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Even after UVA-exposure will nitric oxide protect cells from reactive oxygen intermediate-mediated apoptosis and necrosis

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Abstract

Reactive oxygen species (ROS) play a pivotal role in UVA-induced cell damage. As expression of the inducible nitric oxide synthase (iNOS) is a normal response of human skin to UV radiation we examined the role of nitric oxide (NO) as a protective agent during or even after UVA_1- or ROS-exposure against apoptosis or necrosis of rat endothelial cells. When added during or up to 2 h subsequent to UVA_1 or ROS exposure the NO-donor S-nitroso-cysteine (SNOC) at concentrations from 100–1000 μM significantly protects from both apoptosis as well as necrosis. The NO-mediated protection strongly correlates with complete inhibition of lipid peroxidation (sixfold increase of malonedialdehyde formation in untreated *versus* 1.2-fold with 1 mM SNOC). NO-mediated protection of membrane function was also shown by the inhibition of cytochrome *c* leakage in UVA_1 treated cells, a process not accompanied by alterations in Bax and Bcl-2 protein levels. Thus, the experiments presented demonstrate that NO exposure during or even after a ROS-mediated toxic insult fully protects from apoptosis or necrosis by maintaining membrane integrity and function. *Cell Death and Differentiation* (2001) **8,** 515–527.

Keywords: UVA_1; Rose Bengal; nitric oxide; NO donors; singlet oxygen; reactive oxygen species

Abbreviations: BHT, butylated hydroxytoluene; EC, endothelial cells; iNOS, inducible nitric oxide synthase; NO, nitric oxide; RB/hν, Rose Bengal plus visible light; ROS, reactive oxygen species; SNOC, S-nitrosocysteine; SOD, superoxide dismutase; UVA_1, ultraviolet radiation A_1

Introduction

Ultraviolet radiation is divided into three regions: UVC (190–290 nm), UVB (290–320 nm) and UVA (320–400 nm). Effects on cells by UV radiation of shorter wavelengths, UVB and UVC, have been extensively investigated in terms of direct effects on DNA.[1] UVA was initially perceived as innocuous to skin however, numerous reports recently have demonstrated cytotoxic effects after exposure of eukaryotic cells to UVA radiation as a consequence of oxidative stress. UVA-induced oxidative damage has been reported for several target molecules as well as organelles.[2,3]

A plethora of anti-oxidant and anti-apoptotic defense mechanisms exists in mammalian cells, such as enzymes or compounds which quench or remove reactive oxygen species (ROS), proteins with chaperone activity such as heat shock proteins[4] or proteins of the bcl-2 family which have been shown to serve as potent anti-apoptotic factors.[5] Recent evidence suggests that the signal molecule nitric oxide (NO) can also act as an antiapoptotic agent.[6] NO and equivalent amounts of citrulline are synthesized from the guanidino nitrogen of L-arginine by NO synthases (NOS) found in endothelial cells, neurons and upon activation by proinflammatory stimuli in most cell types. The NOS enzyme family consists of three isoenzymes: constitutively expressed and calcium/calmodulin-regulated are the endothelial (ecNOS) and the neuronal (ncNOS) isoenzymes which produce regulated low amounts of NO for short periods of time, whereas the cytokine-inducible and calcium-independent isoenzyme (iNOS) synthesizes NO for extended periods of time.[7,8]

Cytokines are known modulators of endothelial cell functions during inflammatory processes. One prominent effect that cytokines can exert in endothelial cells is the induction of iNOS and thus high-output NO synthesis.[9] Recently, we have shown that exposure well before UVA_1-irradiation of endothelial cells to NO either endogenously synthesized by iNOS after challenge by proinflammatory cytokines or applied exogenously in the form of a NO-donor fully protects cells from UVA_1-induced cell damage. The protective effect strongly correlated with a NO-induced increase in Bcl-2 protein expression.[10] As in the *vivo* situation NO-generation by iNOS will start after skin exposure to sunlight, we now tested whether and by which mechanisms a protection after UV-irradiation is exerted. We show a strong protective effect of NO even when added during or up to 2 h after the toxic insult. This timing does not allow for increased Bcl-2 expression and we here demonstrate that nitric oxide acts as a powerful inhibitor of ROS-mediated lipid peroxidation with a concomitant arrest of Bax and Bcl-2 independent

mitochondrial cytochrome *c* release. Thus data provide further evidence for the protective role of NO synthesis in skin helping to prevent cellular damage after UV-exposure.

Results

Characterization of endothelial cell death pathways

Irradiation of EC with UVA_1 leading to endothelial apoptotic cell death in a dose-dependent manner has been described previously.[10] Using irradiation with a dose of 10 J/cm^2 UVA, the appearance of apoptotic markers becomes significant after a delay of 4–8 h (Figure 1A) as quantified by Hoechst staining and *in situ* nick translation. In addition, UVA_1-induced cell death is completely inhibited by incubation with the caspase inhibitor Z-VAD (Figure 1B) as additional prove for apoptosis.

We also treated cells with singlet oxygen, generated by photoexcitation after pre-loading with Rose Bengal (RB/hν). Dependent on the concentration of Rose Bengal cell death was maximal at 1.8 μM RB/hν, with half maximal induction of cell death at 0.9 μM RB/hν (Figure 2A). Interestingly, the mode of cell death was necrosis exclusively as evidenced by the rapid onset of cell death which was nearly complete after 2–4 h (Figure 2B) but also by the complete lack of apoptotic nuclear morphology as well as failure of protection by the caspase inhibitor Z-VAD (Figure 2C). Because of its lipophilic character, incubation of endothelial cells with Rose Bengal led to its enrichment into intracellular membranes predominantly[11] (Figure 2D).

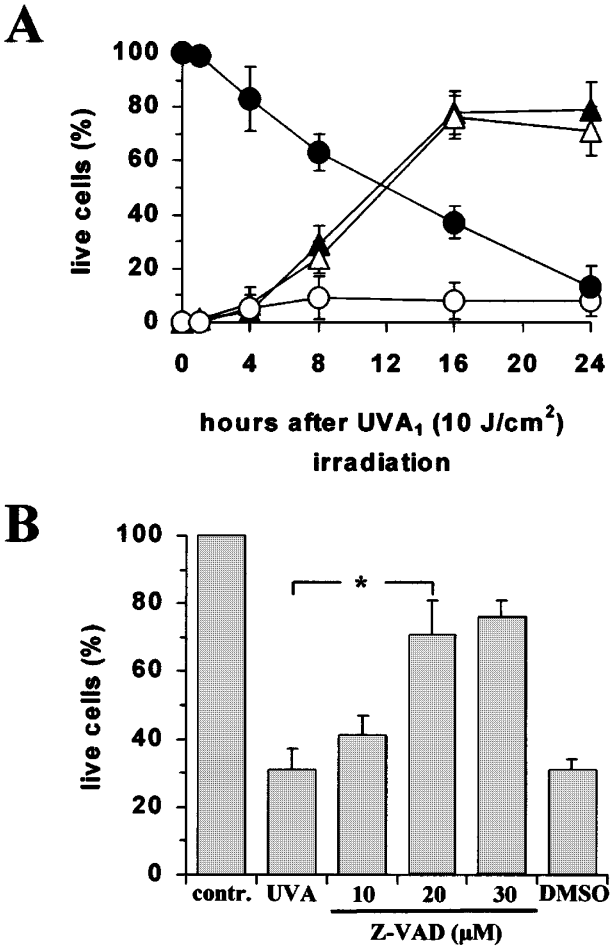

Figure 1 UVA_1 irradiation of endothelial cells leads to cell death via apoptosis. Resting endothelial cells (EC) were exposed to UVA_1 radiation and number of viable (●), apoptotic (△, ▲), and necrotic cells (○) was determined at times indicated. Increasing UVA_1 intensities after 24 h lead to increasing number of cells with nuclear chromatin condensation and nuclear fragmentation as indicators for apoptosis. (**A**), Time-course of UVA_1-induced apoptotic events after UVA_1-irradiation with 10 J/cm^2 as determined by appearance of cells with pyknotic nuclei, nuclear chromatin condensation, and nuclear fragmentation in Hoechst 33343 stained cells (△) or DNA strand breaks visualized by in situ nick translation (▲). At and later than 16 h after UVA_1-irradiation, the number of apoptotic nuclei is significantly increased and 24 h after irradiation with 10 J/cm^2 approximately 80% of cells are apoptotic and about 10% are necrotic as judged by propidium iodide staining. (**B**), Cultures were also incubated with a pan-caspase inhibitor at concentrations indicated prior to UVA_1-irradiation (8 J/cm^2) and the relative number of viable cells was determined by neutral red staining as described in Materials and Methods. Z-VAD fully protects from UVA_1-induced (10 J/cm^2) cell death in a concentration dependent manner. Values are the mean ± S.D. of 3–12 individual experiments *, $P<0.001$

