Autologous nitric oxide protects mouse and human keratinocytes from ultraviolet B radiation-induced apoptosis

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Weller, Richard, Ann Schwentker, Timothy R. Billiar, and Yoram Vodovotz. Autologous nitric oxide protects mouse and human keratinocytes from ultraviolet B radiation-induced apoptosis. Am J Physiol Cell Physiol 284: C1140–C1148, 2003; 10.1152/ajpcell.00462.2002.—Nitric oxide (NO) can either prevent or promote apoptosis, depending on cell type. In the present study, we tested the hypothesis that NO suppresses ultraviolet B radiation-induced keratinocyte apoptosis both in vitro and in vivo. Irradiation with ultraviolet B (UVB) or addition of the NO synthase (NOS) inhibitor 

N\text{-acetyl-penicillamine (SNAP)} immediately after UVB completely abrogated the rise in apoptosis induced by L-NAME. An adenoviral vector expressing human inducible NOS (Adi-NOS) also reduced keratinocyte death after UVB. Caspase-3 activity, an indicator of apoptosis, doubled in keratinocytes incubated with L-NAME compared with the inactive isomer, D-NAME, and was reduced by SNAP. Apoptosis was also increased on addition of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), an inhibitor of soluble guanylate cyclase. Mice null for endothelial NOS (eNOS) exhibited significantly higher apoptosis than wild-type mice both in the dermis and epidermis, whereas mice null for inducible NOS (iNOS) exhibited more apoptosis than wild-type mice only in the dermis. These results demonstrate an antiapoptotic role for NO in keratinocytes, mediated by cGMP, and indicate an antiapoptotic role for both eNOS and iNOS in skin damage induced by UVB.

Ultraviolet radiation; skin; dermis; epidermis

KERATINOCYTES ARE EXPOSED TO MANY ENVIRONMENTAL INSULTS, including ultraviolet radiation, against which they form the body’s first line of defense. Ultraviolet B radiation (UVB) induces apoptosis in all cell types. However, keratinocytes are particularly resistant to UVB, an essential feature in a cell type continually exposed to solar irradiation. Under appropriate conditions, keratinocytes can be stimulated to undergo apoptosis. Death receptor ligand-dependent and -independent pathways act via the FADD/caspase 8 cascade (23, 30, 36). DNA damage upregulates p53 expression, accompanied by G1 arrest and either DNA repair or apoptosis (8). Finally, UVB also activates p38 mitogen-activated protein kinase with resultant mitochondrial cytochrome c release (2).

Nitric oxide (NO) is produced at low levels from the amino acid l-arginine in a calcium-dependent fashion by the constitutive neuronal and endothelial nitric oxide synthases (nNOS and eNOS, respectively) and at higher levels in a calcium-independent manner by the inducible NOS (iNOS). All three NOS isozymes are present in human skin (48). NO release has been described within 1 min of UVB irradiation in a calcium-dependent manner, leading to the production of cGMP produced by the soluble guanylate cyclase (sGC) (9, 32), both features of constitutive NOS. Additionally, iNOS mRNA and protein expression peak 24 h after two minimal erythema doses of UVB in healthy human skin (21).

NO exerts both pro- and antiapoptotic effects (17). In macrophages (1), thymocytes (12), human neutrophils (45), and mesangial cells (11) NO induces apoptosis, often in combination with reactive oxygen intermediates or at high concentrations. In contrast, NO inhibits apoptosis in endothelial cells (42) and hepatocytes (44). This process occurs both directly by nitrosative inactivation of caspases (24) and indirectly via the cGMP pathway (18) and interference with activator protein-1 (AP-1)-mediated upregulation of the CD95L promoter (27). The presence of elevated concentrations of NO in psoriatic (3, 28) and irradiated skin, and the changes in keratinocyte proliferation and apoptosis in these states, suggest that NO can play a pathophysiological role in certain skin disorders.

