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Acute ethanol exposure augments low dose UVB-mediated systemic immunosuppression via enhanced production of Platelet-activating factor receptor agonists.

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Keywords

UVB; platelet-activating factor; immunosuppression

To the Editor: Platelet-activating factor (1-alkyl-2-acetyl glycerophosphocholine; PAF) is a lipid-derived mediator with diverse functions (Shimizu, 2009). Glycerophosphocholines (GPCs) from cell membranes with unsaturated *sn-2* fatty acids can undergo oxidation resulting in the formation of oxidized GPCs (Ox-GPCs) which can act as potent agonists for the PAF receptor (PAF-R) (Konger et al, 2008). Many environmental pro-oxidative stressors from cigarette smoke to ultraviolet B radiation (UVB) can induce systemic immunosuppression via generation of Ox-GPC PAF-R ligands (Sahu et al, 2013, Walterscheid et al, 2002, Wolf et al, 2006, Yao et al, 2009, Zhang et al, 2008). Of interest, UVB-generated PAF-R ligands also augment experimental melanoma tumor growth by suppressing anti-tumor immunity in a process involving mast cells and regulatory T cells (Damiani and Ullrich, 2016; Sahu et al, 2012).

Recently our group has demonstrated that ethanol (EtOH) exposure results in an augmentation of enzymatic PAF synthesis in response to thermal burn injury, in a process involving cytosolic phospholipase A_2 (Harrison et al., 2018). The present study was designed to test the hypothesis that EtOH exposure could generate increased levels of PAF in

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Author Contributions

JBT, JW, JO, CB, CMR, RPS performed experiments and data analysis. JBT supervised the study, and JBT and RPS wrote the manuscript.

Conflict of Interest

The authors state no conflict of interest

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response to UVB, and that UVB will be more immunosuppressive when EtOH intoxicated. This is a relevant concern as the immunosuppressive effects of UVB contribute to (Damiani and Ullrich, 2016), and EtOH exposure is linked to an increased incidence of non-melanoma skin cancer (Fung et al., 2002; Freedman et al., 2003; Jensen et al., 2012).

First, to evaluate whether ethanol can augment UVB-mediated production of PAF-R ligands, we pre-incubated the human keratinocyte-derived cell line HaCaT with 1% EtOH for 30 min, then treated with 1000 J/m^2 UVB using our Philips unit as previously described (Marathe et al., 2005; Yao et al, 2009). Ten minutes after UVB treatment, the lipids were extracted as previously described (Marathe et al., 2005) using the Bligh & Dyer method (Bligh and Dyer, 1959). As multiple glycerophosphocholine species can act as PAF-R agonists, we quantified total PAF-R biochemical activity using PAF-R-expressing KBP cells that produce IL-8 when the receptor is activated (model in Figure 1a) (Pei et al, 1998; Harrison et al., 2018). KBP and PAF-R-negative KBM cells were exposed to lipid extracts from HaCaT cells treated with sham, UVB, EtOH alone, or EtOH + UVB and incubated for 6 hours. The KBP cells were also treated with 1 nM of the metabolically stable PAF-R agonist carbamoyl PAF (CPAF) or 0.1% EtOH as vehicle for positive and negative controls. As shown in Figure 1b, lipid extracts derived from HaCaT cells treated with EtOH + UVB resulted in increased levels of IL-8 release from KBP cells. Yet, this fluence of UVB alone resulted in only a minimal level of increased IL-8 release in KBP cells (which was not statistically significant), and none of the lipid extracts triggered IL-8 release in PAF-R-negative KBM cells with 10 nM of the phorbol ester phorbol myristic acetate used as a positive control (*data not shown*). Of interest, structural analysis of PAF-R agonistic activity following the combination of EtOH + UVB in HaCaT cells by mass spectrometric analysis revealed only enzymatically-generated PAF agonists 1-hexadecyl-2-acetyl GPC and 1-octadecyl-2-acetyl-GPC, not significant amounts of Ox-GPCs (*data not shown*), which we have described are generated following higher fluences of UVB (Marathe et al., 2005). Previously, we have demonstrated that topical application of 20% EtOH to de-identified discarded human skin explants derived from surgical contouring procedures (Exempted study, Wright State University) 30 minutes before a thermal burn injury results in an augmentation of PAF production (Harrison et al., 2018). Using this protocol, we tested if topical EtOH pretreatment can increase PAF following UVB treatment. Of importance, we used a fluence of UVB (1000 J/m^2) that we have previously reported results in only a small reproducible increase in PAF activity (Travers et al., 2010). Ten minutes post UVB/sham, the epidermis was curetted, weighed and lipids extracted. As shown in Figure 1c, pretreatment of skin with EtOH resulted in an exaggerated amount of UVB-generated PAF. We next tested if EtOH intoxication can increase the ability of UVB to generate PAF-R agonists in murine skin in vivo. These studies used a previously published murine model of intraperitoneal injection of 2.4g/kg (approximately 400 μl of 20%) EtOH (Faunce et al., 1997; Harrison et al., 2018) into anesthetized C57BL6 mice followed 30 minutes later by UVB treatment (2500 J/m^2) of an area of 2×2 cm^2 of shaved lower back skin. All animal studies were approved by the institutional review boards of Indiana University and Wright State University. Ten minutes following sham or UVB treatment, the mice were euthanized, and the epidermal skin was removed using a curette and weighed, and lipids extracted. As depicted in Figure 1d, lipid extracts from the skin of UVB-treated intoxicated mice