Protection by antioxidative agents

Adding various antioxidants we elucidated the role of ROS during UVA_1-irradiation or Rose Bengal treatment. Agents were added to cell cultures during UVA_1-irradiation or RB/hν treatment and the numbers of viable cells were determined after 24 h of culture. Deuterium oxide (D_2O) prolonging the lifetime of $^1O_2$ significantly enhanced EC death after UVA_1-irradiation or RB/hν-challenge. Conversely, compounds quenching singlet oxygen, i.e. NaN_3 (20 mM), histidine (40 mM), and imidazole (40 mM) exerted a significant protection (Figure 3A) suggesting the involvement of singlet oxygen generation early in UVA_1-induced cell death, as has been reported previously.[3,12,13] All four compounds gave the same exacerbation or augmentation also in RB/hν-induced toxicity. Next the involvement of superoxide anion generation was screened by adding the $O_2^{\cdot -}$-quencher MnTBAP (80 μM) or DETC (1 mM), an inhibitor of the intracellular SOD. Both, $O_2^{\cdot -}$-depletion by MnTBAP or inhibition of its conversion by SOD to H_2O_2 fully protected cells from induced apoptosis or necrosis (Figure 3B). In contrast, addition of SOD (1000 U/ml) significantly enhanced cell death, while heat-inactivated SOD was without effect (Figure 3B) indicating that H_2O_2 more than $O_2^{\cdot -}$ may represent the toxic agent during UVA-irradiation or RB/hν-treatment. This is supported by our findings that addition of catalase (2000 U/ml) during the toxic inputs was fully protective, whereas the catalase inhibitor 3-amino-1,2,4-triazole (500 μM) enhanced cytotoxicity (Figure 3C). The antioxidant BHT (10 μM), an strong inhibitor of lipid peroxidation, fully protects (Figure 3D) whereas neither DMSO (100 mM) nor mannitol (100 mM) both scavenging of $OH^{\cdot}$ influenced cell death rates indicating that $OH^{\cdot}$ appears not to contribute to UVA_1-or RB/hν-induced cell death (data not shown).

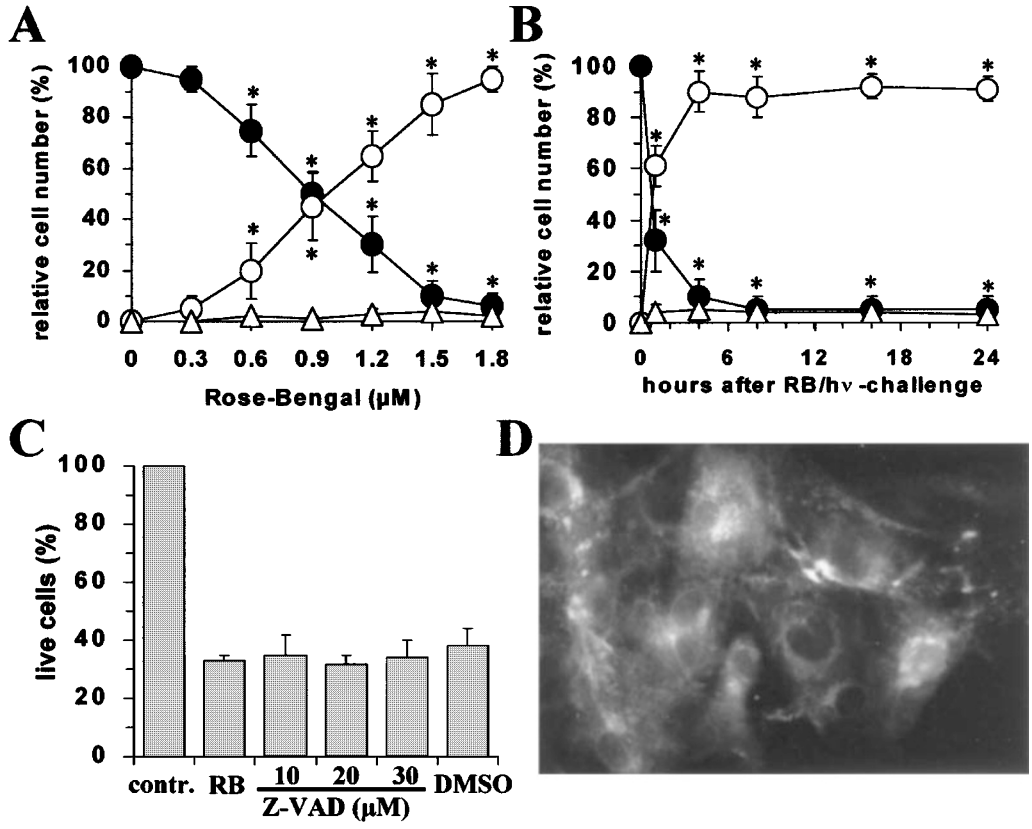

Figure 2 Singlet oxygen-generation in endothelial cells leads to cell death via necrosis. Resting endothelial cells (EC) were pre-loaded with Rose Bengal at concentrations indicated and singlet oxygen generation was induced by irradiation with visible light (RB/hν). The relative number of viable (●), necrotic (○), and apoptotic cells (△) was determined as in Figure 1. (**A**), Endothelial cell death rates correlated with concentration of RB. Death occurred via necrosis as seen by the increase of propidium iodide positive cells (△) and failure to detect apoptotic nuclei with H33342 at any of the RB concentrations used (△). (**B**), Time-course of RB/hν-induced (1.5 μM) cell death shows an early and rapid onset of necrotic cell death (○) with apoptosis in $\leqslant$5% of the cells only (△). (**C**), The caspase inhibitor Z-VAD showed no protection from RB/hν-induced cell death as a further evidence for necrosis. (**D**), A fluorescence photomicrograph of a culture incubated with Rose Bengal clearly demonstrates a strong enrichment of the dye predominantly in membrane of the nucleus, mitochondria, as well as endoplasmatic reticulum. Magnifications: $\times$650. Values are the mean $\pm$ S.D. of 3–8 individual experiments. *, $P<0.001$

Exogenous NO present during the experimental procedure fully protects from both apoptosis caused by UVA_1 and necrosis caused by singlet oxygen

We had shown previously that the presence of NO for 24 h prior to UVA fully protected cells from apoptosis.[10] We now investigated whether NO could also protect when present during treatment and whether it would exacerbate or augment RB/hν-induced necrosis. During UVA treatment cells were incubated with the NO donors SNOC or DETA/NO or with the respective controls cysteine or DETA. SNOC, in a concentration-dependent manner, lowered or completely inhibited UVA_1-induced apoptosis as well as RB/hν-induced necrosis of EC. Concentrations of SNOC $\geqslant$100 μM exerted substantial protection (Figure 4A,B), with best results found with 1 mM SNOC, whereas the control compound cysteine was without effect (Figure 4A,B). Experiments using the NO donor DETA/NO gave similar results (Figure 4C,D).

Time window for protection by nitric oxide or antioxidants added after the insults

To examine whether the various agents tested will also protect when added after UVA_1-irradiation or singlet oxygen treatment, antioxidants or nitric oxide were added to EC cultures at different time points after the treatments.

After UVA_1-irradiation (Figure 5A) or Rose Bengal-challenge (Figure 5B), NaN_3 (20 mM) ceased to protect when added as early as 5 min after UVA_1. MnTBAP (80 μM) showed a significant protection when added up to 15 min after the toxic stimuli. In contrast, catalase (2000 U/ml) or BHT (10 μM) show comparable time-courses of protection which is significant when added to cell cultures as late as 2 h after UVA_1-challenge or 1 h after RB/hν-treatment. Examining the protective time window for NO by addition of the NO-donor SNOC (1 mM) at different time intervals after UVA_1-irradiation (Figure 5A) or RB/hν treatment (Figure 5B) we find a similar time window for protection against UVA_1-induced apoptosis or $^1O_2$-induced

