

NF-E2-related factor 2 regulates the stress response to UVA-1-oxidized phospholipids in skin cells

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ABSTRACT Long-wavelength ultraviolet (UVA-1) radiation causes oxidative stress that modifies cellular molecules. To defend themselves against noxious oxidation products, skin cells produce detoxifying enzymes and antioxidants. We have recently shown that UVA-1 oxidized the abundant membrane phospholipid 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC), which then induced the stress-response protein heme oxygenase 1 (HO-1) in dermal fibroblasts. Here we examined the effects of UVA-1- and UV-oxidized phospholipids on global gene expression in human dermal fibroblasts and keratinocytes. We identified a cluster of genes that were coinduced by UVA-1-oxidized PAPC and UVA-1 radiation. The cluster included HO-1, glutamate-cysteine ligase modifier subunit, aldo-keto reductases-1-C1 and -C2, and IL-8. These genes are members of the cellular stress response system termed “antioxidant response.” Accordingly, the regulatory regions of all of these genes contain binding sites for NF-E2-related factor 2 (NRF2), a major regulator of the antioxidant response. Both UVA-1 irradiation and treatment with oxidized lipids led to increased nuclear accumulation and DNA binding of NRF2. Silencing and deficiency of NRF2 suppressed the antioxidant response. Taken together, our data show that UVA-1-mediated lipid oxidation induces expression of antioxidant response genes, which is dependent on the redox-regulated transcription factor NRF2. Our findings suggest a different view on UV-generated lipid mediators that were commonly regarded as detrimental.—Gruber, F., Mayer, H., Lengauer, B., Mlitz, V., Sanders, J. M., Kadl, A., Bilban, M., de Martin, R., Wagner, O., Kensler, T. W., Yamamoto, M., Leitinger, N., Tschachler, E. NF-E2-related factor 2 regulates the stress response to UVA-1-oxidized phospholipids in skin cells. *FASEB J.* 24, 39–48 (2010). www.fasebj.org

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EXPOSURE TO ULTRAVIOLET (UV) RADIATION is a major cause for damage to the skin that can lead to aging, dysregulation of the immune system, and cancer. Long-wavelength ultraviolet (UVA-1) light generates reactive oxygen species and subsequently causes oxidative modification of various cellular biomolecules (1). In response to oxidative tissue damage, skin cells synthesize detoxifying stress-response proteins, but the mechanisms that trigger this response are only partially understood. Whereas modification of cellular lipids has long been regarded as an end result of UV damage, UV-generated lipids are emerging as specific signaling mediators that can regulate the cellular UV response. Lipid messengers can form directly upon UV radiation from precursors that are abundant in cellular membranes.

1-Palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC) is one of the most abundant membrane phospholipids, and several of its oxidation products have been shown to have important biological functions in inflammation (reviewed in refs. 2, 3), apoptosis (4), endothelial barrier function (5), and angiogenesis (6). Autoxidized PAPC (OxPAPC) (7) is a well-established model for studying the effects of oxidized lipids in biological settings. OxPAPC is a suitable tool for studying global gene expression and as such recently yielded new insights in regulation of inflammation-related and unfolded protein response genes in endothelial cells (8). OxPAPC, which was used as a reference in our study, contains a variety of oxidation products (9). UVA radiation leads to oxidative modification of cellular lipids (10). We recently demonstrated that PAPC can be oxidized by UVA-1 *in vitro* and in living dermal fibroblasts (FBs) (11). The resulting oxidation products (UV-PAPC) all contained a full-length oxygenated

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arachidonoyl moiety in the *sn*-2 position and were biologically active. For instance, 1-palmitoyl-2-(epoxyisoprostane-E2)-*sn*-glycero-3-phosphorylcholine induced expression of heme oxygenase-1 (HO-1). UV-PAPC represents the early stages of phospholipid oxidation, because further oxidation of PAPC to OxPAPC yields oxygenation as well as fragmentation products, resulting in additional biological functions (12, 13).

Activation of NF-E2-related factor 2 (NRF2) and subsequent induction of NRF2-dependent genes is an efficient adaptive response mechanism to electrophilic and oxidant stress (reviewed in ref. 14), as it is found upon UVA radiation (15). NRF2 regulates skin inflammation in wound healing (16) and keratinocyte (KC) differentiation (17). NRF2 activation and expression of the downstream antioxidant genes were shown to prevent chemical-induced skin tumors (18, 19). Under normal conditions, NRF2 is retained in the cytoplasm by its repressor KEAP1, which also promotes ubiquitinylation of NRF2. With oxidative or electrophilic stress, degradation of NRF2 is impeded, allowing it to translocate to the nucleus and activate expression of target genes. Activation of NRF2 signaling can result from modification of cysteines on KEAP1 that leads to impaired ubiquitinylation of NRF2 (20). Alternative pathways involving NRF2 phosphorylation and stabilization (21) or NRF2 itself as the redox sensor have been proposed (22).

We hypothesized that part of the UVA-1-mediated gene expression in dermal FBs would be mediated by UV-oxidized phospholipids. Therefore, we examined whether NRF2 accumulates in the nucleus on stimulation with UV-PAPC. Further, we identified genes that are coregulated in an NRF2-dependent way by UVA-1 and phospholipid oxidation products using microarray analysis. Using bioinformatics, we found that all of them contained an antioxidant response element (ARE), known to function as binding sites for the redox-regulated transcription factor NRF2.

