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Activation of the Epidermal Platelet-Activating Factor Receptor Results in Cytokine and Cyclooxygenase-2 Biosynthesis[1]

Yong Pei,* Lisa A. Barber,* Robert C. Murphy,§ Christopher A. Johnson,§ Susan W. Kelley,* Lady C. Dy,* Richard H. Fertel,¶ Thanh M. Nguyen,¶ David A. Williams,†‡ and Jeffrey B. Travers[2]*†

Recent studies suggest that the lipid mediator platelet-activating factor (PAF) is involved in keratinocyte function and skin inflammation. Indeed, PAF is found in association with inflammatory skin diseases, intradermal injections of PAF induce inflammation, and keratinocytes express functional PAF receptors (PAF-R). One mechanism by which the keratinocyte PAF-R could contribute to epidermal functions and inflammatory states would be through the synthesis of inflammatory regulators, such as PAF, PGs, and cytokines. The ability of the epidermal PAF-R to induce the synthesis of these immunomodulators was tested using a model system created by transduction of the PAF-R-negative human epidermal cell line KB with the PAF-R. Activation of this epidermal PAF-R resulted in arachidonic acid release, and the biosynthesis of PAF and PGE_2. In addition, the KB PAF-R triggered increased levels of mRNA and protein for the inducible isozyme of cyclooxygenase (COX-2) as well as IL-6 and IL-8, both of which have been implicated in skin inflammatory processes. Studies with the human keratinocyte-derived epidermal cell line HaCaT revealed that activation of the endogenous PAF-R led to the increased accumulation of COX-2, IL-6, and IL-8 mRNA similar to that seen with the KB PAF-R model system. Finally, treatment of HaCaT keratinocytes with IL-8 resulted in PAF biosynthesis, indicating the existence of a positive feedback loop between IL-8 and PAF in epidermal cells. These studies suggest involvement of PAF and the PAF-R in the epidermal cytokine network. *The Journal of Immunology,* 1998, 161: 1954–1961.

Platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine)[3] is a family of *sn-2* acetyl phosphocholines with diverse and significant pathophysiologic effects (reviewed in Refs. 1 and 2). Because PAF can both attract and activate granulocytes, it has profound proinflammatory effects. In addition, PAF has trophic effects on fibroblasts and lymphocytic cell lines (3, 4). Although PAF can be metabolized to potentially biologically active neutral lipid or phosphatidic acid species (5–7), the majority of PAF effects are thought to be mediated through a G protein-coupled membrane-associated receptor (PAF-R) (reviewed in Ref. 8). Consistent with the myriad of responses associated with PAF, activation of the PAF-R stimulates numerous signal transduction systems, including phospholipases C, A_2, and D and mitogen-activated protein kinase. In addition to activating these signaling pathways, in monocytes and fibroblasts PAF stimulates the synthesis of numerous cytokines, including TNF-α, IL-1, IL-6, IL-8 (3, 9, 10), and eicosanoids (11).

The perception of the role of the keratinocyte in cutaneous inflammation has changed from one of a passive bystander to that of an active participant. This new understanding of the part that the keratinocyte plays in cutaneous pathophysiology is supported by the ability of these cells to synthesize numerous proinflammatory cytokines, chemokines, and growth factors. Human keratinocytes synthesize TNF-α, IL-1, IL-6, IL-8, as well as PGs (12). Recent evidence suggests that many of these keratinocyte-derived mediators can traverse the basement membrane and have systemic effects (13).

Cyclooxygenase (COX) catalyzes the first committed step in prostanoid synthesis (reviewed in Ref. 14). There are two separate COX enzymes, both of which are found in epidermal cells. COX-1 is constitutively expressed and synthesizes PGs in the endoplasmic reticulum. This isozyme is thought to mediate physiologic responses requiring a rapid and/or constant biosynthesis of PGs. The second isozyme, COX-2, is normally absent from cells, but can be induced in response to cytokines, growth factors, and tumor promoters. Reports of phenotypes of transgenic mice deficient in either COX-1 or COX-2 suggest that these isozymes have different functions (15, 16). The role of COX enzymes in keratinocyte biology is unclear, although treatment of murine epidermis with irritants has been reported to induce COX-2 (17). Several reports that COX-2 has been found to be overexpressed in some skin- and colon-derived carcinomas suggest that this enzyme might be involved in epithelial carcinogenesis (18, 19).

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[3] Abbreviations used in this paper: PAF, platelet-activating factor; COX, cyclooxygenase; GPC, glycerophosphocholine; PAF-R, platelet-activating factor receptor; CPAF, 1-hexadecyl-2-*N*-methyl carbamoyl-glycerophosphocholine; ET-1, endothelin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; PAPC, 1-palmitoyl-2-acetyl glycerophosphocholine; GC/MS, gas chromatography/mass spectrometry; KBP, KB cells transduced with platelet-activating factor receptor; KBM, KB cells transduced with MSCV2.1 retrovirus alone; AA, arachidonic acid.

The pleotropic cytokines IL-6 and IL-8 have been implicated in epidermal function and cutaneous inflammation (reviewed in Ref. 20). Resting keratinocytes do not synthesize appreciable amounts of IL-6 or IL-8, yet will do so in the presence of IL-1α, IL-1β, TNF-α, or PMA. Keratinocytes express functional receptors for these cytokines, and both IL-6 and IL-8 treatments have been reported to induce keratinocyte proliferation in vitro (20, 21). In addition, increased expression of keratinocyte IL-8 (CXC-2) receptors has been recently demonstrated in lesions of psoriasis, a hyperproliferative disease with prominent neutrophil involvement (22).