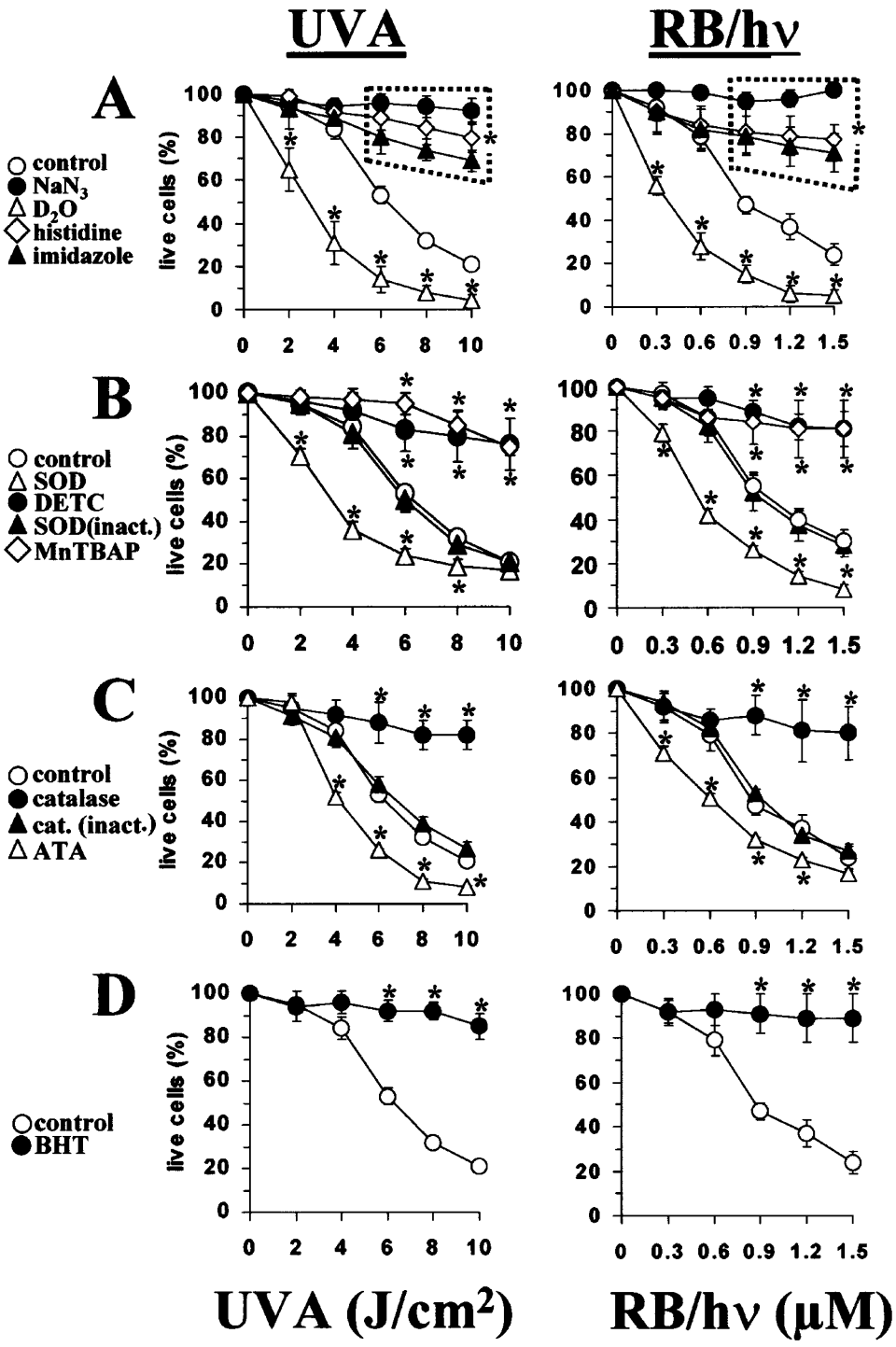

Figure 3 Inhibiting formation of reactive oxygen species will protect from cell death. To examine the role of reactive oxygen species (ROS) during UVA_1-irradiation or RB/hν treatment, various compounds with specific ROS-inhibitory activities were added during UVA_1 or RB/hν and the number of live cells was determined as described in Material and Methods. (**A**), Quenchers of $^1O_2$, i.e. NaN_3 (●; 20 mM), histidine (◇, 40 mM), and imidazole (▲; 40 mM) protected (dotted cage) whereas prolongation of $^1O_2$ half-life (D_2O; △) enhanced cell death. (**B**), Removal of superoxide anions ($O_2^{-\cdot}$) by MnTBAP (◇; 80 μM) and inhibition of intracellular SOD by DETC (●; 1 mM) also protected whereas SOD (△; 1000 U/ml) significantly enhanced cell destruction, while heat-inactivated SOD had no effect (▲). (**C**), Catalase-mediated (●; 2000 U/ml) degradation of H_2O_2 also protects while heat-inactivated catalase (▲) was without effect and inhibition of endogenous catalase by 3-amino-1,2,4-triazole (△; ATA, 500 μM) strongly enhanced cytotoxicity. (**D**), Butylated hydroxytoluene (●; BHT, 10 μM), an inhibitor of lipid peroxidation also fully protects. In all graphs sham treated controls are shown (○). Values are the mean ± S.D. of 3–10 individual experiments. *, $P<0.001$

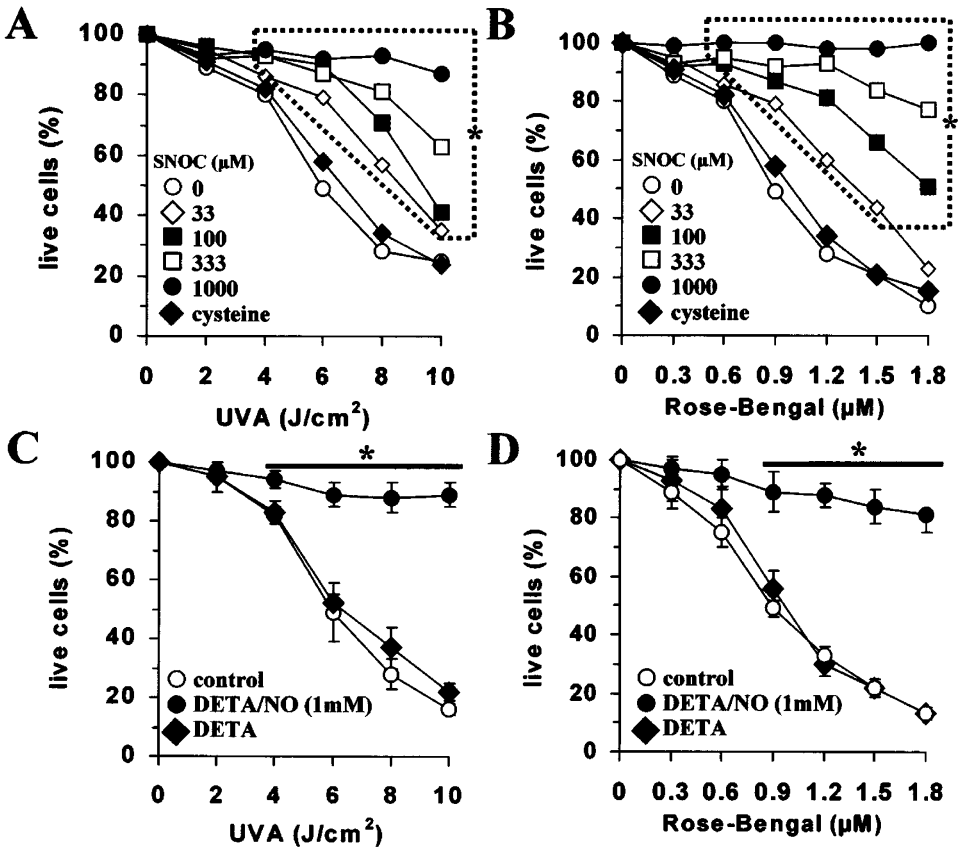

Figure 4 Exogenously generated NO fully protects from UVA_1-induced apoptosis and Rose Bengal-induced necrosis. During UVA_1-irradiation (**A** and **C**) or singlet oxygen-challenge via RB/hν (**B** and **D**) resident endothelial cells (EC) were incubated with S-nitrosocysteine (SNOC) (**A** and **B**) or DETA/NO (**C** and **D**) at concentrations of 0 μM (○), 33 μM (◇), 100 μM (■), 333 μM (□), 1,000 μM (●) or the control compounds cysteine or DETA (◇, 1000 μM each). After 24 h of incubation the number of living cells was determined using Neutral-Red. (**A**, **B**), At concentrations $\geqslant$100 μM SNOC-mediated protection against UVA_1-induced apoptosis or RB/hν-induced necrosis was significant (dotted cage) and a strong correlation between the SNOC concentration used and protection from UVA_1-or RB/hν-induced cell death is found (R^2=0.957 for UVA_1 or R^2=0.979 for RB/hν). Nearly complete protection was seen with 1000 μM SNOC, whereas the control compound cysteine showed no protective effect. (**C, D**), Experiments using the NO donor DETA/NO or the control DETA gave similar results. Values are the mean $\pm$ S.D. of 3–8 individual experiments. *, $P<0.001$

necrosis, which parallels the protection seen after incubation with catalase or BHT.

