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Urate crystals induce macrophage PAF-AH secretion which is differentially regulated by TGFβ1 and hydrocortisone

DARSHNA YAGNIK and FRANK HILLS

Department of Natural Sciences, Biomarker Research Group, School of Science and Technology, Middlesex University, The Burroughs, London NW4 4BT, UK

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Abstract. The aim of the present study was to establish the role of platelet-activating factor acetyl hydrolase (PAF-AH) in the resolution phase of gout using an established in vitro mononuclear cell model. The effects of signalling pathway inhibitors on PAF-AH secretion, as well as the effects of the common treatments hydrocortisone and colchicine, an antibody against the anti-inflammatory cytokine transforming growth factor β1 (TGFβ1), and the transcriptional inhibitor actinomycin D, were also investigated. The effect of recombinant PAF-AH on cytokine secretion by these cells was also determined. Human peripheral blood-derived monocytes were isolated and differentiated into macrophages. Monocytes and macrophages were stimulated with monosodium monohydrate urate (MSU) crystals or lipopolysaccharide in the presence or absence of AEG3482 [a c-Jun N-terminal kinase (JNK) inhibitor], MG132 (a proteasome inhibitor), hydrocortisone or colchicine. Cultures were then analysed for PAF-AH secretion using ELISA. A 6-fold upregulation of PAF-AH secretion was observed following macrophage exposure to MSU crystals for 24 h (29.3±6 vs. 5.4±0.3 ng/ml unstimulated; P<0.05). Following 72 h, PAF-AH levels decreased significantly (11.1±1.8; P<0.01). Secretion was further enhanced following pre-treatment with the JNK protein kinase inhibitor AEG3482 (11.1±1.8; P<0.01). Secretion was further enhanced following pre-treatment with the JNK protein kinase inhibitor AEG3482 prior to MSU crystal stimulation (P<0.05) and was abrogated when cells were preincubated with actinomycin D or the proteasome inhibitor MG132 (50, 100 and 200 µM). The addition of recombinant PAF-AH (2.5-10 ng/ml) to MSU crystal-stimulated immature monocyte cultures significantly decreased pro-inflammatory interleukin (IL)-1β (unstimulated 687±124 vs. stimulated 113±30 pg/ml) and IL-6 secretion (unstimulated 590±50 vs. stimulated 182±19 pg/ml). Treatment of MSU crystal-stimulated macrophages with hydrocortisone (2 µM) also significantly decreased PAF-AH release (P<0.05). Neutralising anti-TGFβ1 addition decreased PAF-AH dose-dependently with the highest inhibition observed at 1 µg/ml (P<0.05). The results implicated that PAF-AH may have an anti-inflammatory role in the resolution phase of gout.

Introduction

Gout was often referred to as an affliction of kings due to its association with a lavish diet and excess alcohol consumption. In fact, Henry VIII was reported to have suffered from gout (1). The last few decades has seen a global rise in new patient diagnoses (2) and the prevalence of the disease has increased particularly in the UK (3). Gout is an inflammatory form of crystalline arthritis characterised by the deposition of excess circulating uric acid in the form of monosodium monohydrate urate (MSU) crystals into intra-articular joints and tissue spaces (4). Patients experience recurrent acute flares of severe joint inflammation. Such attacks involve inflammation caused by the interaction between MSU crystals and the local tissue environment including intra-articular cells, monocytes and macrophages. The active stage of gout is defined by an inflammatory innate response involving monocytes and neutrophils. This response is driven in part by NLRP3 inflammasome activation via NOD-like receptors which activate interleukin-1β (IL-1β) release (5-7). Studies in vitro suggest that MSU crystals may induce tumour necrosis factor α (TNFα), IL-1β, IL-6, platelet-activating factor (PAF) secretion in undifferentiated monocytes which in turn may promote endothelial E-selectin expression and secondary neutrophil adhesion (8-10). Conversely, differentiated macrophages are able to uptake MSU crystals and release transforming growth factor β1 (TGFβ1), a powerful inhibitor of inflammation (11).

