Ultraviolet B Radiation Generates Platelet-activating Factor-like Phospholipids underlying Cutaneous Damage*

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Ultraviolet B light (UVB) causes cutaneous inflammation and cell death, but the agents responsible are not defined. These studies examined the role of the platelet-activating factor (PAF) signaling system in UVB-mediated effects. Expression of the PAF receptor in the PAF receptor-negative epidermoid cell line KB augmented apoptosis in response to UVB irradiation. Overexpression of the PAF receptor in primary human keratinocytes also enhanced UVB-mediated apoptosis in vitro, and it enhanced apoptosis in an in vivo model of human keratinocytes grafted onto severe combined immune-deficient (SCID) mice. To define the mechanism by which UVB activates the PAF receptor, we used mass spectrometry to demonstrate significant amounts of the C4 PAF analogs 1-alkyl-2-arachidonoyl-sn-glycero-3-phosphocholine (HAPC) increased the amount of C4 PAF, and this route is not subject to cellular control. The alkyl phospholipid acetyltransferase activity that transfers an acetyl residue from acetyl-CoA to 1-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine, as well as native PAF in an epidermal cell line after UVB irradiation. Supplementation of the cells with the precursor phospholipid 1-hexadecyl-2-arachidonoyl-sn-glycero-3-phosphocholine (HAPC) increased the amount of C4 PAF. UVB exposure increased HAPC directly and found, even in the absence of a photosensitizer, fragmentation to C4-PAF receptor ligands. We conclude UVB photo-oxidizes cellular phospholipids, creating PAF analogs that stimulate the PAF receptor to induce further PAF synthesis and apoptosis. PAF signaling may participate in the cutaneous inflammation that occurs during photo-aggravated dermatoses.

Platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine; PAF) is an inflammatory phospholipid mediator that exerts its effects through a single, highly specific G-protein-coupled receptor, the PAF receptor (1). The PAF receptor is expressed by cells of the innate immune system (2), but also by keratinocytes of the skin (1, 3, 4). PAF is the most potent phospholipid agonist yet identified, where its high affinity recognition by the PAF receptor depends on its sn-1 ether bond and a short sn-2 acetyl residue (5). PAF can activate cells at sub-picomolar levels (6), and as would be anticipated from this, its synthesis is closely controlled.

PAF is synthesized in response to diverse stimuli, including cytokines, endotoxins, and Ca$^{2+}$ ionophores, and in response to PAF itself (1, 2). The synthetic pathway consists of two enzymes; a phospholipase A$_2$ that generates the lysolipid backbone by releasing the fatty acyl residue esterified at the sn-2 position of alkyl phosphatidylcholines, and a PAF acetyl-transferrase activity that transfers an acetyl residue from acetyl-CoA to this newly generated lysolipid. Both activities are tightly regulated, with increased intracellular Ca$^{2+}$ being the premier agent for their activation.

Ligands that activate the PAF receptor are generated in a second way, and this route is not subject to cellular control. The alkyl phospholipid pool is enriched at the sn-2 position with polyunsaturated arachidonoyl residues (7), and polyunsaturated fatty acids, esterified or not, are susceptible to oxidation. Oxidation of polyunsaturated acyl chains introduces oxy functions (aldehydes, ketones, peroxides, alcohols, and carboxylic acids) to the chain of carbon atoms, rearranges bonds, and fragments carbon–carbon bonds by β-scission (8), all of which generate a myriad of reaction products. Among these products of polyunsaturated phospholipid oxidation are a series of phospholipids with oxidatively fragmented sn-2 acyl residues that terminate with either an ω-oxy function or a methyl group (9), and some, but not all, of which are ligands for the PAF receptor (6, 10–13). The most potent of the non-enzymatically generated PAF analogs are the butanolyl (C$_4$-PAF) and butenoyl (C$_4$,1-PAF) species, which are one-tenth as potent as PAF in PAF receptor-transfected cells (5, 6).

Oxidatively generated PAF analogs have been identified in oxidized low density lipoprotein particles (6, 10, 11), endothelial cells exposed to peroxides (13), the blood of hamsters (14) and humans (15) exposed to cigarette smoke, in atherosclerotic plaque (11), in the plasma of hypercholesterolemic patients (16). Conversely, oxidative (17) liver damage caused by CCL$_4$ intoxication oxidizes membrane phospholipids (18), yet the relevant inflammatory mediator made in response to this oxidative insult is biosynthetic PAF and not oxidatively generated PAF-like lipids (19). It is therefore difficult to predict a priori the actual mediator generated by oxidative stress, and accumulation of chemically generated PAF receptor ligands has not been demonstrated by cells exposed to stimuli they encounter in their environment.

