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Epidermal Platelet-activating Factor Receptor Activation and Ultraviolet B Radiation Result in Synergistic Tumor Necrosis Factor-alpha Production

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Abstract

Ultraviolet B radiation (UVB) is a potent stimulator of epidermal cytokine production which has been implicated in photoaggravated dermatoses. In addition to cytokines such as tumor necrosis factor- α (TNF- α), UVB generates bioactive lipids including platelet-activating factor (PAF). Our previous studies have demonstrated that UVB-mediated production of keratinocyte TNF- α is in part due to PAF. The current studies use a human PAF-receptor (PAF-R) negative epithelial cell line transduced with PAF-Rs and PAF-R-deficient mice to demonstrate that activation of the epidermal PAF-R along with UVB irradiation results in a synergistic production of TNF- α . It should be noted that PAF-R effects are mimicked by the protein kinase C (PKC) agonist phorbol myristic acetate, and are inhibited by pharmacological antagonists of the PKC gamma isoenzyme. These studies suggest that concomitant PAF-R activation and UVB irradiation results in a synergistic production of the cytokine TNF- α which is mediated in part *via* PKC. These studies provide a novel potential mechanism for photosensitivity responses.

INTRODUCTION

Ultraviolet B radiation (290–320 nm; UVB) has profound effects on human skin. UVB exerts many of its effects through its ability to stimulate the production of bioactive proteins and lipids (1,2). Amongst the mediators produced by UVB is the lipid platelet-activating factor (1-O-alkyl-2-acetyl glycerophosphocholine, PAF). PAF is an inflammatory phospholipid mediator that exerts its effects through a single, specific G-protein-coupled receptor, the PAF receptor (3). The PAF receptor is expressed not only by cells of the innate immune system, but also by keratinocytes (4). The PAF-R is linked to numerous signal transduction pathways including activation of protein kinase C (PKC) and can stimulate the synthesis of PAF (4).

PAF is synthesized in response to diverse stimuli including cytokines, endotoxin and calcium ionophores (5). Of note, direct damage to keratinocytes by either heat or cold stimuli result in significant PAF production (6). PAF is produced enzymatically, yet PAF and *sn*-2 short-chained acyl glycerophosphocholines with PAF-R agonistic activity can also be produced *via* free radical-mediated damage (7). Through its ability to act as a potent pro-oxidative stressor (8), UVB has been demonstrated to trigger production of PAF and oxidized glycerophosphocholines (ox-GPC) with PAF-R agonistic activity (9–11).

Several lines of evidence have indicated that keratinocyte-produced tumor necrosis factor- α (TNF- α) is an important mediator of UVB-mediated cutaneous inflammation. UVB irradiation stimulates TNF- α production in keratinocytes *in vitro* and in skin *in vivo* (12,13). TNF- α has been implicated in cutaneous inflammation, especially in photosensitive disorders including lupus erythematosus and phototoxic reactions (14–16). Of significance, a TNF- α promoter polymorphism that greatly enhances the production of this cytokine has been associated with photosensitive forms of lupus erythematosus (17).

A previous report demonstrated that UVB irradiation together with IL-1 β treatment could result in a synergistic production of TNF- α in human fibroblasts (18). These authors found that the levels of TNF- α produced in response to a combination of IL-1 β with UVB were about 30 times more than either stimulus alone. In addition, a more recent study reported that IL-1 α along with UVB irradiation resulted in synergistic TNF- α production in human keratinocytes (19).

As either PAF-R activation or UVB alone can stimulate TNF- α production in epidermal cells (20), the objective of the present studies was to assess whether these two stimuli together can result in a synergistic TNF- α biosynthetic response. These current studies indicate that these stimuli induce exaggerated TNF- α production in human epithelial cells and murine epidermis, and implicate the enzyme PKC in this process. The ability of UVB to synergize with pro-inflammatory stimuli to result in large amounts of TNF- α production could suggest a potential mechanism for photoaggravated dermatoses or photosensitivity.

MATERIALS AND METHODS

Reagents

All PKC pharmacological antagonists were obtained from EMD/Calbiochem (San Diego, CA) except for Go6983 which was obtained from Alexis Biochemicals (San Diego, CA). All other chemicals were obtained from Sigma unless indicated otherwise.