MATERIALS AND METHODS

Cell culture and siRNA transfection

Human neonatal skin FBs were obtained from Lifeline Cell Technology (Walkersville, MD, USA) and grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Gaithersburg, MD, USA) supplemented with 10% FCS and penicillin/streptomycin (1000 U/ml; Life Technologies, Inc.) to subconfluence. Fourth-passage FBs were used at a confluence of 60–70% in 12-well tissue culture plates. Cells were transfected with Stealth small interfering (si) RNAs using Lipofectamine 2000 reagent (both from Invitrogen, Carlsbad, CA, USA); sequences are listed in Supplemental Table 3. For one 12-well plate, Lipofectamine 2000 (30 μ l) was mixed with 60 μ l of a 20 μ M siRNA solution (equimolar mix of both NRF2 siRNA and scramble siRNA controls, respectively) and 3.8 ml of Opti-MEM medium (Invitrogen). After 30 min at room temperature, 300 μ l of the mix was added to 300 μ l of serum-free DMEM (Invitrogen) in each well of a 12-well tissue culture plate and incubated for 24 h;

then the medium was changed to DMEM and incubated for another 24 h before stimulation. Neonatal human epidermal KCs derived from foreskin were obtained from Clonetics (San Diego, CA, USA). KCs were cultured in KC growth medium (Lonza, Walkersville, MD, USA) up to the fifth passage. HaCat cells were cultured in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% FCS and penicillin/streptomycin (1000 U/ml).

UV irradiation and lipid oxidation

UV irradiation of cells was performed as described previously (23). As a light source for UVA-1, a Supersun 5000-type solar simulator (Mutzhas, Munich, Germany) filtered for the emission of UVA-1 (340–390 nm) was used. Cells and skin explants were irradiated with a fluency of 40 J/cm² at ambient temperature. PAPC was oxidized by exposure of the dry lipid to air for 72 h to generate OxPAPC. PAPC dried to a thin film on a glass support was irradiated with UVA-1 with 80 J/cm² to generate UV-PAPC. The extent of oxidation was monitored by electrospray ionization-mass spectrometry as described previously (7).

Microarray hybridization and data analysis

Hybridization to human U133A Plus 2.0 Gene Chips (Affymetrix, Santa Clara, CA, USA) and scanning of the arrays were performed according to the manufacturer's protocols, as described before (24). Arrays were scanned using the GeneArray scanner (Affymetrix). Image analysis was performed with GeneChip software (robust multichip analysis; Affymetrix). Normalization was performed by global scaling, with the arrays scaled to an average intensity of 500. The microarray data were deposited at Gene Expression Omnibus (GEO) (25) as a series record (GSE13606). We restricted further analysis to genes with a fold change of >2 or <0.5 relative to control that were called present in at least one of the treatment conditions.

Gene ontology analysis

Gene ontology analysis was performed using the functional annotation tool of the Database for Annotation, Visualization, and Integrated Discovery (DAVID 2008) program (26). Gene groups with a >2-fold change in expression were analyzed to identify biological processes affected by UVA-1 and/or oxidized lipids. For each probe set, the biological process categories from Gene Ontology (GO:BP) that were significantly overrepresented were determined by using a modified Fisher's exact test [EASE (27) scores to $P < 0.1$ are shown in Supplemental Table 1].

Comparative multiple promoter analysis

We used TOUCAN 2 software (28) for comparative promoter analyses of the coregulated genes. First we extracted proximal promoter sequences (10 kb upstream and 0.2 kb downstream of the transcriptional start sites) from the Ensembl genomic database. We used MotifScanner, which searches the TRANSFAC database, to detect transcription factor binding sites in the sets of sequences. The prior (stringency level) was set to a value of 0.1, and the human promoter set of the Eukaryotic Promoter Database (EPD) was chosen as a third-order background model. The Statistics tool of TOUCAN was applied to the data produced by MotifScanner, in combination with the expected frequencies file (human EPD), to detect overrepresented features in the sets of coregulated genes.

Real-time quantitative PCR (qPCR)

RNA was isolated using TRIzol reagent (Invitrogen). Total RNA (900 ng) was reverse-transcribed with murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and oligo(dT) primers. Primer sequences for qPCR are listed in Supplemental Table 4. qPCR was performed using LightCycler technology and the Fast Start SYBR Green I kit (both from Roche Applied Science, Basel, Switzerland). In all assays, cDNA was amplified using a standardized program (10-min denaturing step; 55 cycles of 5 s at 95°C, 15 s at 65°C, and 15 s at 72°C; melting point analysis in 0.1°C steps). Quantification of target-gene expression was performed using a mathematical model of Pfaffl (29). Results are means \pm SD of target-gene expression that was normalized to expression of β_2 -microglobulin in biological triplicates. Each experiment was performed \geq 3 times.

Immunostaining, data acquisition, and analysis

Human primary dermal FBs were stimulated for 2 h at 37°C and 5% CO₂ with UV-PAPC (100 μ g/ml) or vehicle. Cells from control and treated groups were trypsinized and fixed in 2% paraformaldehyde at room temperature for 15 min. The cells were blocked and permeabilized in 0.3% Triton X-100, 3% fish skin gelatin, and 5% normal donkey serum. Anti-NRF2 antibody (rabbit, 0.4 μ g/ml, sc-722; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was added overnight at 4°C, and anti-rabbit IgG (donkey, Alexa Fluor 488; Molecular Probes, Eugene, OR, USA) was used as secondary antibody. Before analysis, Draq5 (Alexis, Lüufelingen, Switzerland) was added to a final concentration of 25 μ M for nuclear staining.