Ultraviolet radiation both induces apoptosis and causes NO release. We wanted to determine whether the pro- or antiapoptotic effects of NO predominate after exposure of skin to UVB and to examine the mechanisms for this action. Our findings suggest that NO derived from both eNOS and iNOS is antiapoptotic for keratinocytes challenged with UVB and, through isoform-specific patterns of NO release, serves as both an immediate and a sustained mechanism to protect keratinocytes from the toxicity of exposure to UVB.

MATERIALS AND METHODS

Tissue culture. All chemicals were obtained from Sigma (St. Louis, MO), unless otherwise stated. The E6/E7-trans-
formed keratinocyte line CCD 1106 KERTr (American Type Culture Collection, Manassas, VA) was cultured in 175-cm² culture flasks in an equal-parts mixture of medium 154 (Cascade Biologies, Seattle, WA) and keratinocyte-SFM, to each 500 ml of which 2.5 μg of human recombinant EGF and 25 mg of bovine pituitary extract had been added (GIBCO BRL, Grand Island, NY). Fennicillin and streptomycin were added to the culture medium (GIBCO BRL), and the cells were maintained in a humidified incubator at 37°C and 5% CO₂.

Ultraviolet irradiation. For cell culture experiments, two Philips FS20 lamps (Bulbtronics, Farmingdale, NY) were screened with Kodacel (14). Lamp output was measured with a UVP model UVX digital radiometer and UVX-31 probe (UVP, San Gabriel, CA) after the lamps were allowed to warm up for 15 min. At 20 cm from the cells, an irradiance of ~0.5 mW/cm² was measured. Cells were plated onto a six-well tissue culture dish 24 h before irradiation. The relevant treatment was added 18 h later, and after 6 h the culture medium was replaced with 600 μl of Hank's balanced salt solution (containing Ca²⁺ and Mg²⁺; GIBCO BRL) and the cells were irradiated. The irradiation was returned to each well, the cells were re-

Cell treatments. 1-NAM and 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one (ODQ) were obtained from Alexis (San Diego, CA), prepared in keratinocyte growth medium or DMSO, and added to the cells 6 h before irradiation. 8-Bromo-cGMP (8-BrcGMP) was obtained from Sigma. N-propyl-1,3-propanediamine-N-PAO (PAPA-NO) was the kind gift of Dr. Klaus Kroncke (Research Group Immunobiology, Heinrich Heine University, Düsseldorf, Germany). For infection with adenoviral vectors, the cells were washed with three freeze-thaw cycles. Mice were used at the N6 generation and thus were 98.4% homogeneous to the C57Bl/6 mice. iNOS−/− mice were a kind gift from Dr. John Murdett (Merck Research Laboratories, Rahway, NJ); they were received as N10 and backcrossed to N10 (C57Bl/6) 7- to 10-wk-old wild-type females. Accordingly, iNOS−/− mice were 99.91% homogeneous to the C57Bl/6 mice. iNOS−/− and eNOS−/− mice were housed in cages in a room with controlled temperature and humidity and alternating 12:12-h light-dark cycles. Mice were fed with a commercial diet and had water ad libitum. They were anesthetized with isoflurane (Abbott, North Chicago, IL) for shaving of their backs before irradiation and were unrestrained in uncovered cages during irradiation. Mice were euthanized by CO₂ inhalation 24 h after irradiation, the time point at which apoptosis has been shown to be maximal (29). The dorsal skin was removed and placed in 2% paraformaldehyde overnight and then in 30% sucrose for 4 h before being frozen in optimum cutting temperature (OCT) compound (Tissue-Tek, Torrance, CA) and stored at −80°C until analyzed. Single mice were initially irradiated at UV doses ranging from 50 to 1,600 mJ/cm² to determine the dose at which the greatest differences in apoptosis could be discerned (data not shown). Three or four mice per group were then irradiated at 400 and 1,000 mJ/cm², the doses determined to be optimal. Apoptosis assay. Sections (7 μm) were cut and incubated at 37°C for 1 h with a FITC conjugate of the cell-permeant caspase inhibitor VAD-FMK (CaspASE FITC-VAD-FMK, 10 μM; Promega, Madison, WI), which binds to activated caspase sites. The sections were counterstained for 3 min at room temperature with 10 μg/ml of Hoechst 33258 dye, followed by mounting in Gelvatol (23 g poly(vinyl alcohol) 2000), 50 ml glycerol, 0.1% sodium azide to 100 ml PBS). TdT-mediated dUTP nick end labeling (TUNEL), which measures DNA fragmentation, was also performed with a kit (in situ cell death detection kit; Roche, Indianapolis, IN). This analysis produced results analogous to caspase staining, but with a higher number of falsely positive stained cells (data not shown). TUNEL in irradiated skin is not specific for apoptosis (13).

