

# Platelet-activating Factor Receptor Agonists Mediate Xeroderma Pigmentosum A Photosensitivity\*

Received for publication, December 9, 2011, and in revised form, January 31, 2012. Published, JBC Papers in Press, February 1, 2012, DOI 10.1074/jbc.M111.332395

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**Background:** Deficiency of the nucleotide excision repair protein xeroderma pigmentosum type A (XPA) is characterized by photosensitivity.

**Results:** XPA-deficient cells and mice generate increased platelet-activating factor (PAF), oxidized glycerophosphocholines, and skin inflammation following ultraviolet B radiation with the skin inflammation blocked by PAF antagonists.

**Conclusion:** PAF-R signaling mediates photosensitivity seen in XPA deficiency.

**Significance:** These studies provide important insights into the mechanisms of photosensitivity.

To date, oxidized glycerophosphocholines (Ox-GPCs) with platelet-activating factor (PAF) activity produced non-enzymatically have not been definitively demonstrated to mediate any known disease processes. Here we provide evidence that these Ox-GPCs play a pivotal role in the photosensitivity associated with the deficiency of the DNA repair protein xeroderma pigmentosum type A (XPA). It should be noted that XPA-deficient cells are known to have decreased antioxidant defenses. These studies demonstrate that treatment of human XPA-deficient fibroblasts with the pro-oxidative stressor ultraviolet B (UVB) radiation resulted in increased reactive oxygen species and PAF receptor (PAF-R) agonistic activity in comparison with gene-corrected cells. The UVB irradiation-generated PAF-R agonists were inhibited by antioxidants. UVB irradiation of XPA-deficient (*Xpa*<sup>-/-</sup>) mice also resulted in increased PAF-R agonistic activity and skin inflammation in comparison with control mice. The increased UVB irradiation-mediated skin inflammation and TNF- $\alpha$  production in *Xpa*<sup>-/-</sup> mice were blocked by systemic antioxidants and by PAF-R antagonists. Structural characterization of PAF-R-stimulating activity in UVB-irradiated XPA-deficient fibroblasts using mass spectrometry revealed increased levels of *sn*-2 short-chain Ox-GPCs along with native PAF. These studies support a critical role for PAF-R agonistic Ox-GPCs in the pathophysiology of XPA photosensitivity.

Deficiency of the xeroderma pigmentosum type A (XPA)<sup>3</sup> protein is characterized clinically by neurodegeneration, pho-

tosensitivity, and an increased risk of developing skin cancers due to the defect in nucleotide excision repair of ultraviolet (UV) irradiation-induced DNA lesions (1, 2). XPA plays a central role in nucleotide excision repair by recognizing damaged DNA and orchestrating its excision (3–5). The affinity of the DNA-binding domain of XPA for DNA double strand-single strand junctions is thought to allow it to verify damaged DNA, prevent excessive DNA unwinding, and stabilize the preincision complex for the endonucleases XPF and XPG (4). XPA is also needed for the damage incision (6–9). Nucleotide excision repair has been shown to be involved in the removal of certain types of oxidatively mediated DNA damage (3, 10). Of interest, XPA-deficient cells have been shown to display increased susceptibility and reduced repair capacity when subjected to DNA damage induced by pro-oxidative stressors (11–14). In particular, XPA-deficient cells have been reported to have lower levels of the antioxidant enzyme catalase (12, 13).

Ultraviolet B (UVB; 290–320 nm) radiation found in sunlight exerts profound effects on human skin ranging from vitamin D metabolism to skin aging and carcinogenesis (15, 16). UVB exposure is the major cause for non-melanoma skin cancer, the most common type of human cancer. In addition to its ability to damage DNA, UVB irradiation is well known to exert an immunosuppressive effect by inhibiting cell-mediated immune responses that are indispensable for antitumor immunity (17, 18). UVB irradiation also triggers skin inflammation, and diverse diseases ranging from systemic lupus erythematosus to XPA exhibit abnormal responsiveness to this environmental stimulus (19, 20).

Platelet-activating factor (PAF; 1-alkyl-2-acetyl-glycerophosphocholine) is a potent inflammatory lipid mediator that exerts

\* This work was supported, in whole or in part, by National Institutes of Health Grants HL062996 (to J. B. T. and R. L. K.) and HL034303 (to R. C. M.). This work was also supported by grants from the Showalter Memorial Foundation, the Riley Memorial Association, and Veterans Affairs (to J. B. T.).

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<sup>3</sup> The abbreviations used are: XPA, xeroderma pigmentosum type A; CID, collision-induced dissociation; CPAF, 1-hexadecyl-2-N-methylcarbamoyl

glycerophosphocholine; GPC, glycerophosphocholine; MRM, multiple reaction monitoring; Ox-GPC, oxidized glycerophosphocholine; PAF, platelet-activating factor; PAF-R, PAF receptor; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; XPC, xeroderma pigmentosum complementation group C; ROS, reactive oxygen species; CM-H<sub>2</sub>DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester.

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its effects through a single specific G-protein-coupled receptor, the PAF receptor (21). PAF is synthesized enzymatically in response to diverse stimuli, including cytokines, endotoxin,  $\text{Ca}^{2+}$  ionophores, and PAF itself (22–25). In addition, PAF and *sn*-2 short-chain acyl glycerophosphocholines (GPCs) with PAF receptor (PAF-R) agonistic activity can also be produced through free radical-mediated cleavage of membrane GPC (26–29). PAF and oxidized glycerophosphocholines (Ox-GPCs) with PAF activity have been implicated in UVB irradiation-mediated skin inflammation and the systemic immunosuppression known to be a major cause for skin cancers (30–33). Ox-GPCs have been identified in association with other experimental conditions, including chronic alcohol use or cigarette smoke exposure in rodents (34, 35). However, the production of biologically active Ox-GPCs with PAF-R agonistic activity has not been definitively linked to photosensitivity or for that matter shown to cause any other particular disease state.

Because PAF is a potent trigger for skin inflammation, including the production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) associated with photosensitivity (36–39), and there is evidence that XPA-deficient cells have decreased antioxidant defenses (11–14), we hypothesized that the PAF system could be involved in the photosensitivity associated with XPA deficiency. In the present study, we investigated the role of the PAF system in the photosensitivity of XPA using XPA-deficient cells and mice.

### EXPERIMENTAL PROCEDURES

**Reagents and UVB Irradiation Source**—All chemicals were obtained from Sigma-Aldrich unless indicated otherwise. The pan-caspase inhibitor Z-VAD-fmk was from Promega. The UV source was a Philips F20T12/UVB lamp (270–390 nm; containing 2.6% UVC, 43.6% UVB, and 53.8% UVA); a Kodacel membrane was used to remove UVC irradiation (36, 40). The intensity of the UVB source was measured before each experiment using an IL1700 radiometer and an SED240 UVB detector (International Light) at a distance of 8 cm from the UVB source to the monolayer of cells/mice. All chemicals used in the irradiation protocols were first tested to ensure there was no ability to absorb UVB irradiation (*i.e.* sunblock effect) by testing the intensity of UVB irradiation (as measured by the detector) underneath a Kodacel membrane with/without application of the dose used in the *in vitro* or *in vivo* protocol.