UVB radiation (290–320 nm) has profound effects on keratinocytes of human skin, in part because it is a pro-oxidative stressor (20). UVB generates a PAF receptor activity that has not been characterized (20), and stimulation of the PAF receptor of epithelial cells mimics many aspects of UVB on human keratinocytes, including the production of...
tumor necrosis factor-α, interleukin-8 and interleukin-10, and cyclooxygenase-2 production, as well as cytotoxicity (20–22). UVB is immunosuppressive and facilitates progression of non-melanoma skin cancer, and it is proposed (23) that PAF and/or PAF-like lipids mediate this systemic immune suppression. The identity of the one or more actual mediators that couple UVB to cytokine production, immunosuppression, and cell death is unknown. Here we show that UVB photooxidizes polysaturated phosphatidylcholine and identify the PAF receptor ligands generated in this process. We suggest that UVB-generated PAF agonists are involved in the cutaneous inflammation and immunomodulation seen in response to UVB irradiation.

MATERIALS AND METHODS

Cells—The human epidermoid cell line KB was grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Intergen, Purchase, NY) containing 10 μg/ml arachidonic acid. A KB PAF receptor model system was created by transduction of PAF receptor-negative KB cells with the MSCV 2.1 retrovirus encoding the human leukocyte PAF receptor as described previously (21). KB cells stably transduced with the PAF receptor (designated KPB cells) or with fMLP receptor (designated as KBF cells) or control MSCV2.1 retrovirus (defined as KBM cells) were characterized by Southern, Northern, radioligand binding, and calcium transient studies that demonstrate the presence of a functional PAF or fMLP receptor signaling system in these cells (21).

UVB Irradiation Source—Cells were irradiated with UVB as previously described (20). The UV source was a Philips F20T12/UV-B lamp (270–390 nm, containing 2.6% UVC, 43.6% UVB, and 53.8% UVA). The intensity of the UVB source was measured prior to each experiment using an IL1700 radiometer and a SED240 UVB detector (International Light, Newburyport, MA) at a distance of 8 cm from the UVB source to the monolayer of cells/purified lipid.

PAF Receptor Phosphorylation—KB cells were transduced with a PAF receptor construct that contained three FLAG tags at the carboxyl terminus (KBP-Flag3) for these immunoprecipitation studies. KBP-Flag3 cells were grown in 100-mm dishes to confluence and then washed with phosphate-free Dulbecco’s modified Eagle’s medium (Sigma) and incubated for 2 h at 37 °C. The cells were then incubated with 32P-Pi for 2 h before being stimulated by UVB (400 J/m2) or carbamyl-PAF (100 nM) for either 10 or 20 min (24–26). The cells were rapidly washed twice with ice-cold phosphate-buffered saline, and the PAF receptor was immunoprecipitated as described before (26) using anti-FLAG antibody (Sigma) before its phosphorylation was assessed by autoradiography.

EGF Receptor Phosphorylation—Irradiated cells were obtained by exposing KBM and KBP cells to UVB light for the stated time intervals. Following this, the cells were washed twice with ice-cold phosphate-buffered saline and lysed with radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P40) containing 0.5 mM Pefabloc SC (Roche Applied Science) to inhibit plasma PAF acetylhydrolase and 10 mM sodium orthovanadate) for 20 min on ice. The EGFR receptor was immunoprecipitated from cell lysates by incubation with a polyclonal anti-EGF receptor antibody (Ab1, Santa Cruz Biotechnology, Santa Cruz, CA) and protein G plus/protein A-agarose (Calbiochem) overnight at 4 °C. Specific tyrosine phosphorylation of the immunoprecipitated EGF receptor was determined by immunoblotting with anti-phosphotyrosine antibody (Ab-4, Calbiochem) and detecting the reaction product with an ECL kit (Amer sham Biosciences). The immunoprecipitated and electrophoretically resolved proteins were reprobed with anti-EGF receptor antibody to establish that the wells contained equal amounts of material (27). In some experiments, cells were pretreated with (10 μM) Trolox, a water-soluble analog of antioxidant vitamin E, for 30 min (Calbiochem) before exposing the cells to UVB. In other experiments, cells were stimulated with carbamyl-PAF (100 nM, BIOMOL Research Laboratories, Plymouth Meeting, PA), a phospholipase A2-resistant analog of PAF that is a PAF receptor ligand and agonist.

Caspase-3 Assay—Caspase-3 activity was determined in cell lysates derived from primary cultures of human keratinocytes (HK cells) that were transduced with the PAF receptor (HK-PAF-R) or retroviral vector (HK-NIE). Caspase-3 activation was also determined in an epitheloid cell line (KB cells) stably transfected with the PAF receptor (KBP cells) or the seven-transmembrane receptor family member for fMLP (KBF cells). A synthetic fluorogenic substrate (acet-yl-Asp-Glu-Val-Asp-AMC, Alexis Biochemicals, San Diego, CA), which releases fluorescent AMC after hydrolysis, was used to assay activated caspase-3. Fluorescence of AMC was measured by excitation at 380 nm with emission at 460 nm (28). Fluorescence intensity of the reaction products was compared with a standard of AMC (Molecular Probes, Eugene, OR) and normalized to cellular protein using a NanoOrange protein quantitation kit (Molecular Probes).