Human keratinocytes also synthesize PAF (23–25). In addition to synthesizing PAF and other *sn-2* acetyl GPCs, keratinocytes express functional PAF-Rs (26). That human keratinocytes are potential targets of PAF action has significance, since PAF has been found in association with inflammatory skin diseases, including psoriasis (27) and urticaria (28). The functional significance of the keratinocyte PAF-R is not clear. However, the recent report of dermatitis and epidermal hyperplasia as part of the phenotypic abnormalities seen in the PAF-R-overexpressing transgenic mouse (29) suggests that the PAF-R may be involved in cutaneous inflammation. One possible mechanism for PAF-R modulation of cutaneous inflammation could be through PAF-induced keratinocyte cytokine biosynthesis. The ability of keratinocytes to synthesize cytokines in response to PAF-R activation has not been previously examined.

To study the consequences of PAF-R activation in epidermal cells, we have created a model system of the PAF-R by retroviral-mediated gene transduction of the PAF-R-negative human epidermal cell line KB with the human leukocyte PAF-R. The present studies use this novel model system and the human keratinocyte-derived cell line HaCaT (30) to assess the ability of the epidermal PAF-R to stimulate the biosynthesis of COX-2 as well as the cytokines IL-6 and IL-8. Finally, the ability of IL-8 to trigger PAF biosynthesis in epidermal cells was also tested. An understanding of sequelae of the activation of the epidermal PAF-R may provide insights into the role of this lipid mediator in epidermal cell pathophysiology as well as its place in the epidermal cytokine network.

Materials and Methods

Reagents

Solvents (HPLC grade ethanol, methanol, chloroform, hexane, and acetic acid) and reagent grade diethyl ether were obtained from Fisher (Fairlawn, NJ). PAF (1-O-hexadecyl-2-acetyl-GPC), 1-hexadecyl-2-N-methyl carbamoyl-GPC (CPAF), PMA, and fatty acid-free BSA were purchased from Sigma (St. Louis, MO). The PAF-R antagonists were provided as follows: WEB 2086 (31) from Boehringer Ingelheim (Ridgefield, CT), and A-85783 (32) from Dr. James Summers, Abbott Pharmaceuticals (Abbott Park, IL). The peptide growth hormone endothelin-1 (ET-1) was purchased from Peptides International (Louisville, KY). Ethanolic solutions were made with CPAF and WEB 2086; PMA and A-85783 were dissolved in DMSO. Appropriate controls used ethanol and DMSO (final concentrations of all solvents were <0.5%).

KB PAF-R model system

KB cells, a human epidermoid carcinoma originally obtained from a patient with an oral squamous cell carcinoma (33), and the PAF-R-positive (26) human keratinocyte-derived epidermal cell line HaCaT (30) were grown as previously described (24, 26). To create a model system of the PAF-R, the entire human leukocyte PAF-R cDNA (provided by Dr. Takao Shimizu, Tokyo, Japan) was cloned into the *Eco*RI site of the multiple cloning region of the MSCV2.1 retroviral vector (34), and orientation was assessed by restriction endonuclease mapping and sequencing. Infectious amphotropic retroviruses were produced from both MSCV2.1PAF-R and control MSCV2.1 backbone using standard protocols (35). Briefly, the murine ecotropic packaging line GP$^+$ E-86 (E-86) (36) was transfected with the MSCV2.1 construct, and transient supernatants collected 48 h later containing infectious virions were then used to infect the amphotropic packaging line GP$^+$ EnvAm12 (Am-12) (37). Individual colonies of Am-12

were selected with G418 (0.75 mg dry powder/ml; Life Technologies, Grand Island, NY)-containing medium. Viral titers from selected clones were evaluated by infection of NIH-3T3 fibroblasts using dilutions of virus containing supernatants from multiple clones. Clones producing high titer virus and infected and G418-selected NIH-3T3 cells were further analyzed by Southern blot analysis to confirm the integrity of the provirus. Two clones, producing 10^5 G418-resistant colony-forming units/ml (Am12 MSCV2.1PAF-R virus) and 10^6 colony-forming units/ml (Am12 MSCV2.1) of virus, were used to infect KB cells, and clones of transduced KB cells resistant to 1 mg/ml G418 were further characterized by Southern and Northern blot analyses. All experimental protocols were duplicated in at least two separate clones of KBPAF-R (KBP) and two KBMSCV2.1 (KBM) cells.

Genomic DNA isolation and Southern blot analysis

Genomic DNA was isolated from the cultured cells by the method of Sambrook and Maniatus (38) with modification. Briefly, after washing with PBS (Life Technologies, Grand Island, NY) cells were lysed with extraction buffer (10 mM Tris-HCl, 0.1 M EDTA, 0.5% SDS, 20 μg/ml pancreatic RNase, and 100 μg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN)) for >1 h. Following extraction with phenol (pH 8.0), genomic DNA was precipitated with 2 vol of ethanol. After centrifugation, the pellet was washed with 70% ethanol, air-dried, and dissolved in autoclaved sterile water. Ten micrograms of DNA was digested overnight with the appropriate restriction endonuclease, run on an 0.8% agarose gel, and transfered to a positively charged nylon membrane (Boehringer Mannheim). The blot was then probed with ^{32}P-labeled human PAF receptor cDNA probe, or NEO, using the random primed labeling method (Boehringer Mannheim). ExpressHyb Hybridization Solution (Clontech, Palo Alto, CA) was used for both prehybridization and hybridization at 50°C. After hybridization, the blot was washed following the manufacturer's recommendation: 2× SSC-0.05% SDS at room temperature twice for 15 min each time, and 0.2× SSC-0.1% SDS at 50°C twice for 20 min each time. The blot was then exposed to an x-ray film with two intensifying screens at −80°C.