The method for quantifying nuclear translocation of NRF2 was adapted from George *et al.* (30) and relies on the spectral isolation of NRF2 images from nuclear images using ImageStream technology (Amnis Corp., Seattle, WA, USA). Untreated and UV-PAPC-treated dermal FBs were labeled with an antibody for NRF2 (sc-722) and Draq5 for nuclear stain. In the ImageStream cytometer, cells were hydrodynamically focused, excited with 488- and 658-nm laser light, and imaged on a time-delay integration charge-coupled device camera. Single-cell images are optically decomposed into a set of subimages, corresponding to the stains with identical pixel coordinates for each channel (30). These images were analyzed using ImageStream data exploration and analysis software after digital spectral compensation. The degree of nuclear translocation was assessed by quantifying the peak pixel intensity values for NRF2 staining within the digitally masked nuclear region (defined by contour segmentation of the Draq5 image) for the individually treated samples.

Transcription-factor assay

The activity of NRF2 was determined using the TransAM Nrf2 assay (Active Motif, Carlsbad, CA, USA). Nuclear extracts of dermal FBs were obtained according to the manufacturer's protocol. Nuclear extract was quantified, and 4 μ g of nuclear protein was used per condition. In brief, nuclear extracts were incubated with ARE consensus site oligonucleotides (5'-GTCACAGTGACTCAGCAGAATCTG-3') immobilized to 96-well plates. Bound protein was detected by antibody specific to DNA-bound NRF2 and visualized by a colorimetric reaction catalyzed by horseradish peroxidase-conjugated secondary antibody. Absorbance was measured at 450 nm with a reference wavelength of 650 nm; results are means \pm SD, and experiments were done 3 times.

Animals and animal care

All procedures and protocols in this study were approved by the University of Virginia Animal Care and Use Committee. Male C57BL/6 mice (Taconic Laboratories, Germantown, NY, USA) or *Nrf2*^{-/-} mice (31) (the kind gift of Dr. T. Kensler, Johns Hopkins University, Baltimore, MD, USA, and Dr. M. Yamamoto, University of Tsukuba, Tennoudai, Japan) were used for these studies.

Immunohistochemistry and immunofluorescence

We obtained paraffin sections (5 μ m) for immunohistochemistry and stained with HO-1 antibody (SPA-896; Assay Designs, Ann Arbor, MI, USA) at 1 μ g/ml concentration. In brief, after heat-induced antigen retrieval (pressure cooker) with Unmasking Solution (Vector Laboratories, Burlingame, CA, USA), antigen was detected using a Vectastain Elite Kit (Vector Laboratories). Visualization was done with 3,3'-diaminobenzidine (DAB) (DakoCytomation A/S, Copenhagen, Denmark). Counterstaining was done using Azure B (Sigma-Aldrich, St. Louis, MO, USA). Background staining controls were performed by using rabbit IgG in place of the primary antibody. Immunofluorescent stainings for NRF2 were performed using KCs grown on chamber slides that were fixed with methanol. Anti-NRF2 antibody (sc-722) was used at 1 μ g/ml, and Alexa Fluor 546-labeled goat anti-rabbit antibody was used in the second step.

RESULTS

Global gene-expression analysis

We hypothesized that part of the UVA-1-mediated gene expression in dermal FBs would be mediated by UV-oxidized phospholipids. We further hypothesized that treatment of FBs with these lipids would mimic parts of the UVA-1-induced gene regulation. To test these hypotheses, we profiled global mRNA expression levels in FBs that had been treated with either UVA-1 or oxidized lipids and untreated control cells. To investigate the effect of oxidized phospholipids on gene regulation, we used two preparations, which differed in their degree of oxidation: the minimally oxidized UV-PAPC, resulting from UVA-1 irradiation of PAPC, and air-oxidized OxPAPC, which represents the full spectrum of oxidation products (11). We irradiated dermal FBs with UVA-1 (40 J/cm²) or treated them with UV-PAPC, OxPAPC, or native PAPC (100 μ g/ml each). We analyzed global gene expression using microarray technology (Affymetrix U133A Plus 2.0 Gene Chips) 4 h after stimulation. Genes regulated by nonoxidized native PAPC were eliminated from analysis. UVA-1 irradiation increased expression of 334 genes >2-fold; OxPAPC and UV-PAPC treatment increased expression of 89 and 48 genes, respectively. Of the UVA-1-regulated genes, 33 were coregulated by OxPAPC, 12 by UV-PAPC, and 5 by both stages of phospholipid oxidation (Fig. 1). UVA-1 decreased the expression of 143 genes and OxPAPC of 194 genes >2-fold, with an overlap of 13 genes. We hypothesized that coregulated genes would be involved in similar biological processes and