Cellular studies

The human epidermoid cell line KB was grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% Fetal Clone III fetal bovine serum (Hyclone, Logan, UT). A KB PAF-R model system was created by transduction of PAF-R-negative KB cells with the MSCV2.1 retrovirus encoding the human leukocyte PAF-R as described previously (21). KB cells transduced with the PAF-R (KBP) or with control MSCV2.1 retrovirus (KBM) were characterized by Southern and Northern blot analysis and by radioligand binding and calcium mobilization studies to demonstrate that the PAF-R was functional (21).

Trypsinized KB cells were counted (with particle counter CC model Z1) and a total of 200 000 cells were seeded per well (of a 12-well plate Corning 3513) in 1 mL volume. Cells were incubated overnight in a 37°C, 5% CO₂ and 100% humid incubator. The next morning the cells were inspected under the microscope and media were replaced with a 1 mL media containing the final concentration of the agonist or the vehicle as a control. UVB treatment was preceded

by 30 min of pretreatment with the agonist (PMA or CPAF) or ethanol vehicle. Six hours later, cells were re-inspected under the microscope to estimate cellular apoptosis. The plates were then gently shaken to homogenously mix the cytokine contents of the supernatants that were aliquoted into prelabeled microcentrifuge vials and stored at -80°C for a later assay. Afterward, the vials were quickly thawed but kept cool, centrifuged for 20 s and then kept on ice. A human TNF- α EIA 96 well kit (#900-099; Assay Designs, Ann Arbor, MI) was used as instructed per the user manual and the results were read using the VERSAmax microplate reader (Molecular Devices) and SoftMax Pro 4.3.1 LS software utilizing the four-parameter analysis of the plate readout.

Murine studies

C57BL/6 mice (age 6–8 weeks) were purchased from the Jackson Laboratories (Bar Harbor, ME). PAF-R-deficient (PAF-R $^{-/-}$) mice described as previously were a kind gift of Professor Ishii (22). All mice were housed under specific pathogen-free conditions, and procedures were approved by the Animal Care and Use Committee of Indiana University School of Medicine.

The dorsal ears of anesthetized WT or PAF-R $^{-/-}$ mice were sham or UVB-irradiated (1200 J m^{-2}) with a Philips F20T12/UV-B lamp 15 min after administration of 100 ng CPAF in 50 μL PBS (intradermal injection), 10 μg PMA in 10 μL MeOH (topical painting) or vehicle alone. Four hours later, mice were sacrificed and the dorsal halves of ear skin were peeled off using forceps and stored in RNeasy (Qiagen, Valencia, CA) at 4°C until use.

Dorsal ear skin tissue was homogenized and total RNA was isolated using the RNeasy® mini kit (QIAGEN Sciences, Valencia, CA) as described by the manufacturer. cDNA were synthesized with ReactionReady First-Strand cDNA synthesis kit (SuperArray, Frederick, MD) and qRT-PCR was performed by the comparative threshold cycle (ΔC_T) method and normalized to 18S. The primers used for murine TNF- α and 18S were from SuperArray as previously described (23).

TNF- α mRNA measurements

RNA preparation, reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR) was performed as previously described (23). KB cells or epidermal tissue was homogenized and total RNA was isolated with the RNeasy mini kit (QIAGEN Sciences) as described by the manufacturer. RNA was reverse transcribed with ReactionReady™ First Strand cDNA synthesis kit (SuperArray). The quantitative real-time PCR was performed with the TNF- α primer obtained from SuperArray on 7500 Real Time® PCR system with 7500 Real Time® PCR system v.1.3 software (Applied Biosystems, Sunnyvale, CA) at 95°C for 10 min and 40 cycles of (95°C for 15 s and 60°C for 1 min) as described by the manufacturer. The expression levels of TNF- α were normalized to the expression levels of house keeping gene 18S.

Data analysis

Data from the murine studies are presented as mean $\pm$ standard error of the mean (SEM). Student's *t*-tests were used to assess statistical significance for differences in means. Significance was set at $P < 0.05$.

