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Research report

Platelet-activating factor increases prostaglandin E_2 release from astrocyte-enriched cortical cell cultures

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Abstract

The phospholipid mediator platelet-activating factor (PAF) increased the release of prostaglandin E_2 (PGE_2) from astrocyte-enriched cortical cell cultures in a concentration- and time-dependent manner. The nonhydrolyzable PAF analog methylcarbamyl-PAF (mc-PAF), the PAF intermediate lyso-PAF, and arachidonic acid (AA) also produced this effect. In contrast, phosphatidlycholine (PC) and lyso-PC, lipids that are structurally similar to PAF and lyso-PAF, had no effect on PGE_2 production, suggesting that PAF-induced PGE_2 release is not the consequence of nonspecific phospholipid-induced membrane perturbation. Antagonism of intracellular PAF binding sites completely abolished the ability of mc-PAF and lyso-PAF to mobilize PGE_2, and attenuated the AA effect. Antagonism of the G-protein-coupled PAF receptor in plasma membranes had no significant effect on mc-PAF, lyso-PAF or AA-induced PGE_2 release. Based on the present findings, we hypothesize that intracellular PAF is a physiologic stimulus of PGE_2 production in astrocytes.

Theme: Cellular and molecular biology

Topic: Neuroglia

Keywords: Alzheimer's disease; Astrocyte; Inflammation; Platelet-activating factor; Phospholipase; Prostaglandin

1. Introduction

PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a family of structurally related and biologically potent phospholipid mediators. Two major pathways mediate PAF biosynthesis in brain and other tissues [35]. The de novo pathway produces PAF from CDP-choline and alkylacetylglycerol, via a PAF-phosphocholine transferase. The remodeling pathway forms PAF from acetyl CoA and phospholipase A_2 (PLA_2)-derived lyso-PAF (1-*O*-alkyl-*sn*-glycerol-3-phosphocholine), a reaction catalyzed by acetyl-CoA:1-alkyl-*sn*-glycero-3-phosphocholine acetyltransferase (lyso-PAF-AT). The PAF precursor lyso-PAF can also be generated via acetyl-CoA-independent mechanisms [35]. The relative contributions of these distinct pathways to the production of PAF in physiological and pathological conditions has not been fully established, but it has been suggested that, under physiological conditions the de novo pathway mainly contributes to maintaining basal PAF levels, while the remodeling pathway may be more involved in the production of PAF in pathological states [17].

PAF may act as an inter- and intracellular mediator [5]. Two high-affinity intracellular binding sites for PAF have been identified, as well as a low-affinity cell-surface receptor [26]. The cell-surface PAF receptors have been cloned and characterized [9,10], and are coupled to heterotrimeric G-proteins [21,43]. The presence of high-affinity intracellular PAF binding sites [26]and the demonstration of intracellular retention of synthesized PAF in cultured neurons [43]and other cell types [30], suggests that PAF may elicit biochemical actions via intracellular pathways [4,25]. In fact, PAF acting at intracellular binding sites elicits gene expression in neural and glial cell lines [36], including the expression of the inducible cyclooxygenase (COX) enzyme, cyclooxygenase-2 (COX-2) [6], an enzyme responsible for the initial steps in the conversion of free arachidonic acid (AA) to prostaglandins (PGs) during inflammation (for a review see [42]).

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PAF is synthesized in and released by neurons, and PAF subsequently activates surrounding cells, such as glial and endothelial cells [1]. Astrocyte activation occurs during immune reactions [34] and these activated cells synthesize and release such inflammatory compounds as interleukin-1β and prostaglandins (PGs) [13]. Inflammation-related events appear to have a significant role in the progression and propagation of the neurodegenerative process in Alzheimer's disease (AD) [12,28], and amyotrophic lateral sclerosis (ALS) [2]. Interestingly, PGs and/or other enzymes involved in PG production are increased in ALS [2] and AD [37,38]. A role for astrocyte-derived PGs in neuronal cell death has been demonstrated. For example, PGE_2 stimulates astrocytic glutamate release and prevents astrocytes from taking up glutamate [8,31]; the consequent increase in extracellular glutamate is neurotoxic [16]. These results suggest that astrocytes (and the substances they release) may be a critical component in the mechanisms underlying neurodegeneration.