Total RNA extraction and Northern blot analysis

Total RNA was extracted using Tripure (Boehringer Mannheim). After the manufacturer's procedures of purification, an additional phenol (pH 4.2) extraction was performed, followed by ethanol precipitation. The RNA concentration was accessed by UV spectrophotometer. Twenty micrograms of total RNA was separated on a formaldehyde containing gel following the method reported by Sambrook and Maniatis (38) and transfered to a positively charged nylon membrane (Boehringer Mannheim). The blots were then hybridized with various ^{32}P-labeled cDNA probes using ExpressHyb Hybridization Solution (Clontech) following the manufacturer's protocol. The blots were stripped of the probe using 0.5% SDS at 100°C and later probed with human GAPDH to determine equal loading. Human IL-6 (no. 68636) and GAPDH (no. 57091) cDNA clones were purchased from American Type Culture Collection (Rockville, MD); human IL-8, COX-1, and COX-2 clones were gifts from Dr. Jana Stankova (University of Sherbrooke, Sherbrooke, Canada) and were used as previously reported (39).

Radioligand binding studies

Radioligand binding studies using the water-soluble PAF-R antagonist [^{3}H]WEB 2086 (New England Nuclear) were conducted that were similar to those previously described (40). Briefly, parental KB or clones of KB cells transduced with the MSCV2.1 control or MSCV2.1PAF-R virus were seeded onto 24-well tissue culture treated dishes (Falcon, Oxnard, CA) at an initial density of 200,000 cells in 1 ml for 24 h. The cells were washed and treated with various concentrations (1–100 nM) of [^{3}H]WEB 2086 in the presence or the absence of an excess of unlabeled WEB 2086 (2 μM) for 24 h at 4°C. After washing, the cells were treated with Triton X detergent (Sigma) and scraped, and radioactivity was measured by scintillation counting. No displaceable [^{3}H]WEB 2086 binding was detected in either parental KB or KBM clones. Saturation binding isotherms of KBP clones revealed displaceable binding that reached saturation; Scatchard analysis (41) of the binding data revealed a single high affinity binding site. Three KBP clones with similar binding characteristics (K_d of 8.5–11.3 nM; binding capacity of 133–289 fmol/10^6 cells) were used in further studies. Radioligand binding studies of these clones in continuous passage (up to 60 passages) over several months were unchanged, indicating the stability of PAF-R protein expression.

Measurement of COX-2 protein in KB cells

Following incubation with the appropriate experimental treatments, cells were washed with cold PBS (Life Technologies) and lysed with 1 ml of lysis buffer (50 mM Tris-HCl, 1% SDS, 2 mM EDTA, and 2 mM PMSF, pH 7.5). The lysate was scraped and sonicated twice. The protein concentration was determined using the Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA). Twenty-five micrograms of each protein sample was heat denatured, loaded onto a 10% SDS-PAGE gel (Bio-Rad minigel system), and run at 125 V through the stacking gel and 150 V until bromophenol dye run off. The proteins were then transfered to polyvinylidene difluoride Hybond-P transfer membrane (Amersham, Arlington Heights, IL), using a SD semidry electrophoretic cell (Bio-Rad). The membrane was stained with Ponceau Red (Sigma) to determine the effectiveness of transfer and to assess loading. Detection was performed using ECL-Plus (Amersham). Goat-anti COX-2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA; SC 1745) was used at a dilution of 1/7,500. The secondary anti-goat IgG-horseradish peroxidase (SC 2033) was used at a dilution of 1/75,000. Competition assays were also performed using COX-2 control peptide (SC 1745) to ensure the specificity of the Ab.

Intracellular calcium measurements

Intracellular free calcium concentrations ($[Ca^{2+}]_i$) in suspensions of KB cells were assessed using the calcium-sensitive fluorescent dye indo-1/AM as previously described (24, 26).

Measurement of PAF production by epidermal cells

Cell-associated PAF and PAPC production in clones of KBP or KBM cells in response to CPAF and in HaCaT keratinocytes in response to IL-8 (PeproTek, Rocky Hill, NJ) were assessed by GC/MS exactly as previously described (24, 25).

Measurement of arachidonic acid (AA) release and PGE$_2$ production by KB PAF-R cells

KB cells were grown in 10-cm dishes (Costar, Cambridge, MA) until they were approximately 80 to 90% confluent, then were washed three times with HBSS (Sigma) before addition of 10 ml of prewarmed 0.25% BSA in HBSS containing 100 nM of CPAF. At various times, 1 ml of supernatant was removed to measure AA by GC/MS (25) or PGE$_2$ using a specific RIA (42). No more than 4 ml (total) was removed from an individual plate during an experiment. Similarly treated plates of cells were trypsinized and counted (Coulter, Hialeah, FL).

Measurement of IL-6 and IL-8 protein in KB cells

Cells were plated at a density of 100,000 cells in 1 ml on 24-well plates for 24 h, then exposed to media with or without drugs. In experiments using antagonists, cells were treated 1 h before exposure to agonists. The medium was collected 16 h after drug treatment, and IL-6 and IL-8 were assayed using Quantikine ELISA kits (R&D, Minneapolis, MN). Similarly treated cells were trypsinized and counted (Coulter).

Results

The KB PAF-R model system

As described in *Materials and Methods*, PAF-R-negative KB cells (24, 26) were stably transduced with the human wild-type PAF-R cDNA using the MSCV2.1 retrovirus (Fig. 1*A*). The presence of an intact provirus in transduced KB clones was confirmed by Southern blot analysis (Fig. 1*B*), and expression of the integrated provirus was demonstrated by Northern blot analysis (Fig. 1*C*). As outlined in *Materials and Methods*, radioligand binding studies using the PAF-R antagonist [³H]WEB 2086 also revealed PAF-R protein.