Flow cytometry. Apoptosis was assessed by flow cytometric analysis of annexin V with a Becton Dickinson FACSort (San Jose, CA) as described by Schindl et al. (34). Supernatants and cells harvested with 0.05% trypsin were rinsed in PBS and suspended in 100 μl of annexin binding buffer (10 mM HEPES-NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂), 5 μl/100 μl of annexin V, and 5 mg/ml of propidium iodide (PI; BD PharMingen, San Diego, CA). Emission filters used were BP 530/30 nm [fluorescein isothiocyanate (FITC)] and BP 585/42 nm (PI). A minimum of 10,000 cells per sample were recorded, and cell debris was excluded by appropriate forward light scatter threshold setting. Data analysis with CELLQuest software (Becton Dickinson, St. Louis, MO) was performed, and numbers of cells positive for annexin V, PI, or combinations thereof were calculated.
biochemical intermediate of the apoptotic pathway and is more specific in UV-induced apoptosis.

**eNOS immunohistochemistry.** Sections (7 μm) were blocked with 5% normal goat serum in bovine serum albumin for 1 h at room temperature and then probed with a 1:2,000 dilution of anti-eNOS polyclonal antibody (BD Transduction Laboratories, Franklin Lakes, NJ) overnight at 4°C, followed with a 1:3,000 dilution of Cy3-labeled goat anti-rabbit secondary antibody (Jackson, West Grove, PA) for 1 h at 37°C. Omission of primary and secondary antibodies in control wild-type sections confirmed the specificity of eNOS staining.

Sections were photographed with an Olympus Provis epifluorescence microscope connected to an Olympus digital camera (Melville, NY) with 480/535 and 550/570 filters for fluorescein and Cy3, respectively. Total and apoptotic cells were counted with Metamorph software (Universal Imaging, Downingtown, PA), with the epidermis and dermis being differentiated morphologically. Between 6 and 12 high-power fields were counted for each dose of UV, with an average of 800 cells per high-power field.

**Statistics.** All values are means ± SD unless otherwise indicated. Comparisons of multiple groups were done by one-way analysis of variance followed by the Tukey post hoc test, using a 95% confidence interval (P < 0.05) to denote statistical significance.

**RESULTS**

**Effect of NO on UVB-induced keratinocyte apoptosis and survival.** We first determined the effects of NO on UVB-induced apoptosis in human keratinocytes in vitro by treating the cells with UVB in the presence or absence of the nonspecific NOS inhibitor l-NAME. Addition of l-NAME and irradiation with UVB both increased apoptosis, and the combination of l-NAME and UVB produced a higher amount of apoptosis than control (P < 0.001), l-NAME alone (P = 0.009), or UVB alone (P = 0.021). We showed previously (49) that other NOS antagonists also increase apoptosis after UVB. Addition of S-nitroso-N-acetyl-penicillamine (SNAP) immediately after UVB significantly abrogated the rise in apoptosis due to l-NAME (P = 0.012) but not that due to UVB (Fig. 1A). The reduction in keratinocyte cell death is not solely a feature of addition of nitrosothiols such as SNAP. The NONOate PAPA-NO also markedly reduced cell death after UVB irradiation (Fig. 1B). We next infected keratinocytes with AdiNOS at a MOI of 10 pfu/cell. AdiNOS-transduced iNOS protein was functional, because the stable end product of NO, NO₂⁻, was elevated significantly in the culture supernatant of these cultures over untreated cultures, cultures treated with UVB only, or cultures infected with a control adenovirus expressing the bacterial β-galactosidase gene (AdLacZ; Fig. 2A). AdiNOS-infected cultures also exhibited significantly reduced cell death after irradiation (P < 0.001 vs. UVB + AdLacZ or UVB only; Fig. 2B). Interestingly, AdiNOS also increased the viability of keratinocytes not exposed to UVB (P < 0.001 vs. AdLacZ or UVB only).

