Free Radical Research, 2003 Vol. 37 (6), pp. 655–663

The Role of H_2O_2 as a Mediator of UVB-induced Apoptosis in Keratinocytes

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Accepted by Professor H. Sies

(Received 10 July 2002; In revised form 30 September 2002)

Apoptosis is an active form of cell death that is initiated by a variety of stimuli, including reactive oxygen species (ROS) and ultraviolet (UV) radiation. Previously, it has been reported that UVB-irradiation of keratinocytes leads to intracellular generation of hydrogen peroxide (H_2O_2) and that antioxidants can inhibit ROS-induced apoptosis. Although both UVB-irradiation and H_2O_2-incubation led to increased intracellular H_2O_2 levels, the antioxidants catalase and glutathione monoester (GME), inhibited apoptosis only when induced by H_2O_2, not by UVB. Furthermore, extracellular signal-regulated kinase (ERK), a prominent member of the mitogen-activated protein kinase (MAPK) family, was found to be activated by treatment with both UVB and H_2O_2. Inhibition of ERK phosphorylation by pre-treatment with PD98059 resulted in enhanced apoptosis after H_2O_2-exposure. However, no significant difference of apoptosis was observed between cells with and without inhibitor pre-treatment upon UVB-irradiation. DNA damage in the form of cyclobutane pyrimidine dimers was observed after exposure to UVB, but no photoproducts were found in H_2O_2-treated cells. These results suggest a ROS-independent pathway of UVB-induced apoptosis. Although UVB-irradiation causes moderate increase in H_2O_2, the generation of H_2O_2 does not contribute to the induction of apoptosis. Moreover, activation of ERK only blocks H_2O_2-dependent apoptosis but has no impact on UVB-induced apoptosis.

Keywords: Antioxidant; Apoptosis; ERK; H_2O_2; Poly(ADP-ribose); UVB

INTRODUCTION

Apoptosis is an important and well-controlled form of cell death that occurs under a variety of physiological and pathological conditions, such as elevation of intracellular Ca^{2+}, ionizing and non-ionizing irradiation, growth factor deprivation and oxidative stress.[1,2] The skin, which is situated at the interface between the body and its environment, acts as a barrier to harmful effects of ultraviolet (UV) radiation and exogenous chemicals. It has been shown that UVB induces apoptosis in keratinocytes and epithelial cell lines.[3–6] Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and superoxide anion radicals (O_2^-) are known mediators of intracellular signaling cascades. It was demonstrated that ROS and the resulting oxidative stress play an important role in apoptosis.[7] ROS can react with all biological macromolecules, i.e. lipids,[8] proteins,[9] carbohydrates and nucleic acids.[10] This may result in oxidative DNA modifications, protein–protein and protein–DNA crosslink formation.[11–13] Generally, UVA radiation is regarded as the most relevant source of ROS generation in skin.[14] Recent reports suggest, however, that also UVB radiation generates ROS intracellularly in keratinocytes.[15,16] It was shown that, UVB induced generation of ROS, such as H_2O_2, $OH^{\bullet}$ and O_2^-, in keratinocytes.[15,17,18] Furthermore, it was reported that UVB-induced apoptosis can be inhibited by various antioxidants.[19]

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases.[20] The p42/p44 extracellular signal-regulated kinase (ERK1 and 2) are the best characterized of MAPKs. ERK–MAPKs have been shown to play an important role in

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1071576031000094907

cell proliferation, differentiation, survival and apoptosis.[21] Up to date, the role of ERK on apoptosis is discussed controversially. The function of ERK in apoptosis appears to depend on the cell type as well as on the stimulus and the cellular environment.[22–24]

Poly(ADP-ribose) (PAR) formation was previously shown to be an early and very sensitive marker for the detection of apoptosis induced by UVB and H_2O_2 in keratinocytes. PAR formation correlated well with conventional markers of apoptosis, such as cell blebbing, DNA laddering and TUNEL assay.[25] The present study was undertaken to clarify the role of H_2O_2 in UVB-induced apoptosis using PAR formation as an apoptosis marker in keratinocytes, and to investigate the influence of ERK on ROS- and UVB-induced apoptosis. It was demonstrated that UVB induced apoptosis via mechanisms independent of ROS, and that H_2O_2, but not UVB-induced apoptosis is influenced by ERK activation.

MATERIALS AND METHODS

Cell Culture

Human immortalized keratinocytes (HaCaT) were cultured in Dulbecco's modified essential medium (DMEM; Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany), 100 U/ml penicillin/streptomycin and 0.25 μg/ml amphotericin B (Sigma, Munich, Germany) at 37°C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity

To assess cytotoxicity, trypan blue staining was carried out. Dead cells are incapable of excluding trypan blue, therefore, the uptake of this dye was used as a marker of cell death. A 10 μl of trypan blue (Merck, Darmstadt, Germany) was mixed with 50 μl cell suspension. For each sample, at least 100 cells per field were counted with a hemocytometer under a light microscope. Cytotoxicity was defined as the percentage of trypan blue positive cells.

UVB-irradiation and H_2O_2-treatment

HaCaT cells were UVB-irradiated or H_2O_2-treated as described previously,[25] and post-incubated in DMEM medium with 10% FCS for indicated time periods. In order to detect the dose-dependent effect on apoptosis, thymine dimer formation, and ERK phosphorylation by UVB, different doses of UVB (10, 20, 30 mJ/cm^2) were employed. For the antioxidant experiments, the lowest UVB dose which showed clear effects on apoptosis (20 mJ/cm^2) was used.

Catalase Pre-treatment

Catalase was obtained from Sigma (Munich, Germany, 2,000–5,000 U/mg). Prior to UVB- or H_2O_2-exposure, HaCaT cells were pre-treated with 100 ng/ml, 1 μg/ml, or 10 μg/ml catalase in DMEM medium for a period of 24 h, as previously described for neutrophils.[26] Then, the medium was removed and cells were washed twice with PBS. Thereafter, cells were covered with PBS and irradiated with 20 mJ/cm^2 UVB, post-incubated with catalase (at levels matching the pre-incubation periods) for 16 h. Thereafter, PAR immunofluorescence was performed.[25]

For H_2O_2-treatment, cells were pre-incubated with catalase in the same manner as given for UVB experiments. After the catalase pre-incubation, cell culture medium was removed completely, cells were washed twice with PBS, and then exposed to 1 mM H_2O_2 for 16 h. For H_2O_2 experiments, no catalase post-incubation was performed, since this would directly lead to extracellular scavenging of H_2O_2.