To confirm that the PAF-R protein expressed in KBP cells was functional, we examined changes in $[Ca^{2+}]_i$ induced by PAF using the calcium-sensitive fluorescent dye indo-1. Treatment with PAF (in dosages up to 1 μM) did not alter $[Ca^{2+}]_i$ in parental KB (not shown) or in KBM clones (Fig. 1*D*). Treatment of these cell types with 100 nM of the peptide growth hormone ET-1 resulted in an intracellular calcium flux, indicating that these cells have the appropriate cellular machinery to respond to a G protein receptor-mediated signal (Fig. 1*D*). Treatment of KBP clones with 100 nM
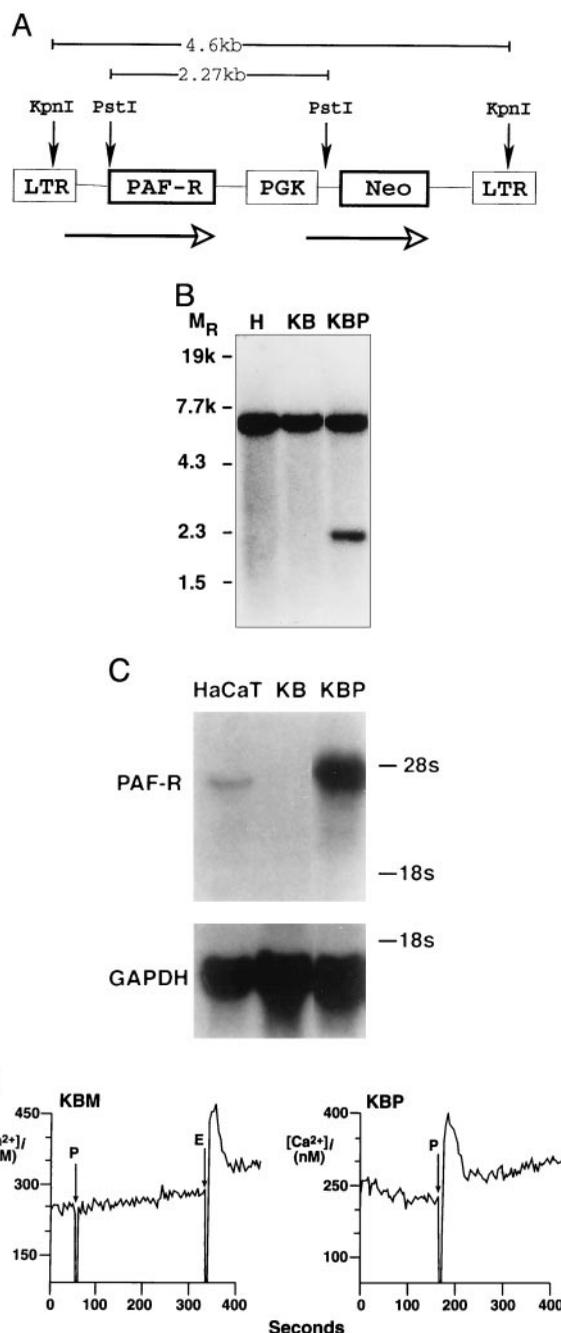

FIGURE 1. The KB PAF-R model system. *A*, Diagram of the MSCV2.1PAF-R retroviral vector. *B*, Southern blot analysis demonstrating incorporation of MSCV2.1PAF-R construct into KBP cells. Genomic DNA was isolated from HaCaT keratinocytes (H), parental KB cells (KB), and a clone of KB cells transduced with the MSCV2.1PAF-R construct (KBP), digested with *Pst*I and hybridized with ³²P-labeled PAF-R cDNA probe. HaCaT and KB cells have only an endogenous genomic PAF-R band (6.8 kb), whereas the transduced KB clones have an additional PAF-R DNA integrated (2.3 kb). KBP genomic DNA was also digested at LTR sites with *Kpn*I and was probed with the PAF-R cDNA probe revealing an expected 4.6-kb band not seen in KB or HaCaT (data not shown). *C*, Northern blot analysis demonstrating PAF-R mRNA in KBPAF-R cells. Total RNA (10 μg) was isolated from HaCaT keratinocytes, parental KB cells, and a KBP clone and hybridized with ³²P-labeled PAF-R cDNA probe. The blot was stripped and reprobed with GAPDH to show equal loading and RNA integrity. In contrast to HaCaT cells, parental KB cells do not express PAF-R mRNA. Transduction of KB cells with the MSCV2.1PAF-R construct resulted in an estimated 4.6-kb PAF-R mRNA. *D*, Intracellular calcium mobilization in KB cells. KBM or KBP cells were loaded with the calcium-sensitive dye indo-1/AM, and changes in $[Ca^{2+}]_i$ in response to treatment with 100 nM PAF (P) or 100 nM ET-1 (E) were assessed using a spectrophotofluorometer.
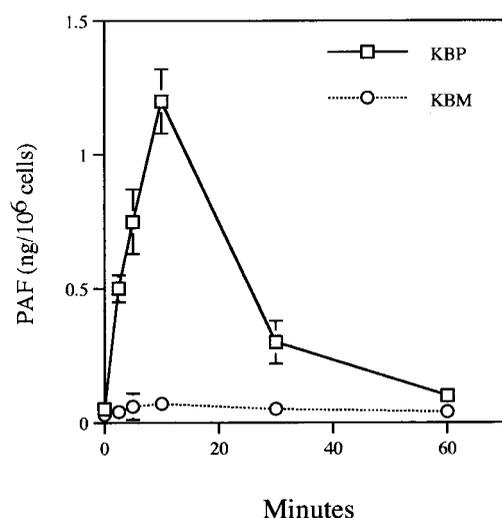
FIGURE 2. Effect of CPAF treatment on PAF biosynthesis in KB cells. KBP or KBM cells were incubated with 100 nM CPAF for various times, and cell-associated 1-hexadecyl-2-acetyl-GPC (PAF) was measured by GC/MS. Each point represents the mean $\pm$ SD from three or four separate experiments.