Protection by NO is not due to reaction with or quenching of ROS

Using dihydrorhodamine 123 (10 μM) a fluorescent dye for H_2O_2 detection we examined the input of NO as potential quencher or reaction partner of the ROS formed after UVA_1-irradiation (8 J/cm^2) or $^1O_2$ generation (1.5 μM RB/hν). UVA_1-exposure of otherwise untreated EC (Figure 6A) led to an increase in intracellular fluorescence (Figure 6B). In contrast, presence of $^1O_2$ quenchers (Figure 6C, histidine, 40 mM), inhibition of endogenous SOD with 1 mM diethyldithiocarbamate (Figure 6D), $O_2^{\cdot -}$ depletion by 80 μM MnTBAP (Figure 6F) or H_2O_2 depletion by 2000 U/ml catalase (Figure 6G) fully inhibited rhodamine 123 formation, whereas heat-inactivated catalase had no inhibitory effect (Figure 6H). In contrast, neither the presence of 10 μM BHT (Figure 6I) nor of 1 mM SNOC (Figure 6J) inhibited the UVA_1-or the RB/hν-induced fluorescence, suggesting that neither BHT nor NO will react directly with the ROS species involved. Control incubations of irradiated cells with SNOC or any of the other substances used (data not shown) did not result in increased cellular fluorescence, while incubation of cells with H_2O_2 (0.3 mM for 15 min) resulted in a pronounced fluorescence signal (Figure 6L). Besides reacting with H_2O_2, dihydrorhodamine 123 may also react with peroxynitrite a reaction product of NO and $O_2^{\cdot -}$.[14] To validate this possibility we incubated UVA or Rose Bengal treated cells with a SOD inhibitor exactly as already shown in Figure 6D but in the presence of the NO-donor SNOC (1 mM). As shown in Figure 6K, no apparent increase in mitochondrial fluorescence could be observed further corroborating the notion that under the conditions used peroxynitrite will not be produced. Results obtained with the RB/hν-system were similar (data not shown).

Nitric oxide fully protects from hydrogen peroxide induced toxicity

Data presented above indicate that H_2O_2 formed enzymatically or non-enzymatically from $^1O_2$ or $O_2^{\cdot -}$ may represent a late ROS product involved in cell death mediated by UVA_1-irradiation or $^1O_2$-challenge. Thus, we examined the effects

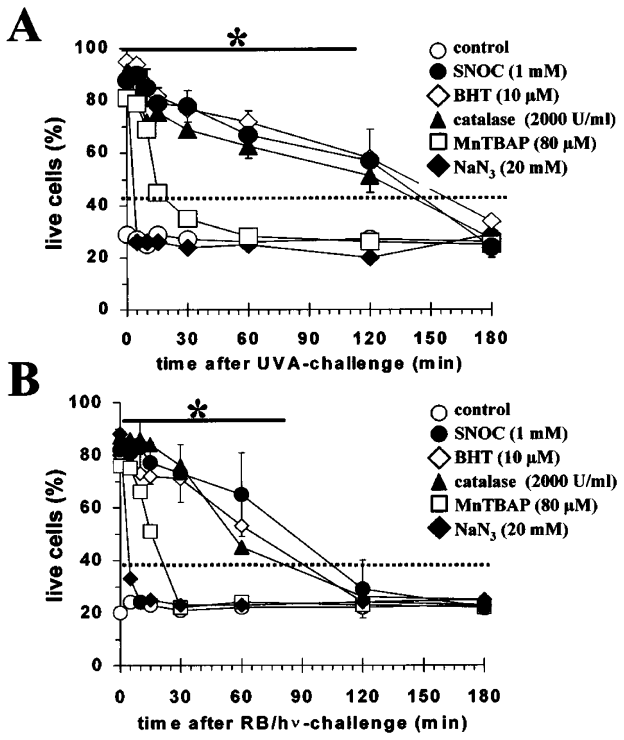

Figure 5 Time window for protection by antioxidants or NO added after the insults. To examine whether the various agents tested will also protect when added after UVA_1-irradiation or singlet oxygen treatment antioxidants used in Figure 3 or the NO-donor S-nitroso-cysteine (1 mM SNOC) were added to the cultures at different time points after the treatments. (**A**), After UVA_1-irradiation (8 J/cm^2), NaN_3 (◆; 20 mM) protect only when present during the treatments. MnTBAP (□, 80 μM) prevented cell death when added up to 15 min later. Catalase (▲; 2000 U/ml) or butylated hydroxytoluene (◇, BHT, 10 μM) show comparable time-courses of protection when added as late as 2 h later. The NO donor S-nitrosocysteine (●; SNOC, 1 mM) showed comparable time-courses of protection as seen with catalase or the lipid peroxidation inhibitor BHT and protects even when added 2 h after UVA_1-challenge. ○, sham treated controls. (**B**), Analogous results were obtained with RB/hν-treated EC albeit with a shortened time scale. Values are the mean ± S.D. of 3–8 individual experiments. Values above the dotted line are statistically significant: *, $P<0.001$

of exogenously applied nitric oxide (1 mM DETA/NO) or catalase (4000 U/ml) on H_2O_2-induced endothelial cell death, respectively. Incubation of endothelial cells with H_2O_2 for 24 h (Figure 7A) led to concentration-dependent cell death. Maximal toxicity was detected after incubation with 0.9 mM, half-maximal with 0.7 mM H_2O_2. Hydrogen peroxide-induced cell death was strongly reduced in the presence of catalase as well as in the presence of DETA/NO, whereas the controls DETA alone or heat-inactivated catalase had no effects.

Furthermore, with the half-maximal toxic dose of 0.7 mM H_2O_2 DETA/NO was protective even when added 90 min after the toxic stimulus, whereas with the maximal dose of 0.9 mM H_2O_2 DETA/NO at a concentration of 1 mM was not efficient enough to protect from cell death (Figure 7B).

NO inhibits lipid peroxidation

The results presented so far suggest lipid peroxidation as a crucial step in cell destruction. Therefore, endothelial cells were treated in the absence or presence of the various additives and lipid peroxidation was analyzed by determining the amount of malonedialdehyde formed. Results show a close correlation between UVA_1- or H_2O_2-induced apoptosis or RB/hν-induced necrosis and lipid peroxidation: As demonstrated in Figure 8A, UVA_1-irradiation as well as $^1O_2$ generation from RB/hν led to a highly significant increase in endothelial lipid peroxidation as evidenced by the amount of MDA formation. All substances which were shown to protect from apoptosis or necrosis (1 mM SNOC, 20 mM NaN_3, 10 μM BHT, 2000 U/ml catalase, 1 mM DETC, 80 μM MnTBAP) fully protected from lipid peroxidation. In addition, promoting induced cell death by addition of SOD or D_2O further enhanced lipid peroxidation, whereas the control compounds did not affect the levels of lipid peroxidation. Furthermore, incubation of endothelial cells with H_2O_2 (0.9 mM) led to an approximately eightfold increase in lipid peroxidation, whereas incubation with hydrogen peroxide in the presence of 1 mM DETA/NO, 10 μM BHT or 2000 U/ml catalase significantly reduced formation of lipid peroxides to control values, respectively (Figure 8B).

We also examined the role of NO as an inhibitor of lipid peroxidation in a cell-free model using liposomes and the $^1O_2$ releasing endoperoxide $NDPO_2$ (20 mM) as an inducer of lipid peroxidation. As exogenous NO source we used the NO donor MAHMA/NO (5 mM). Thermal decomposition of $NDPO_2$ at 37°C led to a significant 6–11-fold increase in lipid peroxidation which was completely inhibited by the NO-donor MAHMA/NO, while MAHMA had no effect (Figure 8C).

Inhibition of lipid peroxidation by NO also protects from UVA_1-induced mitochondrial cytochrome *c* release

For further elucidation of the molecular mechanism responsible for the antiapoptotic effects of NO present during UVA_1-exposure (8 J/cm^2), we examined events upstream of the caspase cascade. Using the Western blot technique we investigated the expression of the apoptosis relevant proteins Bcl-2 and Bax as well as the release of holocytochrome *c* from mitochondria into the cytoplasm as an early and pivotal signal in initiation of the apoptosis program. As shown in Figure 9A, UVA_1-irradiation does not influence the amount of Bax protein. More importantly, during the time interval examined we find no increases of Bcl-2 protein expression and a caspase-mediated cleavage of the antiapoptotic Bcl-2 could not be demonstrated within the first 4 h after UVA_1-irradiation (Figure 9B). In contrast, 2 h after UVA_1-irradiation we found holocytochrome *c* appearing in the cytoplasmic compartment and this remained for up to 4 h after UVA_1-challenge (Figure 10A). Importantly, as shown in Figure 10B, this holocytochrome *c* release was completely blocked when UVA_1-irradiation of endothelial cells was performed in the presence of SNOC or any of the antioxidants, shown to inhibit lipid peroxidation (1 mM SNOC, 40 mM histidine, 80 μM MnTBAP, 2000 U/ml catalase), whereas the control compounds did not block the increases in holocytochrome *c* release.