**Cells and Mice**—XPA-deficient (GM04312) and XPA gene-corrected (GM1587), and xeroderma pigmentosum complementation group C (XPC)-deficient (AG3965) human fibroblast cell lines were obtained from Coriell Cell Repositories of Coriell Institute (Camden, NJ) and grown according to the manufacturer's protocols. Briefly, XPA- and XPC-negative cells were grown in minimum Eagle's medium with 10% FBS, 2 mM glutamine, 100 IU penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin under a 5%  $\text{CO}_2$  atmosphere. XPA-corrected cells were grown in DMEM-high glucose with 10% FBS, 2 mM glutamine, 100 IU penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin under an 8%  $\text{CO}_2$  atmosphere. The human epidermoid cell line KB was grown in DMEM supplemented with 10% FBS (Intergen), 2 mM L-glutamine, and 100 IU penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. 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 receptor as described previously (41). KB cells stably transduced with the PAF receptor (designated as KBP cells) or with control MSCV2.1 retrovirus (defined as KBM cells) were characterized by Southern blot, Northern blot, radioligand binding, and calcium transient studies, which demonstrated the presence of a functional PAF-R receptor signaling system in the KBP but not KBM cells. SKH-1 hairless albino mice (age, 6–8 weeks) were purchased from Charles Rivers Laboratories. *Xpa*<sup>-/-</sup> mice on an SKH-1 background were kindly provided by Dr. W. Glenn McGregor (Department of Pharmacology, University of Louisville, Louisville, KY). PAF-R-null (*Ptafr*<sup>-/-</sup>) mice, originally on a C57BL/6 background (32, 42), were backcrossed seven generations onto the SKH-1 background. All mice were housed under specific pathogen-free conditions at the Indiana University School of Medicine. All procedures were approved by the Indiana University School of Medicine Animal Care and Use Committee.

**Lipid Extraction and Measurement of PAF-R Activity**—Cells grown on 10-cm plates were fed with 5  $\mu\text{M}$  1-hexadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (Avanti) overnight and then washed three to four times with 10 ml of Hanks' balanced salt solution before being UVB-irradiated as described previously (28, 40). Following UVB treatment, the reactions were quenched with ice-cold methanol, and total lipids were extracted using the method of Bligh and Dyer (43). In some experiments, the lipid extracts were treated with PAF acetylhydrolase (10 mg), phospholipase A<sub>1</sub> (5 mg), or PBS overnight at 37 °C, and then lipids were re-extracted (40). The presence of PAF-R agonists in the lipid extracts was measured by their ability to induce an intracellular  $\text{Ca}^{2+}$  mobilization response in KBP cells as described previously (40, 44). In brief, KBP cells were preloaded with the  $\text{Ca}^{2+}$ -sensitive indicator Fura-2-AM (4  $\mu\text{M}$  in Hanks' balanced salt solution) at 37 °C for 90 min followed by washing and resuspending, and cells were maintained in Hanks' balanced salt solution at room temperature before use. Lipid extracts dissolved in ethanol (adjusted to  $2.5 \times 10^6$  cells/20  $\mu\text{l}$ ) were added to an aliquot of KBP cells ( $1.0$ – $1.5 \times 10^6$  cells/2.5 ml) in a cuvette at 37 °C with constant stirring.

Fura-2-AM fluorescence was monitored in a Hitachi F-4010 spectrophotometer with excitation and emission wavelengths of 331 and 410 nm, respectively. The  $\text{Ca}^{2+}$  influx was calculated as described (41) and shown as the percentage of maximal peak calcium flux induced by 1  $\mu\text{M}$  CPAF. To measure the production of PAF-R agonist *in vivo*, back skin of female mice was UVB-irradiated at 1500 J/m<sup>2</sup> under anesthesia. One hour post-UVB treatment, mice were euthanized, and after freezing the skin with liquid nitrogen, the epidermal part of the skin was scraped off and weighed, and lipids were extracted (40, 44). In some experiments, mice were given chow containing 10g/kg vitamin C (Research Diets) *ad libitum* for 10 days (35, 40) before UVB irradiation.

**ROS Measurements**—Intracellular levels of ROS were analyzed by flow cytometry using CM-H<sub>2</sub>DCFDA (Invitrogen) as a fluorescent dye probe (40). Cells loaded with CM-H<sub>2</sub>DCFDA (5  $\mu\text{M}$  for 30 min) were UVB-irradiated after a recovery time of 45

min. In some experiments, cells were pretreated for 30 min with vitamin C (2.5 mM), *N*-acetylcysteine (5 mM), Z-VAD-fmk (10  $\mu$ M), or DMSO vehicle (0.3%) before UVB exposure. Cells without dye loading were used as negative controls. Flow cytometric analysis was performed using a FACSCalibur flow cytometer, and data were analyzed using CellQuest software (BD Biosciences) and are presented as mean fluorescence intensity.

**Inflammation Measurements**—Murine skin inflammation following UVB treatment of the ears was assessed by measurement of ear thickness using calipers (Mitutoyo) before and at various times following UVB irradiation (1500 J/m<sup>2</sup>) of the dorsal ear of anesthetized mice. Ear tissue was removed and bisected. Part was submitted for formalin fixation and paraffin-embedding for H&E staining and TNF- $\alpha$  immunohistochemistry, respectively, as described previously (38, 45). Part of the ear tissue was also placed in RNAlater (Ambion) for measurement of TNF- $\alpha$  mRNA levels as described previously (32). In brief, total RNA was isolated from ear tissue, and cDNAs were synthesized. Quantitative RT-PCR was performed by the comparative threshold cycle ( $\Delta$ CT) method for TNF- $\alpha$  and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Mass Spectrometry Studies**—Following irradiation, lipids were extracted from 4–6  $\times$  10<sup>7</sup> cells using the method of Bligh and Dyer (43). This total lipid extraction was followed by a silica solid phase extraction to isolate the oxidized phosphatidylcholines and eliminate the majority of other lipids. The silica solid phase extraction was carried out as described (46). Butylated hydroxytoluene was added (50  $\mu$ l of 10  $\mu$ M) to the 4 ml of eluate to prevent further oxidation of the isolated GPCs during shipping or storage. The methanol:water (4:1) eluates from solid phase extraction of samples were packed with dry ice and shipped overnight for analysis. Upon arrival, the samples were stored at –70 °C or immediately evaporated to dryness/near dryness under a vacuum in a Savant SpeedVac Plus (SC110A) using the lowest heat setting. The residue was transferred with two 100- $\mu$ l ethanol rinses to a 1-ml glass vial, evaporated again, reconstituted in 100  $\mu$ l with 25% aqueous methanol, and crimp-capped for HPLC/MS/MS analysis.