Apoptosis in Xenografts of HKPAF-R and HKNIE Cells—Primary cultures of human keratinocytes (HKs) obtained from neonatal foreskins were transduced with the PAF-R and xenografts of HKPAF-R and control retroviral-expressing HKNIE cells were established in immunosuppressed NOD/LtSz-scid/scid mice as described before (29). These xenografts were irradiated with 1000 J/m2 UVB, and the tissue was harvested 24 h later, fixed in 10% formalin, and counterstained with methyl green. PARP cleavage, as a measure of caspase activation, was examined with anti-PARP antibody (Cell Signaling Technology, Beverly, MA) at a dilution of 1:100. Caspase-3 activation was determined in parallel using an antibody (BD Pharmingen), which detected only the activated form of caspase-3 at a dilution of 1:100. Secondary rabbit anti-mouse antibody was from Vector Laboratories (Burlingame, CA) and visualized by immunoperoxidase using rabbit immunoCruz staining system (Santa Cruz Biotechnology).

Lipid Manipulation and Analysis—Total lipids from KBM cells exposed or not to UVB were extracted by the method of Bligh and Dyer (30). Phospholipids were isolated with aminopropyl columns (JT Baker Inc., Phillipsburg, NJ) by solid phase extraction and then subjected to reverse-phase HPLC separation as described earlier (6, 10). Isocratic HPLC fractions were collected at 1 ml/min, and fractions containing leukocyte agonists, determined by the bioassay described below, were pooled. In a few experiments, a portion of the pooled HPLC fraction was subjected to extensive treatment with phospholipase A1 (Rhizopus arrhizus, Sigma) to remove diacyl phospholipids (6, 19) before this bioassay. PAF, with its lipase-insensitive sn-1 ether bond, was the negative control for PLA1 treatment, whereas 1-palmitoyl-2-acetyl-sn-glycero-3-phosphocholine (acyl-PAF, BIOMOL Research Laboratories) served as the positive control.

Measurement of Intracellular Ca2+ Flux in Human Neutrophils—Human neutrophils were freshly isolated by dextran sedimentation and centrifugation over Ficoll as described (31). Isolated neutrophils (2.25 × 10⁶/ml) were then loaded with FURA-2-AM (Molecular Probes) as before (6, 19, 32), and changes in intracellular calcium concentration were measured by dual excitation at 340 and 380 nm with emission collected at 510 nm. A graded amount of authentic PAF (BIOMOL) or C₄-PAF analogs (Avanti Polar Lipids, Alabaster, AL) was used to establish the concentration-response relationship of these primary cells. In a few experiments FURA-2-AM-loaded cells were pretreated with the PAF receptor antagonist BNS2021 (20 μM, BIOMOL) and then tested for agonist-stimulated calcium transients. In other experiments, biologically active lipid
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fractions were pretreated with 10 μg of recombinant human PAF acetylhydrolase (ICOS Corp., Bothell, WA) in Hanks’ balanced salt solution containing 0.5% human serum albumin for 1 h at 37 °C before being tested as agonists of the leukocyte PAF receptor.

Oxidation of Synthetic Phospholipids by UVB—Synthetic 1-O-hexadecyl-2-arachidonoyl-sn-glycero-3-phosphocholine (HAPC) was from any of several sources (BIOMOL, Sigma, or Avanti Polar lipids). During these studies we noted that material from Sigma expressed large amounts of pre-existing leukocyte agonists, whereas that from Avanti was devoid of this material. For the cell-free irradiation of HAPC, 1 mg of compound was dried under N₂ gas on a polystyrene tissue culture plate prewashed four times with methanol. Following either sham (non-irradiation, left open on the bench top) or UVB irradiation, the lipid was removed with methanol. The lipids were then extracted (30) and subjected either to mass spectral analysis or bioassy.

Lipids to be used for bioassays were dried under a stream of N₂, resuspended in Hanks’ balanced salt solution containing 0.1% human serum albumin, and then sonicated. Oxygen was excluded in some experiments by placing the UVB source and plates inside a transparent torus-negative KB cells that express a PAF receptor modified at the carboxyl terminus by a trimerized FLAG tag to allow immunoprecipitation (26). We found that addition of the stable PAF analog carbamyl-PAF resulted in the rapid phosphorylation of the modified PAF receptor (Fig.

and the photo oxidation products of HAPC were carried out on an API 3000 triple quadruple mass spectrometer (Applied Biosystems, Foster City, CA). The reversed-phase HPLC system utilized a Column C₁₈ 1-× 150-mm column (Phenomenex, Torrance, CA). The mobile phase solvent system consisted of methanol:20 mM ammonium acetate in water:acetonitrile in a ratio of 80:15:5 (v/v) for solvent A and methanol for solvent B. Initial conditions were 20% B for 5 min, linearly increasing from 20% to 70% B by 18 min, and remaining at 70% B until 26 min, after which it returned to initial conditions of 20% B. The collision-induced decomposition spectra of butanoyl-PAF and butenoyl-PAF were obtained by collisional activation of the acetate adducts in the negative ion mode. The spray voltage was −4000 V with nitrogen as collision gas, and the collision energy was set at −50 V. Multiple reaction monitoring methods, which are sensitive and specific, were developed based on the collision-induced decomposition results to detect and quantify products from photo-oxidation experiments.

