Electrophilic Chemicals but not UV Irradiation or Reactive Oxygen Species Activate Nrf2 in Keratinocytes In Vitro and In Vivo

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The NF-E2-related factor 2 (Nrf2) transcription factor is a potent inducer of cytoprotective genes, which encode – among others – enzymes that detoxify reactive oxygen species (ROS). As we demonstrated a crucial role of Nrf2 in the prevention of skin carcinogenesis, it is of interest to identify Nrf2-activating factors in keratinocytes. For this purpose, keratinocytes from mice transgenic for an Nrf2-responsive reporter gene were analyzed. Electrophilic compounds activated the reporter in keratinocytes, and induced nuclear translocation of Nrf2 and the expression of known Nrf2 target genes. This is biologically relevant, as we show that Nrf2-mediated gene expression protects keratinocytes from the toxicity of these substances. By contrast, hydrogen peroxide, glucose oxidase, UVA, and UVB irradiation had no effect, although these treatments strongly increased the levels of intracellular ROS. To verify these results in vivo, transgenic reporter mice with and without functional Nrf2 alleles were topically treated with electrophilic chemicals or irradiated with UVB. Electrophiles but not UVB activated the reporter in an Nrf2-dependent manner. These results provide the basis for the identification of novel Nrf2 activators in keratinocytes with therapeutic potential for skin tumor prevention.


INTRODUCTION

The skin is frequently exposed to harmful insults such as UV irradiation or toxic chemicals. A major consequence is the formation of intracellular reactive oxygen species (ROS), which damage cellular macromolecules, leading to premature aging or even neoplastic transformation (Cerutti and Trump, 1991). In addition, large amounts of ROS are generated by inflammatory cells in injured tissues as a defense against bacterial infection. Although the antimicrobial defense is beneficial, ROS can damage the inflammatory cells themselves and other cells in their immediate environment. Therefore, cells had to develop strategies to detoxify ROS, including expression of ROS-detoxifying enzymes. Interestingly, several genes encoding such enzymes are under the control of the NF-E2-related factor 2 (Nrf2), a member of the “cap’n’ collar” family of transcription factors (Moi et al., 1994). Upon heterodimerization with other leucine zipper proteins, in particular small Maf proteins, Nrf2 binds to cis-acting elements in the promoters of its target genes, called antioxidant response element (ARE) or electrophile response element (reviewed by Nguyen et al., 2004).

In the absence of a stimulus, Nrf2 is retained in the cytoplasm via interaction with the actin-binding protein Keap1 (Itoh et al., 1999), which also mediates its degradation via the ubiquitin-proteasome pathway (Cullinan et al., 2004; Kobayashi et al., 2004). Activation of Nrf2 occurs in response to electrophilic substances, which react with Keap1 through Michael addition. As a consequence, Nrf2 is stabilized and accumulates in the nucleus, where it activates its target genes (reviewed by Itoh et al., 2004b; Nguyen et al., 2004). In addition, it has been reported that pro-oxidants can also activate Nrf2, possibly via oxidation of Keap1 and/or stimulation of Nrf2 phosphorylation by protein kinase C, mitogen-activated kinases, or phosphoinositide-3-kinase (reviewed by Jaiswal, 2004).

Recent studies revealed an important function of Nrf2 in the skin. It is highly expressed in keratinocytes and macrophages after wounding (Braun et al., 2002; Pedersen et al., 2003), and Nrf2 knockout mice showed a prolonged inflammatory response after skin injury (Braun et al., 2002). Most importantly, transgenic mice expressing a dominant-negative Nrf2 mutant in basal keratinocytes of the epidermis were much more susceptible to chemically induced skin...
Activation of Nrf2 in Keratinocytes

Activation of ARE-dependent gene expression by tBHQ depends on its electrophilic properties but not on the generation of ROS via redox cycling