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generated more PAF-R activity than saline-treated counterparts. Again, none of these lipid extracts from human nor murine skin generated IL-8 release in KBM cells (*data not shown*).

As UVB induces systemic immunosuppression (as measured by inhibition of delayed type hypersensitivity reactions to Candida antigen, or contact hypersensitivity reactions to the allergen dinitrofluorobenzene [DNFB]) in a PAF-R-dependent manner (Walterscheid et al 2002; Zhang et al., 2008), our finding that EtOH augmented UVB-induced PAF production prompted examination of the ability of EtOH intoxication on UVB-induced systemic immunosuppression. To assess the effects of the injury on immune competence, wild-type mice received various fluences of UVB (1000–5000 J/m^2) 30 minutes following EtOH or saline treatment, or control treatments and were then subjected to the well-established delayed-type hypersensitivity protocol (Zhang et al., 2008; Sahu et al., 2013). Briefly, 5 days after treatments, the mice were sensitized with the chemical DNFB applied topically to the shaved non-UVB treated part of the upper back (to assess for systemic immunosuppression) and challenged 9 days later with DNFB applied to the ears. The intensity of the immune response to DNFB was measured by change in the ear thickness before and 24 hours after the delayed challenge. Animals were also injected with various doses of the CPAF or histamine as controls for PAF-R dependent and independent immunosuppression, respectively. PAFR-deficient mice (*Ptafr–/–*) mice only received the high dose of 5000J/m^2, since we have previously reported that these mice do not respond to UVB with systemic immunosuppression (Zhang et al., 2008). As shown in Figure 2a, intoxicated wild-type mice responded to lower fluences of UVB with decreased elicitation responses to DNFB as compared to saline-treated counterparts. Intoxication had no effect alone, nor did it augment the immunosuppressive effects of CPAF. In contrast, UVB and CPAF did not exert immunosuppressive responses in saline nor EtOH treated *Ptafr–/–* mice, yet histamine treatment resulted in a decreased DNFB response (Figure 2b). Application of DNFB to unsensitized wild-type mice only resulted in approximately 10% of the ear thickness reactions found in sensitized mice, and addition of EtOH did not modulate this irritant effect (*data not shown*). These studies indicate that EtOH intoxication can augment the immunosuppressive effects of UVB through augmented PAF generation.