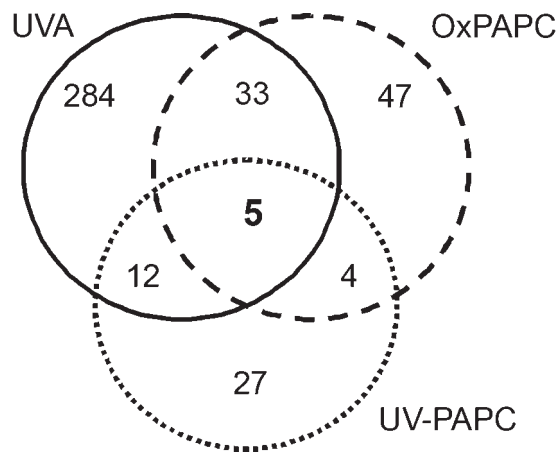


Figure 1. Induction of gene expression by UVA-1 and oxidized lipids in dermal FBs. Numbers of genes induced in primary dermal FBs 4 h after treatment with UVA-1 (40 J/cm²), OxPAPC (100 μg/ml), and UV-PAPC (100 μg/ml) were determined with microarrays and are represented in a Venn diagram. Numbers in regions indicate numbers of genes induced by the treatment; numbers in intersections indicate genes that are up-regulated by both or all three stimuli, respectively.

would share transcription-factor binding sites in their regulatory regions, both of which would indicate that the response to UV irradiation is at least in part mediated by oxidized phospholipids.

Functional annotation

To investigate the biological functions that would be determined by the individual gene groups, we analyzed the data sets using the functional annotation tool of the DAVID bioinformatics resource (26). This functional annotation uses the controlled vocabulary Gene Ontology (GO) to describe functions of gene products and helps to identify shared features in gene groups. The analysis of genes coregulated by OxPAPC and UVA-1 showed that the highest enrichment of biological functions concerned inflammation and immune response (Supplemental Table 1). We thus extracted the genes classified under the GO terms “inflammatory response,” “immune response,” and “immune cell activation” from the groups of genes induced by UVA-1, OxPAPC, or both stimuli (Supplemental Table 2). We found that IL-8, chemokine (C-X-C motif) ligands 2 and 3 (CXCL2 and CXCL3), and all ligands for the IL-8

receptor β (IL-8RB and CXCR2), among the 8 inflammation-related genes (Supplemental Table 2), were coinduced by UVA-1 and OxPAPC in dermal FBs. IL-8 and CXCL3 have been shown previously to be regulated by OxPAPC (32, 33). Interestingly, IL-6 and cyclooxygenase 2 (PTGS2, Cox2), which are inducible by oxidized phospholipids in endothelial cells (33, 34) were induced by UVA-1 but not by OxPAPC or UV-PAPC in dermal FBs (Supplemental Table 2). Genes coregulated by UV-PAPC and UVA-1 showed enrichment in organic acid metabolism and vascular mechanisms (Supplemental Table 1; genes are listed in Supplemental Table 3).

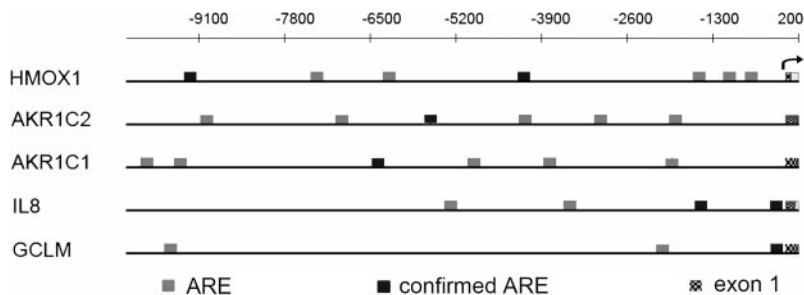
Promoter analysis

Next, we focused our analysis on the subgroup of 5 genes that were coinduced by UVA-1 and OxPAPC and also by UV-PAPC. In addition to heme oxygenase-1 (HMOX1, HO-1), the coregulated genes were the aldo-keto reductase family 1 members C1 and C2 (AKR1C1 and AKR1C2), the glutamate-cysteine ligase modifier subunit (GCLM, γ-GCS), and IL-8. We hypothesized that a concerted regulation of these genes by all three stimuli would be mediated by common regulatory elements in their respective promoter regions. To analyze the regulatory regions of these genes for the presence of shared transcription-factor binding sites, we used the TOUCAN bioinformatics tool (35). We extracted promoter regions (−10 kb to +200 bp) from the European Molecular Biology Laboratory genomic database and analyzed the sequences with the MotifScanner software implemented in TOUCAN. All five genes contained one or more variations of the ARE (Fig. 2), a binding site for the redox-sensitive transcription factor NRF2.

NRF2—the redox-sensitive transcription factor

In response to oxidant, electrophilic, or xenobiotic stress, transcription of human HO-1 (36), AKR1C1 and AKR1C2 (37), and GCLM (38) was shown to be activated by NRF2. Further, IL-8 mRNA was found to be induced by NRF2, which was also shown to stabilize the transcript (39). This finding prompted us to investigate whether oxidized lipids would induce nuclear accumulation of NRF2 and NRF2-dependent response gene expression. Using immunofluorescence microscopy, we

Figure 2. NRF2-binding sites in regulatory regions of the coregulated genes. Boxes indicate ARE sites from −10 kb to +200 bp relative to the transcription start. Gray boxes indicate ARE consensus sites, black boxes indicate binding sites with confirmed functionality. Modified graphic output from TOUCAN 3.0.2.



