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Acute keratinocyte damage stimulates platelet-activating factor production

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Abstract **Recent evidence suggests that the phosphocholine-derived lipid mediator platelet-activating factor (PAF) is involved in keratinocyte function and cutaneous inflammation. PAF is found in various inflammatory skin diseases, and intradermal injection of PAF directly results in cutaneous inflammation. Keratinocytes also synthesize PAF and related 1-acyl species in response to ionophores, cytokines and growth factors, and in response to activation of the epidermal PAF receptor. Since keratinocytes are routinely exposed to potential damage by thermal or oxidative stressors with resultant induction of cutaneous inflammation, the objective of these studies was to assess whether exogenous thermal or oxidative damage can induce the production of PAF and related 1-acyl species. Cells of the immortalized human keratinocyte cell line HaCaT were subjected to acute heat or cold, or treatment with the pro-oxidant lipid tertiary butyl hydroperoxide, and PAF and 1-palmitoyl-2-acetyl-GPC were measured by gas chromatography/mass spectrometry. We report that these diverse toxic stimuli resulted in the accumulation of these biologically active lipids. These studies suggest that the PAF system is involved in the inflammatory response seen following acute epidermal damage.**

Key words Platelet-activating factor · Oxidative stress · Keratinocytes · Mass spectrometry

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Introduction

Because of its ability to synthesize and release numerous proinflammatory and trophic mediators, our understanding of the role of the keratinocyte in cutaneous inflammatory states has changed from that of a passive bystander to that of an active participant. Indeed, human keratinocytes (HK) synthesize interleukin 1 (IL-1), IL-3, IL-8, IL-10 and IL-11 as well as tumor necrosis factor-alpha (TNF-α) (reviewed in reference 1). In addition to their ability to produce protein cytokines, HK can synthesize lipid mediators including hydroxy fatty acids, prostaglandins and platelet-activating factor (1-alkyl-2-acetyl-glycero-3-phosphocholine; PAF) [2–4].

Derived from glycerophosphocholines (GPCs), PAF is a potent activator of many cell types including platelets, monocytes, polymorphonuclear leukocytes, mast cells and vascular endothelium (reviewed in reference 5). PAF also has trophic effects on diverse cell types [6]. PAF interacts with a single G protein-linked transmembrane receptor (PAF-R) (reviewed in reference 7). PAF is the best-characterized ligand for the PAF-R, but other natural products can also bind to and signal through this receptor. These other ligands include oxidized phospholipids derived from low-density lipoproteins [8], lipopolysaccharide and protein A [9], lipotechoic acid moieties on *Streptococcus* species [10], and 1-acyl-2-acetyl GPCs [11, 12]. This diversity of ligands recognized by the PAF-R could potentially allow involvement of this system in a wide range of pathological conditions including oxidative damage and bacterial infection.

Recent studies suggest that the PAF system is involved in keratinocyte function and skin inflammation. PAF and 1-acyl PAF species are found in association with inflammatory skin diseases [12–15]; intradermal injections of PAF also induce inflammation [12, 16]. HKs synthesize both PAF and 1-acyl PAF species as well as express functional PAF-Rs [3, 4, 17, 18]. Activation of epidermal PAF-R leads to the production of PAF, prostaglandins, IL-6, IL-8, and the inducible form of cyclooxygenase (COX-2) [19].

It is not presently known whether the PAF system participates in the inflammation associated with acute epidermal damage. Using the immortalized human keratinocyte-derived cell line, HaCaT, we examined the ability of acute thermal or oxidative stress to induce PAF and 1-palmitoyl-2-acetyl GPC (PAPC) production. We report that HaCaT cells produce PAF and PAPC in response to these diverse noxious stimuli, suggesting that this family of lipid mediators could be involved in epidermal injury.

Materials and methods

Reagents and cells

Routine chemicals, PAF, and fatty acid-free bovine serum albumin (BSA) were obtained from Sigma (St. Louis, Mo.). Growth media and supplements were purchased from Life Technologies (Gaithersburg, Md.) and fetal bovine serum from Intergen (Purchase, N.Y.). The HaCaT cell line [20] was a kind gift from Dr. Petra Boukamp (German Cancer Research Center, Heidelberg, Germany), and was cultured as previously described [4].