PAF and PAF analogs were quantitated using HPLC/MS/MS in multiple reaction monitoring mode with 5 ng of 1-*O*-hexadecyl-2-[2,2,2-<sup>2</sup>H<sub>3</sub>]acetoyl-*sn*-glycero-3-phosphocholine (*d*<sub>3</sub>-PAF) as the internal standard. Samples were analyzed using the AB Sciex (Foster City, CA) QTRAP<sup>®</sup> 5500 hybrid triple quadrupole linear ion trap mass spectrometer equipped with a CTC-PAL autosampler and a Shimadzu HPLC system. The HPLC separation utilized a 150  $\times$  2-mm Gemini 5- $\mu$ m C<sub>18</sub> 110-Å column with a 2-mm Security Guard precolumn (Phenomenex, Torrance, CA). The mobile phase consisted of 80:5:15 (v/v/v) methanol, acetonitrile, water, and ammonium acetate at a concentration of 2 mM (solvent A) and methanol with 2 mM ammonium acetate (solvent B). The gradient used was 25% B held for 0.5 min, increased to 70% B in 15 min, then ramped up to 99% B in 0.1 min, and then held for 18 min to elute non-oxidized GPCs in the sample. The column eluate (0.2 ml/min) was transferred directly into the electrospray ion source with an ion spray voltage of –4200 V to generate negative ions with a turbospray gas flow rate of 10 at 200 °C. The declustering potential was set to –10 V when monitoring collision-induced dissociation (CID)

reactions of [M – H]<sup>–</sup> ions or [M + acetate]<sup>–</sup> adduct ions and to –220 V for monitoring CID reactions of [M – CH<sub>3</sub>]<sup>–</sup> ions from the GPC molecular species. During multiple reaction monitoring (MRM) the collision energy was either –68 V for CID of [M + acetate]<sup>–</sup> adduct ions, –52 V for CID of [M – CH<sub>3</sub>]<sup>–</sup> ions, or –38 V for CID of [M – H]<sup>–</sup> ions. The dwell time of each MRM transition was 30 ms with a 2-s duty cycle. The MRM transition pairs were monitored for species previously identified as PAF receptor agonists (28, 47, 48), including 1-*O*-hexadecyl-2-acetoyl-GPC (16e 2:0; PAF), 1-*O*-hexadecyl-2-butanoyl-GPC (16e 4:0), 1-*O*-hexadecyl-2-butenoyl-GPC (16e 4:1), 1-*O*-hexadecyl-2-(5)oxovaleroyl-GPC (16e 5al), 1-*O*-hexadecyl-2-glutaroyl-GPC (16e 5COOH), 1-palmitoyl-2-acetyl-GPC (16a 2:0), 1-palmitoyl-2-butanoyl-GPC (16a 4:0), 1-palmitoyl-2-butenoyl-GPC (16:0a 4:1), 1-palmitoyl-2-(5)oxovaleroyl-GPC (16a 5al), and 1-palmitoyl-2-glutaroyl-GPC (16a 5COOH).

In the presence of acetate buffer, electrospray of GPC normally produces [M + acetate]<sup>–</sup> adduct ions, which decompose in the ion source to form [M – CH<sub>3</sub>]<sup>–</sup> ions. The extent of this decomposition depends on the magnitude of the declustering potential applied. GPCs that have an acyl group that has been oxidized to a carboxyl moiety at the  $\omega$ -position form [M – H]<sup>–</sup> ions. CID of the [M + acetate]<sup>–</sup> adduct ions results in [M – CH<sub>3</sub>]<sup>–</sup> ions and carboxylate anions from the acyl substituents. CID of [M – H]<sup>–</sup> ions from GPCs containing an  $\omega$ -carboxyl group on an acyl substituent causes the ions to undergo a methyl transfer reaction from the choline to the  $\omega$ -carboxyl before undergoing the loss of the acyl substituent as a carboxylate anion, resulting in an increase of 14 mass units to the carboxylate anion (49). For  $\omega$ -aldehyde species, such as 16e 5al, hemiacetals are formed in the electrospray by reaction with methanol of the HPLC solvent. This results in abundant [M + 32 Da + acetate]<sup>–</sup> ions (50). For most GPC species, MRM transitions were monitored for both the [M + acetate]<sup>–</sup> and the [M – CH<sub>3</sub>]<sup>–</sup> ions to provide additional confirmation of identity. The MRM transition monitored for aldehyde-containing GPCs also included that of the [M + 32 Da + acetate]<sup>–</sup> ion. The MRM transition monitored for  $\omega$ -carboxyl-containing GPCs was that of the [M – H]<sup>–</sup> ion to the [RCOO + 14 Da]<sup>–</sup> ion.

Negative enhanced product ion scans were obtained concurrently with the MRM analysis for some species. The scan rate was 10,000 Da/s with dynamic fill time enabled for the linear ion trap (range, 0.05–100 ms). The declustering potential and collision energy were the same values as for the corresponding MRM transition pair for the species being monitored.

**Statistical Analysis**—In the present study, at least five mice/group were used in all murine experiments. Differences between experimental and control groups were examined by a two-tailed Student's *t* test. Statistical significance was defined as a *p* value <0.05.

## RESULTS

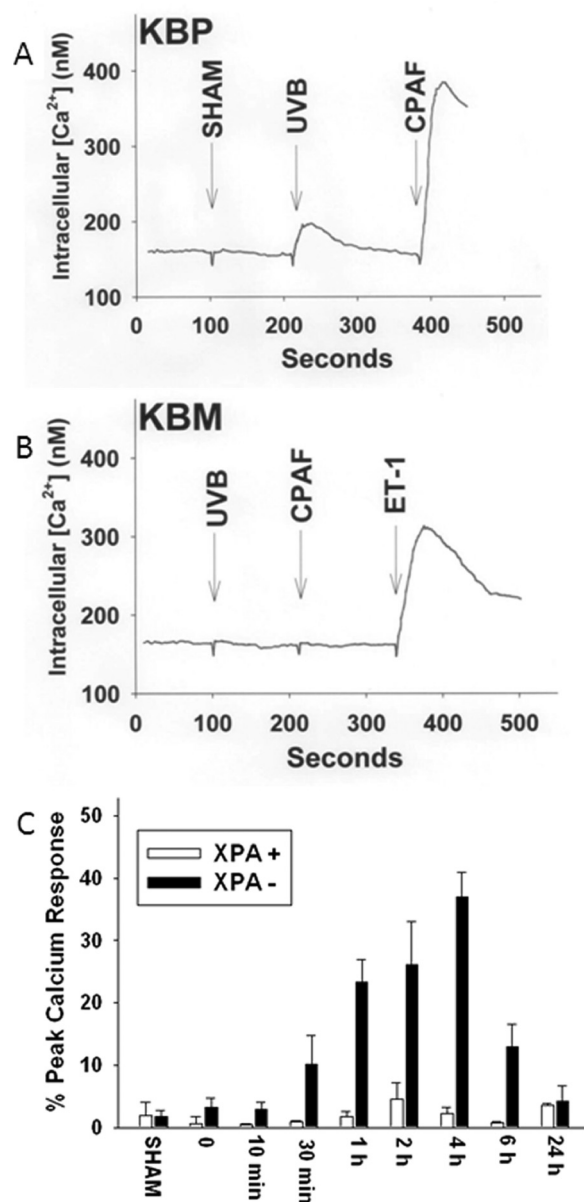
**XPA Deficiency Augments UVB Irradiation-mediated PAF-R Agonistic Activity**—We first examined the ability of fibroblasts deficient in XPA and gene-corrected (XPA+) cells to produce PAF agonists in response to UVB radiation. Our previous studies using mass spectrometry structurally characterized several