Glutathione Monoester (GME) Pre-treatment

Prior to UVB or H_2O_2 exposure, HaCaT cells were pre-treated with 1 and 10 mM GME (Glutathione reduced, ethyl ester, GME) in DMEM medium for 6 h. Thereafter, the medium was removed and cells were washed with PBS twice. Then, cells were covered with PBS and irradiated with 20 mJ/cm^2 UVB, and post-incubated for 16 h. Thereafter, PAR immunofluorescence was performed.

For H_2O_2 treatment experiments, cells were pre-incubated with the same concentrations of GME given for UVB experiments. Afterwards, cell culture medium was removed completely, cells were washed twice with PBS, and exposed to 1 mM H_2O_2 for 16 h.

NAC Pre-treatment

Prior to UVB or H_2O_2 exposure, HaCaT cells were pre-treated with different concentrations of *N*-acetyl-L-cysteine (NAC) (0.5–20 mM) in DMEM medium for 1 h. Thereafter, cells were washed twice with PBS, irradiated with 20 mJ/cm^2 UVB, and post-incubated for 16 h.

For H_2O_2 treatment experiments, cells were pre-incubated with NAC in the same manner as given for UVB experiments. After the NAC pre-incubation, cell culture medium was removed completely, cells were washed twice with PBS, and exposed to 1 mM H_2O_2 for 16 h.

Immunofluorescence of Thymine Dimers

Confluent HaCaT cells grown on coverslips were exposed to 0, 10 or 30 mJ/cm^2 of UVB or to 1 mM

H_2O_2. Cells were analyzed at 30 min, as well as 2, 16 and 24 h after exposure for immunofluorescence of DNA photoproducts. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, kept in freshly prepared 70 mM NaOH in 70% ethanol for 2 min to denature DNA, followed by neutralization for 1 min in 100 mM Tris–HCl (pH 7.5). Cells were then washed once in 70% ethanol and twice in PBS for 5 min each. After washing, cells were incubated with 10% goat serum in PBS for 30 min to prevent non-specific binding. Cells were incubated with 50 μg/ml cyclobutane thymine dimer-specific monoclonal antibody (clone KTM53, Kamiya Biomedical company, Seattle, WA, USA). After incubation for 1 h at 37°C and three washes in PBS, cells were subsequently incubated with a Cy3™-conjugated goat anti-mouse secondary antibody for 1 h at 37°C. Coverslips were then washed in PBS, embedded in Vectashield™ mounting medium and analyzed by BX-40 fluorescence microscopy and, subsequently, densitometric image analysis. Three areas of each slide were randomly selected and the staining intensity per cell was measured and quantified using Analysis 3.0 Software™ (Soft Imaging System, Muenster, Germany).

Fluorescence Measurement of Intracellular Peroxides

H_2O_2 generation was measured using a H_2O_2-sensitive fluorescent probe 2′,7′-dichlorofluorescin-diacetate (DCF-DA) as described previously.[27] Confluent HaCaT cells were treated with 30 mJ/cm^2 UVB or 1 mM H_2O_2 for 30 min, and then incubated with 20 μM DCF-DA (Sigma, Munich, Germany) for additional 30 min at 37°C. After chilling on ice, cells were washed with ice-cold PBS, trypsinized and resuspended at 5×10^5 cells/ml in PBS containing 2% fetal bovine serum and 20 μM DCF-DA. The fluorescence intensities of oxidized form 2′,7′- dichlorofluorescein of 10,000 cells from each sample were analyzed using a flow cytometer (DAKO GALAXY, Hamburg, Germany) with excitation and emission settings of 488 and 525 nm, respectively. Data were analyzed using FloMax Software (Partec, Muenster, Germany). Results are given as the mean peak (MP) of fluorescence intensity.

Activation of ERK

Phosphorylation of ERK was examined by western blot analysis using antibody against phospho-ERK (Cell Signaling Technology, Frankfurt, Germany). After serum starvation for 24 h, cells were subjected to H_2O_2 or UVB treatments as indicated. Thereafter, HaCaT cells were extracted in a lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (1 mM PMSF, 20 μg/ml Aprotinin, 20 μg/ml Leupeptin, 10 mM Benzamidin, 1 mM Vanadate, 50 mM NaF). Protein extracts were resolved by SDS-PAGE on 10% acrylamide gels and were transferred to PVDF membranes (Amersham Pharmacia Biotech, Freiburg, Germany). After blocking with TBS-Tween buffer containing 5% dry milk for 30 min at room temperature, membranes were incubated overnight at 4°C with anti-phospho-ERK antibody diluted 1:2,000 in TBS-Tween buffer containing 1% dry milk. The membranes were washed three times with TBS-Tween, and, subsequently incubated for 1 h at room temperature with HRP-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) (diluted 1:5,000). After three washes in PBS-Tween, proteins were visualized using a ECL detection system (Amersham Pharmacia Biotech) according to the manufacturer's instructions. To ensure the equal loading of proteins, the same stripped membranes were probed with an antibody for panERK (BD Transduction Laboratories, Heidelberg, Germany).

PD98059 Treatment

HaCaT cells were pre-treated with 20 μM of the MEK1 inhibitor PD98059 (Calbiochem, Schwalbach, Germany) for 1 h before exposure to 30 mJ/cm^2 UVB or 1 mM H_2O_2. After the treatments, cells were incubated in the medium with 20 μM PD98059 for 15, 30 and 60 min. Thereafter, western blot for detection of phospho-ERK was performed. For the detection of apoptosis, after pre-treatment with PD98059, cells were irradiated with 10 or 30 mJ/cm^2 UVB and incubated for 4, 8, 16 and 24 h, or exposed to 0.1–1 mM H_2O_2 and incubated for 16 h. Thereafter, immunostaining of PAR was performed.

Statistics

Statistical analysis was carried out using Instat™ (Graphpad, San Diego, CA). Significance test between groups was performed using ANOVA. All data are expressed as mean ± standard error of the mean (Mean ± SEM). Experiments were carried out in triplicates and repeated at least twice.