**Caspase-3 activity after UVB irradiation.** We next wished to determine whether the apparent increase in apoptosis assessed by annexin V staining correlated with increased caspase activity. Caspase-3 activity after irradiation doubled in cells incubated with 1 mM l-NAME compared with caspase-3 activity in cells treated with the inactive isomer, d-NAME (P = 0.021). Addition of 100 μM SNAP partially reduced the rise in caspase-3 activity in both d-NAME- and l-NAME-treated cells (P < 0.001), so that caspase-3 activity in cultures treated with l-NAME + SNAP did not differ significantly from cultures treated with d-NAME (Fig. 3). Caspase activity was not significantly different in control cells, which to neither NAME isomer had been added, and d-NAME-treated cells (control nonirradiated 28.6 ± 1.9 × 10⁻⁴, d-NAME nonirradiated 32.1 ± 1.21 × 10⁻⁴, control irradiated 68.2 ± 9.41 × 10⁻⁴, d-NAME irradiated 68 ± 2.27 × 10⁻⁴ OD·mg protein⁻¹·min⁻¹).

**cGMP activity and apoptosis after UVB.** Keratinocytes become increasingly resistant to UVB-induced...
apoptosis as they become more confluent. Total annexin-positive cells, when cultured at a density of $0.6 \times 10^5$ cells/cm² in a six-well plate and allowed to grow to 70% confluence, increased from 5% to 47% after irradiation with 100 mJ/cm² UVB. This number was reduced to 21% after addition of the nonhydrolyzable cGMP analog 8-Br-cGMP (800 μM; Fig. 4A). The annexin-positive fraction of cells that had grown to 90% confluence increased from 4% to 9% after UVB, and this was increased to 26% after addition of the sGC inhibitor ODQ (Fig. 4B).

Dermal and epidermal apoptosis in wild-type, iNOS−/−, and eNOS−/− mice. Given the potent effects of NO on UVB-induced keratinocyte apoptosis, we next examined whether NO had a similar effect in vivo by examining the effects of UVB on wild-type, iNOS−/−, and eNOS−/− mice. We carried out initial UVB dose-finding experiments in wild-type mice, which were shaved and irradiated with 0, 50, 100, 150, 400, 800, and 1,600 mJ/cm² UVB to determine the doses of UVB required to obtain apoptosis. In these studies, apoptosis was assessed by immunostaining with an antibody to the active site of caspase-3. A clear dose response was seen, with dermal and epidermal apoptosis increasing in proportion to UV dose after 24 h and eNOS−/− mice being most prone to apoptosis (data not shown). On the basis of these results, triplicate mice (wild type, eNOS−/−, or iNOS−/−) were irradiated with 0, 400, and 1,000 mJ/cm², respectively; representative immunostaining for caspase-3 active site is shown in Fig. 5 and quantified in Fig. 6. In the dermis, increasing UVB dose produced increasing apoptosis, with both iNOS−/− ($P < 0.005$) and eNOS−/− ($P < 0.001$) mice exhibiting significantly higher apoptosis at 400 mJ/cm². In the epidermis, eNOS−/− mice was double that of either wild-type or iNOS−/− mice at a UVB dose of 1,000 mJ/cm² ($P < 0.001$; Fig. 6A). In the epidermis, eNOS−/− mice underwent significantly more apoptosis than wild-type mice ($P < 0.001$). iNOS−/− mice showed a tendency to increased apoptosis, but this did not reach statistical significance. There were no differences among groups at 1,000 mJ/cm² (Fig. 6B).

Localization of eNOS. Our studies suggested a profound antiprotective activity of eNOS in the context of UVB irradiation, but it was necessary to localize eNOS in the skin of wild-type mice. Immunohistochemistry was performed with a polyclonal anti-eNOS antibody. Staining for eNOS protein was strong in keratinocyte cytoplasm and in endothelial cells and weaker in dermal fibroblasts. There was no difference in eNOS expression between iNOS−/− and wild-type mice or in irradiated and nonirradiated skin. As expected, no staining was observed in eNOS−/− mice (Fig. 7).

