Role of Phagocyte Oxidase in UVA-Induced Oxidative Stress and Apoptosis in Keratinocytes

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Chronic exposure to ultraviolet radiation including ultraviolet A (315–400 nm) (UVA) may cause photocarcinogenesis and photoaging. The UVA-induced production of reactive oxygen species (ROS) and the resultant oxidative stress exposure play an important role in these biological processes. Here we have investigated the role of phagocyte oxidase (PHOX, gp91phox) in the production of ROS, redox status change, and apoptosis after UVA exposure by using gp91phox-deficient (gp91$^{phox-/-}$) primary keratinocytes. UVA radiation resulted in increased ROS production and oxidation of reduced glutathione (GSH) to its oxidized form (GSSG). The presence of diphenylene iodonium (DPI) inhibited ROS production by UVA. In comparison with wild-type cells, gp91$^{phox-/-}$ cells produced slightly less ROS and GSH oxidation. UVA radiation induced apoptosis in wild-type keratinocytes as detected by phosphatidylserine (PS) translocation, caspase activation, and DNA fragmentation. As compared with wild-type cells, UVA induced less PS translocation in gp91phox-deficient cells. No difference, however, was observed in caspase activation and DNA fragmentation after UVA exposure in wild-type and gp91$^{phox-/-}$ cells. These findings suggest that gp91phox plays a limited role in the UVA-induced ROS production, oxidative stress, and therefore the PS translocation, but has no effect on UVA-induced caspase activation and DNA fragmentation during apoptosis.

Key words: apoptosis/gp91/keratinocytes/NADPH oxidase/reactive oxygen species/UVA
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The ultraviolet (UV) radiation portion of solar radiation reaching the Earth's surface presents a major environmental challenge to the skin and may cause skin damage including skin cancer. Deregulated apoptosis may play a critical role in these processes. Ultraviolet A (315–400 nm) (UVA), which constitutes about 95% of the UV radiation from the sunlight reaching the Earth's surface, has attracted increasing attention over the past 20 years.

Considerable evidence exists that reactive oxygen species (ROS) are involved in some of the deleterious effects of UV light on cutaneous cells and skin (Tyrrell and Pidoux, 1989; Morliere *et al*, 1991; Weber *et al*, 1997; Podda *et al*, 1998; Wondrak *et al*, 2002). ROS and oxidative stress have been clearly linked to apoptosis processes (He *et al*, 2003; Li *et al*, 2003; Qanungo *et al*, 2004). ROS are believed to be involved in many inflammatory skin disorders, skin cancer formation, phototoxicity, and skin aging (Black *et al*, 1985; Trouba *et al*, 2002).

Currently, attention has been increasingly paid to a gene family encoding the catalytic subunit of the superoxide-producing NADPH oxidases (NOX) (Lambeth *et al*, 2000; Lambeth, 2002, 2004;). gp91phox (NOX2), the founding member of the phagocyte NADPH oxidase family, is predominantly expressed in phagocytes such as neutrophils and plays a crucial role in host defense against microbial infection. Recently NADPH oxidase has been shown to be involved in the generation of superoxide (Yang *et al*, 1999), DNA repair (Cho *et al*, 2002), wound healing (Sen *et al*, 2002), and activation of p38 (Chiu *et al*, 2001) in keratinocytes. The role of one member gp91phox in UVA-exposed keratinocytes, however, is not known.

In this study, we investigated the role of phagocyte NADPH oxidase gp91phox in UVA-induced apoptosis in primary epidermal keratinocytes from wild-type (gp91$^{phox+/+}$) and gp91-deficient (gp91$^{phox-/-}$) mice (Pollock *et al*, 1995). We also examined the production of oxidative stress, as detected by 5-(and-6)-chloromethyl-2′,7′- dichlorodihydrofluorescein diacetate (CM-DCFH-DA), and the oxidation of glutathione.

Results

UVA-induced production of ROS and oxidative stress in keratinocytes To determine whether UVA radiation induced the production of ROS and oxidative stress in the cells, primary keratinocytes were isolated from neonatal mouse skin epidermis and irradiated with UVA (16 J per cm^2). Cells were first irradiated with UVA and then labeled with CM-DCFH-DA in the dark. As shown in Fig 1*A*, irradiation with UVA (16 J per cm^2) caused the cell population

Abbreviations: DPI, diphenylene iodonium; EMEM, Eagle's minimum essential medium; FAK, focal adhesion kinase; GSH, reduced glutathione; GSSG, oxidized glutathione; gp91$^{phox-/-}$, phagocyte NADPH oxidase-deficient; PARP, poly (ADP-ribose) polymerase; PHOX, phagocyte oxidase; PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species; UVA, ultraviolet A (315–400 nm); WT, wild-type C57BL/6J (gp91$^{phox+/+}$)