In this study, we examined the effect of PAF and PAF analogs on the release of the proinflammatory mediator, prostaglandin E_2 (PGE_2), from rat cortical cell preparations enriched in astrocytes, an in vitro cell culture system believed to be a model for reactive astrocytes [33]. PAF is readily hydrolyzed by extra- and intra-cellular PAF acetylhydrolases (PAF-AH; for a review see [14]) therefore we also used a nonhydrolyzable analog of PAF, methylcarbamyl-PAF (mc-PAF) [40] for some of our experiments. The synthetic PAF analogs PAF-16 and PAF-18; the PAF precursor lyso-PAF; and the structurally similar lipids phosphatidylcholine (PC) and lyso-phosphatidylcholine (lyso-PC) were also assessed, to better determine the mechanism of PAF action. As PAF has previously been shown to induce the release of AA [4], we also assessed whether co-incubation of AA and mc-PAF could have a synergistic effect on PGE_2 release. Finally, we investigated the potential site(s) of PAF action by examining the effect of specific PAF binding site antagonists on the mc-PAF-induced PGE_2 release. BN 52021 and CV 6209 have previously been shown to be selective for the cell surface PAF receptors, while BN 50730 selectively antagonizes the intracellular PAF binding sites [25,26].

2. Materials and methods

2.1. Cell culture

All experimental protocols were approved by the Massachusetts Institute of Technology institutional review committee and meet the guidelines of the National Institutes of Health. Dissociated astrocytes were cultured from cortices of postnatal day 1–2 rat pups as previously described [27] with minor modifications [23]. In brief, cells from dissociated cortices were plated onto poly-L-lysine coated 35- or 100 mm culture dishes. The initial culture media, minimal essential medium (MEM, Gibco-Life Technologies; Rockville, MD, USA) containing 15% horse serum (BioWhittaker; Walkersville, MD, USA), were aspirated 2–5 h after plating to remove unattached cells and debris, and replaced with MEM containing 5% fetal bovine serum (FBS, BioWhittaker). Half the medium was replaced with MEM–5% FBS twice weekly. Astrocytes were kept at 37 °C in a humidified 5% CO_2–95% air incubator for 9–15 days, by which time the cultures were confluent and could be used for experiments.

Immunohistochemical procedures were carried out to more precisely identify the cell types in our cultures. Cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4) for 10 min, incubated in Chemiblock (Chemicon, Temecula, CA, USA) solution for 1 h, and incubated with primary antibodies (CD-45, NF-145, NF-70 (1:1000) Calbiochem, La Jolla, CA, USA), N-200 and GFAP (1:2000 and 1:3000, respectively; Sigma, St. Louis, MO, USA) overnight at room temperature on an orbital shaker. Cells were then incubated with a biotinylated secondary antibody for 30 min, followed by an incubation with ABC (Vector, Burlingtom, VR) solution for 30 min. Cells were then placed for 6 min in a 0.02% 3,3-diaminobenzadine tetrahydrochloride (DAB) solution containing H_2O_2 for visualization of the bound chromogen.

Most of the cells in this preparation (approximately 85% of cultured cells) were immunopositive for the astrocyte-specific intermediate filament protein glial fibrilary acidic protein (GFAP) and had the characteristics of flat type 1-like astrocytes. It should be noted, however, that endothelial cells might also be immunopositive for GFAP [19]. The only other cells we were able to identify immunologically were microglia (approximately 5% of cultured cells were immunopositive for CD-45). No neurons were detected using neurofilament-specific antibodies. Many of the remaining cells exhibited a morphology reminiscent of radial glia that have not yet assumed the genetic program of mature astrocytes [22].

2.2. Drug preparation

Mc-PAF (Biomol; Plymouth Meeting, PA, USA) was dissolved in methanol at a stock concentration of 10 mM. PAF-16, PAF-18, lyso-PAF, AA (Cayman Chemicals), lyso-PC and PC (Sigma) were dissolved in ethanol at stock concentrations of 10 mM. All stock solutions of lipids were stored at −80 °C and were used within 6 weeks of reconstitution. BN 52021 and CV 6209 (Biomol) were dissolved in ethanol. These PAF antagonists were stored at −20 °C in stock concentrations of 100 mM. BN 50730 (a generous gift from Biomeasure; Milford, MA, USA) was dissolved in 45% hydroxy-β-cyclodextrin (HBC). All agents were diluted in warmed serum-free medium prior to cell stimulation. Equal amounts of vehicle were added to control cells.

2.3. Drug treatments

Cells used for all experiments were established in vitro 9–15 days prior to use in experiments and were over 95% confluent. Serum-containing media were changed every 3–4 days. Cells were serum-deprived 24 h prior to experimental treatments. Where treatment with PAF antagonists is indicated, these compounds were added 30 min prior to the addition of other agents.

2.4. PGE_2 assay

PGE_2 levels were measured by ELISA according to manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI, USA). Since the amount of PGE_2 in fresh medium is negligible [24], direct assays of the PGE_2 concentration in cell-conditioned medium was used as a measurement of PGE_2 secretion by cultured cells. Results are derived from at least three separate experiments, assayed in duplicate or triplicate ($n=6–8$). The reliable detection limit of this assay (i.e. sensitivity) varied across experiments and averaged 14 ± 6 pg of PGE_2.