Therapeutic options for gout management remain limited to the use of anti-inflammatory treatments such as non-steroidal anti-inflammatory drugs (NSAIDs), IL-1β antagonists, adrenocorticotropic hormone (ACTH), colchicine, allopurinol, or hydrocortisone injections following acute flares. A study reviewing patients with tophaceous gout and the risk of complications reported that for all patients there was at least a 25% casual risk of arterial hypertension, hyperlipidaemia, diabetes, renal function impairment and cardiovascular issues.
Even after treatment these patients had at least three gout flares per year (6,7). Mononuclear cells are a vital part of innate immunity and have been identified to secrete multiple enzymes into the microenvironment. However, information regarding the role of enzymes secreted in gout afflicted joints is limited. Recently published guidelines by the European League Against Rheumatism have highlighted the potential of enzymes such as febuxostat (xanthine oxidase inhibitor) and pegloticase (acts as an uricase) which target uric acid metabolism, as therapeutic agents in gout (12,13).

Platelet-activating factor acetyl hydrolase (PAF-AH) has multiple roles in numerous pathologies and synthesis and secretion of PAF-AH are increased during monocyte to macrophage differentiation (14-16). Two PAF-AH isoforms have been identified: plasma PAF-AH and intracytoplasmic PAF-AHII. Plasma PAF-AH is a polypeptide with molecular weight 40 kDa, mostly produced by macrophages and hepatocytes. It is an anti-inflammatory enzyme since it is predominantly involved in degrading inflammatory phospholipids and is found mainly bound to low density lipoprotein (LDL) in the circulation (17-19). Plasma PAF-AH deficiency has been linked to inflammatory reactions such as sepsis, cardiovascular disease and anaphylaxis (20,21).

PAF-AH resolves inflammation through the inactivation of PAF, a potent, inflammatory lipid mediator. Serum PAF concentrations are rigorously controlled by tight regulation of synthesis and degradation. The acetyl sn-2 group on the backbone of PAF is required for biological activity and is the target for PAF-AH action via esterification converting PAF to its inactive lysosomal-PAF form (22). We have previously shown that immature monocytes pre-treated with recombinant PAF-AH showed a dose-dependent inhibition of TNFα synthesis (12). The mechanism by which PAF-AH is regulated is still not fully understood and so we have investigated the effect of common gout therapeutics on PAF-AH expression. The aim of this study was to investigate the effect of MSU crystal stimulation on macrophage PAF-AH secretion in vitro and to determine the factors and pathways that may be involved in this process.

Materials and methods

**Materials.** Hydrocortisone (HC) actin D, lipopolysaccharide (LPS) Dulbecco’s modified Eagle medium (DMEM), dimethyl-ethyl-sulphonyl-oxide, HANKS balanced salt solution, histopaque, phosphate buffer saline (PBS), Colchicine and methanol were purchased from Sigma Aldrich, Poole, UK. Proteasome inhibitor (MG132) and c-Jun N-terminal kinase (JNK) inhibitor (AEGL3482) were purchased from Toecris Biosciences UK. Cyclic adenosine monophosphate inhibitor (cAMPl) and MSU crystals were purchased from Enzo Life Sciences, Germany. Monoclonal mouse IgG1 clone 9016, mouse anti-human TGFβ1 (MAB240), recombinant PAF-AH, IL-1β and IL-6 ELISA kits and cell lysis solution were purchased from R&D Systems, Abingdon, UK. Manufacturer’s instructions were followed for all listed kits and reagents. Mouse anti-human IgG1 monoclonal antibody (MAB3832) was used at 100 μg/ml, mouse anti-human CD163 IgG1 monoclonal antibody (clone 215927) was used at 2.5 μg per 10⁶ cells and were both purchased from R&D Systems, Abingdon, UK. Goat ant-human IgG whole molecule FITC was used at a 1:64 dilution in PBS and purchased from Sigma, Aldrich, Poole, UK.

**Monocyte and macrophage preparation.** The present study incorporated a series of in vitro cell cultures of differentiated monocytes isolated from human blood cones purchased from the National Health Service Blood Bank (Colindale, London, England). Ethical approval was granted by the Middlesex University Institutional Ethics Committee, Department of Natural Sciences (London, England) and this was also a purchase requirement from the blood bank. The present study complied with the ethical standards established in 1964 in the Declaration of Helsinki.