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PAF-R agonists that are produced in response to UVB irradiation in epithelial cells, including 1-hexadecyl-2-acetyl-GPC (native PAF), butanoyl (16e 4:0), and butenoyl (16e 4:1) species (28). These species were also measured upon direct UVB irradiation of purified lipid 1-hexadecyl-2-arachidonoyl-GPC, indicating that their formation can be non-enzymatic (28). In addition, there appear to be many other as yet uncharacterized *sn*-2 short-chain GPCs that act as PAF-R agonists (26–29). Therefore, the present study was designed to measure all PAF-R agonists as a whole in this complex mixture using intracellular calcium mobilization, a sensitive and specific biochemical assay to measure PAF-R activity (40, 41, 44). As shown in Fig. 1A, lipid extracts derived from UVB-irradiated XPA-deficient cells triggered an intracellular calcium mobilization response in PAF-R-expressing KBP cells, whereas extracts from non-irradiated cells (sham) elicited little or no response. In addition, extracts from UVB-irradiated XPA-deficient fibroblasts failed to stimulate a calcium mobilization response in Fura-2-loaded PAF-R-negative KBM cells (Fig. 1B), indicating that this calcium response was due to PAF-R activation. It should be noted that our previous studies indicated that the low level of UVB radiation ( $600 \text{ J/m}^2$ ) used in these studies does not induce PAF-R agonistic activity in epithelial cells or skin explants (28, 40). Similar to our previous findings using higher fluencies ( $>1000 \text{ J/m}^2$ ) in epithelial cells (40, 44), PAF-R agonistic activity of lipid extracts derived from UVB-irradiated XPA-deficient cells was abolished entirely following treatment with serum PAF acetylhydrolase, which degrades PAF- and *sn*-2 oxidatively modified glycerophosphocholines, but was unaffected by phospholipase  $A_1$  (data not shown). These findings indicate that UVB irradiation-mediated PAF-R agonistic activity resides in *sn*-1-alkyl, *sn*-2 short-chain/oxidatively modified acyl glycerophosphocholines.

The next studies compared the time course of PAF-R agonist generation in XPA-deficient *versus* gene-corrected fibroblasts in response to UVB irradiation. As shown in Fig. 1C, a considerable level of PAF-R agonistic activity was apparent by 30 min, was maximal by 1–4 h, and returned to base-line levels by 24 h in XPA-deficient fibroblasts. In contrast, very little PAF-R activity was measured in UVB-irradiated gene-corrected cells. The dose of UV irradiation used in these studies ( $600 \text{ J/m}^2$ ) also selectively triggered an apoptotic response (as measured by caspase 3 enzyme activity) in XPA-negative cells (Fig. 2). Mass spectrometry of the lipid extracts using deuterium-labeled PAF as an internal standard was used to structurally define and quantitate several of the potential Ox-GPCs responsible for UVB irradiation-generated PAF-R activity. As noted in Fig. 3, Ox-GPCs with acetoxy (PAF, 16e 2:0), oxovaleroxy (16e 5al), butanoyl (16e 4:0, 16a 4:0), and glutaroyl (16e 5COOH) moieties at the *sn*-2 position were identified. Greater levels of these Ox-GPCs were present in UVB-irradiated XPA-negative cells than the corresponding sham-treated cells. See the structures of these species and product ion mass spectra of the alkyl species quantified in Fig. 3. The combined measured quantity of 1-alkyl PAF species (16e 2:0, 16e 4:0, 16e 5al, and 16e 5COOH) generated by XPA-negative cells above their base-line level (sham) was  $\sim 2.2$  times the increase above base line detected from XPA-gene corrected cells in response to UVB treatment.



**FIGURE 1. Identification and time course of UVB irradiation-mediated PAF-R agonistic activity in XPA-positive and -negative cells.** A and B, calcium mobilization responses in Fura-2-loaded KBP (A) and PAF-R-negative KBM (B) cells upon addition of lipid extracts from UVB-irradiated ( $600 \text{ J/m}^2$ ) and non-irradiated (sham) XPA-negative cells. Stimulation with  $1 \mu\text{M}$  CPAF or  $1 \mu\text{M}$  endothelin-1 (ET-1) was used as positive controls for KBP and KBM cells, respectively. These studies are representative of at least three separate experiments. C, time course of PAF-R agonistic activity in response to UVB irradiation in XPA-negative and gene-corrected XPA+ cells. Lipids were extracted at various times following UVB irradiation ( $600 \text{ J/m}^2$ ). Data are the mean  $\pm$  S.E. from three independent experiments and are shown as the percentage of maximal peak calcium flux induced by  $1 \mu\text{M}$  CPAF and adjusted for  $2.5 \times 10^6$  cells.

1-Acyl Ox-GPCs with *sn*-2 acetyl (“acyl-PAF”) and butanoyl species were also identified (Fig. 3). There were no consistent differences in lyso-GPC species in response to UVB irradiation in either XPA-deficient or corrected cells (not shown). These studies indicate that XPA deficiency results in an increased level of PAF-R agonistic activity and that Ox-GPCs known to exhibit PAF-R activity are produced in response to UVB irradiation *in vitro*.

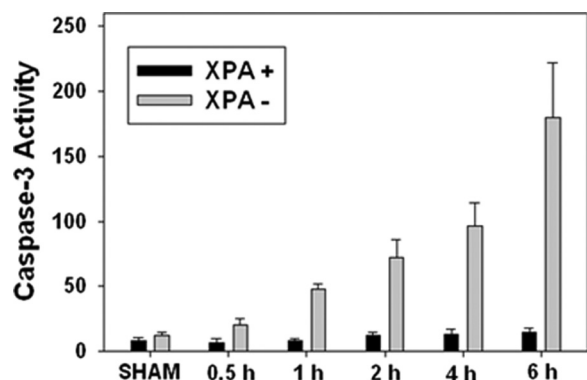


FIGURE 2. UVB irradiation of XPA-deficient cells results in enhanced caspase 3 activity. XPA-negative ( $XPA^{-}$ ) and gene-corrected cells ( $XPA^{+}$ ) were treated with  $600 \text{ J/m}^2$  UVB irradiation. At various times, cells were harvested, and caspase 3 enzymatic activity was assessed (28). The data are the mean  $\pm$  S.D. of caspase 3 enzymatic activity from a representative experiment of duplicate samples from two separate experiments.

**UVB Irradiation-mediated Production of ROS and PAF-R Agonists**—UVB irradiation is a potent inducer of ROS, including superoxide radical, hydrogen peroxide, and hydroxyl radical (51). PAF-R agonists such as *sn*-2 butanoyl, oxovaleroyl, and glutaroyl GPC species as well as PAF itself are produced non-enzymatically via free radical-mediated oxidation of *sn*-2 polyunsaturated fatty acyl glycerophosphocholines (27–29, 47). Because XPA-deficient cells have decreased antioxidant defenses (11–14), we next tested the hypothesis that ROS generation was important to the mechanism for the enhanced PAF-R agonists produced in response to UVB irradiation in the XPA-deficient cells. Intracellular levels of ROS were analyzed by flow cytometry using the fluorescent calcium-sensitive dye probe CM- $\text{H}_2\text{DCFDA}$ . UVB irradiation of XPA-negative fibroblasts resulted in increased levels of cellular ROS in comparison with UVB-irradiated XPA-positive cells (Fig. 4A). Antioxidants vitamin C and *N*-acetylcysteine can effectively scavenge a wide array of ROS and free radicals (52–54). The next set of experiments assessed their abilities to ameliorate UVB irradiation-mediated ROS and PAF-R agonistic activity. XPA-deficient cells were pretreated with vitamin C or *N*-acetylcysteine before irradiation with  $600 \text{ J/m}^2$  UVB irradiation. As shown in Fig. 4, these antioxidants, but not the pan-caspase inhibitor Z-VAD-fmk, inhibited both ROS and PAF-R agonist activity produced by UVB irradiation. These results are consistent with the hypothesis that enhanced UVB irradiation-mediated PAF-R agonists in XPA-negative cells are due to increased ROS.