2.5. Statistical analysis

Data are expressed as means±S.E.M. Statistical analyses were performed using unpaired Student's *t*-tests or ANOVA for comparisons between groups, followed by Fischer's PLSD posthoc comparisons by means contrast. *P* values <0.05 were considered statistically significant.

3. Results

3.1. Effect of solvents on astrocytic PGE_2 release

Addition of either methanol or ethanol (or a combination of both) to astrocyte-enriched cortical cell cultures caused an increases in PGE_2 release (less than a 10% increase) that were not statistically significant; HBC had no effect on PGE_2 release (data not shown).

3.2. mc-PAF increases astrocytic PGE_2 release in a time-dependent manner

Addition of the nonhydrolyzable PAF analog mc-PAF (1 μM) to treatment media caused a time-dependent increase in PGE_2 release from astrocyte-enriched cortical cell cultures (Fig. 1). Within 5 min of mc-PAF incubation, an increase in PGE_2 release was observed ($P<0.05$). The maximum mobilization of PGE_2 occurred at 30 min ($P<0.01$), decreasing gradually by 4 h. A second peak, albeit smaller, was observed at 8 h ($P<0.05$), and levels returned to baseline by 12 h. As the peak release of PGE_2 by mc-PAF was demonstrated to occur at 30 min, we used this time in subsequent studies to assess potential mechanisms of PAF-induced PGE_2 release.

3.3. PAF analogs increase astrocytic PGE_2 release in a concentration-dependent manner

Addition of mc-PAF, lyso-PAF, PAF-16, or PAF-18 to astrocyte-enriched cortical cell cultures resulted in con-

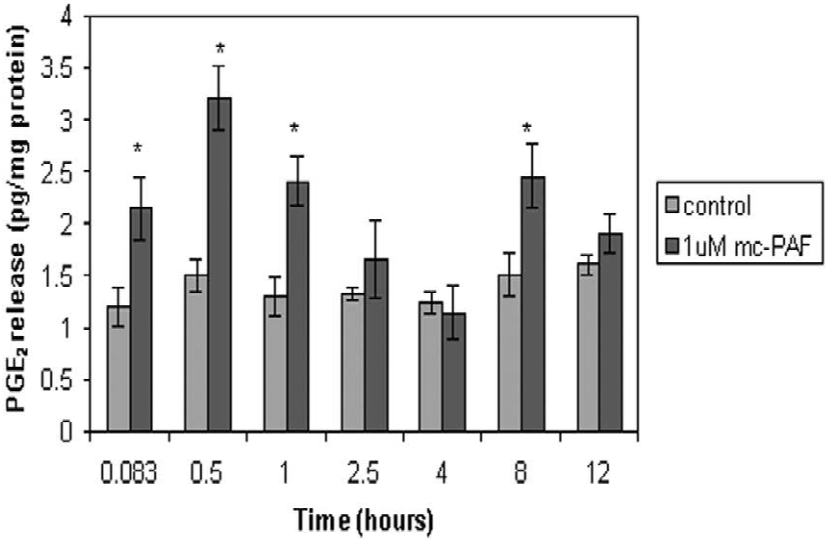

Fig. 1. Time-dependent PGE_2 release induced by mc-PAF from astrocyte-enriched cortical cell cultures. Cells were incubated with 1 μM mc-PAF or vehicle (0.01% methanol) at 37 °C for various times. Media was collected and assayed for PGE_2 (as described in materials and methods). Each point represents the mean±S.E.M. of at least three independent experiments, carried out in duplicate or triplicate. *, Statistically significant ($P<0.05$) differences relative to control.

centration-dependent increases in PGE_2 release into the conditioned media (Fig. 2A). Mc-PAF significantly increased PGE_2 release at concentrations of 0.1 ($P<0.05$), 1 ($P<0.01$), and 10 ($P<0.01$) μM, and lyso-PAF at a concentration of 10 ($P<0.05$) μM. Both PAF-16 and PAF-18 increased PGE_2 release at concentrations of 0.01 ($P<0.05$), and 0.1 ($P<01$) μM, but were less effective at higher concentrations (Fig. 2A).

Though treatment with PAF-16 or PAF-18, caused significant effects these effects were more variable across and within experiments than those produced by lyso-PAF or mc-PAF. For this reason, we used mc-PAF to explore the mechanisms mediating PAF-induced mobilization of PGE_2. Addition to the media of PC or lyso-PC, lipids which are structurally similar to PAF analogs, had no effect on PGE_2 release at any concentration examined (10, 1, 0.1 and 0.01 μM; Fig. 2B).