Leucocyte rich blood cones and blood group AB positive serum were purchased from the NHS Cord blood and transplant bank (Colindale, London, UK). The cones were washed with PBS to and centrifuged on histopaque at 620 x g for 20 mins to harvest leucocytes. The monocyte enriched fraction was obtained from the interface, washed in HANKS balanced solution without calcium or magnesium, after which cells were counted and cultured into 24 well plates at 4x10⁶ per ml in DMEM containing 1% penicillin and streptomycin at 37°C in an atmosphere containing 5% CO₂. The mononuclear cells were allowed to adhere for 1 h at 37°C after which any non-adherent cells were removed by aspiration and washing with PBS. The adherent cells were then cultured in Dulbecco’s media containing 10% AB serum either for seven days to obtain macrophages or overnight for use as monocytes (9-11). These methods were established previously and macrophage differentiation was determined by light microscopy and flow cytometry phenotypic analysis using the macrophage differentiation

![Figure 1. Time course of PAF-AH production. Results are presented as the mean and standard deviation of 3 similar experiments. Peak PAF-AH release was detected at 24-48 h followed by a decline in enzyme release following 48 h. A significant increase in PAF-AH was detected at 24 h when compared to 3.5 h following macrophage MSU crystal stimulation. *P<0.05 and **P<0.001 vs. Media. PAF-AH, platelet-activating factor acetyl hydrolase; MSU, monosodium monohydrate urate crystals.](image-url)
marker CD163 (10). Cell culture media changes were carried out at days 1, 3, 5 and 7 days of culture replacing with fresh DMEM containing 10% AB serum.

Differentiated macrophages were stimulated with LPS, (10 µg/ml) or MSU crystals (0.5 mg/ml) with or without pre-treatment for 30 min with HC (1, 2 and 4 µM) Stimulation was stopped after 24 h of incubation in all experiments. The 0.5 mg/ml MSU concentration and time point (24 h) was used for all experiments since this concentration and time point were previously identified as optimum conditions from previous studies of mononuclear and macrophage cell stimulation (10‑13).

**Figure 2. Effect of hydrocortisone and colchicine on PAF-AH secretion by macrophages stimulated with MSU crystals or lipopolysaccharide.** Differentiated macrophages were pre-treated with HC (1-4 µM) for 30 min prior to the addition of (A) 0.5 mg/ml MSU crystals or (B) 10 mg/ml LPS and (C) 0.5 mg/ml MSU crystals and colchicine for 24 h. Collected supernatants were analysed for PAF-AH by ELISA. Comparisons between macrophages in media only with MSU or LPS stimulation were significantly different. Macrophage MSU crystal-but not LPS-stimulated PAF-AH secretion was significantly decreased in the presence of HC at all doses tested. The results are presented as the mean ± standard error of the mean. *P<0.01 vs. Media; **P<0.01 vs. MSU alone. PAF-AH, platelet-activating factor acetyl hydrolase; HC, hydrocortisone; MSU, monosodium monohydrate urate crystals.

Macrophage stimulation. Macrophages were stimulated MSU crystals (0.5 mg/ml) or LPS (10 µg/ml) with or without pre-treatment for 30 min at 37°C. This was followed by the addition of AEG3482, a JNK inhibitor (12.5, 25 and 50 µM), MG132 a proteasome inhibitor (50 and 100 µM), actin D (2 µM). Incubation was stopped after 24 h in all experiments.

**Treatment with colchicine or hydrocortisone or anti-TGFβ1.** Colchicine was prepared by dissolving in 1 ml ethanol as recommended by manufacturer’s instructions. Further dilutions were carried out in DMEM media at concentrations of 125, 250 or 500 µg/ml. Hydrocortisone was dissolved in 1 ml DMEM and further diluted in media to concentrations of 1, 2 and 4 µM. Mouse anti-TGFβ1 and an isotype matched control IgG1 were both dissolved in PBS and used at 2.5, 5, 10 ng/ml (anti-TGFβ1) and 10 µg/ml (control IgG1) respectively. Macrophages were pre-incubated with colchicine, hydrocortisone, anti-TGFβ1 or IgG1 control for 30 min prior at 37°C to addition of MSU crystals for 24 h after which supernatants were collected and analysed for PAF-AH content.