Induction of keratinocyte damage

HaCaT cells were plated into 10-cm dishes 48 h before use and were 85–95% confluent. The medium was removed and the cells were washed three times with Hank's balanced salt solution (HBSS). For cold treatment, 1 ml HBSS with 0.25% BSA was added, and the cells were then placed into a freezer at –70 °C for 5 min. The plates were then removed from the freezer and placed in a Dubnoff incubator at 37 °C. Once the ice melted, the cells were allowed to incubate for various times and then harvested by the addition of 4 ml ice-cold ethanol. The plates were scraped and the contents placed into tubes containing 2 ng deuterium-labeled 1-hexadecyl and 1-palmitoyl *sn-2* acetyl GPC species. In some experiments, l ml HBSS with BSA was placed into the tubes along with 4 ml ethanol as a procedural blank.

The lipids were isolated and subjected to gas chromatography/mass spectrometry (GC/MS) as previously described [4, 17]. Briefly, water was added to the collected ethanol/water (4 : 1 v/v) to achieve a 1 : 1 v/v ratio, and the extract applied to a 1-ml Varian Bond Elut C18 column (Varian, Harbor City, Calif.) that had been prewashed with 5 ml ethanol followed by 5 ml water. The Bond Elut column was subsequently washed with 5 ml water and water/ethanol (1 : 1 v/v), and eluted onto a Supelclean LC-Si solid phase extraction column (Supelco, Bellefonte, Pa.) that had been prewashed with 8 ml ethanol. The LC-Si column was then washed with 5 ml ethanol, and the sample was eluted with 4 ml methanol/water (4 : 1 v/v). The eluate was taken to dryness in a vacuum centrifugal dryer, and the GPCs were hydrolyzed to diglycerides with 1 U phospholipase C (Sigma). The diglycerides were extracted with hexane and derivatized with pentafluorobenzoyl chloride, and analyzed by select ion monitoring GC/MS. Untreated plates of cells were trypsinized and counted with a particle counter (Coulter, Miami, Fl.). The limit of detection for PAF was approximately 0.1 ng/10^6 cells.

For acute heat injury, cells were incubated for 2 min in a waterbath at 90 °C, then incubated at room temperature for various times before harvesting. To mimic an oxidative stress injury, the cells were treated with 5 ml HBSS containing 100 μ*M* of the lipid pro-oxidant *t*-butyl hydroperoxide (t-BuOOH) for various times before harvesting. Experiments typically were conducted with duplicate samples.

Results

The effects of acute thermal injury on PAF production in HaCaT keratinocytes

Our first studies assessed the ability of acute cold injury to stimulate the accumulation of 1-hexadecyl PAF and PAPC in HaCaT cells. These species were measured because our previous studies had indicated that these are the major PAF molecules produced by HaCaT and primary cultures of HKs [4, 17]. To simulate a cold injury with rewarming, HaCaT cells were subjected to –70 °C for 5 min, then rewarmed to 37 °C to allow rapid thawing of the cells. This protocol resulted in essentially 100% cell death by 24 h as measured by studies using a viability dye test (trypan blue dye exclusion test). As shown in Fig. 1, treatment of HaCaT cells with this acute cold injury resulted in the significant accumulation of both PAF and PAPC. Cold-induced PAF accumulation was maximal at 2.5 min following rewarming, and declined over time. By 30 min PAF levels approached basal values. The accumulation of PAPC was similar to that of PAF, though concentrations of PAPC approached baseline levels by 10 min.

The next studies assessed the ability of acute heat stress to stimulate *sn-2* acetyl GPC biosynthesis in HaCaT cells. HaCaT cells were subjected to 90 °C for 2 min, followed by incubation at room temperature for various times. This also resulted in essentially 100% cell death by 24 h. As shown in Fig. 2, this acute heat injury resulted in the ac-