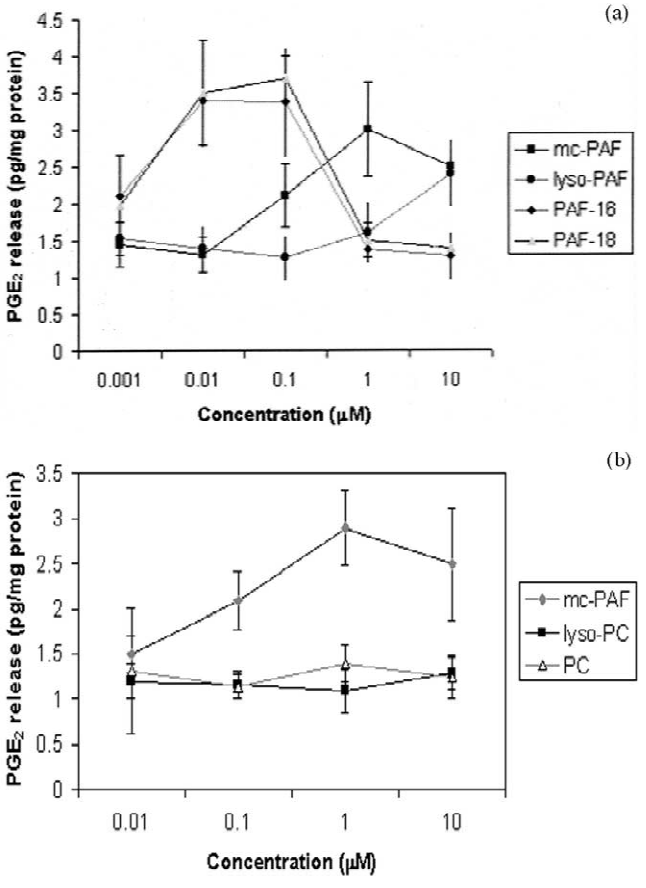

Fig. 2. PGE_2 release from astrocyte-enriched cortical cell cultures exposed to (A) mc-PAF, lyso-PAF, PAF-16, or PAF-18 and (B) mc-PAF, lyso-PC or PC. Cells were incubated in the respective treatments at 37 °C for 30 min, at which time the media was collected and assayed for PGE_2 (as described in materials and methods). Each point represents the mean±S.E.M. of at least three independent experiments, carried out in duplicate or triplicate. The mean±S.E.M. for control cultures was 35.6±7.9 and significant differences are indicated in the Results section.

3.4. Arachidonic acid and mc-PAF act synergistically to increase astrocytic PGE_2 release

Treatment of astrocyte-enriched cell cultures for 30 min with AA (0.01–10 μM) increased PGE_2 release ($P<0.01$; Fig. 3A). Co-administration of AA with mc-PAF (0.1, 1 or 10 μM) caused an additive increase in PGE_2 release with a low arachidonate concentration (0.01 μM) ($P<0.05$; Fig. 3B), but not at a high AA concentration (10 μM) (Fig. 3C). These results suggest a 'ceiling effect' may block added responses to higher AA concentrations (i.e. no synergism), perhaps mediated by limits in the availability of downstream enzymes responsible for AA conversion to PGE_2 (e.g. cyclooxygenases).

3.5. Effect of intracellular PAF binding site antagonists on PAF analog- and AA-induced PGE_2 release

Prior exposure of cells to BN 50730 (0.1–100 μM) attenuated mc-PAF-induced PGE_2 release (Fig. 4A). Prior administration of BN 50730 also significantly attenuated the increase in PGE_2 release induced by lyso-PAF (Fig. 4B). These results suggest that intracellular PAF binding sites are essential for the PAF analog-induced effect on PGE_2 mobilization. Prior exposure of cells to BN 50730 also significantly attenuated the release of PGE_2 induced by AA (Fig. 4C), suggesting that exogenous AA may increase intracellular PAF.

3.6. Effect of cell surface PAF antagonists on PAF analog- and AA-induced PGE_2 release

BN 52021and CV 6209, two structurally distinct antagonists to cell surface PAF receptors, had no significant effect on mc-PAF-induced PGE_2 release (Fig. 5A and B, respectively) at concentrations previously shown to effectively block the plasma membrane receptors [26]. At higher concentrations both antagonists attenuated by 20–25% the mc-PAF-induced increase in PGE_2; this effect could be caused by blockade at intracellular sites. These agents had no effect on the PGE_2 release caused by lyso-PAF-or AA (data not shown). When BN 52021 or CV 6209 were administered alone (i.e. no mc-PAF), PGE_2 release was increased, perhaps by shunting endogenous PAF to intracellular binding sites.