PAF resulted in changes in $[Ca^{2+}]_i$ similar to those described in other PAF-R-expressing cell types (11, 26, 40). Lyso-PAF (1 μM) treatment of KBP clones did not affect $[Ca^{2+}]_i$, indicating that the response did not result from the physical effects of a phospholipid (data not shown). Pretreatment of KBP cells with the structurally distinct PAF-R antagonists WEB 2086 (10 μM) and A-85783 (10 μM) inhibited 100 nM PAF-induced calcium mobilization, yet had no effect on that induced by 100 nM ET-1 (data not shown).

PAF biosynthesis in KB PAF-R cells

Activation of the PAF-R has been reported to induce PAF biosynthesis in both neutrophils (43) and keratinocytes (24). We tested the ability of the metabolically stable PAF-R agonist CPAF (43) to stimulate PAF biosynthesis in our KBPAF-R model system. As shown in Figure 2, CPAF treatment resulted in PAF biosynthesis in KBP, but not KBM, cells. CPAF treatment of KBP cells also induced the biosynthesis of comparable amounts of the PAF-R agonist PAPC (data not shown).

Arachidonic acid release and PGE₂ biosynthesis in KB PAF-R cells

As PAF could be generated from AA-enriched 1-alkyl-2-acetyl-GPC precursors, the ability of PAF-R activation to stimulate the release of AA was next examined. As shown in Figure 3A, CPAF treatment of KBP cells resulted in the rapid release of AA, with an initial time course resembling that of PAF production (Fig. 2). However, unlike PAF, which was metabolized to baseline levels, the AA content in the supernatant remained elevated at 60 min. CPAF did not induce AA release in KBM cells (Fig. 3A) or in parental KB cells (data not shown), consistent with the lack of a functional PAF-R on these cells.

Inasmuch as activation of the KB PAF-R induced the release of precursor AA, the next experiments examined the ability of CPAF to stimulate PGE₂ production in KBP cells. As demonstrated in Figure 3B, CPAF treatment resulted in PGE₂ accumulation in KBP, but not KBM, clones. In contrast to CPAF-stimulated AA release, which was maximal by 10 min, significant PGE₂ accumulation above baseline levels was not seen until after 1 h.
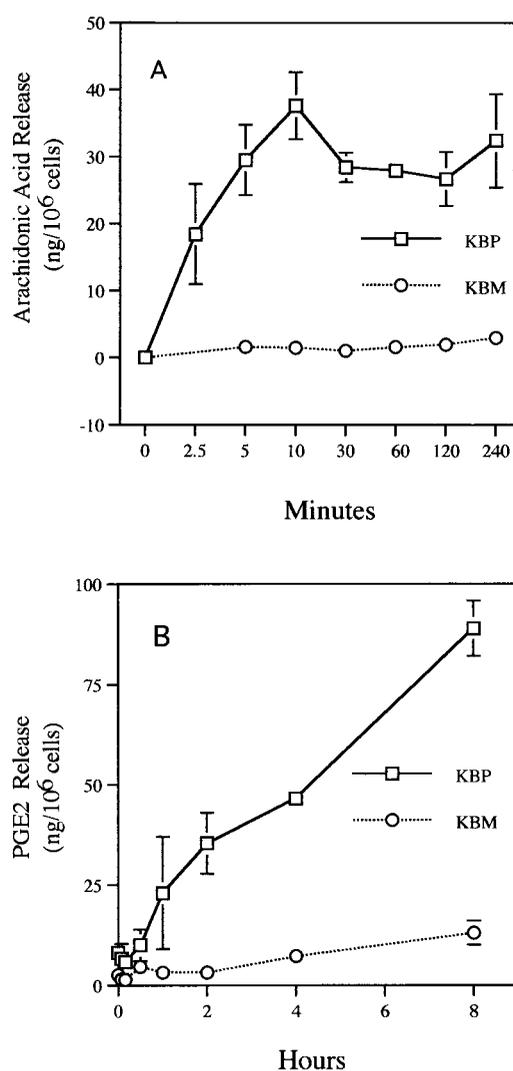

FIGURE 3. Effect of CPAF treatment on arachidonic acid release and PGE₂ biosynthesis in KB cells. KBP or KBM cells were incubated with 100 nM CPAF, and 1-ml aliquots of supernatant were removed at various times to assay for AA (*A*) or PGE₂ (*B*) as described in *Materials and Methods*. Each point is the mean $\pm$ SD of a typical experiment from two or three experiments performed using duplicate samples.

COX-2 biosynthesis in KB PAF-R cells

Our finding that the time course of CPAF-induced PGE₂ production lagged behind arachidonate release suggested the possibility that PAF-R activation could induce COX-2. To test this, KBP or KBM clones were treated with CPAF for various times, and total RNA was isolated. As shown in Figure 4, CPAF treatment of KBP cells resulted in an accumulation of COX-2 mRNA. The increased COX-2 mRNA was first seen at 30 min and persisted at 6 h after CPAF treatment. These findings contrast with the lack of effect of CPAF treatment on KBM clones (Fig. 4). Treatment of the PAF-R-positive (26) human keratinocyte cell line HaCaT with CPAF also resulted in an increased accumulation of COX-2 mRNA (Fig. 4). CPAF treatment did not result in an increase in mRNA encoding the COX-1 enzyme in KBP or HaCaT keratinocytes.