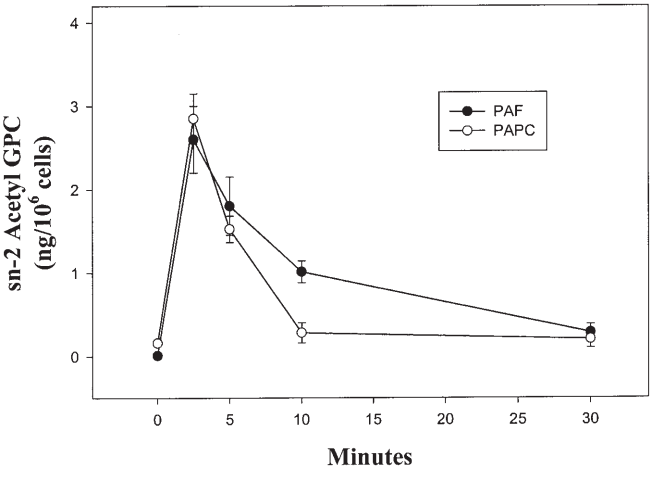

Fig. 1 The effect of acute cold stress on *sn-2* acetyl GPC biosynthesis in HaCaT cells. HaCaT cells were subjected to an acute cold stress as outlined in Materials and methods, and the amounts of 1-hexadecyl (PAF) and 1-palmitoyl (PAPC) *sn-2* acetyl GPC measured by GC/MS over time. Each point is the mean ± SD of the amounts of PAF/PAPC from five separate experiments

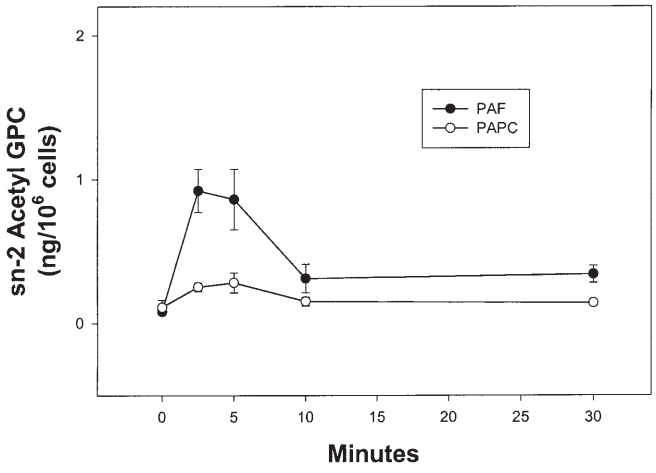

Fig. 2 The effect of acute heat stress on *sn-2* acetyl GPC biosynthesis in HaCaT cells. HaCaT cells were subjected to an acute heat stress as outlined in Materials and methods, and the amounts of 1-hexadecyl (PAF) and 1-palmitoyl (PAPC) *sn-2* acetyl GPC measured by GC/MS over time. Each point is the mean ± SD of the amounts of PAF/PAPC from three separate experiments

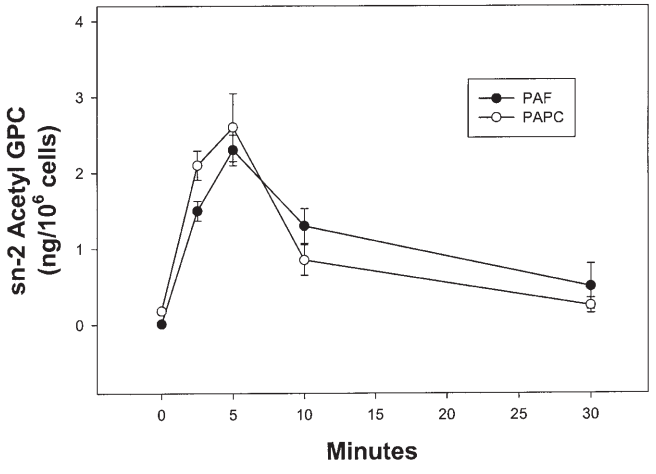

Fig. 3 The effect of t-BuOOH on *sn-2* acetyl GPC biosynthesis in HaCaT cells. HaCaT cells were treated with 100 μ*M* t-BuOOH and the amounts of 1-hexadecyl (PAF) and 1-palmitoyl (PAPC) *sn-2* acetyl GPC measured by GC/MS over time. Each point is the mean ± SD of the amounts of PAF/PAPC from four separate experiments

cumulation of these PAF species, though to a lesser extent than in our cold injury model. Significant amounts of PAF were measured 2.5 and 5 min following heat damage. By 30 min PAF levels approached basal values. Though the amounts of PAPC were increased over baseline, these values were not statistically significant (unstimulated, 0.11 ± 0.05 ng/10^6 cells; 2.5 min, 0.25 ± 0.05 ng/10^6 cells; 5 min, 0.28 ± 0.8 ng/10^6 cells; means ± SD). These studies indicate that acute thermal injury can result in the accumulation of these biologically active lipids in an HK cell line.