To confirm the presence of butanoyl-PAF in UVB-irradiated KB cells, lipids were extracted (30). Phospholipids were further purified by solid phase extraction on a silica column (Supelco), subjected to the above reversed-phase HPLC separation, and 30-s fractions from 10 to 25 min were collected in a 96-well plate. These fractions were analyzed with a Nanomate (Advion Biosciences, Ithaca, NY) nanospray interface coupled to a 4000 Q Trap (Applied Biosystems). Negative ion full scan confirmed the presence of ions consistent with PAF-like compounds; these were then collisionally activated in the product ion mode to generate collision-induced decomposition spectra. The conditions were as follows: the Nanomate spray pressure was 0.35 p.s.i. with a spray voltage of 1.35 kV, the 4000 Q Trap used nitrogen as the collision gas, and the collision energy was −59 V. PAF and PAF-like lipids were also quantitated by negative ion gas chromatography-mass spectroscopy after hydrolysis with phospholipase C and derivatization of the liberated diacylglycerols with pentafluorobenzyl chloride as described previously (6).

RESULTS

UVB Light Activates PAF Receptor Signaling—Our first studies tested whether the PAF receptor of epidermal cells was activated by UVB irradiation. We first determined whether the receptor became phosphorylated, a characteristic of the activated PAF receptor (25, 33), after UVB irradiation. To detect this modification, we employed PAF receptor-negative KB cells that express a PAF receptor modified at the carboxyl terminus by a trimerized FLAG tag to allow immunoprecipitation (26). We found that addition of the stable PAF analog carbamyl-PAF resulted in the rapid phosphorylation of the modified PAF receptor (Fig.

FIGURE 1. UVB irradiation activates the PAF receptor of epidermal cells and thereby enhances tyrosine phosphorylation of the EGF receptor. A, UVB irradiation leads to the phosphorylation of the PAF receptor. An epithelial cell line, KBP-Flag3, that stably expresses a FLAG-tagged PAF receptor, was incubated with [32P]orthophosphate and then treated with buffer, with the stable PAF agonist carbamyl-PAF (cPAF) at 100 nM, or was irradiated with 400 J/m² UVB for 10 or 20 min. Phosphorylation of the PAF receptor was then assessed following FLAG antibody immunoprecipitation of 32P-labeled protein. B, expression of the PAF receptor leads to enhanced EGF receptor phosphorylation. PAF receptor-expressing KB cells and control KBM cells were irradiated for 4 h with 400 J/m² UVB. The EGF receptor was immunoprecipitated from the cellular lysates, resolved by SDS-PAGE, and the amount of total and phospho-EGF receptor determined by autoradiography MS/MS analysis of synthetic phospholipids, butanoyl-PAF,

FIGURE 2. Effect of UVB irradiation on caspase-3 activation in KB cells expressing seven-transmembrane receptors. KB cells stably transfected with a control vector (KBM), the human PAF receptor (KBP), or the receptor for fMLP (KBF) were irradiated with UVB at a flux of 400 J/m². At the stated time, the cells were recovered, and hydrolysis of the synthetic fluorogenic substrate (Ac-Asp-Glu-Val-Asp-AMC) for caspase-3 substrate was determined as described under “Materials and Methods.”
We also found that this ectopic PAF receptor was rapidly and extensively phosphorylated when the cells were exposed to low levels of UVB irradiation (400 J/m²), indicating that UVB irradiation activates the PAF receptor.

We used another, although indirect, measure of PAF receptor activation to assess the effect of UVB light on PAF signaling. Activation of the PAF receptor releases an EGF receptor ligand, in a matrix metalloprotease-dependent way, that then stimulates EGF receptor function (27). Thus, PAF receptor activation leads to EGF receptor autophosphorylation (27). We irradiated control KBM cells or KBP cells that overexpress the PAF receptor to find (Fig. 1B) that UVB stimulated phosphorylation of the EGF receptor in both cell lines, but that expression of the PAF receptor greatly increased EGF receptor phosphorylation. We determined whether activation of the EGF receptor was downstream of UVB-induced formation of reactive oxygen species by preincubating the cells with Trolox, a water-soluble antioxidant analog of vitamin E. The data (Fig. 1C) show that Trolox completely inhibited UVB-mediated EGF receptor phosphorylation, but not that induced by the stable PAF receptor ligand carbamoyl-PAF. Other antioxidants such as N-acetylcysteine (10 mM) and resveratrol (10 mM) had similar effects (not shown). These data suggest that stimulation of the EGF receptor by UVB light is secondary to oxidative stress and potentially oxidative formation of PAF receptor ligands.