4. Discussion

These data show that PAF enhances PGE_2 release from cortical astrocytes; that mc-PAF and lyso-PAF share this effect; that related phosphatides (PC, lyso-PC) fail to enhance PGE_2 release; that AA synergizes the effect of mc-PAF on PGE_2 production; and that intracellular PAF

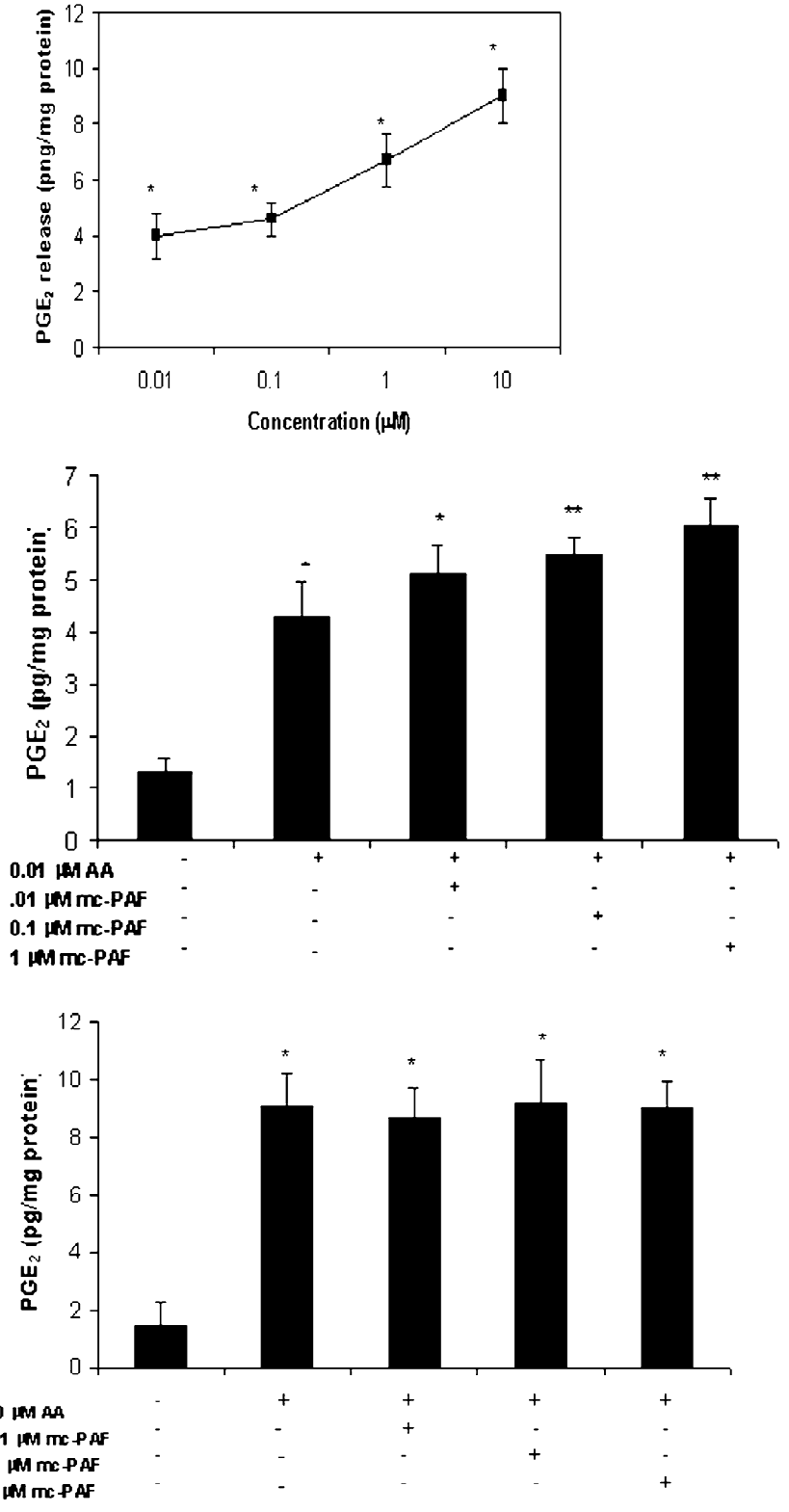

Fig. 3. Concentration-dependent PGE_2 release from media of astrocyte-enriched cortical cell cultures exposed to (A) AA (0.01–10 μM) and (B) AA (0.01 μM) with or without mc-PAF (0.01–1 μM) and (C) AA (10 μM) with or without mc-PAF (0.01–1 μM). Cells were incubated in various concentrations of AA (with or without mc-PAF) or vehicle (0.01% ethanol, 0.01% methanol or both) at 37 °C for 30 min, at which time the media was collected and assayed for PGE_2 (as described in materials and methods). Each point represents the mean±S.E.M. of at least three independent experiments, carried out in duplicate or triplicate. *, Statistically significant ($P<0.05$) differences relative to control and **, relative to AA alone.