RESULTS

PAF-R activation and UVB result in synergistic cytokine production in KB cells

Both PAF-R activation and UVB irradiation of epidermal cells are known to induce production of cytokines including TNF- α (12,20). Thus, our first studies assessed whether the combination of PAF-R activation and UVB irradiation modulated the release of this cytokine using our KB

PAF-R model system (21). PAF-R-expressing KBP and control PAF-R-negative KBM cells were treated with 100 nM of the metabolically stable PAF-R agonist CPAF, 500 nM of the phorbol ester PMA, 500 nM of the calcium ionophore A23187, 600 J m⁻² UVB, or the combination of UVB with either CPAF, ionophore or PMA. At 6 h after final treatment, supernatants were collected and levels of TNF- α protein were measured by ELISA. As shown in Fig. 1, CPAF treatment alone stimulated cytokine production in KBP but not KBM cells, whereas PMA stimulated cytokine production in both cell types. It should be noted that ionophore A23187, at concentrations which induce a calcium mobilization response, did not significantly alter levels of these cytokines. As previously reported (20,23), UVB irradiation of KBP resulted in an augmentation of TNF- α in KBP over KBM cells. Stimulation of KB cells with both UVB and CPAF resulted in a tremendous augmentation of cytokine production only in KBP cells. UVB + PMA treatment resulted in similar effects in both KB types. However, combination of ionophore and UVB did not result in enhanced TNF- α production. It should be noted that the increased levels of the TNF- α release in response to UVB + PMA/CPAF were approximately 20-fold over the individual stimuli, indicating a synergistic response.

The next studies examined TNF- α mRNA levels in KB cells in response to CPAF, PMA, UVB and UVB + CPAF or PMA. As shown in Fig. 2, the levels of TNF- α mRNA levels followed the same qualitative trends as the protein measurements. As depicted in Fig. 3, the synergistic response of greatly increased levels of TNF- α mRNA production in KBP cells treated with CPAF + UVB appeared by 4 h. These findings suggest that the synergistic effect of PAF-R activation and UVB irradiation on cytokine production involves increased transcription.

UVB triggers synergistic production of cytokines *in vivo*

The next studies were designed to assess whether the synergistic effect of UVB + PAF-R or PKC activation could result in the synergistic production of cytokines *in vivo*. To that end, the ears of C57BL6 mice were treated with either intradermal injections of CPAF or BSA vehicle and then 15 min later irradiated with UVB. Four hours later the tissue was harvested and the epidermis removed, RNA isolated and subjected to qRT-PCR. As shown in Fig. 4, UVB + CPAF treatment resulted in an increased TNF- α mRNA expression in wild-type mice. It should be noted that in PAF-R-deficient mice CPAF did not have a significant effect on epidermal TNF- α mRNA levels (Fig. 4). However, topical application of PMA 15 min before UVB irradiation resulted in a synergistic TNF- α response in PAF-R-deficient mice. These studies indicate that UVB + PAF-R or PKC activation results in enhanced TNF- α cytokine levels *in vivo*.

Involvement of PKC in synergistic cytokine production

The phorbol ester PMA, a known direct activator of PKC mimicked the CPAF TNF- α production response in a PAF-R-independent fashion. This suggested the possibility that PKC mediated this PAF-R effect. Thus, we examined the ability of pharmacological inhibitors of various PKC isoenzymes to affect the UVB-CPAF synergistic response. The classical PKC isoenzymes (alpha [α], beta [β] and gamma [γ]) are Ca²⁺-dependent and can be activated by diacylglycerol. The novel isotypes, PKC delta (δ), PKC epsilon (ϵ), PKC eta (η) and PKC theta (τ), are Ca²⁺-independent, whereas the two atypical PKCs (zeta [ζ] and lambda [λ]) lack the Ca²⁺-binding region and are not activated by diacylglycerol (24,25). KB cells have been reported to express the α , γ , δ , ϵ , ζ , τ , λ and μ PKC isoenzymes (26). As shown in Table 1, pretreatment with 500 nM Ro-31-8425, 50 μ M Rottlerin (but not 5 μ M Rottlerin), 200 nM of UCN01 and 200 nM Go6983 all blocked the ability of CPAF + UVB and PMA + UVB to stimulate TNF- α protein production in KBP cells. It should be noted that all of the pharmacological inhibitors that were effective in blocking the synergistic production of TNF- α protein had in common the ability to inhibit the PKC ϵ isoenzyme. The use of the PKC inhibitor Rottlerin was particularly instructive as it had no inhibitory effect at 5 μ M, yet was a

potent inhibitor at 50 μM . It should be noted that the IC_{50} for the $\text{PKC}\gamma$ isoenzyme is 40 μM . Altogether, these studies suggest that PKC, most likely the $\text{PKC}\gamma$ isoenzyme, mediates the synergistic response seen with UVB and PAF-R activation.