Alcohol exerts profound effects in humans. The combination of EtOH and UVB is common, and studies have described alcohol use as a risk factor for skin cancer (Fung et al., 2002; Freedman et al., 2003; Jensen et al., 2012). The present studies demonstrate that EtOH exposure can increase PAF synthesis in response to UVB, and that an outcome of this clinically relevant combination is augmented UVB-mediated systemic immunosuppression leading to increased risks of EtoH-associated morbidity and skin cancer.

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Abbreviations used in this paper:

CHS contact hypersensitivity

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WT	wild type
PAF-R KO	PAF-R-deficient mice
KBP	PAF-R-expressing human epithelial KB cell line
KBM	PAF-R-non-expressing human epithelial KB cell line
COX-2	cyclooxygenase type 2
UVB	ultraviolet B
IL-8	interleukin-8
CPAF	1-hexadecyl-2-*N*-methylcarbamoyl glycerophosphocholine
DNFB	dinitrofluorobenzene
PAF	platelet-activating factor
GPC	glycerophosphocholine
Ox-GPC	oxidized GPC
PAF-R	PAF receptor
i.p.	intraperitoneal injection
s.c.	subcutaneous injection

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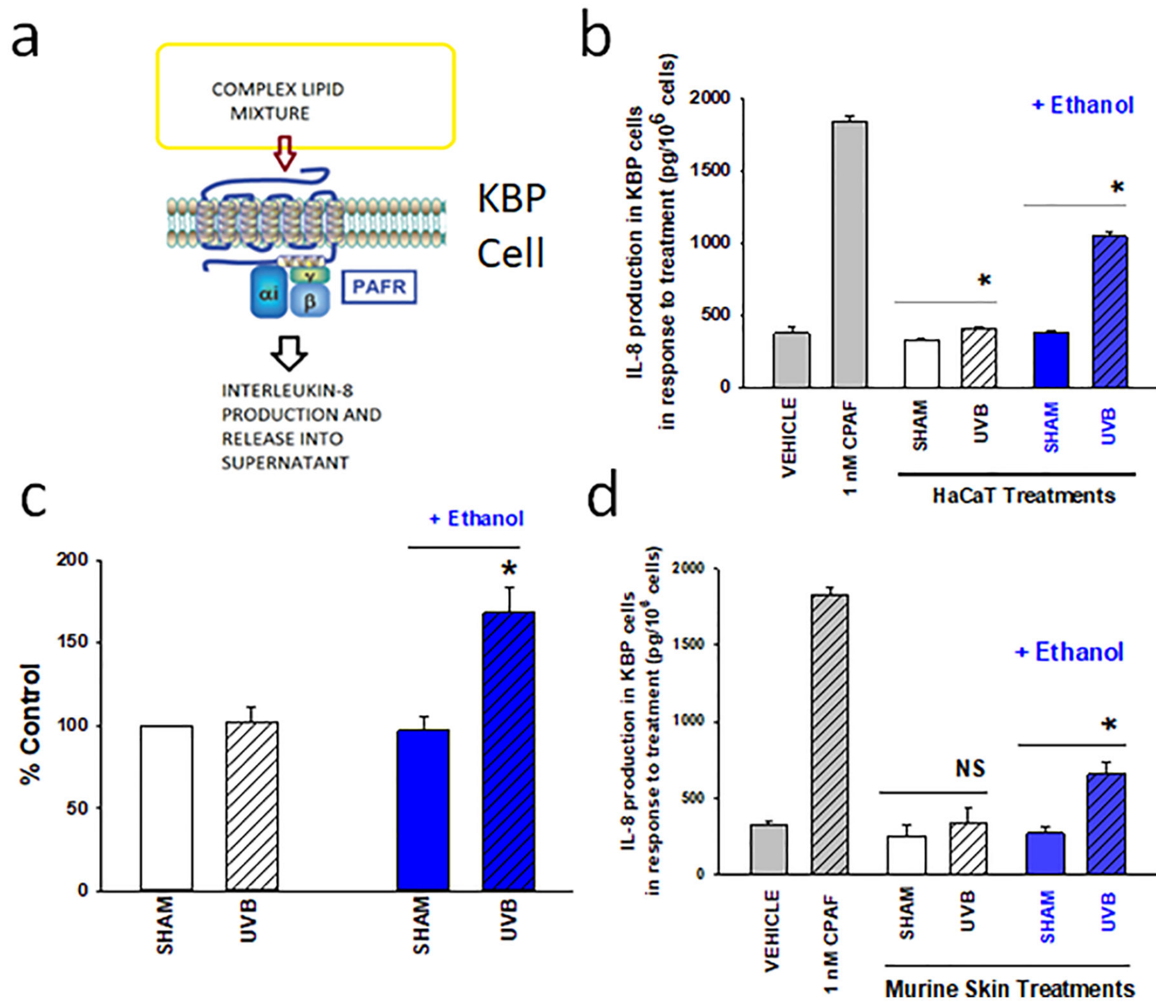