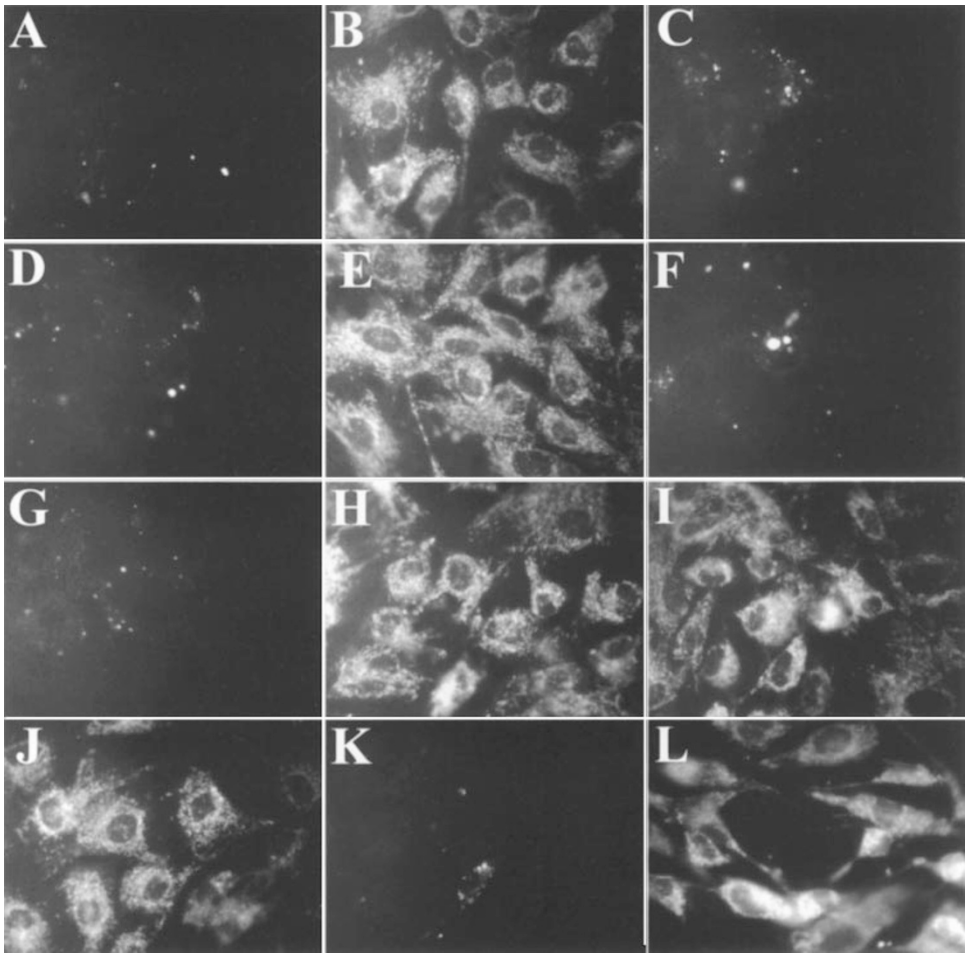

Figure 6 Dihydrorhodamine 123 labeling shows that NO does not directly quench ROS. We examined the input of UVA_1-irradiation or singlet oxygen-challenge on ROS mediated H_2O_2 formation in dihydrorhodamine 123 (DHR, 10 μM) labeled cells. Additionally, the role of NO as potential scavenger or reaction partner for the ROS formed was examined. Photomicrographs from experiments with UVA_1 (8 J/cm^2) are shown. Results obtained with the RB/hν-system were similar (data not shown). (**A**), Negative control, non-irradiated cells. (**B**), UVA_1-irradiation leads to an increase in mitochondrial fluorescence. (**C**), Presence of histidine (40 mM) or (**D**), inhibition of SOD activity with DETC (1 mM) or (**F**), depletion of superoxide by MnTBAP (80 μM) as well as (**G**), peroxide depletion by catalase (2000 U/ml) fully inhibited fluorochrome formation. (**E**), Addition of SOD (1000 U/ml) during the treatments gave no apparent alterations in fluorescence activity, and (**H**) heat-inactivated catalase had no inhibitory effect on UVA-induced fluorochrome formation. (**I**), Neither BHT (10 μM) nor (**J**), SNOC (1 mM) inhibited the UVA_1-induced fluorescence. (**K**), Cells treated as in (D) but additionally in the presence of the NO-donor SNOC (1 mM) show no increase in fluorochrome formation indicating that peroxynitrite is not produced. (**L**), positive control, 30 min incubation with 0.3 mM H_2O_2. In the absence of UVA_1-irradiation neither SNOC nor any of the other antioxidants lead to increased fluorescence due to rhodamine 123 formation (data not shown). Magnifications: $\times$ 650

Discussion

The cytotoxic action of UVA_1-radiation on mammalian cells is known to depend on the presence of oxygen. Multiple evidence exist that reactive oxygen species are involved.[15] Studies with chemical probes have implicated the involvement of H_2O_2,[16] $O_2^{-\cdot}$,[17] $OH^{\cdot}$,[18] and an important role for $^1O_2$[13] in UVA_1-mediated cytotoxicity. In biological environments singlet oxygen is highly reactive with a sufficiently long half-life to reach specific compartments.[19] All ROS are potentially harmful to cells as they interact with and modify a broad spectrum of biomolecules.[20] Thus, exposure of cells to ROS results in progressive cell damage by oxidative modification of various bio-molecules.[21]

Free-radical-induced damage of membranes results in loss of membrane function caused by UVA_1-radiation as well as radical-induced membrane damage using insults other than UVA_1 radiation[22,23] correlates with cell death via apoptosis or necrosis. We recently observed that UV-challenge of normal human skin leads to the induction of iNOS expression in keratinocytes and capillary endothelial cells.[24] High amounts of NO as produced by iNOS are known inducers of cell death via apoptosis or necrosis determined by the energy status of the respective cell.[25,26] Thus, it was tempting to speculate that iNOS expression after UV challenge may contribute to cellular skin damage.

However, NO may also exert antiapoptotic activity and indeed we have shown recently, that the presence of endogenously or exogenously formed NO prior to UVA_1-

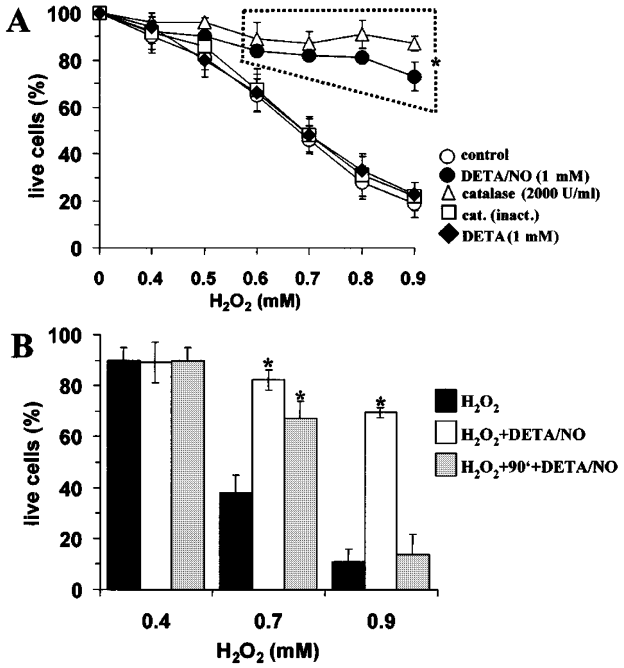

Figure 7 Nitric oxide fully protects from hydrogen peroxide induced toxicity. We examined the effects of exogenously applied nitric oxide (1 mM DETA/NO) or catalase (4000 U/ml) as well as the controls DETA (1 mM) or heat inactivated catalase on H_2O_2 induced endothelial cell death, respectively. (**A**) Incubation of endothelial cells with H_2O_2 (○) leads to concentration-dependent cell death. Hydrogen peroxide induced cell death was significantly reduced in the presence of catalase (△) as well as DETA/NO (●), whereas neither DETA alone ◆ nor heat inactivated catalase (□) had any effects. Values in the dotted cage are statistically significant. (**B**) When incubating endothelial cells with the half-maximal toxic dose of 0.7 mH H_2O_2, DETA/NO significantly protected from cell death even when added 90 min after the toxic stimulus, whereas with 0.9 mM H_2O_2 DETA/NO at that concentration had no protective effects (gray bars). Black bars, incubation with H_2O_2 alone. White bars, incubation with H_2O_2 in the presence of 1 mM DETA/NO. Values are the mean ± S.D. of 3–6 individual experiments. *, $P<0.001$, as compared to H_2O_2 treated cell cultures (○, in **A** or black bars in **B**)

irradiation results in a strong increase of Bcl-2 protein expression correlating with full protection from UVA_1-induced apoptosis.[10] We now provide evidence for protection even when NO is added up to 2 h after irradiation and also present data showing that here a different mechanism upstream of the apoptotic machinery is at work.