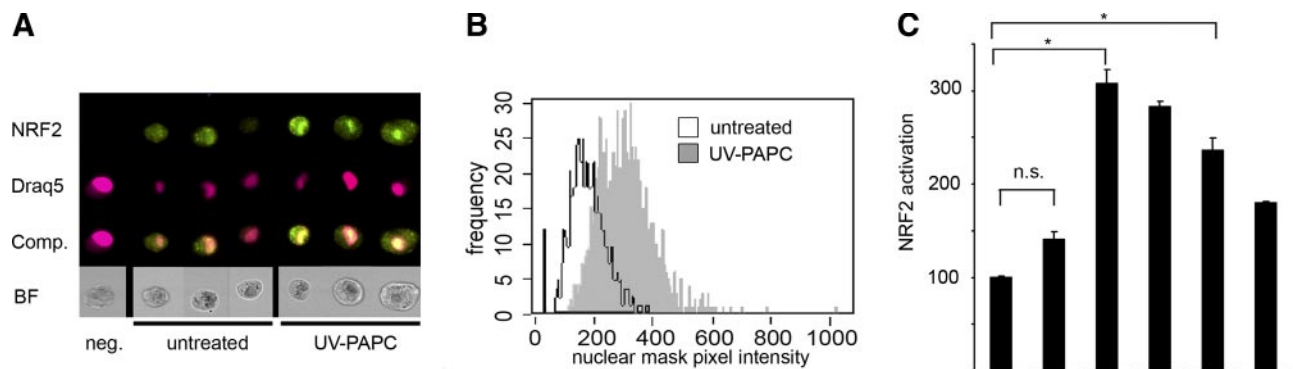


Figure 3. Nuclear accumulation of NRF2 on UV-PAPC treatment. *A*) ImageStream fluorescent imaging of UV-PAPC-treated and untreated control FBs (ctrl). NRF2 (green), Draq5 (magenta), composite NRF2 + Draq5, and bright-field (BF) (white) images of 3 representative cells of each (of >750 imaged) are shown. *B*) Similarity histogram overlay of control (black contour) and UV-PAPC-treated cells (gray fill). *x*-Axis values represent NRF2 peak intensity within the masked nuclear region. *C*) NRF2 activity assay of dermal fibroblast nuclear extract (4 µg/well). ELISA-based quantification of NRF2 bound to the consensus ARE sequence 3 h after stimulation with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (100 µg/ml) or UV-PAPC (100 and 50 µg/ml) or irradiation with UVA-1 (40 and 30 J/cm²). Data are expressed as means ± sd of absorbance at 450 nm; untreated controls were set to 100. **P* < 0.05 vs. control cells.

found increased nuclear accumulation of NRF2 in cultured dermal FBs and KCs 2 h after stimulation with UV-PAPC (100 µg/ml) (Supplemental Fig. 1), which also was followed by induction of HO-1 and GCLM mRNA expression in KCs (Supplemental Fig. 2). Because translocation of NRF2 on lipid treatment was pronounced but not complete, we quantitatively analyzed changes in cellular distribution of NRF2 using ImageStream technology. This technology couples features of flow cytometry with immunofluorescence microscopy (30) and thus allows quantification of nuclear localization based on the spectral isolation of target gene staining from nuclear staining (40). We adapted this experimental setup to quantify lipid-induced changes in nuclear intensities of NRF2 staining in large numbers of sample cells (>750 focused cells). We found strongly increased nuclear staining of NRF2 in dermal FBs 2 h after treatment with UV-PAPC (100 µg/ml), compared with untreated cells (representative cells in Fig. 3A; histogram in Fig. 3B). The mean peak pixel intensities for nuclear NRF2 staining increased from 179 ± 63 in the untreated cells to 308 ± 94 in the UV-PAPC-treated cells (Supplemental Table 5). UVA-1 treatment resulted in a less pronounced increase (216 ± 71). We performed an NRF-2 activity assay to investigate whether treatment with UV-PAPC and UVA-1 would increase binding of NRF-2 to the ARE in dermal FBs. We observed significant and dose-dependent activation of NRF2 3 h after treatment with UV-PAPC and fluency-dependent activation of NRF2 3 h after irradiation with UVA-1 (Fig. 3C). These results strongly suggest that UV-PAPC, like UVA-1 (15), leads to increased translocation and activation of NRF2 in dermal FBs.

Knockdown of NRF2 selectively inhibits the induction of the antioxidant gene cluster

To explore whether NRF2 was essential for induction of the response genes by UVA-1 and oxidized phospholip-

ids, we transfected primary dermal FBs with stealth siRNAs directed against NRF2 or the respective scrambled siRNAs and then treated the cells with OxPAPC, UV-PAPC, and UVA-1. As shown in Fig. 4A, siRNA knockdown efficiently reduced NRF2 mRNA and protein (inset in Fig. 4A) expression 48 h after transfection. The knockdown resulted in significant reduction of HO-1, GCLM, and IL-8 mRNA 4 h after treatment with oxidized lipids (UV-PAPC and OxPAPC) and UVA-1 (Fig. 4B–D). The induction of Cox2 and IL-6 mRNA expression by UVA-1 was not affected by the knockdown (Fig. 4E, F), demonstrating that the cells