**UVB Irradiation Generates Increased PAF-R Agonists in XPA<sup>-/-</sup> Murine Skin in Vivo**—XPA-deficient ( $Xpa^{-/-}$ ) mice were next used to assess the role of the XPA protein in UVB irradiation-mediated production of PAF species *in vivo*. To that end, the back skin of immobilized  $Xpa^{-/-}$ , heterozygous  $Xpa^{+/-}$ , and control mice on an albino hairless SKH-1 background were treated with UVB irradiation at a dose ( $1500 \text{ J/m}^2$ ) and time (1 h) for which our previous studies using mice and human skin explants demonstrated a minimal amount of PAF-R agonistic activity (40, 44). UVB irradiation of  $Xpa^{-/-}$  mice resulted in enhanced PAF-R agonistic activity in skin lipid extracts in comparison with wild-type or  $Xpa^{+/-}$  counterparts (Fig. 5A). Measurable levels of PAF-R activity were not

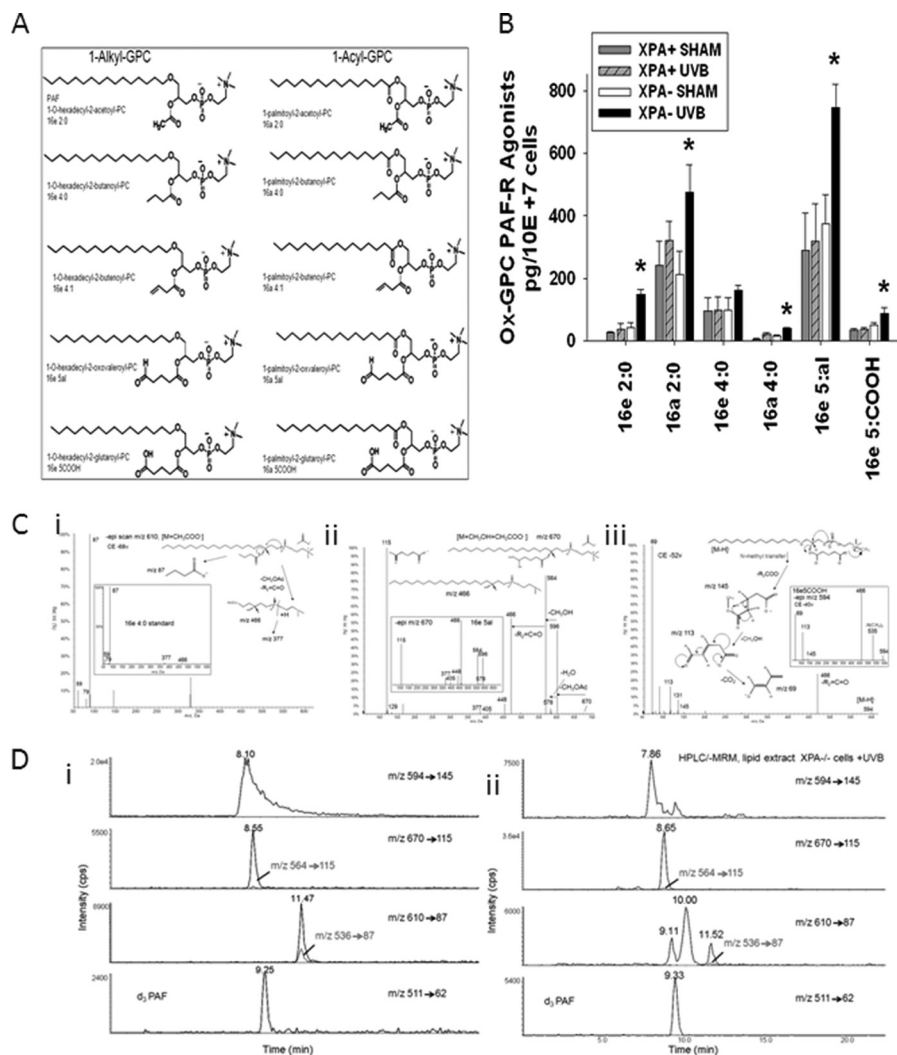
identified in non-irradiated skin. Again, lipid extracts obtained from skin of UVB-irradiated XPA-deficient mice triggered a calcium response in KBP but not in PAF-R-negative KBM cells, thus indicating that these calcium fluxes were due to PAF-R activity (data not shown). Next, the ability of systemic treatment with antioxidant vitamin C to modulate UVB irradiation-generated PAF-R agonistic activity was tested. The protocol of vitamin C-enriched (10 g/kg) chow has been demonstrated to inhibit cigarette smoke- and UVB irradiation-mediated PAF-R agonistic activity *in vivo* (35, 40). As shown in Fig. 5B, mice fed vitamin C-enriched chow for 10 days before UVB irradiation produced lesser levels of PAF-R agonistic activity in comparison with those fed standard diet.

**UVB Irradiation-mediated Exaggerated Skin Inflammation in XPA-deficient Mice Involves ROS and PAF Agonists**—Humans deficient in XPA exhibit profound photosensitivity, and this feature has been faithfully replicated in  $Xpa^{-/-}$  mice (55–57). Given that PAF is a potent stimulator of skin inflammation (38, 58, 59), the next studies were designed to assess the role of the PAF system in the photosensitivity exhibited by  $Xpa^{-/-}$  mice. To that end, the dorsal side of ears of  $Xpa^{-/-}$  and wild-type SKH-1 mice were treated with UVB irradiation, and inflammation was assessed by daily measurement of ear thickness. As shown in Fig. 6, UVB irradiation-mediated skin inflammation was maximal at 48 h, and statistically significant increased ear swelling was noted in  $Xpa^{-/-}$  mice in comparison with control mice. Of note, the least amount of skin inflammation was found in PAF-R-deficient ( $Ptafr^{-/-}$ ) mice (Fig. 6B).

To define the role of ROS and the PAF system in the exaggerated inflammatory response in  $Xpa^{-/-}$  mice, animals were pretreated with PAF-R antagonists or fed vitamin C-enriched chow before UVB treatment of ears, and ear thickness was measured at 48 h. Both antioxidant supplementation (Fig. 6D) and treatment with three structurally distinct PAF-R antagonists (Fig. 6E) inhibited the enhanced UVB irradiation-mediated inflammatory responses in the  $Xpa^{-/-}$  mice. Consistent with the increased skin thickness measurements, enhanced expression of TNF- $\alpha$  mRNA and protein were found in UVB-treated  $Xpa^{-/-}$  mouse skin over that found in the skin of irradiated control mice. Of note, the dose of UVB irradiation ( $1500 \text{ J/m}^2$ ) used also induced a statistically significant increase in TNF- $\alpha$  mRNA in SKH-1 mice, although the amounts were much lesser than those seen in their XPA-deficient counterparts. The increased TNF- $\alpha$  mRNA and protein levels in UVB-irradiated  $Xpa^{-/-}$  mice were also inhibited by pretreatment with PAF-R antagonists (Fig. 7). These studies indicate that Ox-GPC PAF-R agonists play a pivotal role in the exaggerated UVB inflammatory response characteristic of XPA deficiency.