As tBHQ generates ROS via redox cycling, we wondered if the tBHQ-mediated Nrf2 activation is based on its electrophilic properties or on its potency to generate ROS. For this purpose, we determined the levels of intracellular ROS using flow cytometry of cells that had been preincubated with 2′,7′-dichlorodihydrofluorescein diacetate, a membrane permeable dye, which forms a fluorescent product upon oxidation. A 2-hour treatment with tBHQ was sufficient to induce the expression of the Nrf2 target genes gclc and nqo1, and PG-J2 activated the gclc gene within this time frame (Figure 2a). Consistent with the fact that tBHQ generates ROS via redox cycling, which requires a latency period (van Ommen et al., 1992), the concentration of intracellular ROS was not elevated within 2 hours after addition of this electrophile (Figure 2c). By contrast, a 2-hour treatment with H2O2 and in particular with glucose oxidase strongly enhanced the levels of intracellular ROS (Figure 2c), but failed to activate Nrf2 target genes at any time point (Figure 2b). The tBHQ- and PG-J2-mediated induction of gclc and nqo1 gene expression was further enhanced within 2–6 hours after removal of the electrophiles. This may be due to ROS generated by redox cycling of the internalized electrophile. To test this possibility, we performed a second control

cancer, demonstrating that Nrf-dependent gene expression in keratinocytes has a cancer-preventive effect (auf dem Keller et al., 2006). Furthermore, activation of Nrf2 in the epidermis by topical application of broccoli sprout extract, which includes the electrophilic Nrf2 activator sulforaphane, protected mice against UV-induced skin carcinogenesis (Dinkova-Kostova et al., 2006). Therefore, it is of particular interest to determine the mechanisms of Nrf2 activation in keratinocytes and to identify potent and specific substances, which activate Nrf2 in these cells. In this study, we determined the ability of different electrophilic chemicals, ROS-inducing substances, and UV irradiation to activate Nrf2 in keratinocytes in vitro and in vivo.

RESULTS

Electrophiles but not ROS-inducing chemicals activate Nrf2 in cultured keratinocytes

To identify Nrf2 activators in keratinocytes, we established primary keratinocytes from mice transgenic for an ARE-hPAP (human placental alkaline phosphatase) reporter gene. The latter allow monitoring of ARE activation by staining for alkaline phosphatase activity. As shown in Figure 1a, a 16-hour treatment with the synthetic electrophile tert-butylhydroquinone (tBHQ) strongly activated the reporter. A weaker activation was observed in response to sulforaphane, PG-J2 (Figure 1a, lower panel), and glucose oxidase, which results in the continuous formation of H2O2. However, this treatment also failed to activate the reporter.

As ARE activation requires nuclear translocation of Nrf2, we analyzed nuclear lysates from untreated, tBHQ-treated, H2O2-treated, and glucose oxidase-treated immortalized murine keratinocytes for the presence of Nrf2 by Western blotting. Nrf2 was only detected in nuclear lysates of tBHQ-treated cells (Figure 1b). This result was obtained with two independent cell clones (data not shown).

Figure 1. Activation of an ARE reporter gene in keratinocytes and nuclear accumulation of Nrf2 in response to electrophilic chemicals. (a) Primary keratinocytes from ARE-hPAP transgenic mice (ARE-hPAP) were treated for 16 hours with tBHQ, PG-J2, sulforaphane, glucose oxidase, or H2O2 as described in Materials and Methods and subsequently stained for hPAP activity. DMSO-treated ARE-hPAP keratinocytes as well as keratinocytes from ARE-hPAP mice, which lack Nrf2 (Nrf2−/−) were used as controls. (b) Immortalized murine keratinocytes were treated for 30 minutes with tBHQ, glucose oxidase, or H2O2. Nuclear lysates from untreated and treated cells were analyzed by Western blotting using an Nrf2-specific antibody and a lamin A antibody as a loading control.

Activation of ARE-dependent gene expression by tBHQ depends on its electrophilic properties but not on the generation of ROS via redox cycling

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experiment to determine if the electrophilic properties of tBHQ and/or the generation of ROS by this compound are responsible for its potency to activate ARE-dependent gene expression. For this purpose, keratinocytes were treated with tBHQ for 8 hours with or without a 1-hour pretreatment with the ROS scavenger N-acetylcysteine (NAC). At this time point, tBHQ-treated cells showed strongly elevated levels of intracellular ROS, which were completely suppressed by NAC (Figure 2d). However, activation of the\textit{nqo1} and\textit{gclc} genes still occurred in the presence of NAC (Figure 2e). The slight reduction in the tBHQ-mediated expression of\textit{nqo1} and\textit{gclc} genes by NAC may result from direct covalent modification of tBHQ through the free sulfhydryl group of NAC.