The time-course experiments using various antioxidants implicate a ROS cascade starting with UVA_1-or Rose Bengal-induced $^1O_2$ formation, followed by generation of $O_2^{-\cdot}$, then H_2O_2 accumulation, and the latest step found to occur in our experiments was lipid peroxidation. Interestingly, and despite the close similarity of the chain of ROS formation both ultimately resulting in lipid peroxidation, the mode of cell death differs for the two types of treatment. This may be due to the lipophilic character of Rose Bengal which readily incorporates into cellular membranes. Thus, $^1O_2$ formation and subsequent reactions will occur within bio-membranes and may thus lead to rapid and irreversible destruction of membrane integrity, which is regarded as the hallmark event of necrosis. In contrast, UVA_1 irradiation will lead to $^1O_2$ formation in many compartments of the cell mostly dependent on the presence of intracellular chromophores responsible for $^1O_2$ release.[27,28] As long as energy equivalents are not leaking from the cytoplasm as is the case with necrosis, cells can undergo apoptosis as has been reported.[25] It thus appears that here the decision for death by necrosis or apoptosis is not dominated by the type of molecular insult, but rather by the site of the damaging event.

Lipid peroxidation, found to represent a late step in the cascade, was decreased by the various antioxidants used as well as by exogenously applied NO donors, all of which inhibit the onset of necrosis or apoptosis. Examining events downstream of lipid peroxidation demonstrated a close positive correlation between the peroxidation of lipids and mitochondrial cytochrome *c* leakage. Within the time frame given, UVA_1-irradiation in the presence or absence of NO does neither decrease nor increase Bcl-2 protein expression or caspase mediated Bcl-2 cleavage, nor does the late addition of NO allow for gene up-regulation. Thus, in this experimental setup protection from cytochrome *c* release by acute NO treatment strongly correlates with, and thus appears to be dominantly mediated by inhibition of lipid peroxidation.

Lipid peroxidation results from the net abstraction of an allylic hydrogen atom of an unsaturated fatty acid by an initiating radical species to generating a lipid radical. The lipid radical then reacts with O_2 to generate an alkylperoxy radical ($LOO^{\cdot}$) which can further react with another lipid to form another lipid radical that can also react with O_2 and so on.[29] Thus, a single initiating event can lead to the destruction/modification of numerous lipid molecules resulting in loss of membrane integrity. As shown, lipid peroxidation can be avoided or limited by inhibiting the generation of or quenching the initiating radical species by antioxidants, however, data presented do not corroborate such a role for NO but rather suggest a radical-chain terminating activity. The NO-radical ($^{\cdot}NO$) reacts rapidly via simple radical-radical combination reactions with species possessing unpaired electrons as $^{\cdot}O_2$, O_2 and $O_2^{\cdot-}$. The ability of NO to quench other radical species also allows it to terminate radical chain reactions.[30] The reaction of $^{\cdot}NO$ with $LOO^{\cdot}$ species readily predominates over the much slower initiation of secondary peroxidation propagation reactions by $LOO^{\cdot}$ with vicinal unsaturated lipids, and when inhibiting lipid peroxidation propagation reactions, NO undergoes an initial termination reaction with organic peroxyl radicals to form organic peroxynitrates (LOONO).[31]

In summary, we present evidence that NO can prevent cell death as a consequence of UVA-induced onset of lipid peroxidation and ROS-formation. This finding bears significance in understanding protective responses in human skin exposed to excessive doses of sunlight. As during the first exposure to UV skin cells are usually not expressing iNOS, it is important to understand the protective mechanisms exerted by subsequent NO synthesis in the skin, a process which will help to minimize skin cell destruction.

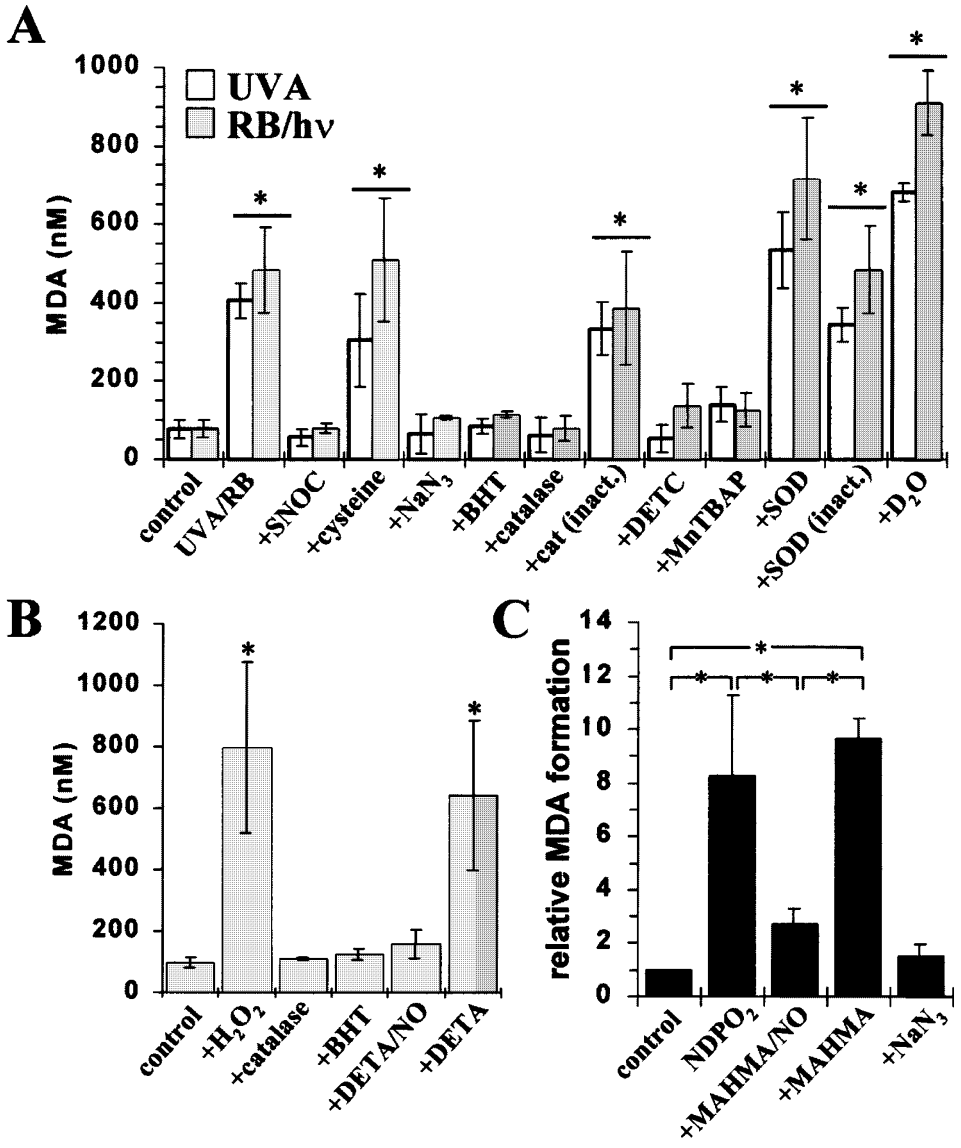

Figure 8 NO blocks lipid peroxidation. (**A**), Endothelial cells were irradiated with UVA_1 (white bars, 10 J/cm^2) or treated with RB/hν (gray bars, 1.5 μM) or (**B**), were incubated with H_2O_2 (0.9 mM) in the absence or presence of the respective additives and lipid peroxidation was assayed by measuring malonedialdehyde (MDA) formation. Additionally, lipid peroxidation was examined in liposomes (**C**) after treatment with the endoperoxide $NDPO_2$. (**A**), UVA_1-irradiation or RB/hν-challenge led to lipid peroxidation and compounds that protected cells from apoptosis or necrosis (1 mM SNOC, 20 mM NaN_3, 10 μM BHT, 2000 U/ml catalase, 1 mM DETC, 80 μM MnTBAP) also protected from UVA_1- or RB/hν-induced lipid peroxidation as seen by the reduction of MDA-levels to control values. All compounds shown to increase cell death such as SOD (1000 U/ml) or D_2O enhanced lipid peroxidation. The control compounds cysteine (1 mM), heat-inactivated catalase or SOD did not alter MDA formation. (**B**), As compared to the controls, incubation of endothelial cells with H_2O_2 led to an approximately eightfold increase in lipid peroxidation. Incubation with hydrogen peroxide in the presence of DETA/NO (1 mM) or catalase (4000 U/ml) significantly reduced formation of lipid peroxides to control values, where as the control agent DETA alone had no effects. (**C**), In liposomes, thermal decomposition (37°C) of the endoperoxide $NDPO_2$ (20 mM) led to a 6–11-fold increase in lipid peroxidation, which was inhibited by NaN_3 (20 mM) or by the NO donor MAHMA/NO (5 mM) whereas the control compound MAHMA showed no effects. Values are the mean $\pm$ S.D. of 3–6 individual experiments. *, $P<0.001$