RESULTS

UVB-irradiation and H_2O_2-incubation Induce Generation of Intercellular H_2O_2

As shown in Fig. 1, in response to both stressors, UVB-irradiation and H_2O_2-exposure,

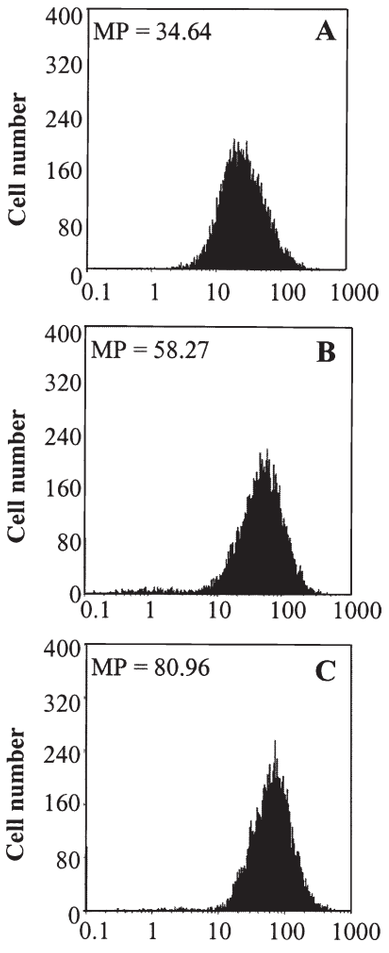

FIGURE 1 UVB- and H_2O_2-exposure increase intracellular levels of H_2O_2 in keratinocytes. HaCaT cells were treated either with 30 mJ/cm^2 of UVB or 1 mM H_2O_2 for 30 min, subsequently incubated with 20 μM 2′,7′-dichlorfluorescencein diacetate (DCF-DA) for 30 min at 37°C, and analyzed by flow cytometry. Control without treatment (A); 30 min after 30 mJ/cm^2 UVB-irradiation (B); 30 min after 1 mM H_2O_2 incubation (C). MP = mean peak. MP values are given for the respective scan from a representative experiment.

the fluorescence intensity was significantly increased as compared with baseline levels of control cells (MP = 34.64). 30 min after UVB-irradiation with 30 mJ/cm^2 or after incubation with 1 mM H_2O_2, the intracellular H_2O_2 concentration increased rapidly (MP = 58.27 and 80.96, respectively).

Catalase and GME Pre-treatment Inhibit H_2O_2-induced, but not UVB-induced Apoptosis

H_2O_2-induced apoptosis was significantly inhibited by pre-incubation with catalase. Both, morphological features (data not shown) and PAR formation showed a significant decrease in the number of apoptotic cells. In contrast, pre- and post-incubation with the same concentrations of catalase did not show any protective effect against UVB-induced apoptosis (Fig. 2A). Moreover, pre-incubation of HaCaT cells with another H_2O_2 scavenger, GME, inhibited H_2O_2-induced apoptosis, but had no effect on UVB-induced PAR formation (Fig. 2B). Similar results were obtained in NAC treated cells. Pre-incubation with NAC at 2.5 and 5 mM for 1 h protected the cells from H_2O_2-induced apoptosis. However, similar to catalase and GME, pre-treatment with the same concentrations of NAC did not show any protective effect against UVB-induced apoptosis (data not shown). Both antioxidants, catalase and GME, had no effect on cytotoxicity, as detected by trypan blue staining (data not shown).

Both UVB and H_2O_2 Induce ERK Phosphorylation

It was previously described that ERK is highly activated by treatment with oxidants.[28,29] In this study, the activation of ERK after treatment with UVB and H_2O_2 was evaluated. Levels of phosphorylated ERK were examined from 15 to 60 min after exposure to 10–30 mJ/cm^2 UVB or 0.1–1 mM H_2O_2. Within 15 min of both UVB- and H_2O_2-treatment, ERK phosphorylation was maximally upregulated (Figs. 3A and 4A). By 30 min after treatment, the activity declined. Total ERK protein levels at all time points were equivalent as measured by immunoblot of the same stripped membranes using a pan-ERK antibody. Phospho-ERK and pan-ERK antibodies detect both ERK1 and ERK2, however, the band which is visible depends on the size of the gel, the concentration of acrylamide, and the time of running the gel. The band shown in Figs. 3 and 4 is mostly ERK1. The resolution of the gel did not separate both isoforms.

Inhibition of ERK Phosphorylation Results in Enhanced Apoptosis after H_2O_2-treatment, but not after UVB-irradiation

The inhibition of ERK activation induced by UVB or H_2O_2 by MEK inhibitor PD98059 was determined. HaCaT cells were pre-incubated with 20 μM PD98059 for 1 h, and exposed to 30 mJ/cm^2 UVB or 1 mM H_2O_2 for 15–60 min. Both UVB- and H_2O_2-stimulated ERK phosphorylation were inhibited by PD98059 pre-treatment (Figs. 3B and 4B).

Thereafter, the effect of ERK activation on HaCaT cell apoptosis was investigated. After pre-treatment with 20 μM PD98059 for 1 h, HaCaT cells were exposed to 10–30 mJ/cm^2 UVB or 0.1–1 mM H_2O_2. As shown in Fig. 5B, 16 h after incubation with 0.5 and 1 mM H_2O_2, PAR formation increased significantly in cells pre-treated with PD98059. However, no significant difference in PAR formation was observed between cells with and without PD98059 pre-treatment, followed by UVB irradiation (Fig. 5A). PD98059 had no effect on cytotoxicity, as detected by trypan blue staining (data not shown).