**Nrf-dependent gene expression protects keratinocytes from tBHQ-mediated cell death**

To determine the biological consequences of the Nrf-mediated expression of cytoprotective genes, we compared the cytotoxicity of tBHQ for primary keratinocytes from newborn wild-type mice and from transgenic littermates expressing a dominant-negative Nrf2 mutant (dnNrf2) in the epidermis. In the latter, Nrf2-mediated gene expression in response to tBHQ is abrogated (auf dem Keller et al., 2006). Morphological abnormalities of dnNrf2-expressing keratinocytes were already observed under normal growth conditions (data not shown), and in particular in the presence of tBHQ. The overall growth of dnNrf2-expressing cells was reduced compared to wild-type cells. This was not due to a lower proliferation rate (data not shown), but to an enhanced rate of apoptosis as shown by TdT-mediated dUTP biotin nick end labeling staining. This difference was strongly enhanced when the cells were stressed by tBHQ (Figure 3a), and the rate of apoptotic dnNrf2-expressing keratinocytes reached a level of almost 60%. By contrast, wild-type cells did not show a comparable alteration in morphology or in the rate of apoptosis under stress conditions. Consistent with the enhanced rate of apoptosis, the number of dnNrf2-expressing...
keratinocytes/well was much lower compared to wild-type cells at day 8 after plating as reflected by the values obtained with the 3-[(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation/viability assay (Figure 3b, left bars). The remaining cells were much more susceptible to the toxicity of tBHQ (Figure 3b, right bars).

**ARE-dependent gene expression is not activated by UVA or UVB irradiation**

We next irradiated primary keratinocytes from ARE-hPAP mice with doses of UVA or UVB, which are within the physiological range (5-20 J/cm² UVA or 10-40 ml/cm² UVB). However, neither UVA nor UVB activated the reporter gene at any dose tested (Figure 4a and data not shown). Consistent with this finding, expression of *nqo1* and *gclc* was not induced within 2-8 hours after UVB irradiation of spontaneously immortalized murine keratinocytes (Figure 4c), although the same dose of UVB caused a significant increase in the concentration of intracellular ROS (Figure 4b) and also enhanced the levels of intracellular oxidized proteins (data not shown).

**Electrophiles but not UVB activate an ARE reporter gene in keratinocytes *in vivo***

To verify our *in vitro* data for the *in vivo* situation, we analyzed ARE activation in the skin of ARE-hPAP reporter mice. We first evoked a hyperproliferative response of the epidermis, as Nrf2 can only be activated in hyperproliferative skin (auf dem Keller *et al.*, 2006). Back skin of mice was topically treated with 12-O-tetradecanoylphorbol-13-acetate, which stimulates keratinocyte proliferation (Marks *et al.*, 1991). Forty-eight hours later mice were topically treated with tBHQ or irradiated with UVB. tBHQ strongly activated the reporter in keratinocytes as well as in cells of the underlying dermis in an Nrf2-dependent manner, whereas the solvent DMSO had no effect (auf dem Keller *et al.*, 2006 and Figure 5a, left panels). By contrast, irradiation of the skin with 100 or 300 ml/cm² UVB neither activated the reporter in keratinocytes nor in fibroblasts (Figure 5a, third panel and data not shown). The efficiency of the UVB irradiation was verified by analysis of apoptotic sunburn cells in the epidermis of irradiated mice (data not shown). These results demonstrate that electrophiles, but not ROS activate the Nrf2 transcription factor in keratinocytes *in vitro* and *in vivo*.

**DISCUSSION**

Recent studies from our laboratory revealed a potent tumor-suppressive effect of Nrf-dependent gene expression in the epidermis (auf dem Keller *et al.*, 2006), suggesting that Nrf2 activators could be used for skin protection under stress conditions.
conditions. Therefore, it is of major interest to identify the mechanisms of Nrf2 activation in keratinocytes. In this study, we identified electrophilic chemicals as potent inducers of Nrf2-mediated gene expression in mouse keratinocytes in vitro and in vivo, whereas ROS and UV irradiation had no effect.