## DISCUSSION

Photosensitivity, the abnormal reaction to sunlight, has numerous causes and is a source of considerable morbidity (19, 20). Although there has been substantial research in this area, the mechanisms by which photosensitivity occur are still for the most part elusive. The present studies implicate the PAF system in the abnormal UVB responsiveness associated with XPA deficiency. We demonstrate that UVB irradiation of human XPA-



**FIGURE 3. Structural characterization of UVB irradiation-generated Ox-GPC PAF-R agonists.** *A*, structures of Ox-GPC PAF-R agonists. *B*, lipid extracts obtained from XPA-deficient and gene-corrected XPA+ fibroblasts treated with UVB irradiation ( $600 \text{ J/m}^2$ ) or non-irradiated (sham) (1-h incubation) were quantified by mass spectrometry using deuterium-labeled PAF internal standard as outlined under "Experimental Procedures." The data are the mean  $\pm$  S.E. from a single experiment run in duplicate and are representative of four to five separate experiments with similar results. \* denotes statistically significant ( $p < 0.05$ ) differences in Ox-GPCs between UVB- versus sham-treated XPA-negative cells. *C*, negative ion enhanced product ion spectrum of 16e 4:0 (*i*), 16e 5al (*ii*), and 16e 5COOH (*iii*) from UVB-irradiated XPA-negative cells. *D*, total ion current of MRM traces for 16e 5COOH, 16e 5al, 16e 4:0, and  $d_3$ -PAF from standards (*i*) and lipid extracts from XPA-deficient cells + UVB irradiation (*ii*).

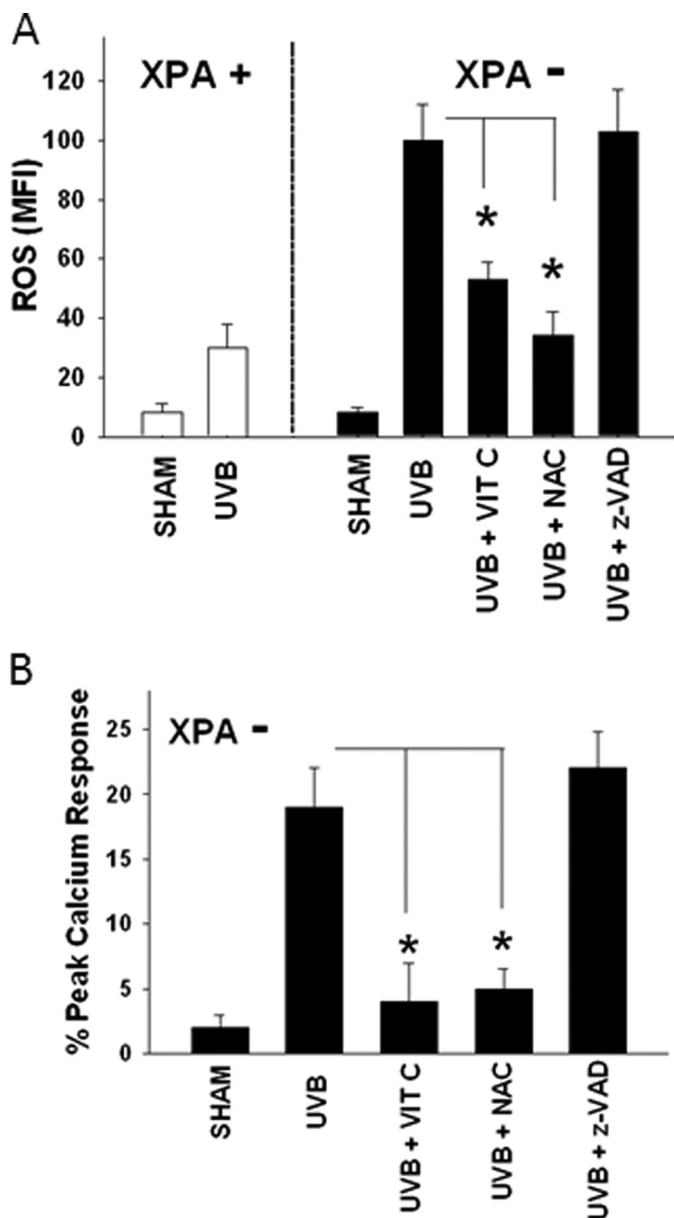
deficient fibroblasts in comparison with XPA gene-corrected cells resulted in increased levels of ROS and PAF-R agonistic activity, both of which were inhibited by antioxidants vitamin C and *N*-acetylcysteine.

The XPC protein is important in recognizing initial damaged DNA in global genome repair. In contrast, XPA protein plays a critical role in both global genome repair and transcription-coupled repair (60). Notably, UVB irradiation of fibroblasts deficient in XPC (obtained from Coriell Cell Repositories of Coriell Institute) did not result in exaggerated ROS formation (data not shown) or increased levels of PAF-R agonists. (XPC-deficient cells exhibit  $4.5 \pm 2\%$  of maximal PAF-R agonistic activity at 1 h post-UVB treatment (data not shown), whereas XPA-negative cells exhibit  $23.5 \pm 2.3\%$  of maximal PAF-R agonistic activity (see Fig. 1C)). This finding is congruent with our hypothesis implicating the PAF system in photosensitivity as XPC deficiency does not exhibit the profound photosensitivity seen in XPA deficiency (61). Moreover, unlike XPA-deficient

cells, XPC-deficient cells do not have decreased levels of the antioxidant enzyme catalase (12, 13).

Consistent with the biochemical calcium mobilization studies, structural studies using mass spectrometry revealed the presence of not only increased levels of native PAF but elevated levels of novel 1-hexadecyl GPC species with *sn*-2 chains of four and five carbon groups in XPA-deficient cells in response to UVB irradiation. Of importance, recent studies have demonstrated that the non-PAF Ox-GPCs we measured and other Ox-GPCs chemically synthesized have significant PAF-R agonistic activity *in vitro* (48). It should be noted that the low amount of UVB irradiation used in the *in vitro* studies ( $600 \text{ J/m}^2$ ) that generates  $\sim 2$ -fold increased levels of these compounds does not generate Ox-GPCs in other cell types such as epithelial cells (28, 40), which fits with the concept that the increased responsiveness of XPA-deficient cells to UVB irradiation is mediated at least in part by the resulting Ox-GPCs. The difference between the amount of PAF-R agonistic activity

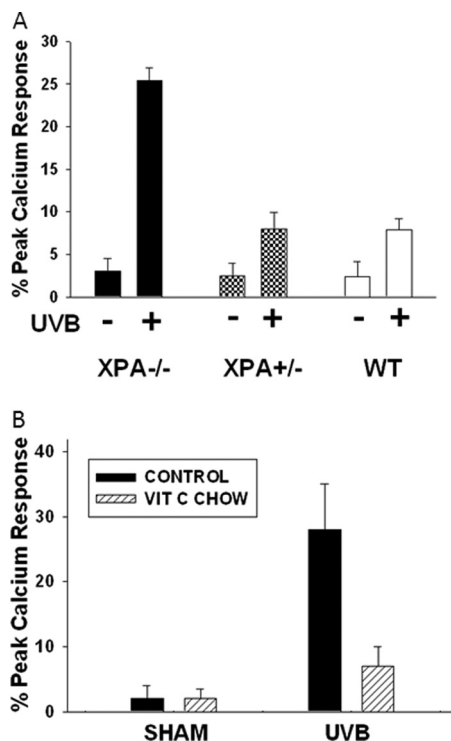




**FIGURE 4. Augmented UVB irradiation-mediated ROS and PAF-R agonist formation in XPA-deficient cells.** XPA-negative cells were pretreated with vitamin C (VIT C; 2.5 mM), N-acetylcysteine (NAC; 5 mM), Z-VAD-fmk (z-VAD; 10  $\mu$ M), or DMSO vehicle 30 min before UVB irradiation (600 J/m<sup>2</sup>). A, ROS levels were measured at 30 min post-UVB irradiation by flow cytometry using CM-H<sub>2</sub>DCFDA as a fluorescent dye probe and are shown as mean fluorescence intensity (MFI). B, PAF-R agonistic activity was measured in lipid extracts obtained 1 h post-UVB irradiation using KBP cells as in Fig. 1. Data are shown as the percentage of maximal peak calcium flux induced by 1  $\mu$ M CPAF and adjusted for  $2.5 \times 10^6$  cells. Data are expressed as the mean  $\pm$  S.E. from three to four independent experiments. \* denotes statistically significant ( $p < 0.05$ ) differences.