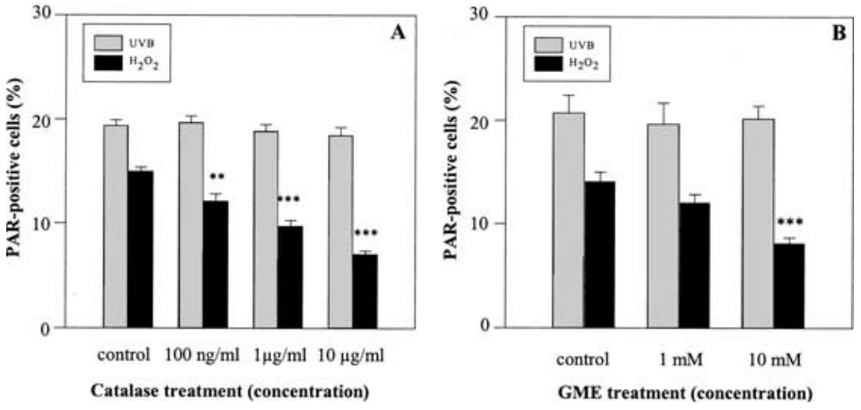

FIGURE 2 Catalase and GME pre-treatment inhibit PAR formation in H_2O_2-treated, but not in UVB-irradiated cells. (A) HaCaT cells were pre-incubated with different concentrations of catalase, exposed to 20 mJ/cm^2 of UVB, and post-incubated with catalase for 16 h prior to PAR immunofluorescence. For the H_2O_2 treatment, cells were pre-treated with catalase for 24 h and incubated with 1 mM H_2O_2 for 16 h prior to PAR immunofluorescence. (B) HaCaT cells were pre-incubated with different concentrations of GME, exposed to 20 mJ/cm^2 of UVB or 1 mM H_2O_2, and post-incubated for 16 h prior to PAR immunofluorescence. "control": cells were subjected to the same procedure, only without GME, but the same amount of vehicle (water). $^{**}p < 0.01$, $^{***}p < 0.001$.

UVB but not H_2O_2 Induces Formation of Cyclobutane Thymine Dimers in Nuclear DNA of HaCaT Cells

As shown in Fig. 6, UVB-exposure induced formation of cyclobutane thymine dimers in nuclear DNA of HaCaT cells. However, no significant difference of photoproducts was found between control and H_2O_2-treated cells (Fig. 6D, H, and I). As early as 30 min after 30 mJ/cm^2 UVB-exposure, thymine dimer formation increased significantly, and even 10 mJ/cm^2 of UVB resulted in detectable levels of thymine dimers (Fig 6B, C, F, and G).

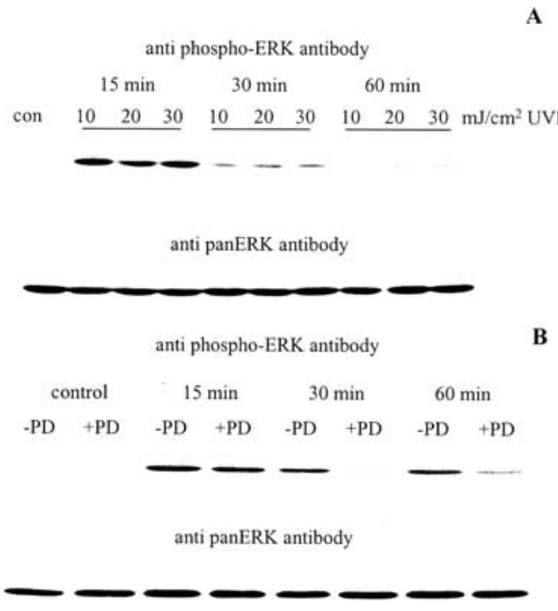

FIGURE 3 UVB induces ERK phosphorylation and inhibition of ERK phosphorylation by PD98059. HaCaT cells were irradiated with 10–30 mJ/cm^2 UVB, and post-incubated for 15–60 min (A). HaCaT cells were pre-treated with 20 μM PD98059 for 1 h, exposed to 30 mJ/cm^2 UVB and post-incubated for 15–60 min (B). Total proteins were extracted and separated by SDS-PAGE gel electrophoresis, transferred to PVDF membrane and visualized with anti phospho-ERK antibody. Equal loading of protein was monitored by immunoblot with anti panERK antibody using the same stripped membrane. ERK phosphorylation was maximally activated 15 min after irradiation, and was inhibited by PD98059. "-PD": instead of PD98059, the same concentration of DMSO was employed as control.

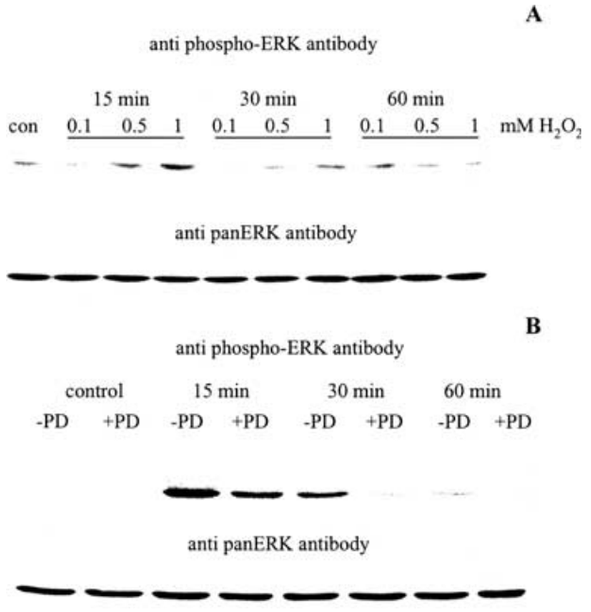

FIGURE 4 H_2O_2 induces ERK phosphorylation and inhibition of ERK phosphorylation by PD98059. HaCaT cells were exposed to 0.1–1 mM H_2O_2 for 15–60 min (A). HaCaT cells were pre-treated with 20 μM PD98059 for 1 h, exposed to 1 mM H_2O_2 for 15–60 min (B). Immunoblotting with anti–phospho-ERK antibody was performed. Equal loading of protein was monitored by immunoblotting with anti-panERK antibody using the same stripped membrane. ERK phosphorylation was maximally activated 15 min after H_2O_2 treatment, and was inhibited by PD98059. "-PD": instead of PD98059, the same concentration of DMSO was employed as control.

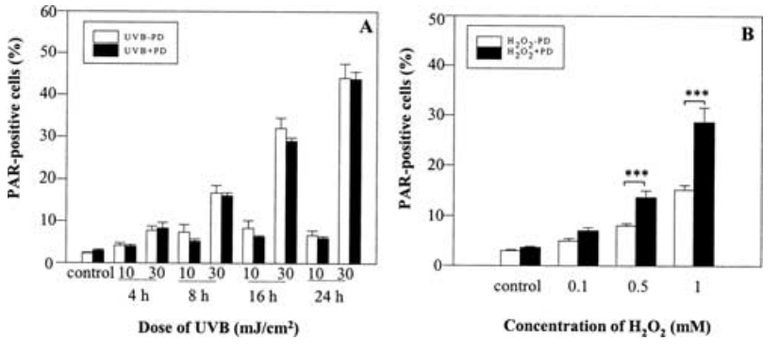

FIGURE 5 Pre-treatment of HaCaT cells with PD98059 enhances H_2O_2-induced apoptosis, but has no effect on UVB-induced apoptosis. (A) Cells were pre-incubated with 20 μM PD98059 for 1 h before exposure to 10 or 30 mJ/cm^2 UVB. Apoptosis was assessed by PAR immunofluorescence at 4, 8, 16 and 24 h after the radiation. (B) Cells were pre-treated with 20 μM PD98059 for 1 h before the addition of different concentrations of H_2O_2 (0.1, 0.5 and 1 mM). PAR formation was detected 16 h after the exposure. "-PD": instead of PD98059, the same concentration of DMSO was employed as control. $^{**}p < 0.01$, $^{***}p < 0.001$.