The activation of Nrf2-mediated gene expression by electrophiles is consistent with results from previous studies that identified these substances as Nrf2 activators in different cell types (reviewed by Itoh et al., 2004b). The most likely mechanism underlying this activation is the direct reaction of electrophiles with the Nrf2 inhibitor Keap1 via Michael addition (Dinkova-Kostova et al., 2002; Zhang and Hannink, 2003; Wakabayashi et al., 2004), which results in inhibition of the Keap1-mediated proteasomal degradation of Nrf2 and accumulation of stabilized Nrf2 in the nucleus (Eggl et al., 2005; Kobayashi et al., 2006). However, electrophiles also induce the formation of ROS via redox cycling (Nakamura et al., 2003), and this was confirmed biochemically (this study). Thus, in addition to the electrophilic properties, cellular ROS generated in response to treatment with electrophiles may be involved in the Nrf2-activating effect of these chemicals. However, two observations argue against this possibility: (i) a 2-hour treatment with tBHQ was sufficient to induce the expression of known Nrf2 target genes, but was insufficient to enhance the levels of intracellular ROS, (ii) ARE-mediated gene expression in keratinocytes by tBHQ was not abrogated when the cells were pretreated with the antioxidant NAC, although this treatment completely inhibited the tBHQ-mediated increase in intracellular ROS. Thus, tBHQ and other electrophiles are likely to mediate Nrf2 activation in keratinocytes exclusively via their electrophilic properties. Consistent with this finding, Nrf2-dependent ARE activation by tBHQ was independent of oxidative stress in a neuroblastoma cell line (Lee et al., 2001).

Whereas the activation of Nrf2 by electrophiles is generally accepted, the role of ROS in Nrf2 activation is still under debate. Several investigators reported on ARE activation by ROS, for example, in response to hyperoxia or to H2O2, glucose oxidase or ROS generated by particles of diesel exhaust (Rushmore et al., 1991; Ishii et al., 2000; Baulig et al., 2003; Papaiahgari et al., 2004). Furthermore, H2O2 enhanced the expression of the Ets-1 transcription factor via activation of an ARE in the promoter of this gene (Wilson et al., 2005). By contrast, different concentrations of H2O2 and even glucose oxidase failed to activate the ARE-hPAP reporter as well as classical Nrf2 target genes in mouse keratinocytes (this study). The discrepancy between the published results and our data may reflect cell-type specific differences in Nrf2 activation. In support of this hypothesis, we found that primary murine fibroblasts and macrophages from ARE reporter mice already expressed hPAP under normal culture conditions in the absence of electrophiles or exogenous ROS (data not shown). This further suggests fundamental differences in ARE activation between keratinocytes and other cell types. Surprisingly and in contrast to this hypothesis, activation of Nrf2 in the human immortalized HaCaT keratinocyte cell line occurred in response to inorganic arsenic, and this effect was shown to involve H2O2. Furthermore, H2O2 alone induced nuclear accumulation of Nrf2 (Pi et al., 2003). However, it remains to be determined if Nrf2 target genes are indeed activated in response to H2O2 in HaCaT cells.

In addition to H2O2, UVA and UVB irradiation also failed to activate the ARE-hPAP reporter in keratinocytes, although elevated levels of ROS were observed under these conditions. Again, this selectivity appears to be cell-type dependent, as UVA induced nuclear translocation and accumulation of Nrf2 in cultured fibroblasts (Hirota et al., 2005).

The electrophile-mediated expression of Nrf2 target genes was shown to be biologically important. Thus, treatment of normal primary mouse keratinocytes with tBHQ induced the expression of various ROS-detoxifying enzymes and other cytoprotective proteins, and this induction was abrogated in keratinocytes from transgenic mice expressing a dominant-negative Nrf2 mutant (auf dem Keller et al., 2006 and this study). Interestingly, this correlated with a reduced viability of the mutant cells and enhanced susceptibility to the toxicity of tBHQ. Thus, it seems likely that tBHQ induces the expression of cytoprotective proteins, which in turn protects from the electrophilic/oxidative stress exerted by this substance.