(8–10 $\times$  base line) versus the modest 2-fold increase in the amounts of the Ox-GPCs actually measured in UVB-irradiated XPA-deficient cells suggests that there are likely numerous biologically active Ox-GPCs other than those we have structurally identified and measured in this complex mixture.

As has been reported previously (55–57), UVB irradiation of XPA-deficient mice with a relatively low dose of UVB irradiation resulted in increased skin inflammation as measured by ear thickness in comparison with control mice. The present studies

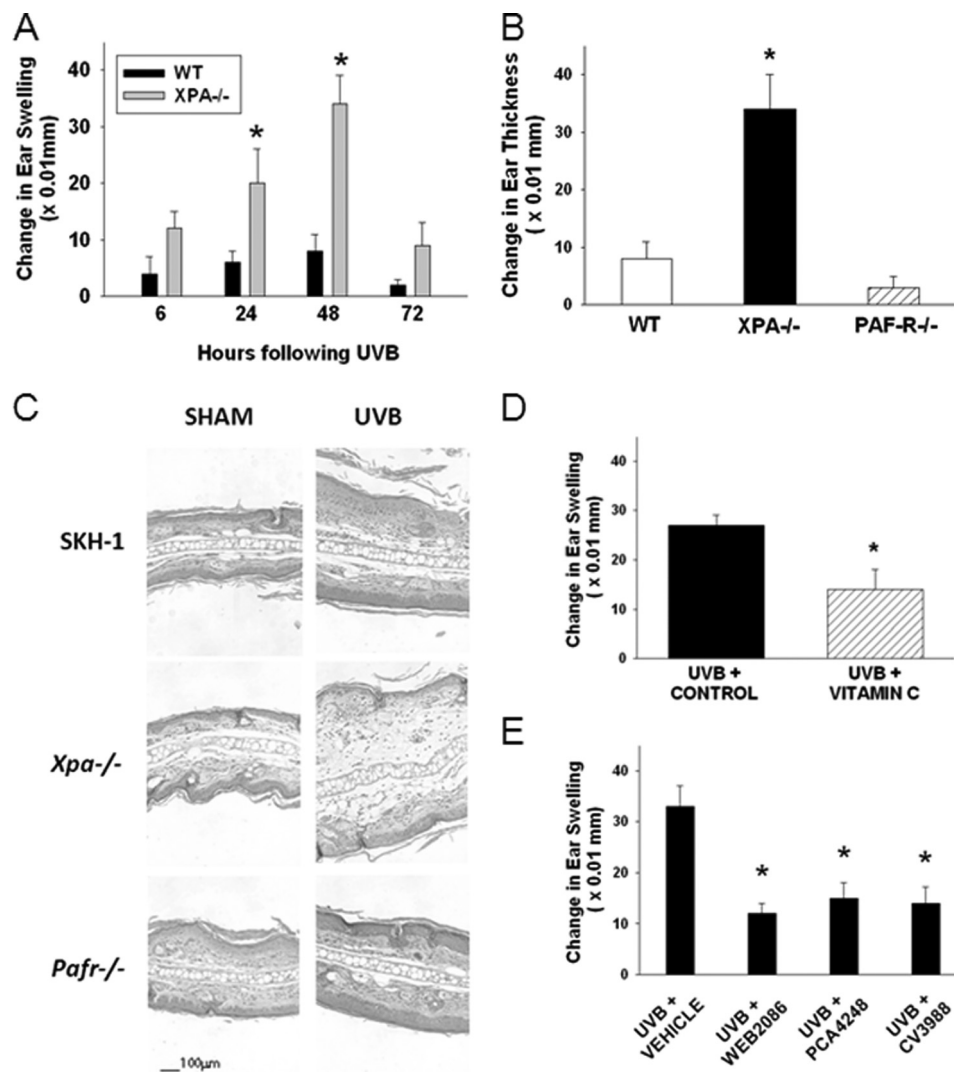


**FIGURE 5. UVB irradiation of XPA-deficient mice results in increased PAF-R agonistic activity susceptible to antioxidants.** A, wild-type (SKH-1; WT), *Xpa*<sup>+/-</sup> heterozygotes, or *Xpa*<sup>-/-</sup> mice were treated with 1500 J/m<sup>2</sup> UVB irradiation or unirradiated (sham). B, *Xpa*<sup>-/-</sup> mice were fed with standard (CONTROL) or vitamin C-enriched (VIT C CHOW) chow for 10 days before sham treatment or UVB irradiation (1500 J/m<sup>2</sup>). Treated epidermis was removed 1 h after UVB irradiation via curettage, and the lipids were extracted from weighed tissue and tested for the ability to trigger a calcium mobilization response in Fura-2-loaded KBP cells. Data are the mean  $\pm$  S.E. percentage of maximal peak calcium flux induced by 1  $\mu$ M CPAF and adjusted for 5-mg tissue weight from duplicate specimens obtained from six to eight mice in each group.

demonstrate that this abnormal UVB inflammatory response in XPA-deficient mice was susceptible to systemic treatment with the antioxidant vitamin C at doses that have been previously reported to block the formation of PAF-R agonists in blood following exposure to the potent pro-oxidative stressor cigarette smoke (35). More relevant to the current studies, we have recently demonstrated that this vitamin C-supplemented diet blocks systemic immunosuppression by UVB irradiation that is also mediated by Ox-GPC PAF-R agonists (40).

Coherent with the histological and ear thickness measurements, UVB irradiation of XPA-deficient (*Xpa*<sup>-/-</sup>) mice also resulted in the enhanced expression of TNF- $\alpha$ , a major cytokine implicated in photosensitivity (19, 20, 39), over that in control SKH-1 mice. This augmentation of UVB irradiation-induced skin inflammation and TNF- $\alpha$  expression in XPA-deficient mice was suppressed by PAF-R antagonists, indicating that the increased PAF-R agonists generated in *Xpa*<sup>-/-</sup> mice upon UVB irradiation were biologically relevant. Of interest, our previous studies have demonstrated that UVB irradiation-mediated epidermal TNF- $\alpha$  expression in epithelial cells and murine skin involves PAF (36, 38). That the augmented UVB inflammatory response in *Xpa*<sup>-/-</sup> mice was also blocked by systemic antioxidant treatment implicates PAF-R agonistic Ox-GPCs produced via ROS.