DISCUSSION

It is well known that UVB-irradiation induces intracellular ROS generation,[15,30] and that both ROS and UVB induce apoptosis[17,18] and ERK phosphorylation[31] in keratinocytes. Some reports also propose that the effects of UVB-irradiation are largely mediated by H_2O_2 and other ROS.[16,31,32] In the present study using PAR as a marker for keratinocyte apoptosis, we were able to demonstrate that (i) UVB-induced apoptosis is not dependent on intracellular H_2O_2 generation, and (ii) H_2O_2, but not UVB-induced apoptosis is influenced by ERK phosphorylation. These results indicate that UVB- and H_2O_2-induced apoptosis in HaCaT cells employed different mechanisms.

It has been reported that ROS and the resulting cellular redox changes regulate signal transduction pathways during apoptosis, and accumulation of ROS plays an important role in mediating the apoptotic effect of UV irradiation.[30] It has also been shown that UVB-irradiation of keratinocytes leads to dose-dependent intracellular production of H_2O_2,[16] and that the effects of UVB-irradiation, such as NF-kappa B activation[32] and phosphorylation of EGF receptors,[16] may be mediated, at least in part, by H_2O_2.[31] Thus, ROS were regarded as the second messenger to regulate biological effects in response to UVB irradiation.[31,32] Based on these findings, it was hypothesized that UVB-induced apoptosis in HaCaT cells is mediated by intracellular H_2O_2 generation. However, although in our system UVB-irradiation led to a formation of H_2O_2 in keratinocytes, neither pre-incubation of keratinocytes with catalase (Fig. 2A) nor GME (Fig. 2B) inhibited UVB-induced PAR formation. In contrast, apoptosis induced by H_2O_2 could be counteracted by pre-treatment with catalase and GME, and H_2O_2-induced PAR formation was reduced by almost 50% (Fig 2A and B). Antioxidants catalase and GME are powerful H_2O_2 scavengers, the thiol-compound NAC has antioxidant activities both as a direct scavenger of oxidant radicals and as a precursor of glutathione (GSH) synthesis. Catalase reacts very efficiently with H_2O_2 to form water and molecular oxygen. GSH serves as a substrate in the reduction of peroxides catalyzed by glutathione peroxidase (GSHPx) which converts H_2O_2 to water and yields oxidized glutathione (GSSG). These findings indicate that although UVB is capable of inducing increased intracellular H_2O_2 level and apoptosis, UVB-induced apoptosis in keratinocytes is mediated mainly by mechanisms independent of H_2O_2. Similarly, Bush *et al.* reported that pre-treatment with non-toxic doses of NAC showed no significant protection against UVB-induced apoptosis in keratinocytes.[33] These data suggest that oxidative stress may only play a minor role in UVB-induced apoptosis in keratinocytes.

Recently, studies provided evidence that ROS, and in particular H_2O_2, function as prominent mediators in the activation and regulation of UVB-induced ERK signal transduction pathways in normal human keratinocytes. The role of ERK activation in apoptosis remains controversial and seems to depend on cell lines and treatments used.[22,23,34] To further characterize mechanisms that are involved in apoptosis mediated by UVB and H_2O_2, ERK activation was evaluated. It was shown that both UVB- and H_2O_2-treatment resulted in ERK phosphorylation, and that the potent kinase inhibitor PD98059 inhibited ERK activation upon UVB-irradiation and H_2O_2-incubation, indicating that ERK activation was mediated via classical Raf-MEK-ERK pathway. However, PD98059 treatment increased H_2O_2-induced apoptosis, but had no effect on UVB-induced PAR formation (Fig. 5). These results suggest that UVB-induced apoptosis is not mediated via the activation of the ERK signaling pathway. Similarly, it was reported by