Figure 1: EtOH exposure augments PAF-R agonistic activity in response to UVB in HaCaT cells in vitro, human skin ex vivo, and murine skin in vivo.

(a) Model system of KBP-IL-8 assay where a complex mixture (lipid extracts) containing PAF agonists are incubated with PAF-R-positive KBP cells or PAF-R negative KBM cells and IL-8 released into medium measured as a surrogate for PAF-R activation, using PAF-R agonist CPAF as a positive control. (b). HaCaT cells were treated with 1000 J/m^2 UVB or sham-treated either under normal conditions or following a 30-minute pre-incubation with 1% EtOH. Ten minutes post-treatment, the lipids were extracted and normalized to 5 × 106 HaCaT cells. (c) Human skin explants were treated with topical solution of 20% EtOH in PBS (100 μl per 2 × 2 cm^2 area) for 30 minutes, then treated with sham or 1000 J/m^2 UVB and 10 minutes post-treatment epidermis harvested, weighed and lipids extracted, and normalized to 25mg of skin. (d) The shaved dorsal back skin of groups of 5–6 anesthetized C57BL6 mice were subjected to sham or 2500 J/m^2 UVB treatment after a 30 minute exposure to intraperitoneal PBS or 2.4g/kg EtOH (blue). Ten minutes following sham/UVB treatment to mice, the skin was harvested and lipids extracted and normalized to 10mg weight of epidermis. The data are: (b) mean ± SE IL-8 production in KBP cells (pg/10^6 KBP cells) from three separate experiments; (c) mean ± SE % control human skin PAF-R agonistic activity from four separate experiments; (d) mean ± SD IL-8 production in KBP

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cells (pg/106 KBP cells) from 5–6 mice in each group. *Denotes statistically significant ($P < 0.05$) changes by Student *t* test using Prism 6.