Overexpression of the PAF Receptor in Keratinocytes Enhances UVB Light-induced Apoptosis—Keratinocytes, the epithelial cells of skin, must be protected from solar radiation by pigmented melanocytes, FIGURE 3. Overexpression of the PAF receptor in keratinocytes enhances UVB-induced apoptosis. A, primary cultures of human keratinocytes overexpressing the PAF receptor (HK-PAF-R cells, dark bars) or vehicle control cells (HK-NIE cells, open bars) were irradiated with 400 J/m² UVB, and caspase-3 enzymatic activity assessed 8 h later as described under “Materials and Methods.” B–E, xenografts of HK-NIE or HK-PAF-R cells established in SCID mice were UVB irradiated (1000 J/m²) in vivo, and 24 h later the tissue was harvested and fixed in formalin. The fixed tissues were counterstained, and then cleaved PARP (B and C), or activated caspase-3 (D and E) was visualized by immunohistochemistry that generates a brown reaction product.

FIGURE 4. UVB irradiation of KB cells generates PAF receptor agonists. KBM cells were irradiated, or not, with 400 J/m² UVB light before their total lipids were extracted. The presence of PAF receptor ligands in the lipid extracts was detected using FURA-2AM loaded PMN where changes in intracellular Ca²⁺ concentrations are reflected by corresponding changes in the emission after excitation at 340 nm and 380 nm. The total lipid extract from UVB-irradiated cells (B), but not from control (A) cells, induced Ca²⁺ mobilization. The subsequent addition of a low concentration of PAF showed each cell population was capable of a response. Phospholipids isolated from total lipid extract of UVB irradiated KBM cells were fractionated by reversed phase HPLC column and PAF receptor agonists eluted in fractions five to seven. These pooled fractions induced a Ca²⁺ mobilization response (C) that was completely suppressed (D) by pre-exposing the FURA-2-labeled PMN to the PAF receptor antagonist BN52021 (20 μM). BN52021 is a competitive antagonist, and its effect was overcome by adding a high (1 μM) concentration of PAF (D, arrow). The PAF receptor agonists in the partially purified lipid extracts of irradiated KBM cells are sensitive to hydrolysis by PAF acetylhydrolase pretreatment (E), but are insensitive to phospholipase A₁ pretreatment (F) that cannot attack the ether bond of alkyl phospholipids.
because keratinocytes undergo apoptosis when irradiated with UVB light (34). UVB induces cell death of the cultured human epidermal PAF receptor-negative cell line KB, and this is augmented when these cells are transduced with a retrovirus to express the PAF receptor (KBP) (20). We found that irradiation of KBP cells resulted in a rapid and profound activation of the effector protease caspase-3 (Fig. 2) compared with control KBM cells. The enhanced sensitivity to UVB was specific for cells expressing the PAF receptor, because cells stably transfected with the related receptor for the small peptide f-Met-Leu-Phe (KBF) behaved just as control KBM cells when exposed to UVB (Fig. 2).

To assess the effects of PAF receptor overexpression on UVB-induced apoptosis in human keratinocytes, we established primary human keratinocytes (which express the PAF receptor (3)) that either overexpressed the PAF receptor (HK-PAF-R) or contained a control vector (HK-NIE cells) (29). We verified that overexpressing the PAF receptor augmented caspase-3 activation in the HK-PAF-R cells compared with the control KBM cells when exposed to UVB (Fig. 2).

To determine whether UVB irradiation of KB cells generates PAF receptor agonists, we next assessed whether this occurred through the production of soluble PAF receptor agonists. To that end, we irradiated control KBM cells (to avoid PAF receptor-stimulated PAF biosynthesis (21)), extracted the lipids, and tested for the presence of PAF receptor stimulatory activity using intracellular calcium mobilization in neutrophils as a sensitive bioassay. We found that the total lipid extracts derived from untreated KBM cells contained little PAF receptor stimulatory activity. This was true even in this sensitive bioassay where mobilization of calcium in response to the addition of just 10 nM PAF, added at the end of the experiment, was readily detected (Fig. 4A). In contrast, lipid extracts from an identical number of UVB-treated cells rapidly mobilized calcium from neutrophil intracellular stores (Fig. 4B).