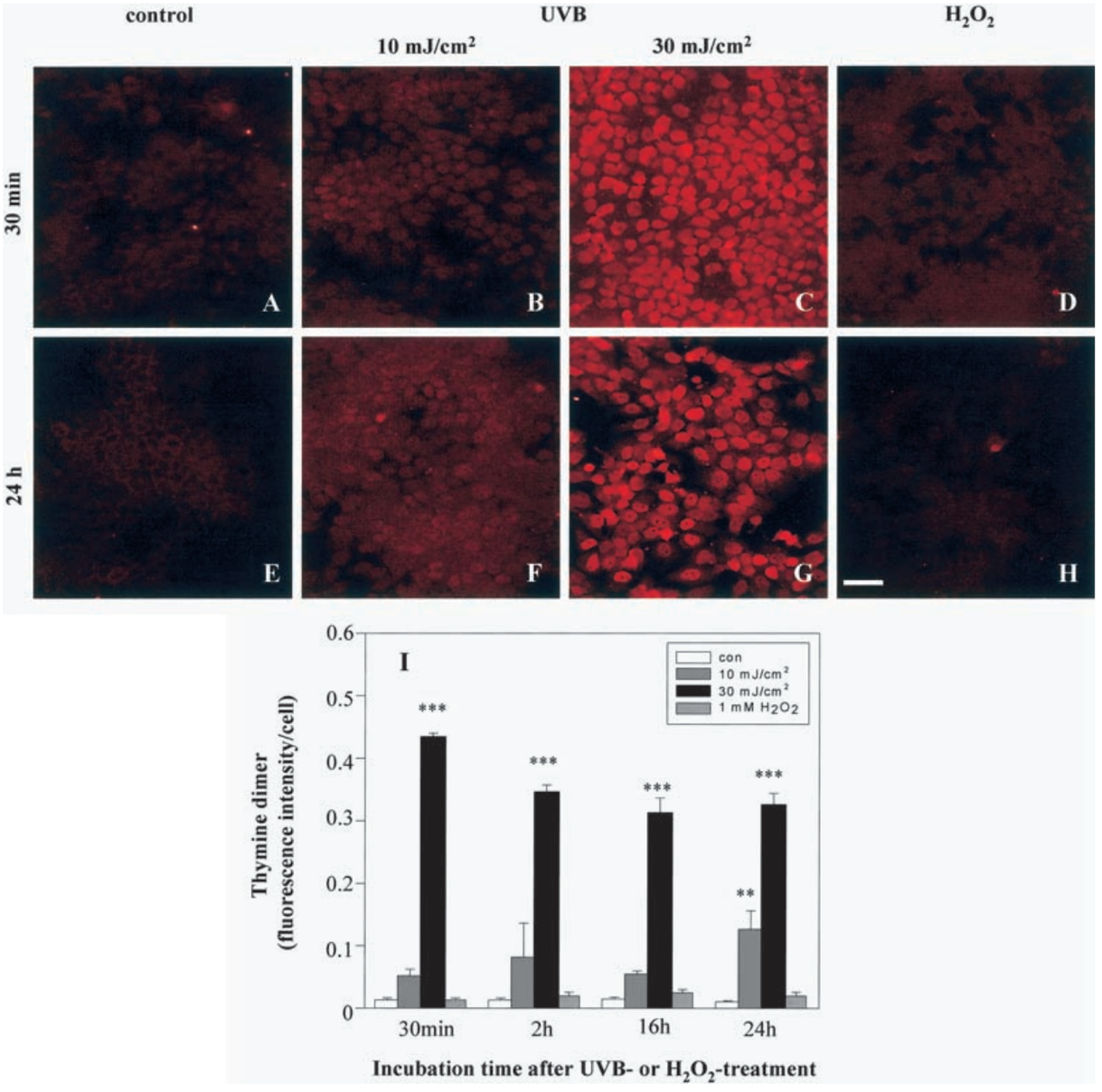

FIGURE 6 Dose-dependent generation of cyclobutane pyrimidine dimers (CPD) by UVB-irradiation, but not by H_2O_2-treatment. Cells were either irradiated with 10 and 30 mJ/cm^2 UVB and post-incubated between 30 min and 24 h (B, C, F, and G), or incubated with 1 mM H_2O_2 between 30 min and 24 h (D and H), A and E: control. After treatment cells were incubated with an anti-CPD antibody and analyzed by immunofluorescence staining (A–H). Fluorescence intensity was quantified by Analysis 3.0 Software™ (Soft Imaging System, Muenster, Germany) (I). $^{**}p < 0.01$, $^{***}p < 0.001$.

Nakamura *et al.*[35] that UVB-irradiation induced a slight increase of ERK phosphorylation in SV40-transformed human keratinocytes, and UVB-induced cell death was not significantly affected by PD98059. In contrast to the data presented in this study, Peus *et al.* reported inhibition of ERK phosphorylation by PD98059 resulted in enhanced cell death 24 h after 40 mJ/cm^2 UVB irradiation in normal human keratinocytes.[31] Differences between the studies might depend on cells and the spectrum of UV-light which were used. These findings indicate that the anti-apoptotic contribution of ERK may be cell type and stimulus dependent. Moreover, higher UVB doses may generate higher concentration of intracellular H_2O_2, which might have a more notable impact on apoptosis.

Downstream events of ERK activation for its ability to suppress apoptosis are still not well understood. However, the following findings may explain the anti-apoptotic role of ERK described here

for H_2O_2-induced apoptosis. It is known that mitochondrial dysfunction, including loss of mitochondria transmembrane potential,[36] release of cytochrome *c* to the cytoplasm,[37] and loss of bcl-2,[38] plays a central role in ROS-induced apoptosis. Caspase 9 is activated by cytochrome *c* release[39] and, in turn, activates the key cytosolic downstream caspase, caspase 3, thereafter, promoting cell death. Erhardt *et al.* have shown that ERK activation inhibits cytosolic caspase-3 activation following release of cytochrome *c* from the mitochondria.[40] A further possible target of the ERK pathway is the pro-apoptotic Bcl-2 family protein Bad.[41] Binding of Bad inhibits the anti-apoptotic function of Bcl-2/Bcl-X_L, thereby, promoting cell death. With Bad sequestered in the cytosol upon its phosphorylation, the anti-apoptotic Bcl-2/Bcl-X_L protein can prevent the release of cytochrome *c* and activation of the caspase cascade,[42,43] and, subsequently, inhibits apoptosis. Scheid and Duromio have shown that inhibition of ERK phosphorylation by PD98059 inhibits Bad phosphorylation, suggesting that phosphorylation of Bad requires activation of ERK.[41]

Mechanisms that are less sensitive to cellular redox control, such as UVB-induced formation of cyclobutane pyrimidine dimers and ⟨6–4⟩ photoproducts may prevail in UVB-induced apoptosis.[44] Therefore, we compared cyclobutane dimer formation in H_2O_2- and UVB-treated cells. DNA damage in the form of cyclobutane thymine dimers appeared as early as 30 min after UVB-irradiation in a clearly dose-dependent fashion, and thus preceded to the detection of apoptosis (Fig. 6). Importantly, no cyclobutane thymine dimers were detected in H_2O_2-treated cells up to 24 h after exposure (Fig. 6). UVB-induced apoptosis in keratinocytes may be mediated by rapidly formed DNA photoproducts, rather than by H_2O_2. These findings are in agreement with a similar study, in which HeLa cells that were exposed to UVB showed significantly reduced levels of both cyclobutane pyrimidine dimers and apoptosis when incubated with the DNA repair enzyme photolyase immediately after UVB-exposure.[45]

Recently, Kulms *et al.* have reported that UVB induced apoptosis can be induced by three independent pathways: DNA damage, death receptor activation and ROS. At first sight, this appears to be conflicting with our findings in that our results suggest that H_2O_2 is not involved in UVB induced apoptosis. A number of methodological differences may account for this: While Kulms *et al.* used serum starvation for 24 h before stimulation of HeLa cells with a UVB dose of 40 mJ/cm^2, the present study was carried out in HaCaT cells that were not serum starved prior to stimulation, and the UVB dose was only 20 mJ/cm^2 for the antioxidant experiments. Furthermore, the most potent antioxidant used by Kulms *et al.* for ROS inhibition, pyrrolidine-dithiocarbamate (PDTC), is not specifically scavenging H_2O_2, but has recently been demonstrated to be a powerful HOCl scavenger preventing from oxidative protein damage,[46] while that antioxidant catalase, used in the present study, is a specific H_2O_2 scavenger. Taken together, these findings suggest, that ROS other than H_2O_2 may account for the redox-sensitive pathway described for UVB induced apoptosis.