Consistent with the Northern blotting data, CPAF treatment of KBP cells resulted in increased COX-2 protein in KBP clones (Fig. 5, *lane 5*). CPAF-induced COX-2 protein was seen by 2 h and remained elevated at 24 h (data not shown). As shown in Figure 5 (*lane 7*) for an 8-h period, pretreatment of KBP cells with 10 μM

FIGURE 4. Effect of CPAF treatment on COX mRNA levels in KB cells and HaCaT keratinocytes. KBP, KBM, or HaCaT keratinocytes were incubated with 100 nM CPAF, and RNA was extracted at various times and subjected to Northern blot analysis using probes for COX-1, COX-2, or GAPDH. The results shown are typical for at least two separate KBM or KBP clones.
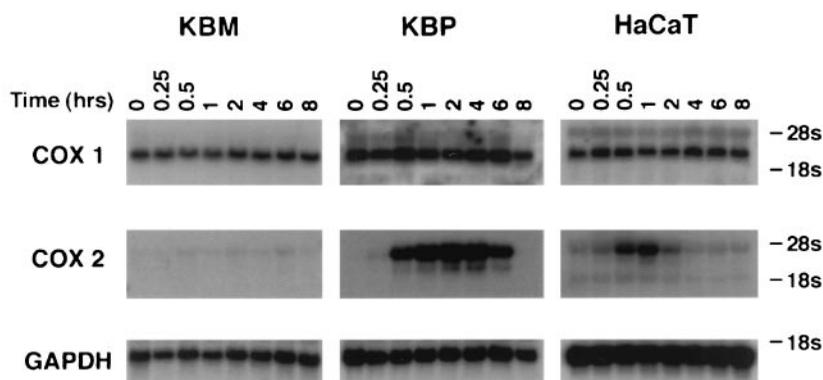
of the PAF-R antagonist A-85783 resulted in an inhibition of CPAF-induced COX-2 protein. Similarly, preincubation of KBP cells with the PAF-R antagonist WEB 2086 (10 μM) inhibited subsequent CPAF-stimulated COX-2 protein (data not shown). As expected, significant amounts of COX-2 protein were not seen in KBM cells following CPAF treatment. However, treatment of these cells with the phorbol ester PMA (10 nM) induced COX-2 protein (Fig. 5). Competition assays using a COX-2 control peptide demonstrated the specificity of the Ab (data not shown). These studies indicate that activation of the epidermal PAF-R results in increased COX-2 protein biosynthesis.

IL-6 and IL-8 biosynthesis in KB cells

Keratinocytes have been shown to synthesize both IL-6 and IL-8 in response to various stimuli, including phorbol esters and TNF-α (12, 20, 21). The next experiments assessed the ability of PAF-R activation to generate increased amounts of these cytokines. As shown in Figure 6, incubation of KBP, but not KBM, cells with CPAF resulted in an increased accumulation of mRNA encoding both IL-6 and IL-8. With both cytokines, increased mRNA was first seen at 30 min, was maximal at 1 h, and returned to baseline by 6 h. Unlike KB cells, significant levels of both IL-6 and IL-8 mRNA were detected in unstimulated HaCaT keratinocytes. However, CPAF treatment of HaCaT keratinocytes resulted in an increased accumulation of mRNA from these two cytokines above baseline levels with a time course similar to that seen in KBP cells (Fig. 6).

Consistent with the Northern blotting data, CPAF treatment of KBP cells resulted in an increase in immunoreactive IL-6 and IL-8 protein secretion as shown in Figure 7. CPAF treatment of KBM cells did not result in an increase in IL-6 or IL-8 protein. However, treatment of KBM cells with PMA resulted in IL-6 and IL-8 protein release. The PAF-R antagonists A-85783 (Fig. 7) and WEB 2086 (10 μM; not shown) inhibited CPAF-induced cytokine biosynthesis in KBP cells, yet did not affect cytokine synthesis induced by PMA in KBP. In addition, higher levels of IL-8 were

consistently seen in baseline (i.e., unstimulated) KBP cells compared with those in KBM cells.

PAF production in response to IL-8 in HaCaT keratinocytes

Since IL-8Rs are linked to an intracellular calcium mobilization response, which is a known stimulus for PAF biosynthesis, we next examined whether IL-8 can act on epidermal cells to stimulate PAF biosynthesis. As shown in Figure 8, treatment of HaCaT keratinocytes with IL-8 (100 ng/ml) stimulated PAF and PAPC production. IL-8-induced PAF and PAPC biosynthesis was seen at 1.5 min, was maximal by 2.5 min, and returned to baseline by 5 min. The amounts of PAF and PAPC biosynthesis induced in these cells by IL-8 treatment at 2.5 min were 170 and 320 pg/10^6 cells, respectively. These studies indicate that PAF and IL-8 can act to stimulate the biosynthesis of each other in epidermal cells that express receptors for both lipid mediator and chemokine.

Discussion

Although not considered an immune cell per se, keratinocytes synthesize numerous molecules that can serve to modulate immune cell function. Among these immunomodulatory molecules synthesized by human keratinocytes is the phosphocholine derivative PAF (23–25). Human keratinocytes also express functional PAF-Rs, indicating that these cells are targets of PAF action (26).

Although the majority of PAF effects are thought to be mediated by the PAF-R, PAF is metabolized to potentially biologically active molecules (5–7). Structurally similar lipids such as lysophosphatidylcholines have also been described as having biologic activity. For example, lysophosphatidylcholine has been reported to stimulate IL-6 release from rat anterior pituitary cells in vitro (44). Thus, PAF-R-independent effects exerted by PAF metabolites and other lipids might result in misleading findings attributable to activation of the PAF-R.