UVB Irradiation of KB Cells Generates PAF Receptor Agonists—Given our findings that UVB activates the PAF receptor, we next assessed whether this occurred through the production of soluble PAF receptor agonists. To that end, we irradiated control KBM cells (to avoid PAF receptor-stimulated PAF biosynthesis (21)), extracted the lipids, and tested for the presence of PAF receptor agonistic activity using intracellular calcium mobilization in neutrophils as a sensitive bioassay. We found that the total lipid extracts derived from untreated KBM cells contained little PAF receptor stimulatory activity. This was true even in this sensitive bioassay where mobilization of calcium in response to the addition of just 10 nM PAF, added at the end of the experiment, was readily detected (Fig. 4A). In contrast, lipid extracts from an identical number of UVB-treated cells rapidly mobilized calcium from neutrophil intracellular stores (Fig. 4B).
The leukocyte agonist(s) accumulating in KBM cells following UVB irradiation acted through the PAF receptor, because the selective antagonist BN52021 blocked all of the response to the lipid agonists generated by UVB light (Fig. 4D). Addition of a 10,000-fold molar excess (10^{-6} M) of synthetic PAF at the end of the assay showed that the PMNs still respond to PAF and that the inhibition by the competitive antagonist BN52021 was reversible. Additionally, treatment of the lipid extracts with recombinant human plasma PAF acetylhydrolase, an enzyme that selectively hydrolyzes PAF and oxidatively fragmented phospholipids (35), abolished the positive response to the partially purified lipids of irradiated cells (Fig. 4E). PAF and the most potent of its analogs all have a phospholipase A_{1}-resistant sn-1 ether bond, and lipid extracts from UVB-irradiated cells were resistant to phospholipase A_{1} treatment (Fig. 4F). The acyl analog of PAF, which is active at high concentrations, was sensitive to phospholipase A_{1}, whereas PAF was resistant to this treatment, which served as positive and negative controls in these experiments (not shown).

### TABLE ONE

| PAF species | Sham (ng/mg) | High UVB (2000 J/m²) (ng/mg) |
|-------------|-------------|-----------------------------|
| PAF         | 0.25 ± 0.07 | 2.8 ± 0.8                   |
| Butanoyl PAF| 1.05 ± 0.95 | 18.4 ± 2.4                  |
| Butenoyl PAF| Not detected | 16.9 ± 3.8                  |

**UVB irradiation of synthetic 1-hexadecyl-2-arachidonoyl-sn-glycero-3-phosphocholine generates PAF and PAF analogs**

The synthetic phospholipid was irradiated as described under “Materials and Methods.” The values are ± S.E. of nanograms/mg 1-hexadecyl-2-arachidonoyl-sn-glycero-3-phosphocholine from 4–5 independent experiments.

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**FIGURE 6.** Collision-induced dissociation of the negative ion m/z 610.6 present in the reversed phase-HPLC fraction collected between 18 and 18.5 min from phospholipids extracted from UVB irradiated KBM cells. The inset presents the negative ion molecular ion species of components in this HPLC fraction including the ion at m/z 610.6. The origin of abundant product ions following collisional activation of m/z 610.6 is depicted in the structure of 1-hexadecyl-2-butenoyl-glycerophosphocholine acetate adduct ion [M + OAc]^{-}.
UVB Generates PAF Agonists

| TABLE TWO |
| UVB irradiation of KB cells generates PAF and PAF analogs |

KB cells were cultured in media (A) or media supplemented with 10 μM 1-hexadecyl-2-arachidonylglycero-3-phosphocholine for 24 h prior exposure to UVB light at the stated flux. Lipid extracts from these cells were subjected to gas chromatography-mass spectroscopy, and the quantity of material was determined through the use of deuterated internal standards. Values are mean (± S.E.) of picograms per 10^6 cells from four independent experiments.

| PAF-species | Sham | Low UVB (400 J/m²) | High UVB (2000 J/m²) |
|-------------|------|-------------------|---------------------|
| A) Media alone | | | |
| PAF | 46 ± 12 | 123 ± 60 | 160 ± 31 |
| Butanoyl-PAF | 7 ± 4 | 92 ± 23 | 630 ± 105 |
| Butenoyl-PAF | 3.5 ± 5 | 39 ± 12 | 380 ± 95 |
| B) Media supplemented with 1-hexadecyl-2-arachidonyl-sn-glycero-3-phosphocholine | | | |
| PAF | 70 ± 15 | 170 ± 60 | 470 ± 53 |
| Butanoyl-PAF | 39 ± 13 | 550 ± 30 | 2890 ± 60 |
| Butenoyl-PAF | 47 ± 9 | 873 ± 100 | 3920 ± 125 |

(2000 J/m²) flux of UVB light, isolated the phospholipid oxidation products, and assayed them as PAF receptor agonists. We found that UVB irradiation of this synthetic phospholipid created leukocyte agonists (Fig. 5A) and that all of the UVB-induced agonistic activity was abolished by the competitive PAF receptor antagonist BN52021 (Fig. 5B) or by pre-treatment of the recovered polar phospholipids with PAF acetylhydrolase (data not shown). Molecular oxygen was required for the formation of the agonist(s), because irradiation in an argon atmosphere failed to generate leukocyte agonists (not shown). We analyzed the HPLC fractions that contained the polar phospholipid agonists by mass spectrometry and found that UVB photo-oxidation generated both PAF and the PAF-like analogs butanoyl-PAF and butenoyl-PAF (Fig. 5, C and D). The quantity of PAF generated by UVB irradiation (TABLE ONE) of the synthetic phospholipid was just 7% of the combined quantities of the two C₄-homologs, so the reactions giving the shorter fragment are less efficient than those that fragment the acyl residue just proximal to the first double bond between carbon atoms 5 and 6 of the arachidonoyl residue esterified at the sn-2 glycerol position.