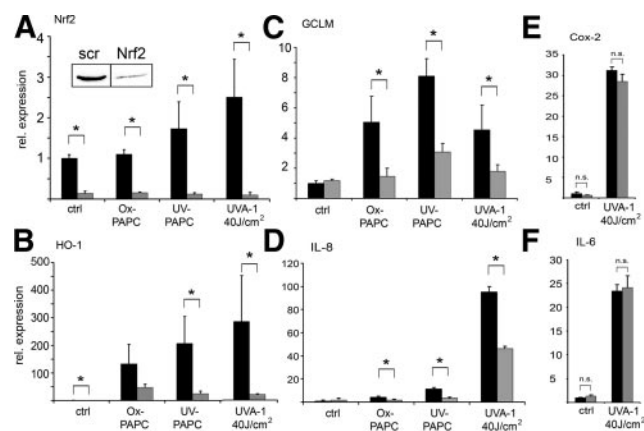


Figure 4. Silencing of NRF2 expression inhibits ARE-dependent but not other UVA-1 regulated genes. Primary human dermal FBs were transfected with NRF2 siRNA (gray bars) or scrambled siRNA (black bars). *A*) NRF2 knockdown in unstimulated and lipid- and UVA-1-treated FBs on mRNA and protein levels. Inset: Western blot. *B–F*) Expression of HO-1 (*B*), GCLM (*C*), IL-8 (*D*), Cox2 (*E*), and IL-6 (*F*) mRNA expression 4 h after induction with the indicated stimuli was assayed using qPCR. Results are means ± sd of target-gene expression normalized to β_2 -microglobulin expression. **P* < 0.05; unpaired Student's *t* test. ctrl, control.

were not generally refractory to transcriptional activation by UVA-1 after transfection with NRF2 siRNA.

Nrf2 deficiency abrogates HO-1 and GCLM induction by UVA-oxidized lipids and UVA in mice

To test Nrf2-dependent gene regulation in the skin, we used skin explants from *Nrf2*^{-/-} mice or wild-type mice, which were either irradiated with 40 J/cm² UVA-1 or treated with 100 μg/cm² UV-PAPC. Our data show that expression of HO-1 and GCLM mRNA was induced by both UV-PAPC and UVA-1 in the wild-type mice 4 h after treatment but was abrogated in the *Nrf2*^{-/-} mice. In accordance with the siRNA data, induction of *Cox2* by UVA-1 was not affected by the knockdown (Fig. 5A–F). On topical application of UV-PAPC, we observed HO-1 protein induction by immunohistochemical staining in the epidermal KCs of wild-type mice 6 h after treatment (Fig. 5G, H), which was abrogated in the *Nrf2*^{-/-} mice

(Fig. 5I, J). Further, HO-1 induction by UV-PAPC was detected in bone marrow-derived monocytes from wild-type mice but abrogated in cells from *Nrf2*^{-/-} mice (not shown). Thus, we conclude that UVA-1-oxidized phospholipids induce the antioxidant response to UVA-1 irradiation and that this transcriptional response is dependent on NRF2. Modification of membrane phospholipids is a novel mechanism for activation of the antioxidant response and a novel function for phospholipid photoproducts.

DISCUSSION

The consequences of UV irradiation to the skin have been well studied and include skin aging, perturbation of the immune system, and ultimately the promotion of serious diseases such as skin cancer. At the same time, ways to use ultraviolet light as a valuable treatment for

