean (s.e. mean) values expressed as nmol (lyso-PAF and PAF) or μmol (arachidonic acid).

spectively, PAF 1.12 ± 0.35 and 1.97 ± 0.52 ; lyso-PAF 152 ± 25 and 230 ± 43 ($P > 0.05$); arachidonic acid 5560 ± 1510 and $5300 \pm 630 \text{ nmol l}^{-1}$.

Discussion

A previous study (Wong *et al.*, 1986) has shown that application of 0.05% clobetasol propionate under a similar protocol, but without occlusion, inhibits the release of arachidonic acid from psoriatic lesions. In

addition, it has been shown that the systemic administration of the corticosteroid, prednisolone, inhibits the stimulated release of arachidonic acid into suction blisters on inflamed skin but does not significantly affect its levels in normal skin (Kobza Black *et al.*, 1982). In this study, treatment of normal skin by clobetasol propionate, also had no effect on arachidonic acid release. The inhibition of lyso-PAF, but not of arachidonic acid release by the clobetasol propionate suggests that the production of lyso-PAF in normal human skin from its precursor ether-phospholipid is more sensitive to PLA_2 inhibition than is that of arachidonic acid. The diminution of lyso-PAF concentration by pre-treatment with a potent steroid, while significant, was not of sufficient magnitude to have made a discernible suppression of the production of PAF in skin exudates.

The substantially greater amounts of arachidonic acid compared with PAF and lyso-PAF measured in skin exudates in this study suggests sources for the release of arachidonic acid independent of PAF synthesis in human skin. This observation is in accord with the occurrence in cells of many further potential sources of arachidonic acid present in high concentrations, such as diacyl phosphatidyl cholines, from which PAF cannot be released. The time course study showed that the synthesis of PAF and PGE_2 , the end-products of mediator formation, are independently regulated and are not conditional upon any potential linked release of arachidonic acid and lyso-PAF from the parent ether phospholipid. The decrease in PGE_2 recovery with time is in good agreement with our previous study (Barr *et al.*, 1988).

The failure of PAF concentrations to reflect changes in lyso-PAF concentrations confirms the hypothesis that the acetyl transferase enzyme which converts lyso-PAF to PAF, is rate limiting in human skin.

The novel methods described here permit the extraction of PAF and lyso-PAF from biological matrices followed by their simultaneous purification by h.p.l.c. and their quantitative analysis by GC-MS. The use of stable isotope substituted hexadecyl PAF and lyso-PAF as internal standards compensated for any variation in recoveries of PAF and lyso-PAF during the analytical procedures. The hexadecyl species of PAF and lyso-PAF were chosen for analysis because many previous studies have shown that the hexadecyl PAF is the major species derived from human PMN (Clay *et al.*, 1984; Oda *et al.*, 1985), scale from psoriatic lesions (Mallet & Cunningham, 1985), human dermal fibroblasts (Michel *et al.*, 1988) and human epidermal cells (Michel *et al.*, 1990).

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