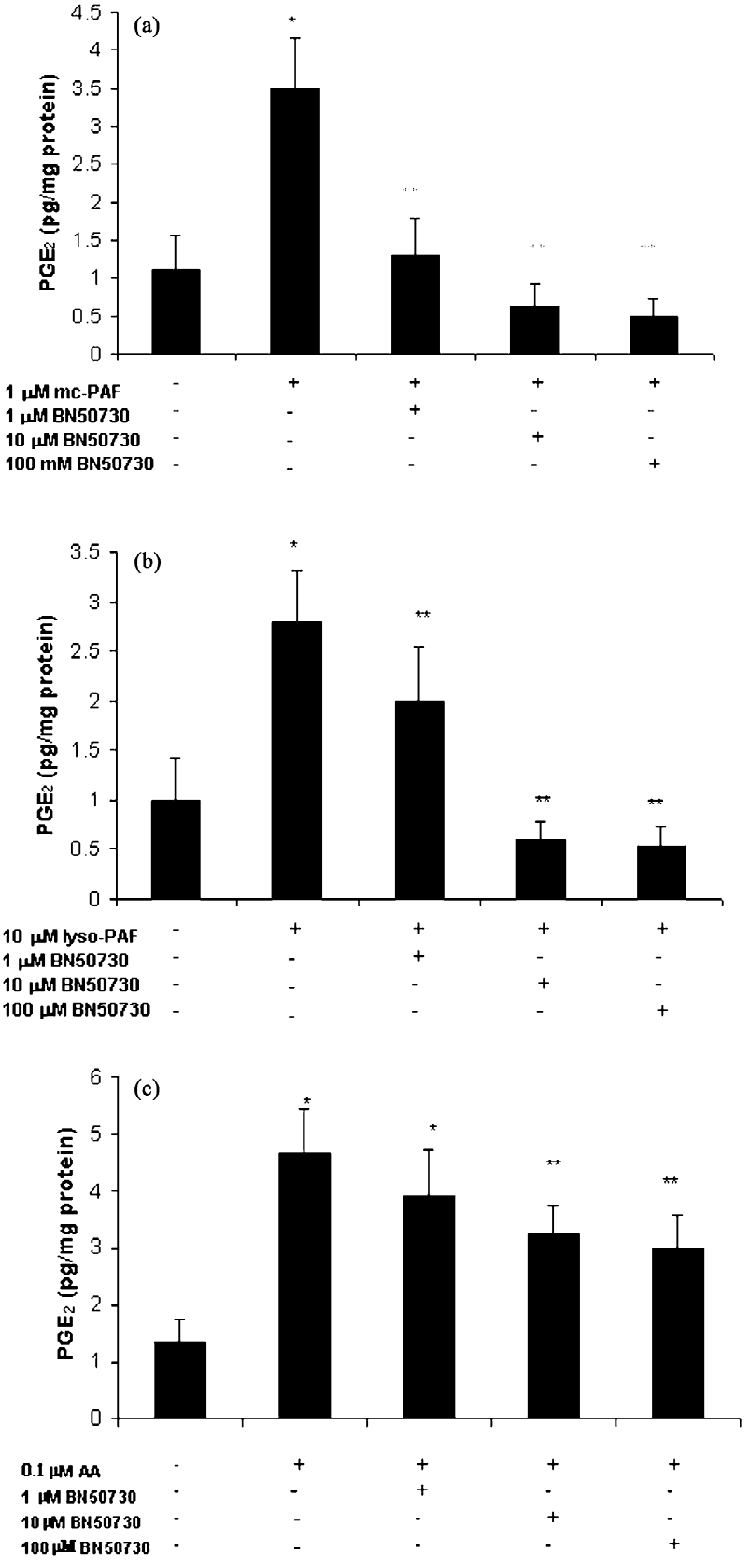

Fig. 4. PAF antagonist, BN 50730 attenuates the (A) mc-PAF-, (B) lyso-PAF- and (C) AA-induced PGE_2 release in astrocytes in concentration-dependent manners. Cells were incubated at 37 °C for 30 min with various concentrations of BN 50730 before addition of mc-PAF (1 μM). After 30 min, media was collected and assayed for PGE_2 (as described in Materials and methods). Each point represents the mean±S.E.M. of at least three independent experiments, carried out in duplicate or triplicate. *, Statistically significant ($P<0.05$) differences relative to control and **, relative to mc-PAF, lyso-PAF or AA alone.