In conclusion, the findings of the present study demonstrate that, although both UVB and H_2O_2 induce apoptosis in HaCaT cells, different pathways are involved. While UVB generates increased levels of intracellular H_2O_2 in keratinocytes, H_2O_2 appears not to be a major mediator of UVB-induced apoptosis.

Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Th-620/2-1, Th-620/2-2).

References

[1] Martensson, J., Jain, A., Stole, E., Frayer, W., Auld, P.A. and Meister, A. (1991) "Inhibition of glutathione synthesis in the newborn rat: a model for endogenously produced oxidative stress", *Proc. Natl Acad. Sci. USA* **88**, 9360–9364.
[2] Murphy, G., Young, A.R., Wulf, H.C., Kulms, D. and Schwarz, T. (2001) "The molecular determinants of sunburn cell formation", *Exp. Dermatol.* **10**, 155–160.
[3] Casciola-Rosen, L.A., Anhalt, G. and Rosen, A. (1994) "Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes", *J. Exp. Med.* **179**, 1317–1330.
[4] Schwarz, A., Bhardwaj, R., Aragane, Y., Mahnke, K., Riemann, H., Metze, D., Luger, T.A. and Schwarz, T. (1995) "Ultraviolet-B-induced apoptosis of keratinocytes: evidence for partial involvement of tumor necrosis factor-alpha in the formation of sunburn cells", *J. Invest. Dermatol.* **104**, 922–927.
[5] Benassi, L., Ottani, D., Fantini, F., Marconi, A., Chiodino, C., Giannetti, A. and Pincelli, C. (1997) "1,25-dihydroxyvitamin D3, transforming growth factor beta1, calcium and ultraviolet B radiation induce apoptosis in cultured human keratinocytes", *J. Invest. Dermatol.* **109**, 276–282.
[6] Gniadecki, R., Hansen, M. and Wulf, H.C. (1997) "Two pathways for induction of apoptosis by ultraviolet radiation in cultured human keratinocytes", *J. Invest. Dermatol.* **109**, 163–169.
[7] Kannan, K. and Jain, S.K. (2000) "Oxidative stress and apoptosis", *Pathophysiology* **7**, 153–163.
[8] Thiele, J.J., Traber, M.G. and Packer, L. (1998) "Depletion of human stratum corneum vitamin E: an early and sensitive *in vivo* marker of UV induced photo-oxidation", *J. Invest. Dermatol.* **110**, 756–761.
[9] Thiele, J.J., Hsieh, S.N., Briviba, K. and Sies, H. (1999) "Protein oxidation in human stratum corneum: susceptibility of keratins to oxidation *in vitro* and presence of a keratin oxidation gradient *in vivo*", *J. Invest. Dermatol.* **113**, 335–339.
[10] Stadtman, E.R. (1992) "Protein oxidation and aging", *Science* **257**, 1220–1224.
[11] Griffiths, H.R. (2000) "Antioxidants and protein oxidation", *Free Radic. Res.* **33**, S47–S58.
[12] Floyd, R.A. (1990) "Role of oxygen free radicals in carcinogenesis and brain ischemia", *FASEB J.* **4**, 2587–2597.
[13] Cadet, J., Ravanat, J.L., Buchko, G.W., Yeo, H.C. and Ames, B.N. (1994) "Singlet oxygen DNA damage: chromatographic