The effects of acute oxidative injury on PAF production in HaCaT cells

Keratinocytes are subjected to oxidative stress from both endogenous as well as exogenous sources. Exogenous sources include ultraviolet radiation as well as granulocytic cells as part of diverse inflammatory responses. Previous studies have demonstrated that lipid peroxidation is a potent stimulus for PAF production in endothelial cells [21]. To assess whether a pro-oxidative stimulus can stimulate *sn-2* acetyl GPC accumulation in keratinocytes, HaCaT cells were treated with 100 μ*M* of the lipid t-BuOOH for various times. This stimulus typically induces cell apoptosis in HaCaT cells within 8 h as measured by caspase-3 measurements and morphological studies [22]. Significant amounts of both PAF and PAPC were seen following t-BuOOH treatment (Fig. 3). Again, the pattern of *sn-2* acetyl GPC accumulation indicated maximal production at 2.5 and 5 min. By 30 min levels of both PAF and PAPC approached baseline values. These studies indicate that acute oxidative stress is a potent stimulus for keratinocyte *sn-2* acetyl GPC production.

Discussion

Acute keratinocyte damage resulting in the disruption of the cutaneous barrier function is seen in response both to inflammatory dermatoses and to exogenous toxic insults including thermal or chemical injury. At present, the mediators involved in inflammation associated with epidermal damage are not known. In these studies a very sensitive and specific GC/MS protocol was used to demonstrate that acute heat, cold, or pro-oxidative stress results in the accumulation of PAF and PAPC in a human keratinocyte-derived cell line. These findings provide evidence that the PAF system may be involved in the keratinocyte acute damage response.

HaCaT cells are a nontumorigenic human keratinocyte-derived cell line with many similarities to human basal keratinocytes [20]. Our previous studies have demonstrated that HaCaT cells synthesize PAF and 1-acyl PAF analogues in response to PAF-R activation, as well as after treatment with the peptide growth factor endothelin-1 or the calcium ionophore A23187 [6]. Similarly, we now report that thermal or oxidative damage can stimulate the production of PAF and PAPC. Of note, the levels of PAF generated by our acute cold or oxidative stress models approach the amounts seen following treatment of HaCaT cells with the potent stimulus A23187 (about 2.5 ng/10^6 cells) [4]. In view of the fact that PAF can exert its effects at picomolar concentrations, the levels of PAF seen in our in vitro models of acute damage could potentially have pathophysiological effects in vivo.

The role of the PAF system in keratinocyte function/cutaneous inflammation is not clear. However, the epidermal PAF-R can trigger the production of other proinflammatory and cytotoxic mediators including IL-6, IL-8, pro-

staglandin E_2 and TNF-α [19, 23]. Activation of the epidermal PAF-R also induces the production of PAF, suggesting a potential positive-feedback loop which could serve to augment the inflammatory and cytotoxic effects of agents that induce PAF production [4, 19]. The report by Ono et al. of protective effects of the PAF-R antagonist TCV-309 in a rabbit burn model [24] is consistent with the notion that PAF could be involved in thermal injury.

In summary, a sensitive and specific GC/MS protocol was used to demonstrate that acute thermal or oxidative injury can induce the production of PAF species. These findings have potential clinical significance given the potent proinflammatory and toxic effects of this family of lipid mediators. Toxic injuries such as thermal burns can be associated with significant morbidity and even mortality. Thus, an understanding of the mediators involved in epidermal cytotoxicity/cutaneous inflammation could result in improved treatment strategies. The finding that the PAF system is involved in these processes could serve as a rational basis for the use of inhibitors of PAF production/PAF-R to treat patients with acute toxic injuries.

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