To overcome the potential problem of non PAF-R-induced PAF effects and to account for the diverse non-PAF PAF-R agonists that can signal through this receptor (45–47), our laboratory has developed the KB PAF-R model system. Although often used as a model for human keratinocytes because of a similar cytokine production profile, KB cells differ from human keratinocytes and the epidermal cell lines HaCaT and A-431 in that they do not express functional PAF-Rs. KB cells do not express PAF-R mRNA (Fig. 1*C*) and lack PAF-R protein, as shown by radioligand binding studies with [^{3}H]PAF and [^{3}H]WEB 2086 (data not shown) and immunohistochemical studies using a specific PAF-R polyclonal Ab (26). In addition, KB cells do not respond to PAF-R agonists by an intracellular calcium mobilization, AA release, or PAF biosynthesis (24). KB cells were transduced with the human leukocyte
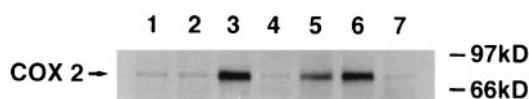
FIGURE 5. Measurement of COX-2 protein following treatment of KB cells with CPAF or PMA. KBM (*lanes 1–3*) or KBP (*lanes 4–7*) cells were incubated with ethanol vehicle (*lanes 1 and 4*), 100 nM CPAF (*lanes 2 and 5*), and 10 nM PMA (*lanes 3 and 6*) or were preincubated for 1 h with 10 μM A-85783 followed by 100 nM CPAF (*lane 7*) for 8 h, protein was extracted, and cells were immunoblotted using a COX-2 Ab. The results shown are typical for two separate KBM or three KBP clones.

FIGURE 6. Effect of CPAF treatment on IL-6 and IL-8 mRNA levels in KB cells and HaCaT keratinocytes. KBP, KBM, or HaCaT keratinocytes were incubated with 100 nM CPAF, and RNA was extracted at various times and subjected to Northern blot analysis using probes for IL-6, IL-8, or GAPDH. The results shown are typical for at least two separate KBM or KBP clones.
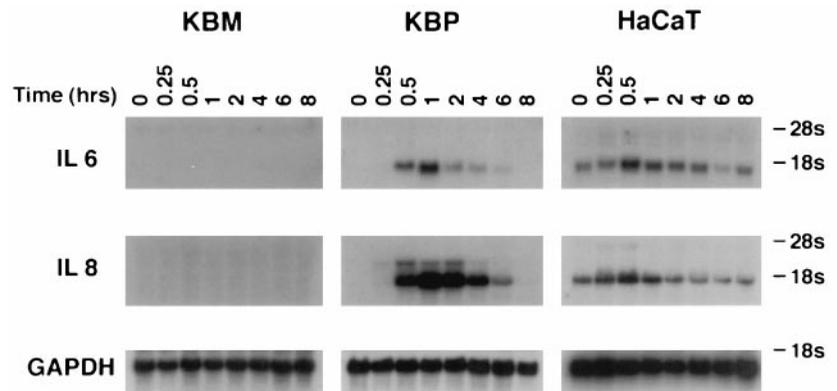
PAF-R using a replication-deficient retrovirus. To control for possible confounding effects due to the integration of a retrovirus into genomic DNA, KB cells were also transduced with the empty retrovirus alone (KBM). With both PAF-R-negative and -positive cells, this unique KB PAF-R model system can complement existing pharmacologic tools (i.e., PAF-R antagonists) to study the effects of PAF-R.

Activation of the KB PAF-R resulted in the biosynthesis of PAF (Fig. 2). Similarly, our laboratory has shown that CPAF treatment of human HaCaT keratinocytes results in PAF biosynthesis (24). The ability of PAF-R activation to both induce PAF synthesis as well as increase transcription of the PAF-R (48) indicates the existence of a positive feedback loop resulting in enhanced PAF-R-induced effects in a cell such as a keratinocyte that can both synthesize and respond to this mediator. Positive feedback loops between PAF and the PAF-R may have potential clinical significance, especially in populations exhibiting decreased activity of the major PAF-metabolizing enzyme acetylhydrolase (49).

Activation of the KB PAF-R resulted in AA release and PGE_2 production (Fig. 3). Similarly, CPAF treatment of human keratinocytes results in AA release (25). PAF treatment of feline tracheal cells (50) and rabbit corneocytes (51) has previously been shown to increase eicosanoid biosynthesis. In KBP cells, the lag time in PGE_2 biosynthesis compared with that of arachidonate release suggested the induction of COX-2. Indeed, PAF-R activation of KBP cells resulted in increased COX-2 mRNA (Fig. 4) and protein (Fig. 5) expression. Treatment of HaCaT keratinocytes with CPAF also resulted in an increase in baseline COX-2, but not COX-1, mRNA levels, suggesting that the KB PAF-R model system resembles the endogenous epidermal PAF-R. The fact that unstimulated HaCaT keratinocytes were found to express COX-2 mRNA yet KB cells did not (Fig. 4) is compatible with previous findings that some carcinomas constitutively express COX-2 (18, 19).