Figure 5. Activation of ARE-hPAP activity in hyperproliferative mouse skin by tBHQ but not by UVB irradiation. Mouse back skin was topically treated with 12-O-tetradecanoylphorbol-13-acetate to induce hyperproliferation, followed by either DMSO or tBHQ treatment or UVB irradiation as indicated. Animals were killed 6 hours after the treatment. Frozen sections of treated back skin were analyzed by (a) alkaline phosphatase staining or (b) hematoxylin/eosin staining. D: Dermis, E: epidermis, HF: hair follicles. Bar = 200 μm.
The exclusive activation of the ARE-hPAP reporter in keratinocytes by electrophiles but not by ROS was also demonstrated in vivo. In these experiments, tBHQ but not UVB activated the reporter in keratinocytes of hyperproliferative mouse skin. Consistent with this finding, we only found ARE activation in cells of the granulation tissue of skin wounds, which most likely represent inflammatory cells (Auf dem Keller et al., 2006). By contrast, no activation was observed in keratinocytes, which are also exposed to large amounts of inflammatory cell-derived ROS in the wound environment (Clark, 1996). This suggests that keratinocytes have developed mechanisms to prevent ROS-induced Nrf2 activation. This could be an important prerequisite for their survival, as continuous activation of Nrf2 in keratinocytes appears to be deleterious as demonstrated by the epidermal scaling and enhanced keratinocyte differentiation observed in Keap1 knockout mice (Wakabayashi et al., 2003). Although the mechanisms underlying such differences in Nrf2 activation between keratinocytes and other cell types remain to be determined, our study unequivocally demonstrates that electrophiles, but not ROS activate ARE-mediated gene expression in keratinocytes. This result will provide an important basis for the identification of novel Nrf2 activators in this cell type.

MATERIALS AND METHODS

Establishment of primary mouse keratinocytes, treatment with different chemicals, and UV irradiation

Epidermal keratinocytes were isolated from adult mice (Yano and Okochi, 2005). To obtain spontaneously immortalized mouse keratinocytes, primary cells were isolated from newborn mice (Caldelari et al., 2000), with the exception that cells were seeded at a density of 2.5 × 10^5 cells/cm² on collagen IV (2.5 μg/cm²) coated dishes. They were cultured in defined keratinocyte serum-free medium (Invitrogen, Carlsbad, CA) supplemented with 10 ng/ml of epidermal growth factor and 10^{-10} M cholaer toxin. After spontaneous immortalization and multiple passaging, they were treated with tBHQ (50 μM; Acros Organics, Morris Plains, NJ), sulforaphane (5 μM; Sigma, Munich, Germany), hydrogen peroxide (H₂O₂; 0.5–1 mM, Merck, Darmstadt, Germany), glucose oxidase (20–100 mM/mL; Sigma), or PG-J2 (2 μM; Alexis Corporation, Lausen, Switzerland). Alternatively, they were irradiated with UVB (10–40 ml/cm²) or UVA (5–20 ml/cm²). For this purpose, 20 mJ/m² (H₂O₂; 0.5–1 mM, Merck, Darmstadt, Germany), glucose oxidase (20–100 mM/mL; Sigma), or PG-J2 (2 μM; Alexis Corporation, Lausen, Switzerland). Alternatively, they were irradiated with UVB (10–40 ml/cm²) or UVA (5–20 ml/cm²). For this purpose, 20 mJ/m² or glucose oxidase for 30 minutes. Nuclear extracts were prepared from untreated and treated cells as described by Andrews and Faller (1991) and analyzed by Western blotting for the presence of nuclear Nrf2 using a rabbit polyclonal antibody against Nrf2 (Santa Cruz, Santa Cruz, CA) and an alkaline phosphatase detection system. Probing of the membrane with a goat polyclonal antibody against lamin A (Santa Cruz) was used as a loading control.