**Monocyte stimulation with recombinant PAF-AH.** Recombinant PAF-AH was dissolved in 0.5 ml of PBS and further diluted in DMEM media (2.5-10 ng/ml). Freshly isolated monocytes were pre-treated with recombinant PAF-AH for 20 min prior to the addition of MSU crystals and then incubated at 37°C for 24 h after which supernatants were collected for cytokine analysis.

**Enzyme linked immunosorbent assays (ELISAs).** IL-1β and IL-6 concentrations in cell culture supernatants were determined by sandwich ELISA using matched antibodies (DuoSet: R&D Systems, Abingdon, UK). PAF-AH levels were determined using a solid phase quantikine ELISA (DPLG70 from R&D Systems) with sensitivity (0.8-50 ng/ml) in conditioned cell culture supernatants. All samples were measured in duplicates using manufacturer’s instructions without any deviations. Results were expressed as the mean ± SEM cytokine concentration (ng/ml) from at least 4 experiments.

**Statistical analysis.** All samples were measured in duplicate from at least three experiments, with results expressed as the mean ± standard deviation. Statistical analysis was carried out by applying one-way analysis of variance with Dunnett’s post hoc test or Student’s t-test where appropriate. P<0.05 was considered to indicate a statistically significant difference. All data were analysed using Excel (2016 version; Microsoft Corporation, Redmond, WA, USA).

**Results**

**PAF-AH secretion by MSU crystal stimulated macrophages.** MSU crystal stimulation resulted in the upregulation of PAF-AH secretion by in vitro differentiated macrophages compared to unstimulated macrophages. Peak enzyme release was detected at an incubation time of 24-48 h which was approximately six-fold higher than unstimulated macrophages (mean ± SD; 0.4±0.1 ng/ml vs. 0.02±0.001 ng/ml respectively, P<0.001) after which a decline in enzyme secretion occurred with levels reverting to near baseline levels after 72 h (Fig. 1).

**Effect of hydrocortisone, colchicine, LPS, anti-TGFβ1 and pathway inhibitors on macrophage PAF-AH secretion.**
Co-incubation of macrophages with hydrocortisone (1-4 µM) significantly decreased MSU crystal stimulated PAF secretion (Fig. 2A). Interestingly, LPS-stimulated macrophages also release PAF-AH after 24 h of LPS stimulation. However when we added equivalent concentration of HC to LPS stimulated macrophages we did not observe a significant decrease in PAF-AH secretion (Fig. 2). Also colchicine did not significantly alter the levels of PAF-AH at the doses used. In the next stage of our investigation we tested a variety of pathway inhibitors to investigate the signalling mechanisms involved in this model. Notably, the JNK inhibitor AEG3482 did not decrease MSU crystal-mediated PAF-AH secretion. In fact, PAF-AH release was enhanced following incubation with this inhibitor (Fig. 3). Inclusion of a proteasome inhibitor to the cultures significantly reduced PAF-AH release at the doses used (Fig. 4). In contrast, PAF-AH secretion was not significantly altered by the addition of cyclic AMP inhibitor (Fig. 5) implying that this mode of intracellular signalling was not involved in MSU-mediated PAF-AH secretion. The addition of actin D into the co-cultures resulted in complete amelioration of PAF-AH detection in this pathway as seen in Fig. 4 suggesting that MSU crystal de novo synthesis of PAF-AH is required in macrophages. Addition of neutralising anti-TGFβ1 resulted in a dose dependent reduction in PAF-AH at concentrations of 0.25, 0.5 and 1 µg/ml whereas an isotype matched IgG control antibody at the same concentrations had no effect on PAF-AH secretion (Fig. 5).