and mass spectrometric analysis of damage products", *Methods Enzymol.* **234**, 79–88.
[14] Klotz, L.O., Holbrook, N.J. and Sies, H. (2001) UVA and singlet oxygen as inducers of cutaneous signaling events. *Curr. Probl. Dermatol.* **29**, 95–113.
[15] Lawley, W., Doherty, A., Denniss, S., Chauhan, D., Pruijn, G., van Venrooij, W.J., Lunec, J. and Herbert, K. (2000) "Rapid lupus autoantigen relocalization and reactive oxygen species accumulation following ultraviolet irradiation of human keratinocytes", *Rheumatology (Oxford)* **39**, 253–261.
[16] Peus, D., Vasa, R.A., Meves, A., Pott, M., Beyerle, A., Squillace, K. and Pittelkow, M.R. (1998) "H_2O_2 is an important mediator of UVB-induced EGF-receptor phosphorylation in cultured keratinocytes", *J. Invest. Dermatol.* **110**, 966–971.
[17] Baba, T., Hanada, K. and Hashimoto, I. (1996) "The study of ultraviolet B-induced apoptosis in cultured mouse keratinocytes and in mouse skin", *J. Dermatol. Sci.* **12**, 18–23.
[18] Henseleit, U., Rosenbach, T. and Kolde, G. (1996) "Induction of apoptosis in human HaCaT keratinocytes", *Arch. Dermatol. Res.* **288**, 676–683.
[19] Takahashi, H., Hashimoto, Y., Aoki, N., Kinouchi, M., Ishida-Yamamoto, A. and Iizuka, H. (2000) "Copper, zinc-superoxide dismutase protects from ultraviolet B-induced apoptosis of SV40-transformed human keratinocytes: the protection is associated with the increased levels of antioxidant enzymes", *J. Dermatol. Sci.* **23**, 12–21.
[20] Seger, R. and Krebs, E.G. (1995) "The MAPK signaling cascade", *FASEB J.* **9**, 726–735.
[21] Su, B. and Karin, M. (1996) "Mitogen-activated protein kinase cascades and regulation of gene expression", *Curr. Opin. Immunol.* **8**, 402–411.
[22] Gunn-Moore, F.J., Williams, A.G., Toms, N.J. and Tavare, J.M. (1997) "Activation of mitogen-activated protein kinase and p70S6 kinase is not correlated with cerebellar granule cell survival", *Biochem. J.* **324**, 365–369.
[23] Aikawa, R., Komuro, I., Yamazaki, T., Zou, Y., Kudoh, S., Tanaka, M., Shiojima, I., Hiroi, Y. and Yazaki, Y. (1997) "Oxidative stress activates extracellular signal-regulated kinases through Src and Ras in cultured cardiac myocytes of neonatal rats", *J. Clin. Investig.* **100**, 1813–1821.
[24] Goillot, E., Raingeaud, J., Ranger, A., Tepper, R.I., Davis, R.J., Harlow, E. and Sanchez, I. (1997) "Mitogen-activated protein kinase-mediated Fas apoptotic signaling pathway", *Proc. Natl Acad. Sci. USA* **94**, 3302–3307.
[25] Chang, H., Sander, C.S., Muller, C.S., Elsner, P. and Thiele, J.J. (2002) "Detection of poly(ADP-ribose) by immunocytochemistry: a sensitive new method for the early identification of UVB- and H_2O_2-induced apoptosis in keratinocytes", *Biol. Chem.* **383**, 703–708.
[26] Aoshiba, K., Yasui, S., Hayashi, M., Tamaoki, J. and Nagai, A. (1999) "Role of p38-mitogen-activated protein kinase in spontaneous apoptosis of human neutrophils", *J. Immunol.* **162**, 1692–1700.
[27] Yu, R., Tan, T.H. and Kong, A.T. (1997) "Butylated hydroxyanisole and its metabolite tert-butylhydroquinone differentially regulate mitogen-activated protein kinases. The role of oxidative stress in the activation of mitogen-activated protein kinases by phenolic antioxidants", *J. Biol. Chem.* **272**, 28962–28970.
[28] Guyton, K.Z., Liu, Y., Gorospe, M., Xu, Q. and Holbrook, N.J. (1996) "Activation of mitogen-activated protein kinase by H_2O_2. Role in cell survival following oxidant injury", *J. Biol. Chem.* **271**, 4138–4142.
[29] Wang, X., Martindale, J.L., Liu, Y. and Holbrook, N.J. (1998) "The cellular response to oxidative stress: influences of mitogen-activated protein kinase signaling pathways on cell survival", *Biochem. J.* **333**, 291–300.
[30] Chan, W.H. and Yu, J.S. (2000) "Inhibition of UV irradiation-induced oxidative stress and apoptotic biochemical changes in human epidermal carcinoma A431 cells by genistein", *J. Cell. Biochem.* **78**, 73–84.
[31] Peus, D., Vasa, R.A., Beyerle, A., Meves, A., Krautmacher, C. and Pittelkow, M.R. (1999) "UVB activates ERK1/2 and p38 signaling pathways via reactive oxygen species in cultured keratinocytes", *J. Invest. Dermatol.* **112**, 751–756.
[32] Flohe, L., Brigelius-Flohe, R., Saliou, C., Traber, M.G. and Packer, L. (1997) "Redox regulation of NF-kappa B activation", *Free Radic. Biol. Med.* **22**, 1115–1126.
[33] Bush, J.A., Ho, V.C., Mitchell, D.L., Tron, V.A. and Li, G. (1999) "Effect of *N*-acetylcysteine on UVB-induced apoptosis and DNA repair in human and mouse keratinocytes", *Photochem. Photobiol.* **70**, 329–333.
[34] Sheng, Z., Knowlton, K., Chen, J., Hoshijima, M., Brown, J.H. and Chien, K.R. (1997) "Cardiotrophin 1 (CT-1) inhibition of cardiac myocyte apoptosis via a mitogen-activated protein kinase-dependent pathway. Divergence from downstream CT-1 signals for myocardial cell hypertrophy", *J. Biol. Chem.* **272**, 5783–5791.
[35] Nakamura, S., Takahashi, H., Kinouchi, M., Manabe, A., Ishida-Yamamoto, A., Hashimoto, Y. and Iizuka, H. (2001) "Differential phosphorylation of mitogen-activated protein kinase families by epidermal growth factor and ultraviolet B irradiation in SV40-transformed human keratinocytes", *J. Dermatol. Sci.* **25**, 139–149.
[36] Zamzami, N., Hirsch, T., Dallaporta, B., Petit, P.X. and Kroemer, G. (1997) "Mitochondrial implication in accidental and programmed cell death: apoptosis and necrosis", *J. Bioenerg. Biomembr.* **29**, 185–193.
[37] Reed, J.C. (1997) "Cytochrome *c*: can't live with it—can't live without it", *Cell* **91**, 559–562.
[38] Kane, D.J., Sarafian, T.A., Anton, R., Hahn, H., Gralla, E.B., Valentine, J.S., Ord, T. and Bredesen, D.E. (1993) "Bcl-2 inhibition of neural death: decreased generation of reactive oxygen species", *Science* **262**, 1274–1277.
[39] Li, S. and Sedivy, J.M. (1993) "Raf-1 protein kinase activates the NF-kappa B transcription factor by dissociating the cytoplasmic NF-kappa B-I kappa B complex", *Proc. Natl Acad. Sci. USA* **90**, 9247–9251.
[40] Erhardt, P., Schremser, E.J. and Cooper, G.M. (1999) "B-Raf inhibits programmed cell death downstream of cytochrome *c* release from mitochondria by activating the MEK/Erk pathway", *Mol. Cell. Biol.* **19**, 5308–5315.
[41] Scheid, M.P. and Duronio, V. (1998) "Dissociation of cytokine-induced phosphorylation of Bad and activation of PKB/akt: involvement of MEK upstream of Bad phosphorylation", *Proc. Natl Acad. Sci. USA* **95**, 7439–7444.
[42] Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B. and Korsmeyer, S.J. (1995) "Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death", *Cell* **80**, 285–291.
[43] Pettmann, B. and Henderson, C.E. (1998) "Neuronal cell death", *Neuron* **20**, 633–647.
[44] Kulms, D. and Schwarz, T. (2000) "Molecular mechanisms of UV-induced apoptosis", *Photodermatol. Photoimmunol. Photomed.* **16**, 195–201.
[45] Kulms, D., Poppelmann, B., Yarosh, D., Luger, T.A., Krutmann, J. and Schwarz, T. (1999) "Nuclear and cell membrane effects contribute independently to the induction of apoptosis in human cells exposed to UVB radiation", *Proc. Natl Acad. Sci. USA* **96**, 7974–7979.
[46] Zhu, B.Z., Carr, A.C. and Frei, B. (2002) "Pyrrolidine dithiocarbamate is a potent antioxidant against hypochlorous acid-induced protein damage", *FEBS Lett.* **532**, 80–84.