DISCUSSION

The present studies indicate that activation of the PAF-R along with UVB irradiation results in the synergistic production of the primary cytokine $\text{TNF-}\alpha$. Exposure to a PAF-R agonist along with UVB irradiation resulted in a synergistic increase in $\text{TNF-}\alpha$ in both human KB cells as well as in murine skin. Given that $\text{TNF-}\alpha$ has potent effects on skin as well as systemic effects, these findings have possible clinical importance in photosensitive disorders.

UVB-mediated $\text{TNF-}\alpha$ production in epidermal cells has been reported to be regulated by both transcriptional and posttranscriptional mechanisms (27). With regard to the synergistic $\text{TNF-}\alpha$ seen in response to UVB + $\text{IL-1}\alpha$ in human keratinocytes, Bashir et al. recently reported that this treatment does not affect the half-life of $\text{TNF-}\alpha$ mRNA (19). Their findings indicate that enhanced gene transcription appears to be the major mechanism by which synergistic $\text{TNF-}\alpha$ production in response to UVB + $\text{IL-1}\alpha$ occurs.

The ability of the phorbol ester PMA to mimic PAF-R activation in its ability to combine with UVB to stimulate exaggerated $\text{TNF-}\alpha$ production suggests that the PAF-R exerts its effects *via* PKC activation. This is compatible with previous reports that the PAF-R is linked to PKC (28,29). That UVB along with both PAF-R and PKC activation was blocked by pharmacological inhibition of PKC also fits with this notion that PKC mediates this PAF-R effect.

The downstream transduction pathway(s) by which UVB radiation can synergize with PAF-R or PKC to induce cytokine production is unclear. Activation of the epidermal PAF-R is linked to numerous signal transduction pathways, including phospholipase A_2 , phospholipase C, phospholipase D, ERK and p38 MAP kinase cascades, nuclear factor kappa-B ($\text{NF}\kappa\text{B}$) and adenylate cyclase (21,30). Both molecular and pharmacologic strategies are being utilized to begin to define which signal transduction pathway(s) could be involved. Our previous studies have shown that PAF-R-mediated $\text{NF}\kappa\text{B}$ activation protects against proapoptotic stimuli including $\text{TNF-}\alpha$ through production of inhibitor of apoptosis proteins (31). Our ongoing studies using KBP cells transduced with the mutant $\text{I}\kappa\text{BM}$ that does not allow $\text{NF}\kappa\text{B}$ activation (31,32) suggest that the $\text{NF}\kappa\text{B}$ pathway is not appreciably involved in the synergistic cytokine response seen with UVB + PAF-R or PKC agonists (data not shown).

The significance of the present findings that UVB and PKC activation can result in a synergistic epithelial $\text{TNF-}\alpha$ response both *in vitro* as well as *in vivo* is unclear. It should be noted that PKC activation is a common downstream target for many cytokine receptors and other stimuli (24). Hence, PAF-R activation is probably not unique in its ability to synergize with UVB to stimulate $\text{TNF-}\alpha$ production. Inasmuch as $\text{TNF-}\alpha$ has been implicated in UVB cutaneous immune responses, especially photosensitivity, these findings could provide a novel mechanism by which UV could exert exaggerated inflammatory and immunomodulatory effects.

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Figure 1.

Effect of various stimuli along with UVB on TNF- α protein production in KB cells. PAF-R-expressing KBP and control PAF-R-negative KBM cells were treated with 100 nM of the metabolically stable PAF-R agonist CPAF, 500 nM of the phorbol ester PMA, 500 nM of the calcium ionophore A23187, 600 J m⁻² UVB, ethanol vehicle control (CON) or the combination of UVB 30 min after either vehicle, CPAF, ionophore or PMA. At 6 h after treatment, supernatants were collected and levels of TNF- α protein were measured by ELISA. The data represent mean $\pm$ SD TNF- α protein of duplicate samples from a representative experiment from four separate experiments.