## Ox-GPCs and XPA Photosensitivity



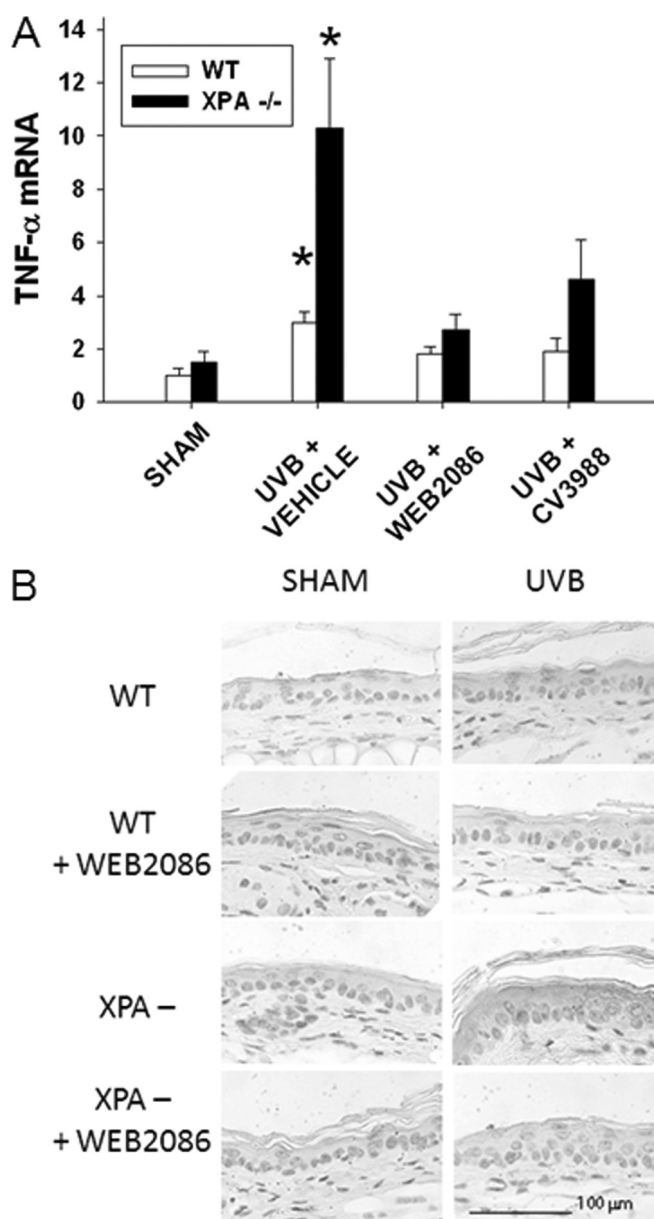
**FIGURE 6. UVB irradiation results in enhanced skin inflammation in XPA-deficient mice susceptible to antioxidants and PAF-R antagonists.** *A*, SKH-1 (WT), or *Xpa*<sup>-/-</sup> mice were treated with 1500 J/m<sup>2</sup> UVB irradiation to one dorsal ear, and the ear thickness was measured at various times. *B*, SKH-1 (WT), *Xpa*<sup>-/-</sup>, or PAF-R-null mice were treated with 1500 J/m<sup>2</sup> UVB irradiation to one dorsal ear, and the ear thickness was measured 48 h post-treatment and compared with pretreatment values. *C*, representative H&E-stained sections from mice 48 h following UVB or sham treatment. *D*, *Xpa*<sup>-/-</sup> mice were fed with control or vitamin C-enriched chow for 10 days before sham treatment or UVB irradiation (1500 J/m<sup>2</sup>), and ear thickness was measured at 48 h and compared with pretreatment values. *E*, *Xpa*<sup>-/-</sup> mice were pretreated with intraperitoneal injections of PAF-R antagonists WEB2086 (550 nmol), CV3988 (500 nmol), and PCA4248 (500 nmol) or ethanol vehicle 30 min before UVB irradiation (1500 J/m<sup>2</sup>), and ear thickness was measured 48 h later and compared with pretreatment values. The data are the mean ± S.E. difference in ear thickness from preirradiated values and are from six to eight mice in each group. \* denotes statistically significant ( $p < 0.05$ ) differences.

The biological effects of UV irradiation occur as a consequence of the absorption of electromagnetic energy by certain molecules within the cell or tissue. The exact target(s) for UVB irradiation in cells is not entirely clear, although DNA, the amino acid tryptophan, and urocanic acid have all been implicated (15, 17). Two separate mechanisms by which UVB irradiation can stimulate the non-enzymatic production of PAF-R agonists have been demonstrated. First, UVB irradiation can generate the delayed production of ROS through a process involving NADPH and the epidermal growth factor receptor (40, 62, 63). A second mechanism for Ox-GPC generation appears to be the direct absorption of UVB irradiation by lipids and is immediate (28, 29, 40). Although GPCs do not absorb light in the UVB range, we have shown that Ox-GPCs do absorb in this range (29). Thus, small amounts of Ox-GPCs that are found in cells at any one time can serve as targets for UVB

irradiation. The results of the time course studies and use of NADPH oxidase inhibitor both suggest that the first mechanism mediates the augmentation of PAF-R agonist formation in response to low levels of UVB radiation in XPA-deficient cells. We propose that the lack of antioxidant defenses (e.g. catalase) reported for XPA-null cells allows an increased ROS and Ox-GPC PAF-R agonist response to low levels of UV radiation. The augmented Ox-GPC PAF-R agonist formation thus mediates the increased skin inflammation response. Inasmuch as Ox-GPCs have been demonstrated to act as potent inducers of cellular apoptosis (64), these findings could also potentially explain the keratinocyte apoptosis associated with photosensitivity.

It should be noted that native PAF is likely the most potent PAF-R agonist. As PAF can be synthesized via enzymatic as well as non-enzymatic means (28), it is possible that the augmenta-





**FIGURE 7. UVB irradiation results in enhanced TNF- $\alpha$  expression in XPA-deficient mice susceptible to PAF-R antagonists.** SKH-1 (WT) or *Xpa*<sup>-/-</sup> mice were pretreated with the PAF-R antagonists WEB2086 (550 nmol) and CV3988 (500 nmol) or ethanol vehicle 30 min before 1500 J/m<sup>2</sup> UVB or sham treatment to one dorsal ear. *A*, 24 h post-treatment, RNA from the ears was subjected to quantitative RT-PCR to measure TNF- $\alpha$  mRNA. The values are mean  $\pm$  S.E. -fold induction of TNF- $\alpha$  mRNA normalized to GAPDH mRNA from groups of six mice. *B*, representative sections of immunohistochemical staining of 24-h post-treatment ear tissue to assess TNF- $\alpha$  protein levels. \* denotes statistically significant ( $p < 0.05$ ) differences in TNF- $\alpha$  mRNA between UVB- versus sham- and PAF-R antagonist-treated *Xpa*<sup>-/-</sup> mice and UVB- versus sham-treated WT mice.

tion of PAF-R agonistic activity reported here could be via enzymatic PAF synthesis. However, the ability of antioxidants to block both the UVB irradiation-mediated augmented formation of PAF-R agonistic activity in XPA-negative cells and *Xpa*<sup>-/-</sup> mice and the downstream inflammation is compatible with the hypothesis that the non-enzymatic process of PAF-R agonist formation is critical to XPA photosensitivity.

The present studies demonstrating that the photosensitivity associated with XPA deficiency is mediated by Ox-GPCs with

PAF-R agonistic activity provide the first clear linkage of these novel lipids to a disease process. These studies have clinical relevance as they potentially provide a mechanism by which antioxidants have efficacy in the treatment of photosensitivity disorders ranging from lupus erythematosus to porphyrias (65–67). More importantly, these studies could provide the impetus for testing direct inhibitors of the PAF system in photosensitivity diseases.

*Acknowledgments*—We thank Qiaofang Yi and Sonia DaSilva for technical assistance and Dr. Mark Kaplan for reviewing the manuscript.

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