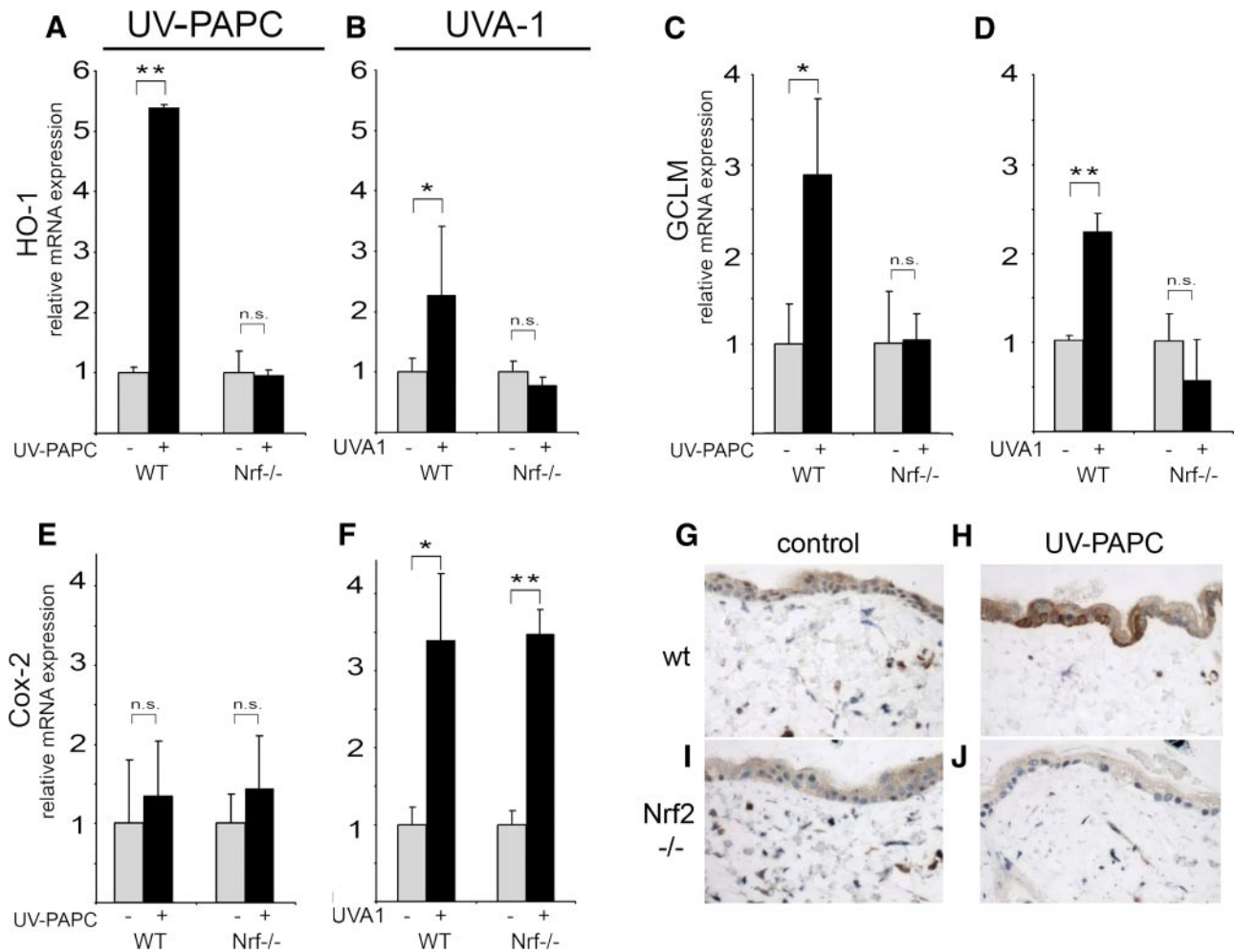


Figure 5. mRNA and protein expression in skin explants of wild-type (WT) and NRF2-deficient mice. A–F) Skin explants were treated with UV-PAPC (100 μg/cm²; A, C, E) or UVA-1 (40 J/cm²; B, D, F), and RNA was extracted after 4 h. Expression of HO-1 (A, B), GCLM (C, D), and *Cox2* (E, F) mRNA was measured using qPCR. Results are means ± SD of target-gene expression normalized to β₂-microglobulin expression. **P* < 0.1, ***P* < 0.01; unpaired Student's *t* test. G–J) Skin explants from wild-type (G, H) and *Nrf2*^{-/-} (I, J) mice were treated with vehicle (ethanol-acetone, 1:1; G, I) or UV-PAPC (100 μg/cm²; H, J) for 6 h and fixed in paraffin for immunohistochemistry. HO-1 protein was detected with anti-HO-1 SPA-896 antibody, visualized with DAB, and counterstained with Azure B.

skin and also systemic diseases have emerged. UVA-1 light (340–390 nm) can penetrate deep into the skin and is used for treatment of several inflammatory skin diseases (reviewed in ref. 41). Most of the photoproducts that form in cells after UV irradiation have been identified as unwanted or noxious effects. Surprisingly, our data indicate that early lipid oxidation products mediate potentially beneficial effects of UVA-1 irradiation. UVA-1-oxidized lipids activate the NRF2-dependent antioxidant response pathway, a target for many novel therapeutic agents (reviewed in refs. 19, 42, 43). We have recently discovered UVA-1 oxidation products of the membrane phospholipid PAPC (UV-PAPC) as novel phospholipid mediators for UVA-mediated HO-1 expression (11). The aim of the current study was to investigate a possible contribution of UV-oxidized PAPC to changes in global gene expression in dermal FBs after UV radiation and to identify the underlying mechanism. UVA-1 irradiation induced in dermal FBs expression of >300 genes that are involved in biological processes spanning from metabolism to cell death. The full spectrum of oxidation products of the membrane phospholipid PAPC, OxPAPC, induced 89 genes, and the UVA-1 oxidation products of PAPC induced 48 genes. These findings indicated that only a part of the UVA-1 response can be mediated by the signaling of oxidized phospholipids. When focusing on genes regulated by OxPAPC and UVA-1, GO analysis showed enrichment for the terms “immune response” and “inflammation.” However, when we focused further on genes that were also coregulated by the early UVA-1 oxidation products of PAPC, we identified a set of genes with mostly cytoprotective and detoxifying properties. This finding indicated that the early UV oxidation products lack most of the proinflammatory activity elicited by advanced lipid oxidation products but strongly induce the antioxidant-response genes. We show that both UV-PAPC and UVA-1 treatment induced these genes by activating the redox-sensitive transcription factor NRF2. Both treatments led to a marked increase of NRF2 nuclear accumulation and its binding to the ARE. Depletion of NRF2 using siRNA but also deficiency of Nrf2 in the skin of respective knockout animals abolished the induction of antioxidant genes by both stimuli. At the same time, other transcriptional responses to either stimulus were unaltered; therefore, the majority of genes that are not involved in the antioxidant response are regulated by UVA-1 independent of NRF2. This finding indicates that NRF2 is a specific regulator of the adaptive response to UV stress mediated by oxidized phospholipids. The genes induced by UV-PAPC *via* NRF2 are key players in the oxidative and xenobiotic stress response (44). They have well-defined functions for attenuating oxidative damage in the skin and probably also in an adaptive response to stress that is targeted in particular against harmful lipid oxidation products. Accordingly, activation of NRF2 was shown to confer photoprotection to retinal cells (45) and dermal FBs (15).