Figure 2.

Effect of various stimuli along with UVB on TNF- α mRNA production in KB cells. PAF-R-expressing KBP and control PAF-R-negative KBM cells were treated with 100 nM of the metabolically stable PAF-R agonist CPAF, 500 nM of the phorbol ester PMA, 600 J m⁻² UVB, ethanol vehicle control (CON) or the combination of UVB 30 min following either CPAF or PMA. At 4 h after treatment, mRNA was isolated and qRT-PCR for TNF- α and 18S performed. The data represent mean amount of TNF- α mRNA relative to 18S $\pm$ SD of duplicate samples from a representative experiment from three separate experiments.

Figure 3.

Time course of CPAF along with UVB on TNF- α mRNA production in KBP cells. PAF-R-expressing KBP cells were treated with ethanol vehicle or 100 nM of the metabolically stable PAF-R agonist CPAF followed by sham or 600 J m⁻² UVB 30 min later. At various times, mRNA was isolated and qRT-PCR for TNF- α and 18S mRNA performed. The data represent mean amount of TNF- α mRNA relative to 18S $\pm$ SD of duplicate samples from a representative experiment from two separate experiments.

Figure 4.

Effect of CPAF along with UVB on TNF- α mRNA production in murine epidermis. The dorsal ears of wild-type and PAF-R^{-/-} mice were treated with either intradermal injection of 50 μ L of either CPAF or 0.5% ethanol control in PBS. In some experiments PAF-R^{-/-} mice were treated with topical application of 10 μ g of PMA in 10 μ L or ethanol vehicle on the dorsal ears. Fifteen minutes following the treatments, the ears were treated with 1200 J m⁻² UVB. Four hours later mRNA was extracted from the auricular epidermis, and homogenized and total RNA was isolated. RNA was reverse transcribed and qRT-PCR performed. The expression levels of TNF- α mRNA were normalized to the expression levels of 18S. The data listed are the mean $\pm$ SEM normalized TNF- α mRNA from duplicate samples from $n = 6-8$ mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ in comparison with vehicle-treated skin.

Table 1

Effect of PKC inhibitors on TNF- α production induced by CPAF + UVB or PMA + UVB in KBP cells. KBP cells were pretreated for 30 min with the various PKC inhibitors or ethanol vehicle control before addition of 100 nM of the metabolically stable PAF-R agonist CPAF or 500 nM of the phorbol ester PMA. Thirty minutes later the cells were treated with 600 J m⁻² UVB. At 6 h after treatment, supernatants were collected and levels of TNF- α protein were measured by ELISA and adjusted per cell number. The data represent mean $\pm$ SEM percentage of stimulated TNF- α protein from two separate experiments using duplicate samples.

PKC inhibitor and dosage (in μ M)	PKC inhibitor isoenzyme specificity (IC ₅₀ in μ M)	CPAF + UVB (% stimulated)	PMA + UVB (% stimulated)
Ro31-8425 (0.5)	α (0.008), β I- β II (0.008), γ (0.014), ϵ (0.039)	36 $\pm$ 12	3 $\pm$ 1
Rottlerin (5)	α (30), β (30), γ (40), δ (3)	101 $\pm$ 6	102 $\pm$ 5
Rottlerin (50)	α (30), β (30), γ (40), δ (3)	4 $\pm$ 1	35 $\pm$ 11
UCN01 (0.2)	α (0.03), β I (0.034), γ (0.03), ϵ (0.04)	76 $\pm$ 15	62 $\pm$ 5
Go6983 (0.2)	α (0.007), β (0.007), γ (0.006), δ (0.01), ζ (0.06)	61 $\pm$ 15	8.5 $\pm$ 2
Go6976 (0.2)	α (0.002), β I (0.06), ρ (0.02)	114 $\pm$ 8	86 $\pm$ 17
PKC- β inh (0.2)	α (0.331), β I (0.021), β II (0.005)	91 $\pm$ 9	113 $\pm$ 8
PKC- ϵ inhibitory peptide (10)	ϵ (1)	86 $\pm$ 16	96 $\pm$ 6

PKC = protein kinase C; TNF- α = tumor necrosis factor- α .