DISCUSSION

One of the primary functions of the skin is to protect the body from the external environment. UVB radiation is a potent carcinogen, causing DNA damage,
immunosuppression, and apoptosis (7). Keratinocytes are relatively resistant to the apoptogenic properties of UVB, and they form an effective barrier to the transmission of UVB to the underlying tissues. Here, we demonstrate that the resistance of keratinocytes to UVB-induced apoptosis is a consequence of NO release by eNOS, acting through the stimulation of cGMP production.

Previous reports demonstrated the release of NO in normal skin but did not ascribe a clear function to this NO. Romero-Graillet et al. (32) described the release of NO and activation of cGMP within 1 min of UVB irradiation of cultured human keratinocytes, implicating an eNOS-like enzyme. Additionally, iNOS in the skin releases NO with a peak 18–24 h after UVB irradiation (47). Our experiments in gene knockout mice extend these prior observations, demonstrating that keratinocyte apoptosis is greater in both eNOS−/− and iNOS−/− mice compared with wild-type mice after irradiation with 400 mJ/cm² UVB. We found that eNOS−/− mice are markedly more sensitive to UVB than wild-type or iNOS−/− mice. They undergo a greater degree of apoptosis in both the dermis and the epidermis, as well as exhibiting a higher degree of apoptosis at both 400 and 1,000 mJ/cm² UVB. This effect produced by eNOS is consistent with the rapid release of NO from this constitutive NOS in the skin. Together, these data raise the possibility that the coordinated release of NO from eNOS and iNOS serves both as an immediate and a sustained mechanism to protect keratinocytes from the toxicity of exposure to UVB.

Keratinocyte apoptosis occurs by several partially independent pathways: activation of the TNF-α/Fas death receptor, release of reactive oxygen species, mitochondrial damage with subsequent release of cytochrome c, and DNA damage with resultant upregulation of p53 (22). Relative resistance to UV-induced apoptosis has been demonstrated in stem cell-enriched keratinocytes via an integrin signaling pathway (43), and in normal keratinocytes stabilization of wild-type p53 after UV irradiation occurs by p38-mediated phosphorylation (5). NO can inhibit apoptosis directly by S-nitrosylation of cysteine groups on a number of caspases in hepatocytes (25), indirectly via cGMP-dependent pathways (18, 37), and by quenching of reactive oxygen species released after UV irradiation (40). The antiapoptotic effect of NO in keratinocytes was consistently reduced by the sGC inhibitor ODQ and replicated by addition of the nonhydrolyzable cGMP analog 8-BrcGMP. Caspase-3 activity after irradiation was increased by blocking NOS activity and reduced on addition of an NO donor. These observations suggest that the protective role of NO in keratinocytes involves, at least in part, cGMP.

The prototypic target for cGMP is protein kinase G (PKG), whereas protein kinase A (PKA) is one of the main targets of cAMP. Both cGMP and cAMP can cross talk through the activation of PKG or PKA (15, 19). Previous studies from our laboratory suggest that both cyclic nucleotides prevent hepatocyte apoptosis through the activation of PKA (26). Similar to NO, cell-permeant cGMP analogs suppressed caspase-8 activation, loss of mitochondrial membrane potential, cytochrome c release, and caspase-3 activation. Nonhydrolyzable analogs of cAMP were even more potent in the inhibition of hepatocyte cell death. The protective effects of both cGMP and cAMP were dependent, in part, on PKA. Furthermore, NO, cGMP, and cAMP all activated the Akt/protein kinase B (PKB) pathway in cultured hepatocytes (26). Future studies will be aimed at elucidating whether this same paradigm holds in