Materials and Methods

Reagents

Endothelial cell growth supplement (ECGS), Neutral Red, deuterium oxide (98%), rabbit anti-human von Willebrand Factor (vWF) antiserum, monoclonal mouse anti-α-tubulin antibody, Rose Bengal, histidine, mannitol, imidazole, dimethyl sulfoxide (DMSO), sodium azide, catalase, diethyldithiocarbamate (DETC), 3-amino-1,2,4-triazole (ATA), H_2O_2, and Hoechst dye H33342 were purchased from Sigma (Deisenhofen, Germany), rabbit anti-rat Bcl-2 antibody or rabbit anti-rat Bax antiserum from Pharmingen (San Diego, CA, USA), peroxidase-conjugated porcine anti-rabbit IgG from DAKO (Hamburg, Germany), and peroxidase-conjugated goat anti-mouse IgG from Zymed Laboratories (San Francisco, CA, USA), manganese (III) tetrakis(4-benzoic acid)porphyrin (MnTBAP), and manganese (III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) from Alexis

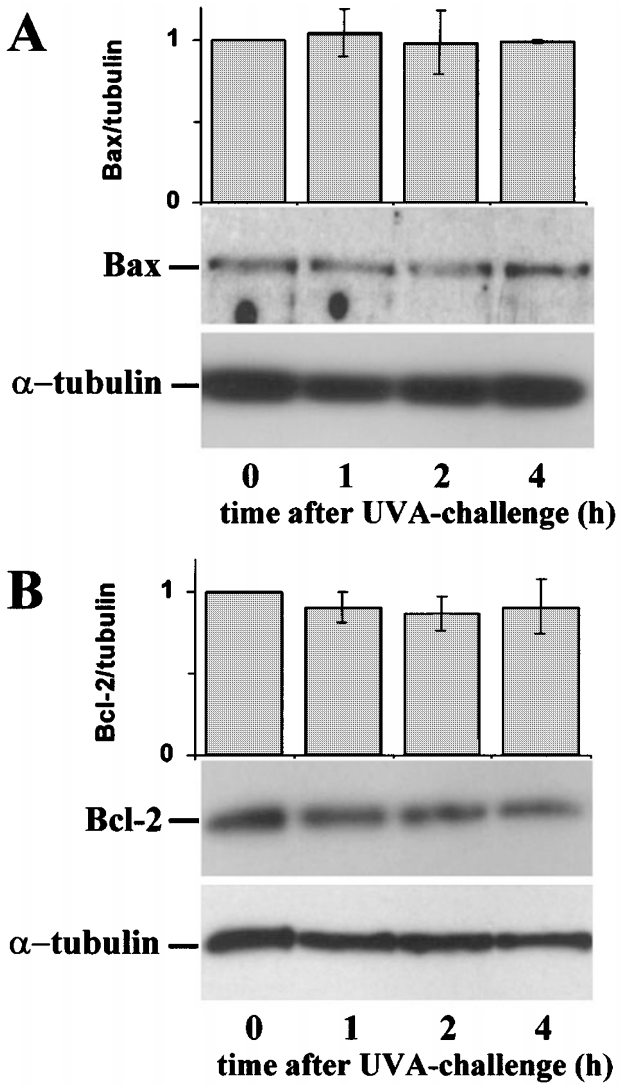

Figure 9 UVA_1-irradiation did not alter Bax or Bcl-2 protein expression. Endothelial cells were irradiated with UVA_1 (8 J/cm^2) and within the first 4 h, expression of the apoptosis-relevant proteins Bcl-2 and Bax were studied by Western blot. Bax protein expression (**A**) or Bcl-2 protein expression (**B**) were not altered. Bars represent the relative value of the Bax/α-tubulin- or Bcl-2/α-tubulin-ratios obtained by densitometric analysis of visualized protein-bands from three individual experiments. Photomicrographs shown represent one out of three identical experiments

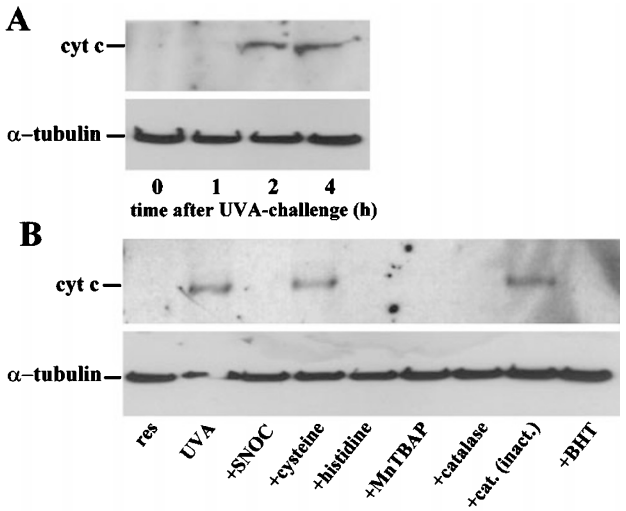

Figure 10 Nitric oxide prevents UVA_1-induced mitochondrial cytochrome *c* leakage. To correlate UVA_1-induced apoptosis with lipid peroxidation, cells were irradiated with UVA_1 (8 J/cm^2) in the presence or absence of the NO donor SNOC or antioxidants. Within the first 4 h after irradiation, release of holocytochrome *c* from mitochondria into the cytoplasm was studied by Western blot. (**A**), Two hours after UVA_1-irradiation holocytochrome *c* appeared in the cytoplasm and remained at comparable level at 4 h. (**B**), As determined 4 h after UVA-irradiation, cytoplasmic appearance of holocytochrome *c* was completely inhibited in the presence of SNOC (1 mM) or any of the agents previously shown to inhibit cell death and lipid peroxidation (40 mM histidine, 80 μM MnTBAP, 2000 U/ml catalase), whereas control compounds (1 mM cysteine, heat-inactivated catalase) had no effects on mitochondrial holocytochrome *c* release. Photomicrographs shown represent one out of three identical experiments

(Läufelfingen, Switzerland), diethylenetriamine and 2,[6]-di-tert-butyl-p-cresol (butylated hydroxytoluene, BHT) from Aldrich (Steinheim, Germany), dihydrorhodamine 123 (DHR) from Molecular Probes (Eugene, OR, USA), the monoclonal antibody O × 43 from Serotec (Camon, Wiesbaden, Germany), and the monoclonal mouse anti-holocytochrome *c* antibody from R&D Systems (Heidelberg, Germany), protease inhibitor mix, trypsin, EDTA, fetal calf serum (FCS, endotoxin free), RPMI-1640 (endotoxin free), NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase from Boehringer Mannheim (Mannheim, Germany) or Gibco Laboratories (Eggenstein, Germany), and the ICE-inhibitor Z-VAD from Enzyme Systems (Livermore, CA, USA). The endoperoxide 3,3′-(1,4-naphthylidene)dipropionate ($NDPO_2$) was synthesized as described by Di Mascio and Sies.[32] The NO donors (Z)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA/NO), (Z)-1-{*N*-methyl-*N*-[6-(*N*-methylammoniohexyl)amino]}diazen-1-ium-1,2-diolate (MAHMA/NO), and S-nitrosocysteine (SNOC) were synthesized as described previously.[33,34] The iNOS inhibitor L-N^5-(1-iminoethyl)-ornithin (NIO) was a kind gift from Boehringer Mannheim. As UVA_1-source we used a Sellas-2,000 or Sellas-4000 lamp (Sellas Medizinische Geräte, Gevelsberg, Germany) emitting the UVA_1-spectrum (340 – 390 nm).

Endothelial cells

Endothelial cells (EC) were isolated by outgrowth from male Wistar rat aortic rings as described previously.[9] Briefly, aortic segments were placed on top of a collagen gel (1.8 mg collagen/ml) in 24-well tissue culture plates and incubated in RPMI 1640/ 20% FCS supplemented with 100 μg ECGS/ml for 5 days. Aortic explants were then removed, cells detached with 0.25% collagenase in HBSS and replated onto plastic culture dishes in RPMI 1640/ 20% FCS. Cells were subcultured for up to eight passages, and removal from culture dishes for each passage was performed by treatment with trypsin/EDTA. Cellular characterization of cultured endothelial cells was performed by determination of the endothelium characteristic antigenic phenotype using the anti-vWF antiserum, the endothelium-specific monoclonal antibody O × 43, and the respective peroxidase-conjugated secondary antibodies as described previously.[35]

Singlet oxygen production

Intracellular release of singlet oxygen was achieved by photoexcitation of solutions containing endothelial cells and varying concentrations of

Rose Bengal (RB/hν) with a commercially available white lamp from a fixed distance.[36] Briefly, endothelial cells were incubated for 15 min with Rose-Bengal in the dark at 37°C in RPMI 1640 without FCS and phenol red. Cells were then irradiated for 5 min using the 500 W white light source in a fixed distance of 40 cm. Alternatively, singlet oxygen was generated by chemiexcitation via thermodecomposition of the endoperoxide $NDPO_2$.[12,32]

Experimental design

All measurements were performed with cells from passages 2–8. Endothelial cells were cultured in 12-well tissue culture plates (2×10^5) or on 8-well chamber-Tec glass slides (1×10^4) in a humidified incubator at 37°C in RPMI 1640/ 20% FCS. Endothelial apoptosis was induced by irradiation with UVA_1 (2–10 J/cm^2) or H_2O_2 challenge (0.1–0.9 mM) whereas necrosis was achieved by singlet oxygen generated from Rose Bengal at the concentrations indicated plus light-treatment (RB/hν). During or at indicated time points after UVA_1 irradiation, RB/hν challenge or hydrogen peroxide incubation resident endothelial cells were incubated with the respective additives at concentrations indicated and 24 h after irradiation the relative number of living endothelial cells was detected by neutral red staining as described previously.[10] Additionally, viability of endothelial cells was routinely controlled at the beginning and the end of every experiment using the trypan blue exclusion assay or propidium iodide staining. Viable cells were defined as cells excluding trypan blue or propidium iodide and positive for respiratory activity as determined by neutral red.