Analysis of intracellular ROS levels

Spontaneously immortalized murine keratinocytes were grown to confluence and subsequently incubated in fresh medium containing 50 μM 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes, Leiden, The Netherlands). After a 1-hour incubation, cells were treated with different chemicals or UV irradiated, washed with phosphate-buffered saline/EDTA and incubated in phosphate-buffered saline/EDTA at 37°C for 10 minutes. Cells were trypsinized, resuspended in growth medium containing 10% fetal calf serum, pelleted, and analyzed by flow cytometry using the FL1 channel of a FACSCalibur™ (BD Biosciences, San Jose, CA), which detects fluorescence at 505–535 nm.

TaqMan real-time reverse transcriptase-PCR

Total cellular RNA was reverse transcribed using SuperScript™ II reverse transcriptase (Invitrogen, Paisley, UK) and subjected to mRNA quantification using pre-designed gene expression assays for GCLC and NQO1 (Applied Biosystems, Foster City, CA). As internal control, assays specific for eukaryotic 18S or L32 RNAs were used. Assays were performed in triplicate and evaluated by the ΔΔct-method as described by the manufacturer (Applied Biosystems).

Histological analysis

Back skin was frozen in tissue-freezing medium (Jung, Nussloch, Germany). Sections (7 μm) were fixed in 4% paraformaldehyde in phosphate-buffered saline and stained with hematoxylin/eosin. Sections were postfixed with 4° formaldehyde (10–40 mJ/cm²) or UVA (5–20 J/cm²). For this purpose, 20 mJ/m² or glucose oxidase for 30 minutes. Nuclear extracts were prepared from untreated and treated cells as described by Andrews and Faller (1991) and analyzed by Western blotting for the presence of nuclear Nrf2 using a rabbit polyclonal antibody against Nrf2 (Santa Cruz, Santa Cruz, CA) and an alkaline phosphatase detection system. Probing of the membrane with a goat polyclonal antibody against lamin A (Santa Cruz) was used as a loading control.

Genetically modified mice

Transgenic ARE reporter mice (Johnson et al., 2002) harbor a reporter construct in their genome that includes a 51 bp ARE-containing fragment of the rat NQO1 promoter upstream of an initiator element (Inr) containing a minimal promoter, followed by the hPAP cDNA. For some experiments, these transgenic mice were mated with Nrf2 knockout mice (Chan et al., 1996) to obtain ARE reporter mice lacking Nrf2. Transgenic mice expressing a dominant-negative Nrf2 mutant in the epidermis were recently described (Auf dem Keller et al., 2006).

hPAP histochemistry

hPAP immunocytochemistry/histochemistry was performed as described (Auf dem Keller et al., 2006).

Cell proliferation/viability assay

Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline and analyzed using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Penzberg, Germany) according to the manufacturer’s instructions. Cell proliferation/viability was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. For this purpose, 25 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/ml in 1 × phosphate-buffered saline) were added to the culture medium and incubated for 2 hours. The supernatant was removed, and cells were lysed in 100 μl of 0.04 M hydrochloric acid. After 10 minutes
incubation at room temperature, 100 μl H2O was added and the absorption was measured at 590 nm.

Treatment of hyperproliferative mouse skin with tBHQ or UVB
Mice (10–12 weeks of age) were anesthetized by intraperitoneal injection of ketamine/xylazine. The back of the mice was shaved and depililated (Pilca, GlaxoSmithKline, Buehl, Germany). To induce keratinocyte hyperproliferation, the skin was topically treated with 100 nm of 12-O-tetradecanoylphorbol-13-acetate (Sigma) in 200 μl acetone. Forty-eight hours later two areas of approximately 1 cm² on the mouse back were treated iteratively up to three times with a 2:1 mixture of tBHQ (100 nm dissolved in DMSO) and hydrophilic cream or with DMSO only. Two hours after the last application, the animals were killed, and the treated skin was embedded in tissue-freezing medium without prior fixation. Alternatively, mice were irradiated with 100 or 300 mJ/cm² UVB 48 hours after application of 12-O-tetradecanoylphorbol-13-acetate using the UVB light source described above. At different time points after UVB exposure mice were killed, and the irradiated skin was frozen in tissue-freezing medium. All animal experiments were performed with permission from the local veterinary authorities, Zurich, Switzerland.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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