![Fig. 4. cGMP reduces keratinocyte apoptosis after UVB. A: keratinocytes were cultured to 70% confluence in 6-well plates, and 800 μM 8-bromo-cGMP (8-BrcGMP) was added to half of the cells 8 h before irradiation with 100 mJ/cm² UVB. Cells were harvested 12 h later, and apoptosis was measured by flow cytometry with annexin V-FITC and PI counterstaining. Representative of 3 independent experiments. B: keratinocytes were cultured to 90% confluence, and the soluble guanylate cyclase (sGC) inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 100 μM) was added 8 h before irradiation. Cell harvesting and flow cytometry were performed as in A. Representative of 3 independent experiments.](http://ajpcell.physiology.org/.../10.220.32.246 on June 22, 2017)
keratinocytes. Future studies will also determine whether the mechanisms outlined here in a transformed keratinocyte cell line are still operative in primary cells. This is especially important because Jackson et al. (16) have shown that human papillomavirus reduces post-UV apoptosis by reducing Bak expression, whereas Simbulan-Rosenthal et al. (39) have shown that E6/7 increases sensitivity to UV-induced apoptosis.

At higher doses of UVB (≥1,000 mJ/cm²), the anti-apoptotic role of eNOS in the dermis is striking, whereas neither iNOS nor eNOS appears to be able to
protect the epidermis from apoptosis. This blurring of the effect of NO in the epidermis may be due to changes in the redox environment at these higher radiation doses. For example, very small differences in flux of reactive oxygen species and NO can markedly alter the balance between oxidative and nitrosative effects (33, 52). We previously described (49) how the presence of superoxide dismutase enhances the antiapoptotic effects of NO in irradiated keratinocytes. In that study, we suggested that limiting formation of proapoptotic reactive nitrogen intermediates such as peroxynitrite, or quenching of NO by formation of superoxide, shifts the balance toward the antiapoptotic effects of NO (49). The loss of the antiapoptotic effect of eNOS-derived NO at 1,000 mJ/cm² could be due either to the inability of these levels of NO to overcome the proapoptotic effects induced by UVB through the mechanisms outlined above or to the presence of proapoptotic reactive nitrogen intermediates formed at higher doses of UVB (10).

In the dermis, we observed a direct linear relation between UVB dose and apoptosis, with eNOS⁻/⁻ mice exhibiting significantly increased apoptosis at all doses of UVB. Apoptosis was not counted in adnexal structures and hair follicles because apoptosis in these structures is dependent on hair cycle rather than purely on UV radiation. The cells in which apoptosis was measured are thus predominantly fibroblasts. The persistently high apoptosis in eNOS⁻/⁻ mice, even at 1,000 mJ/cm², might be due to differences in cell type-specific responses to NO and reactive nitrogen intermediates or to the reduced levels of UVB reaching the dermis compared with the epidermis. We demonstrated eNOS staining intensely in the cytoplasm of keratinocytes and diffusely in fibroblasts of iNOS⁻/⁻ and wild-type mice, in accord with the findings of other authors studying isolated cells from human skin (20, 38). There was no change in distribution or intensity of eNOS protein observed after irradiation, and eNOS was not seen in eNOS⁻/⁻ mice.

Besides eNOS, we define a novel role for iNOS in the protection of keratinocytes from UVB-induced apoptosis. Higher levels of NO output would be expected from iNOS, which peaks in human skin 24 h after irradiation in both keratinocytes (21) and dermal endothelial...
cells (41). The time course of this iNOS upregulation correlates with marked skin erythema (46, 47). A lower-grade but more persistent erythema is seen for up to 3 wk after irradiation (51). Because erythema is a good marker for NO and cGMP production (6), we believe that a prolonged production of lower concentrations of NO after irradiation may continue to exercise an anti-apoptotic effect.

The aberrant regulation of NO may have important pathophysiological sequelae. In psoriasis, plaque NO levels are persistently elevated (50), which may at least partially account for the excess keratinocyte numbers and reduced apoptosis (4) characteristic of this disease. In systemic and localized scleroderma, reduced dermal eNOS protein and NO production correlates with reduced endothelial cell growth (51). In conclusion, our findings extend to keratinocytes the antiapoptotic role of NO and raise the clinical possibility of NOS inhibition for the treatment of hyperproliferative skin disorders such as psoriasis.

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