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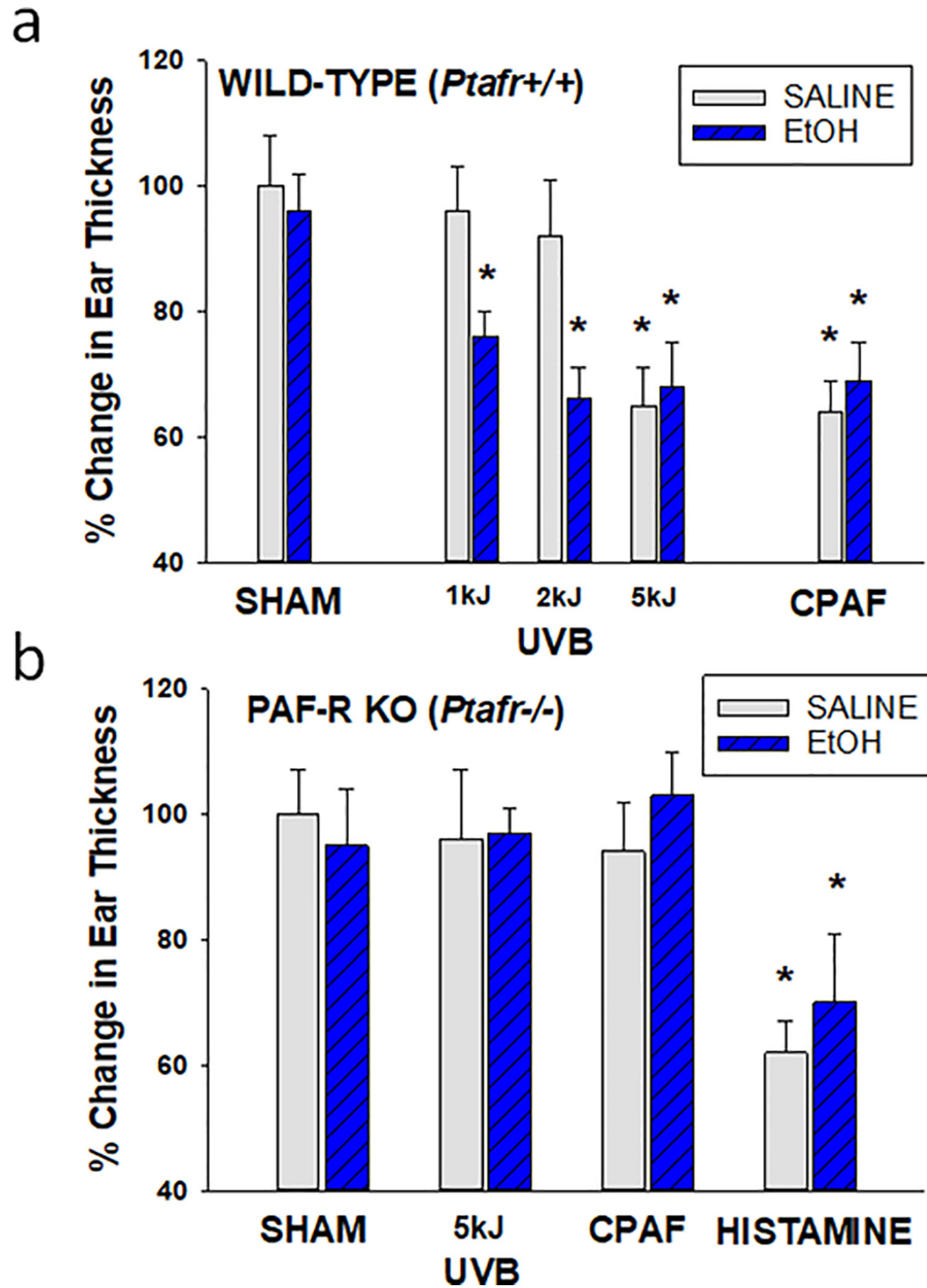

Figure 2: Combining EtOH and UVB results in an enhanced inhibition of contact hypersensitivity in a PAF-R-dependent manner.

Wild-type (*Ptafr+/+*) (a) or PAF-R-deficient (*Ptafr−/−*) (b) mice underwent treatment with either vehicle or EtOH, then 30 min later subjected to UVB or Sham treatment. Some mice were treated with 250ng CPAF i.p. or 200 μg Histamine (HIS) s.c. as positive controls for inhibition of contact hypersensitivity reactions to allergen DNFB (systemic immunosuppression). Five days later the mice were sensitized on non-irradiated back skin with DNFB, and 9 days later ear elicitation reactions to DNFB were obtained. The data are the Mean ± SE % change in ear thickness of the elicitation reactions obtained from sham-

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treated animals at 24 hours using 7–9 mice in each group, totaled from smaller experiments using 3 mice per group. *Denotes significant ($P<0.05$) differences between sham control and EtOH exposed by Student *t* test using Prism 6. Please note only Histamine treatment resulted in an inhibition of CHS reactions to DNFB in *Ptafr–/–* mice.