Detection of apoptotic or necrotic cells

At different time points (1–24 h) after UVA_1-irradiation (2–10 J/cm^2), RB/hν-challenge or H_2O_2 incubation endothelial cells grown in 12-well culture plates were washed with PBS, stained with Hoechst dye H33342 (8 μg/ml) and/or propidium iodide (0.5 μg/ml) for 5 min and nuclei or necrotic cells were visualized using a Zeiss fluorescence microscope, respectively. In each sample a minimum of 400 cells was counted and condensed or fragmented nuclei as well as necrotic cells were expressed as per cent of total cells.

Detection and quantification of nuclear DNA fragmentation

DNA strand breaks of cells grown on 8-well chamber-tec slides were visualized by the in situ nick-translation method[37] 1–24 h after UVA-irradiation (2–10 J/cm^2). In acetone fixed cells endogenous peroxidase activity was blocked with methanol+0.3% H_2O_2 for 30 min. The nick-translation mixture contained 3 μM biotin-dUTP, 5 U/100 μl Kornberg polymerase, 3 μM each dGTP, dATP, dCTP, 50 mM Tris-HCL, pH 7.5, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, and reaction was performed at room temperature for 20 min. Slides were washed in PBS and processed for immunocytochemical detection of biotin-labeled UTP by peroxidase-labeled avidin, followed by an enzyme reaction using DAB as substrate. In each sample a minimum of 500 cells were counted and labeled nuclei were expressed as per cent of total nuclei.

Use of antioxidants

For examining the role of reactive oxygen species (ROS) in cytotoxicity during or at time points indicated after UVA_1-irradiation, RB/hν challenge or H_2O_2 incubation, endothelial cells were incubated with inhibitors of the respective ROS class.

Inhibition of singlet oxygen ($^1O_2$) action was achieved by addition of sodium azide (20 mM), histidine (40 mM), or imidazole (40 mM). In contrast, increasing of $^1O_2$ half-life and thus its action was achieved in the presence of deuterated water (D_2O, 99% atom).[38–40] These agents were added to the cells 10 min prior to, were present during, and for 5 min after the respective procedure (10/present/5). To deplete superoxide anions ($O_2^{\cdot-}$) SOD (1000 U/ml; 60/present/5) but also the $O_2^{\cdot-}$ quencher MnTBAP (80 μM; 0/absent/30) or MnTMPyP (150 μM; 0/absent/30) [41,42] were used. As a control, heat-inactivated SOD (10 min at 100°C) was used, and to inhibit the cytosolic Cu/Zn SOD the powerful inhibitor diethyldithiocarbamate (1 mM; 30/present/5) was used.[43] Hydrogen peroxide (H_2O_2) was degraded by catalase (2000 U/ml; 60/absent/5), and as a control, heat-inactivated catalase (20 min at 100°C) was used, and intracellular catalase activity was inhibited using 3-amino-1,2,4-triazole (500 μM; 30/present/5).[44] Inhibition of lipid peroxide formation was achieved with BHT (10 μM; 30/present/5),[45] and for hydroxyl radical ($OH^{\cdot}$) quenching DMSO (100 mM; 30/present/5) or mannitol (100 mM; 30/present/5) were used.[46,47] In experiments examining H_2O_2 toxicity the respective agents (1 mM DETA/NO, 1 mM DETA, 4000 U/ml catalase, heat-inactivated catalase) were added 10 min prior to the addition of hydrogen peroxide and were present during the 24 h of incubation.

In experiments examining the time-course of ROS action the respective agents were added for 30 min to the cell cultures at the different time points indicated after the toxic insult. In the case of incubation with H_2O_2 the respective agents (1 mM DETA/NO, 1 mM DETA, 4000 U/ml catalase, heat-inactivated catalase) were added to the cell cultures for 30 min after 90 min of incubation with 0.4, 0.7 or 0.9 mM H_2O_2, then cell cultures were washed twice with PBS and grown in culture medium for 24 h. At the concentrations used, none of the compounds mentioned above showed any cytotoxic effects towards the EC within the incubation time.

Detection of intracellular H_20_2

Intracellular H_2O_2 formation was visualized using the fluorescent dye dihydrorhodamine 123 (DHR). Endothelial cells (1×10^5) in 12 well tissue culture plates were loaded with DHR (10 μM) for 45 min and washed with PBS before treatment with UVA_1 or RB/hν. During the intracellular release of H_2O_2, reduced DHR is irreversibly oxidized and converted to the fluorescent compound rhodamine 123.[48] Between 20 and 120 min after UVA_1-irradiation rhodamine 123 formation was constant and was visualized using a Zeiss fluorescence microscope.

Detection of lipid peroxidation in UVA_1-irradiated endothelial cells and measurement of microsomal lipid peroxidation

Resting endothelial cells (1×10^6) in 6-well tissue culture plates were irradiated with UVA_1 (8 J/cm^2), were exposed to RB/hν (1.5 μM) or incubated with H_2O_2 (0.9 mM) in the absence or presence of the respective additives at the concentrations indicated. One hundred and twenty or 60 min after irradiation lipid peroxidation was stopped by addition of BHT (10 μM), respectively. Cells were harvested and lysed by repeated freezing and thawing. Malonedialdehyde (MDA) equivalents were estimated via the formation of thiobarbituric acid (TBA)-reactive substances using ε_{535}=156 mM^{-1}cm^{-1}.[49]

Additionally, in a cell-free system lipid peroxidation was initiated in liposomes (0.5 mg protein/ml) by singlet oxygen generated by thermal decomposition from $NDPO_2$ (20 mM) with premixed ADP (1 mM)/ $FeCl_3$ (10 μM), and NADPH regenerating system, containing NADPH (0.4 mM), glucose-6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (5 units/ml)[50] in the presence or absence of the NO donor

MAHMA/NO (5 mM; 1–2 min half-life for NO release at 37°C), denitrosylated MAHMA/NO (MAHMA, 5 mM), sodium azide (20 mM) or NDP (20 mM) alone. Lipid peroxidation was assayed as described above.

Western-blot analysis

Resting or UVA_1-irradiated (8 J/cm^2) endothelial cells (4×10^7), irradiated in the presence or absence of the compounds indicated, were detached after an incubation time of 1, 2 or 4 h by trypsin treatment, and washed twice with ice-cold PBS. The cell pellets were suspended in three volumes of ice-cold isolation buffer (0.25 M saccharose, 10 mM triethanolamine/HCl, 5 mM KH_2PO_4, 20 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, pH 7.0) supplemented with the protease inhibitor mix from Boehringer. After 15 min on ice, the cells were disrupted by douncing in a 2 ml glass douncer. Intact cells and nuclei were separated from cytosolic fraction by two centrifugation steps (10 min at 2000 $\times$ *g* and 25 min at 16 500 $\times$ *g*). Pellets or mitochondria-free supernatants were mixed with the sample buffer (Novex, San Diego, CA, USA). Proteins (50 μg per lane taken from the pellet fraction for Bcl-2 or Bax determination or 100 μg per lane taken from the mitochondria-free supernatant for holocytochrome *c* determination) were separated by electrophoresis in a gradient (4–12%) bis-tris-polyacrylamide gel with MOPS-SDS running buffer (Novex) and transferred to nitrocellulose membranes. Further incubations of the blots were: 1 h with blocking buffer (2% BSA, 5% non-fat milk powder, 0.1% Tween 20 in PBS-buffer), 1 h with a 1 : 1000 dilution of the monoclonal mouse anti-holocytochrome *c* antibody or the rabbit anti-Bcl-2 or rabbit anti-Bax antiserum, washed, 1 h with a 1 : 1000 dilution of the secondary horseradish peroxidase conjugated goat anti-mouse IgG antibody or horseradish peroxidase conjugated porcine anti-rabbit IgG antibody. Finally, blots were incubated for 5 min in ECL reagent (Pierce, Rockford, IL, USA), placed into a plastic bag and exposed to an enhanced autoradiographic film. To control equal loading of total protein in all lanes, blots were stained with a 1 : 2000 solution of the mouse anti α-tubulin antibody. The secondary horseradish peroxidase conjugated goat anti-mouse IgG antibody was diluted 1 : 2000 prior to use.

Statistical analysis

Data are given as means $\pm$ S.D. Values were calculated using analysis with Student's *t*-test (two-tailed for independent samples).

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