Identification of PAF-like Oxidized Phospholipids in UVB-irradiated Cells—To identify the bioactive lipids present in the UVB-irradiated KBM cells, tandem mass spectrometry was employed. Extraction of lipids was carried out using the Bligh and Dyer extraction protocol (30) and then fractionated by normal phase/solid phase extraction to isolate phospholipid-like molecules. Phospholipids were then subjected to reversed-phase HPLC separation and fraction collection using the same conditions employed in the studies of the photo-oxidation studies above. Electrospray ionization (negative ions) and mass spectrometric analysis of each fraction were then carried out as detailed under “Materials and Methods.” A major component at m/z 610.5 (negative ions) as well as additional lipids with molecular ions or molecular adduct ions at m/z 566.5 and 568.4 eluted at 18 min (Fig. 6, inset). Tandem mass spectrometry of the ion at m/z 610.5 revealed characteristic ions at m/z 536 [M-15]^−, 466 [M-15-C₄H₂O]^-, 87, and 59 (Fig. 6). As the acetate adduct, 16:0:4/0-glycerophosphocholine [M+Ac]^- would appear at m/z 610, which is known to undergo collision-induced decomposition to the abundant product ion at m/z 87 as well as m/z 466 and 536 as observed for the photo-oxidation of 16:0/20:4-glycerophosphocholine (Fig. 5C).

Subsequent quantitation of the 1-hexadecyl-2-butanoyl/butenyl glycerophosphocholines was carried out using a gas chromatography/MS and a stable isotope dilution protocol after degradation of extracted phospholipids to corresponding diglycerides followed by derivatization. For these experiments, lipids from KBM cells irradiated with UVB or not, were extracted as before, isolated by an aminopropyl column, then subjected to reversed-phase HPLC separation, the pooled bioactive fractions were extensively treated with phospholipase A₁ to remove less active diacyl phospholipids, and then subjected to gas chromatography-mass spectroscopy as described previously (6). This analysis detected significant levels (TABLE TWO) of 1-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine, the common molecular species of PAF, before and especially after UVB irradiation. In addition to PAF, UVB irradiation of KBM cells also caused cellular accumulation of 1-hexadecyl-2-butanoyl/butenoyl phosphatidylcholine (C₄₋PAF analogs), just as we found following the in vitro oxidation of purified low density lipoprotein by Cu²⁺ (6). Notably, only 1-hexadecyl-2-butanoyl/butenyl glycero-phosphocholines increased with the level of UVB light, whereas PAF did not vary with the flux of UVB (TABLE TWO).

We supplemented KB cells with 10 μM HAPC, a precursor (6) for oxidatively generated PAF-like lipids and lys-PAF, to assess whether this precursor was limiting. We found (TABLE TWO, Part B) that accumulation of PAF and PAF-related molecules was largely unaffected by this supplementation in the absence of UVB exposure and that accumulation of PAF after irradiation also was only modestly affected by this supplementation. In contrast, accumulation of the two C₄₋PAF analogs was greatly stimulated by the supplement in a way dependent on UVB flux. This differential effectiveness of the common precursor suggests PAF accumulation differs from the reactions that produce C₄₋PAF analogs.

Relative Contribution of PAF and PAF-like Lipids to the Inflammatory Material in UVB-irradiated Cells—UVB irradiation of HAPC produces lipid oxidation products that stimulate a concentration-dependent and saturable effect on leukocytes (Fig. 7A). Comparison of the effect of increasing concentrations of synthetic butanoyl-PAF and butenoyl-PAF with PAF show the two phospholipid oxidation products are equivalent and are 10-fold less potent than PAF (Fig. 7B). The relative amounts of these lipids in irradiated cells (TABLE TWO) means that PAF accounts for 98%, 90%, and 61% of the total PAF receptor agonists from cells exposed to 0, 400, or 2000 J/m² UVB, respectively.

DISCUSSION

Keratinocytes are the cells primarily affected by damaging incident light. They have an integral role in cutaneous inflammation and are progenitor cells in squamous cell carcinoma. The high energy fraction of ultraviolet light, UVB (290–320 nm), penetrates a short distance into the cutaneous layer to cause photoperoxidation of cellular lipids (36), formation of radicals and other reactive oxygen species (37), and DNA damage (38). UVB irradiation of keratinocytes alters their milieu through the stimulated production of inflammatory cytokines and lipid mediators (Ref. 39, and references therein). UVB light indirectly activates the receptor for EGF, which in turn is implicated in UVB-mediated apoptotic death (40). Moreover, UVB irradiation-mediated immunosuppression is inhibited by PAF antag-
onists and mimicked by systemic treatment with PAF agonists (23). Although accumulating evidence implicates the PAF system in UVB-mediated cytotoxicity, cytokine production, and immunosuppression, the identity of the PAF ligand(s) generated in response to UVB light has until now been undefined, because ligands could arise both from regulated biosynthesis (1) and from oxidative attack on polyunsaturated ether phospholipids (41). We find that UVB fragments synthetic phospholipids to C4-PAF-like agonists even in the absence of a photo-sensitizer, and we find that a similar process occurs in cultured cells. This is the first identification of oxidatively generated PAF-like lipids in cells exposed to an ambient stress, and the data indicate that the same oxidative generation of PAF receptor ligands occurs in the skin of animals exposed to UVB.