**Treatment with recombinant PAF-AH inhibits pro-inflammatory cytokine secretion by immature monocytes stimulated with MSU crystals.** We investigated whether the activity of recombinant PAF-AH could also modify IL-1β and IL-6 secretion. Previously, we had reported that recombinant PAF-AH decreases TNFα cytokine secretion from immature human monocytes in a similar experimental model (9).

Pre-treatment of undifferentiated, inflammatory monocytes with recombinant PAF-AH at doses of 2.5, 5 and 10 ng/ml and then MSU crystals for 24 h resulted in a dose dependent decrease in IL-1β and IL-6 secretion. The highest inhibition was achieved at 10 ng/ml recombinant PAF-AH (P=0.02 for IL-1β and P=0.003 for IL-6 respectively, Fig. 6).

**Discussion**

To the best of our knowledge this is the first study to identify that macrophage uptake of MSU crystals leads to an upregulation in secretion of the enzyme PAF-AH. Little is known about the enzymes involved in the inflammation resolution phase of gout with research focussing mainly on caspase-1 activating NALP3 (23,24). PAF-AH is actually an anti-inflammatory phospholipase that is found as a plasma isoform complexed mainly with LDLs (25). Low levels of PAF-AH seem to correlate with a number of modalities. A study involving patients with acute allergy reactions reported that those patients with the lowest levels of plasma PAF-AH were at high risk of severe anaphylaxis (26). Also, mast cells derived from rat bone marrow secrete PAF-AH upon direct IgE activation indicating PAF-AH may have a potentially crucial function in pathological and physiological defence. It is postulated that the likely function of PAF-AH may be as a safety biosensor during inflammation. This is due to its ability to neutralise the effects of inflammatory PAF and oxidised phospholipids which are upregulated in pathologies such as thrombosis, allergy, sepsis as well as gout (26,27).

A local increase in harmful lipids often accompanies sustained inflammation. Thus upregulation of PAF-AH takes place in cells to deactivate and degrade PAF. In fact joint inflammation in gout is accompanied by a local increase in PAF, prostaglandin E2 and leukotrienes (28,29). In this study we have demonstrated that recombinant PAF-AH treatment...
may tip the inflammatory balance in favour of inflammation resolution as it was able to decrease monocyte derived IL-1β and IL-6 secretion due to MSU crystal stimulation. These results are consistent with our previous study in which PAF was upregulated by monocytes in response to MSU crystals (9) and recombinant PAF-AH administration downregulated TNFα secretion. The exact form of PAF was not characterised; we propose that this was probably inert lysosomal PAF. Platelet-activating factor acetyl hydrolase; IgG, immunoglobulin G; MSU, monosodium monohydrate urate crystals.

**Figure 5.** TGFβ1 is involved in macrophage PAF-AH secretion upon MSU crystal uptake. Differentiated macrophages were cultured in media containing a neutralising antibody to TGF-β prior to MSU crystal stimulation for 24 h and the collected supernatants were analysed for PAF-AH by ELISA. Results are expressed as the mean ± standard deviation of at least 3 experiments. **P<0.01 vs. MSU only. TGFβ1 activity using a neutralising antibody resulted in a dose dependent increase in PAF-AH secretion. The anti-inflammatory activity of TGFβ1 is well established in gout inflammation resolution (13). Our results suggest that this effect may be attributed in part by the upregulation of PAF-AH. Essentially both PAF-AH and TGFβ1 share a transcription binding site involved in macrophage regulation, mediating effects through canonical Sp1 (16,34). Hydrocortisone is known for its anti-inflammatory properties through suppression of vascular permeability, vasodilation and leukocyte migration into inflamed sites (35). Unexpectedly in our study hydrocortisone decreased PAF-AH secretion by MSU crystal stimulated macrophages. However, there is evidence that hydrocortisone may also have pro-inflammatory properties. For example, hydrocortisone can interact with anti-inflammatory drugs such as aspirin hydrolysis where it mediates esterase activity (36). Furthermore, when normal subjects were injected with 300 mg of hydrocortisone, there was an increase in toll like inflammatory receptor 2, 5, 9, high mobility group box-1 and matrix metalloproteinase-9 expression occurred (37). Perhaps the long term use of hydrocortisone therapy in gout or arthritis could result in inadvertently driving the inflammatory response rather than limiting it.