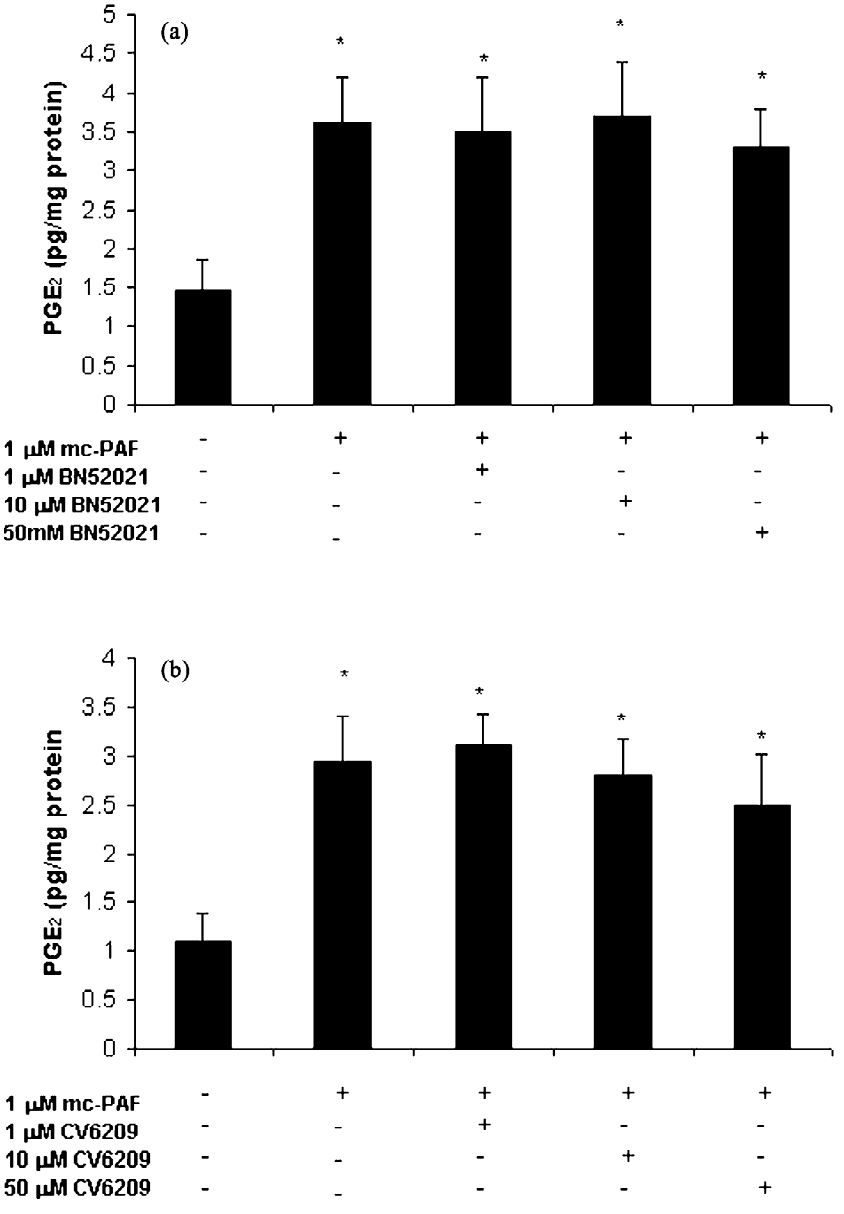

Fig. 5. PAF antagonists, (A) BN 52021 (1–50 μM) and (B) CV 6209 (1–50 μM) do not attenuate the mc-PAF-induced PGE_2 release in astrocytes in concentration-dependent manners. Cells were incubated at 37 °C for 30 min in the respective antagonists before addition of mc-PAF (1 μM). After 30 min, media was collected and assayed for PGE_2 (as described in Materials and methods). Each point represents the mean±S.E.M. of at least three independent experiments, carried out in duplicate or triplicate. *, Statistically significant ($P<0.05$) differences relative to control.

antagonists can attenuate the PGE_2 response elicited by PAF analogs and AA.

Increasing the concentration of mc-PAF (0.001–10 μM) caused an increase in the amounts of PGE_2 released into the media (Fig. 2A). The highest concentration of mc-PAF used in this study (10 μM) increased PGE_2 release, however greater variability was observed with some cultures displaying no increases in PGE_2 release. Incubation of cells with this high concentration for 24 h causes cytologic evidence of toxicity (unpublished observations). As 1 μM mc-PAF did not appear to have toxic effects and produced a reliable PGE_2 increase that varied very little across cultures (relative to other concentrations), we used this concentration to explore the site of PAF action. In contrast to mc-PAF's concentration–response effect on PGE_2 release, peak PGE_2 release was observed with 0.1 μM PAF-16 or PAF-18, and higher and lower concentrations of these compounds elicited less release (Fig. 2A). Based on the present results, we cannot determine the reason for the lack of effect of higher concentrations of synthetic PAF. Although it is unlikely that cell death occurred within 30 min, it is possible that these higher concentrations elicited a cellular program distinct from the physiological program activated by lower concentrations.