Nrf2-dependent genes in the UV response

The most strongly induced NRF2 target gene, HO-1, is protective against UV-induced damage (46) and strongly regulated by oxidized phospholipids (47). Its metabolites CO and bilirubin have antioxidant and anti-inflammatory properties (48). HO-1 is essential to prevent potentiation of lipid peroxidation through heme (49), which is set free on UVA exposure (50). Thus, induction of HO-1 is a potent negative feedback mechanism to counteract UV-induced lipid peroxidation. Glutathione (GSH) is a thiol-containing antioxidant that is essential for cellular redox homeostasis and is synthesized by glutamate cysteine ligase (GCL, γ GCS). The regulatory and catalytic subunits of GCL are transcriptionally regulated on electrophilic stress (reviewed in 51). Skin FBs of GCLM-deficient mice are highly susceptible to oxidative stress (52), and GSH is essential in the defense against UV damage (reviewed in ref. 53). In addition, here a direct feedback mechanism to UV-mediated lipid oxidation is present because GSH can be conjugated to isoprostanoid lipid peroxidation products (54), which also induce its expression (55). AKR1C1 and -C2 belong to a family of NAD(P)H-dependent oxidoreductases that are essential in steroid metabolism and also in the detoxication of lipid aldehydes (56, 57) that are found on UV stress. Both enzymes are expressed in skin cells (58, 59), but regulation by UV or oxidized lipids has not been described so far. IL-8 stands out in this list of molecules with mostly anti-inflammatory properties. IL-8 was found to be redox-regulated in an NRF2-dependent way (39) and is a major chemokine for recruitment of neutrophils. Attraction of neutrophils may represent a protective feedback mechanism to UV damage, because neutrophils participate in skin wound healing (60). The concerted induction of the antioxidant-response enzymes can thus be regarded as a specific reaction to potentially dangerous lipid oxidation products as well as an adaptive mechanism that provides antioxidants to avert future damage.

Lipid photoproducts in the UV response

There is accumulating evidence for bioactivation of lipids by UV radiation (61) and for crosstalk between the signaling of these lipids and the UV-PAPC/NRF2 response. Platelet-activating factor (PAF)-like lipids were recently discovered to be formed directly by UVB irradiation from precursor alkyl phospholipids (61, 62). PAF antagonists can inhibit endothelial barrier modulation by oxidized acyl phospholipids (63) but not by HO-1 regulation. Another recent discovery was UVB-dependent formation of ligands for the cytoplasmic aryl hydrocarbon receptor (AhR) (64). Intriguingly, this receptor can also be activated by oxLDL, a major source of oxidized phospholipids (65), and AhR and NRF2 signaling interact bidirectionally (66). Cox2, a central enzyme in lipid metabolism and strongly induced by UVA-1, was one of the genes that was, to our surprise, not significantly induced by UV-PAPC. Valencia *et al.* (67) recently described the NADPH oxidase Nox1 as a central activator of

Cox2 and other inflammatory mediators in UVA-irradiated KCs. Because there are also recent reports of involvement of the electron transport chain in the induction of NRF2 by lipids (68), it will be interesting to investigate the crosstalk between these seemingly independent pathways that both address the regulation of stress response, immune regulation, and lipid metabolism.

The mechanism of how UV-PAPC activates NRF2 was beyond the scope of this study and needs further investigation. The newest findings about NRF2, UVA-1, and oxidized phospholipids suggest to us two models that could coexist. In the first one, the oxidized and reactive lipid interacts (possibly *via* a receptor) with the plasma membrane electron transport chain complex, which in turn promotes NRF2 activation (68). The second one is direct interaction of the active lipid with KEAP1 and subsequent activation of NRF2. This model is supported by findings that oxidized eicosapentaenoic acid and docosahexaenoic acid can interact directly with KEAP1 and initiate NRF2-dependent transcription (69). Nitrated unsaturated fatty acids (70) and 15-deoxy- Δ 12,14-prostaglandin J₂, which bears similarities to structures in oxidized PAPC, can directly modify KEAP1 and thereby activate NRF2 and induce downstream genes (71).

Aging NRF2-deficient mice develop a severe inflammatory disease resembling systemic lupus erythematosus (SLE) (72, 73). The severity of this disease is strongly correlated with levels of oxidized lipids that can be detected with the E06 antibody (74), which also recognizes epitopes in UV-PAPC, as we have shown recently (11). Interestingly, UVA-1 phototherapy has a favorable effect on disease severity in patients with SLE and in mouse models of SLE (75). Future studies are needed to show whether the beneficial effects of UVA-1 phototherapy in inflammatory skin diseases are attributable to activation of NRF2-dependent genes. Further research on this topic is also needed to evaluate the use of lipid mediators based on structures found in UV-PAPC as potential drugs for diseases that can be treated with phototherapy. These studies will have to clarify whether the beneficial effects from NRF2-dependent gene regulation can outweigh (or protect from) potential damage resulting from advanced lipid oxidation *in vivo*. Our finding that UVA-1-oxidized lipids are inducers of NRF2-dependent gene expression sheds new (UV) light on the link between oxidative stress, lipid oxidation, NRF2, and the pathological changes that are associated with these parameters at several levels. **FJ**

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