**Figure 6.** Downregulation of IL-1β and IL-6 by recombinant PAF-AH in immature monocytes stimulated with MSU crystals. Inclusion of exogenous recombinant PAF-AH resulted in a dose dependent reduction in IL-1β release by monocytes. Statistical significance was observed at 5 ng/ml (IL-1β, P=0.03; IL-6, P=0.003) and at 10 ng/ml (IL-1β, P=0.008; IL-6, P=0.003) with respect to recombinant PAF-AH pre-treatment of macrophage cultures. Results are expressed as the mean ± standard deviation of at least 3 experiments. *P<0.05 and **P<0.01 vs. MSU only. PAF-AH, recombinant platelet-activating factor acetyl hydrolase; IL, interleukin; MSU, monosodium monohydrate urate crystals.

It is well established in gout inflammation resolution (13), our results suggest that this effect may be attributed in part by the upregulation of PAF-AH. Essentially both PAF-AH and TGFβ1 share a transcription binding site involved in macrophage regulation, mediating effects through canonical Sp1 (16,34). Hydrocortisone is known for its anti-inflammatory properties through suppression of vascular permeability, vasodilation and leukocyte migration into inflamed sites (35). Unexpectedly in our study hydrocortisone decreased PAF-AH secretion by MSU crystal stimulated macrophages. However, there is evidence that hydrocortisone may also have pro-inflammatory properties. For example, hydrocortisone can interact with anti-inflammatory drugs such as aspirin hydrolysis where it mediates esterase activity (36). Furthermore, when normal subjects were injected with 300 mg of hydrocortisone, there was an increase in toll like inflammatory receptor 2, 5, 9, high mobility group box-1 and matrix metalloproteinase-9 expression occurred (37). Perhaps the long term use of hydrocortisone therapy in gout or arthritis could result in inadvertently driving the inflammatory response rather than limiting it.

Co-incubation of MSU-stimulated macrophages with the proteasome inhibitor MG132 resulted in a significant decrease in PAF-AH secretion. This suggests that this pathway involves protein degradation (38). Conversely, colchicine had no significant effect. Colchicine is routinely prescribed as a prophylactic treatment for gout at similar doses tested in our study. It has been shown that colchicine inhibits leukocyte migration into inflamed sites (35). Unexpectedly in our study hydrocortisone decreased PAF-AH secretion by MSU crystal stimulated macrophages. However, there is evidence that hydrocortisone may also have pro-inflammatory properties. For example, hydrocortisone can interact with anti-inflammatory drugs such as aspirin hydrolysis where it mediates esterase activity (36). Furthermore, when normal subjects were injected with 300 mg of hydrocortisone, there was an increase in toll like inflammatory receptor 2, 5, 9, high mobility group box-1 and matrix metalloproteinase-9 expression occurred (37). Perhaps the long term use of hydrocortisone therapy in gout or arthritis could result in inadvertently driving the inflammatory response rather than limiting it.

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the synovial fluid samples obtained from gout patients during active disease compared to quiescent joints. The efficacy of PAF-AH treatment for gout patients should also be explored.

In conclusion, we have demonstrated that PAF-AH enzyme secretion is upregulated by macrophages following MSU crystal uptake and involves anti-inflammatory TGFβ1. This research expands our understanding of an important anti-inflammatory enzyme which could be functioning as a biosensor responding to local microenvironmental conditions.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

DY designed all of the experiments, collected and analyzed the data, performed statistical analysis and wrote the manuscript. FH contributed to data analysis and writing the manuscript.

Ethics approval and consent to participate

The present study incorporated a series of in vitro cell cultures of differentiated monocytes isolated from human blood cones purchased from the National Health Service Blood Bank (Colindale, London, England). Ethical approval was granted by the Middlesex University Institutional Ethics Committee, Department of Natural Sciences (London, England) and this was also a purchase requirement from the blood bank. The present study complied with the ethical standards established in 1964 in the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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