Also, higher concentrations of synthetic PAF may have resulted in poor solubility or extracellular micelle formation causing less PAF to enter the cells.

PC and lyso-PC, which have similar abilities to perturb membranes, failed to affect PGE_2 release (Fig. 2B), suggesting that the PAF, mc-PAF and lyso-PAF effects were a result of specific actions on PAF binding sites, rather than nonspecific membrane perturbation. As lyso-PAF does not activate cell surface PAF receptors [7], this lipid may cause PGE_2 release by its conversion to intracellular PAF. Several lines of evidence support this hypothesis. First, lyso-PAF levels may be limiting in the remodeling pathway for PAF biosynthesis [17]. Second, PAF and related analogs increase intracellular Ca^{2+} levels in astrocytes [18], and lyso-PAF-AH is fully active at the μM Ca^{2+} concentrations [17] induced by these compounds. Finally, lyso-PAF is able to enter cells by diffusion through plasma membranes [11]. In fact, as the G-protein coupled PAF receptors do not appear to be critical for the effect of the PAF-analogs on PGE_2 release, these effects may be due in part, to hydrolysis to lyso-PAF, which might prevent the lipids from reaching intracellular sites.

BN 50730, a competitive antagonist to intracellular PAF binding sites, prevented mc-PAF-induced PGE_2 release (Fig. 4A). This finding is consistent with a previous study that suggested a role for intracellular PAF in the promotion of PGE_2 synthesis [39]. Lipopolysaccharide (LPS) rapidly increases both PGE_2 release [3] as well as intracellular PAF levels in macrophage cell lines [39]. Accumulation of PAF was accompanied by initial activation of $cPLA_2$ (within 5 min), followed by lyso-PAF-AT activation [39]. These findings not only support a role for PAF in PGE_2 release, but also suggest that the enzyme responsible for lyso-PAF conversion to PAF is also activated early in LPS-induced PGE_2 release.

BN 50730 also attenuated PGE_2 release induced by lyso-PAF (Fig. 4B) and AA (Fig. 4C). While BN 50730 completely abolished lyso-PAF and mc-PAF generated PGE_2 release, it only attenuated the AA-induced PGE_2 release. This suggests that intracellular PAF bindings sites are required for the effects of mc-PAF and lyso-PAF on PGE_2 release, but not for those of AA. As AA can induce PGE_2 release even when these intracellular PAF binding sites are blocked, this suggests that the sites are not necessary for PGE_2 production when exogenous AA is made available to cells. However, the blockade of intracellular PAF binding sites does attenuate some of the mobilization of PGE_2 by AA. This attenuation may be explained by other actions of exogenous AA on cells. For instance, AA has previously been shown to increase $cPLA_2$ activation [15]. Thus, besides providing the necessary substrate for PGE_2 synthesis, exogenous AA can also produce more AA (and PAF) by activating $cPLA_2$. It is of interest to note, that in the case of lyso-PAF and mc-PAF, that higher concentrations of BN 50730 not only attenuated the PAF analog-induced PGE_2 release but also reduced the basal release of PGE_2, suggesting that endogenous intracellular PAF has a role in basal PGE_2 release.

CV-6209 and BN 52021, which are structurally distinct antagonists to PAF's plasma membrane receptors, do not significantly influence mc-PAF-induced PGE_2 release (Fig. 5A and B). Administration of these agents alone increased PGE_2 release (data not shown). From the present findings, we cannot establish the physiological basis of this increase. This effect may be caused by a compensatory increase in PAF synthesis and/or a shunting of endogenously produced PAF to intracellular sites.

Our observation that PAF increases the release PGE_2 is in accordance with the results of previous studies. For instance, PAF increased the release of PGE_2 in trout astrocytes (2 h incubation; [41]), and PAF was shown to increase the release of other eicosanoids in mammalian astrocytes (15 min incubation; [32]). Marked increases in AA levels and eicosanoids (including PGE_2) have been observed in association with brain inflammation [29] and in degenerative disorders like HIV dementia [20], ALS [2] and AD [37,38]. Thus, the PAF-induced PGE_2 release might initiate an inflammatory cascade in astrocytes that can be detrimental to central nervous system function.

Acknowledgements

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