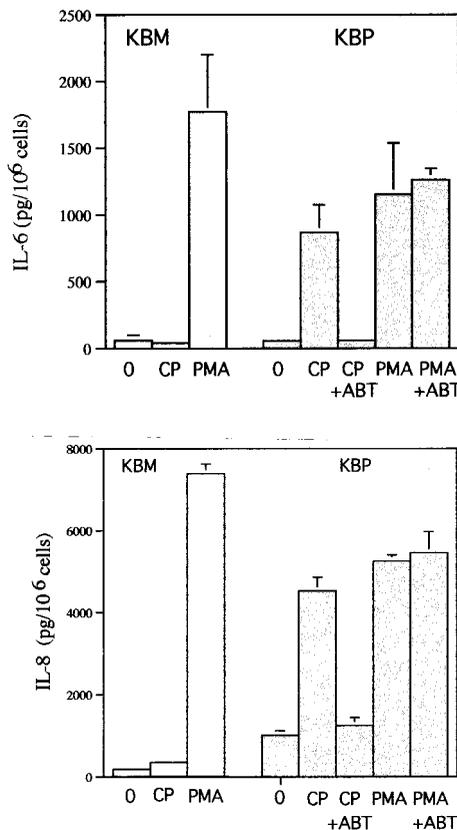**FIGURE 7.** Measurement of IL-6 and IL-8 protein following treatment of KB cells with CPAF or PMA. KBP or KBM cells were incubated with ethanol vehicle (0), 100 nM CPAF (CP), or 10 nM PMA (PMA) or were preincubated for 1 h with 10 μM A-85783 followed by 100 nM CPAF (CP + ABT) or 10 nM PMA (PMA + ABT) for 16 h, and supernatant was removed and assayed for immunoreactive IL-6 or IL-8 protein using specific ELISAs. The results shown are the mean $\pm$ SD of duplicate samples of a representative experiment and are typical of at least two separate KBM or KBP clones.
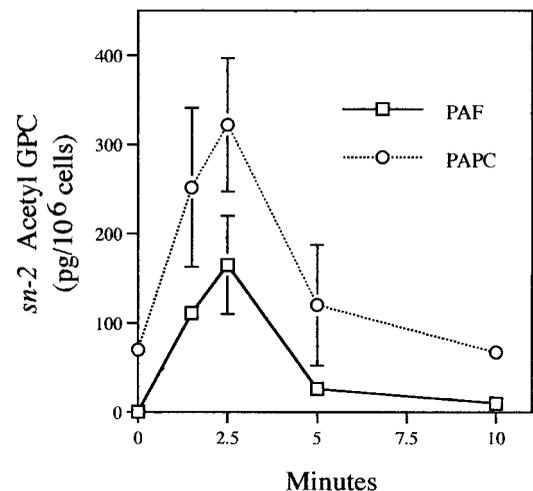

FIGURE 8. Effect of IL-8 on *sn-2* acetyl GPC biosynthesis in HaCaT keratinocytes. HaCaT cells were incubated with 100 ng/ml IL-8 for various times, and cell-associated 1-hexadecyl-2-acetyl-GPC (PAF) and PAPC were measured by GC/MS. Each point represents the mean $\pm$ SD from four or five separate experiments.

Our studies with KB and HaCaT keratinocytes indicate that PAF-R activation alone is an adequate stimulus for COX-2 biosynthesis in epidermal cells. In contrast, PAF alone does not stimulate rat alveolar macrophage COX-2 biosynthesis, but can act to enhance LPS-stimulated COX-2 biosynthesis through transcriptional activation (39). The recent studies by Marcheselli and Bazan (52) indicate that pretreatment of rat brains with the PAF-R antagonist BN 50730 can inhibit kainic acid- and electroconvulsive shock-induced COX-2 induction. Their findings suggest involvement of the PAF-R in the regulation of this enzyme in vivo.

The significance of PAF-R-mediated PG production in epidermal cells is not clear. Depending upon the experimental conditions, PGE_2 has been reported to have mitogenic effects (53) or induce differentiation of human keratinocytes (54). In addition to direct effects on keratinocytes, PGE_2 can inhibit monocyte/macrophage function (55). Thus, epidermal cell-derived PGE_2 could potentially modulate cutaneous immunity through its effects on Langerhans' cells. Finally, the reported ability of COX inhibitors to enhance and of exogenous PGE_2 to inhibit both macrophage PAF production (56) and PAF-R transcription (48) suggest that this eicosanoid could be an endogenous negative regulator of PAF function.

Activation of the epidermal PAF-R was also found to result in an increased accumulation of the cytokines IL-6 and IL-8. These data suggest that the epidermal PAF-R resembles the PAF-R in human lung fibroblasts that synthesize IL-6 and IL-8 in response to PAF challenge (3). These cell types differ from rat macrophages, as PAF alone does not stimulate IL-6 production but will augment muramyl dipeptide-induced IL-6 biosynthesis (57).

Both IL-6 and IL-8 have been reported to stimulate PAF biosynthesis in human neutrophils (58, 59). As shown in Figure 8, IL-8 can also induce PAF biosynthesis in human epidermal cells. The amount of cell-associated PAF produced in response to 100 ng IL-8 was 0.17 $ng/10^6$ cells at 2.5 min, which is less than what we have previously reported for 1 μM A23187 or 250 nM CPAF stimulation (2.2 vs 1.7 $ng/10^6$ cells at 2.5 min, respectively) of HaCaT keratinocytes (24). That IL-8 and PAF can both induce the biosynthesis of each other indicates the possible presence of a "vicious cycle" that may result in the propagation of the inflammatory response, especially in situations characterized by the overexpression of epidermal IL-8Rs (22) or lack of PAF metabolism (49).

These studies demonstrate that activation of the epidermal PAF-R results in increased biosynthesis of PAF, eicosanoids, COX-2, and the cytokines IL-6 and IL-8. These observations have significance, since both eicosanoids and these ILs have been implicated in keratinocyte biology and cutaneous inflammation. Together, these findings indicate that PAF could be an important member of the epidermal cytokine network in part through its ability to stimulate these other mediators. An understanding of the role of the PAF system in the cytokine network of the skin could have potential therapeutic implications given the availability of PAF-R antagonists.

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