Lipid hydroperoxides are common products of polyunsaturated fatty acyl group oxidation generated by both enzymatic and non-enzymatic mechanisms. They accumulate in cellular and extracellular complex lipids to varying extents. These carbon-centered peroxides can be photo-dissociated by UVB light (42) to yield alkoxyl radicals that then may fragment into the numerous products we previously described (6). Photo-peroxidation of the polyunsaturated fatty acid arachidonate initially gives monohydroperoxide products, followed by secondary oxidation to dihydroperoxides, and finally results in oxidative fragmentation of the fatty acid chain (43). Indeed, we found that irradiation of the complex polyunsaturated lipid 1-hexadecyl-2-arachidonoyl phosphatidylcholine with UVB oxidized and fragmented the polyunsaturated sn-2 acyl residue. Among the products so generated were two PAF receptor ligands, 1-alkyl-2-butanoyl-sn-glycero-3-phosphocholine and 1-alkyl-2-butenoyl-sn-glycero-3-phosphocholine previously identified in low density lipoprotein oxidized in vitro with Cu2+ (6).

UVB irradiation of keratinocytes generates H2O2 (44), and the phos-
UVB Generates PAF Agonists

pholipids of keratinocytes seem to be at particular risk to UVB photo-oxidation and subsequent photo-dissociation. In fact, keratinocyte catalase absorbs incident UVB light, in a way dependent on the ligand pocket and heme group of the enzyme, to generate reactive oxygen species (37). This study also shows that increasing catalase expression increases the production of reactive oxygen species, whereas antisense oligonucleotides to catalase suppress protein expression and radical production. The authors postulate UVB-induced proton abstraction from H₂O (37), and either OH⁻ from this reaction, or that arising from an iron-catalyzed Fenton reaction (36), generate lipid and peroxoy radicals. β-Scission of these peroxoy lipids fragments the carbon backbone of the polyunsaturated sn-2 fatty acyl residue to produce a series of short-ened reaction products.

UVB irradiation of human skin generates isoprostanes (chemically generated arachidonate products), although these are three orders of magnitude less abundant compared with the formation arachidonoyl hydroperoxides that fragment to PAF-like lipids (45). Isoprostanes are exclusively formed from arachidonyl residues esterified in phospholipids (46), and two biologically active isoprostane-containing phospholipids have been reported (47) that are sensitive to phospholipase digestion. Phospholipase A₁ hydrolyzes isoprostane-containing phospholipids while preserving the PAF-like activity formed in parallel (19). We found that the HPLC-purified phospholipids recovered from UVB-irradiated KB cells were resistant to phospholipase A₁ treatment, and so are PAF-like lipids rather than biologically active isoprostane-containing phospholipids.

The effect of UVB light on keratinocytes, in addition to promoting oxidizing chemical reactions, includes stimulated PAF biosynthesis. UVB irradiation of keratinocytes (20), or for that matter exposing them to exogenous peroxides (48), stimulates PAF synthesis by the remodeling pathway. The remodeling pathway to PAF formation consists of two or more enzymatic steps (depending on how the lypo-PAF is generated) to generate lyso-PAF that is then acetylated by acetyl-coenzyme A. PAF synthesis by keratinocytes in response to UVB light is greatly enhanced by overexpression of the PAF receptor (20), and PAF-induced PAF synthesis has also been observed in endothelial cells (49), where it may serve as a positive feedback signal. PAF synthesis in keratinocytes subsequent to UVB exposure is fully suppressed by a PAF receptor antagonist and by anti-oxidants (20). Either reactive oxygen species couple PAF receptor signaling to the PAF synthetic pathway, or oxidative fragmentation of cellular phospholipids creates PAF receptor ligands that interact with the PAF receptor to stimulate the remodeling pathway for PAF synthesis. This PAF receptor-induced PAF synthesis expands the initial response to the oxidatively generated PAF receptor ligands.

Keratinocytes express message for the PAF receptor (50), and they express the receptor on the cell surface where it participates in autocrine signaling (3). Overexpression of the PAF receptor in cultured keratinocytes induces EGF receptor-dependent cellular proliferation (27), crine signaling (3). Overexpression of the PAF receptor in cultured keratinocytes induces EGF receptor-dependent cellular proliferation (27), and PAF receptor antagonists is a relevant strategy to blunt the photo-oxidation that occurs during cutaneous inflammation, as suggested by the demonstration (52) that the PAF receptor antagonist WEB2086 inhibits UVB dermatitis in human skin.

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UVB Generates PAF Agonists
Ultraviolet B Radiation Generates Platelet-activating Factor-like Phospholipids underlying Cutaneous Damage

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