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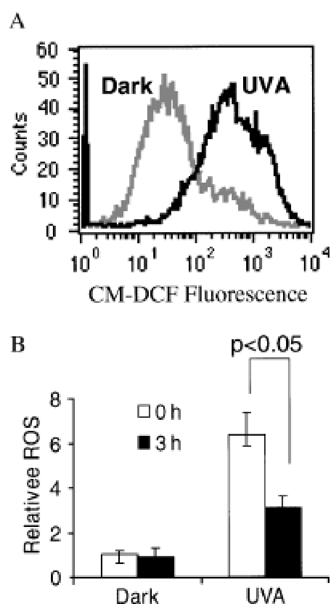

Figure 1
Ultraviolet A (315–400 nm) (UVA) radiation induced the production of reactive oxygen species (ROS) in wild-type primary keratinocytes. (*A*) Wild-type primary keratinocytes were washed with phosphate-buffered saline (PBS), exposed to UVA (16 J per cm^2) in PBS and then immediately incubated with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-DCFH-DA) in serum-free medium at 37°C. After trypsinization and resuspension, cells were analyzed by flow cytometry. The histogram shown is representative of three independent experiments (n = 3). (*B*) Same as in *A* except that cells were stained with CM-DCFH-DA immediately or at 3 h after UVA exposure from three independent experiments (n = 3). Values are means ± SEM. The relative ROS in *B* represents the ratio of the fluorescence of the cells treated or kept in the dark to the cells kept in the dark at 0 h.

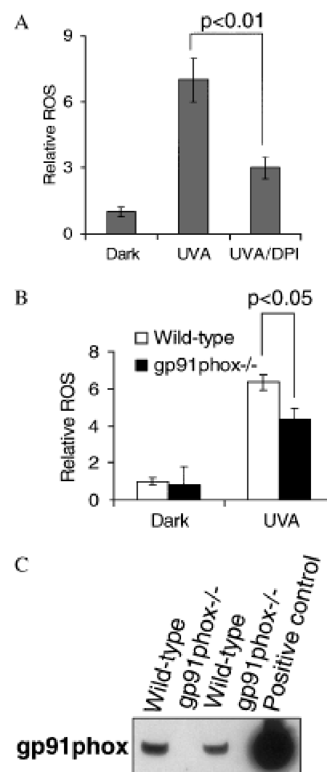

Figure 2
Pretreatment with diphenylene iodonium (DPI) or gp91 deficiency reduced the production of reactive oxygen species (ROS). (*A*) Cells were pretreated with DPI (10 μM) for 30 min, irradiated with ultraviolet A (315–400 nm) (UVA) (16 J per cm^2) and then analyzed as in Fig 1*A*. (*B*) Wild-type or $gp91^{phox-/-}$ keratinocytes were irradiated with UVA or kept in the dark and then analyzed as in Fig 1*A*. The relative ROS in *A* and *B* represents the ratio of the fluorescence of the wild-type and $gp91^{phox-/-}$ cells treated or kept in the dark to the wild-type cells kept in the dark. (*C*) Wild-type cells and $gp91^{phox-/-}$ cells were lysed and then subjected to electrophoresis, transferred to membranes, and immunoblotted with detection with anti-gp91phox antibody. Biological duplicates were used for the immunoblotting. Mouse macrophage lysate was used as positive control. The data are representative of three independent experiments (n = 3). Values are means ± SEM.

to shift to higher fluorescence intensity in wild-type mouse keratinocytes, indicating an increased production of ROS and oxidative stress. Three hours after UVA exposure, however, the oxidative stress produced by UVA exposure was attenuated as demonstrated by the smaller shift of cell population (Fig 1*B*), suggesting that cells recovered partially from UVA-caused oxidative stress.

Effect of diphenylene iodonium (DPI) on the ROS production To determine whether NADPH oxidase is involved in the production of ROS, the cells were pretreated with DPI, a widely used inhibitor of NADPH oxidase. After UVA irradiation, the production of ROS was determined as in Fig 1. The presence of DPI attenuated the formation of ROS by 50% (Fig 2*A*), suggesting that NADPH oxidase might play an important role in UVA-induced oxidative stress.

Role of gp91phox in the ROS production gp91phox is one member of the NADPH oxidase family that plays a critical role in the production of ROS, such as superoxide (Babior, 1999; Bokoch and Diebold, 2002; Gao *et al*, 2003). Primary keratinocytes were isolated from wild-type (WT, $gp91^{+/+}$) and $gp91^{phox-/-}$ mice. In $gp91^{phox-/-}$ cells, UVA radiation also induced the production of ROS (Fig 2*B*). The formation of ROS and oxidative stress in $gp91^{phox-/-}$ cells, however, was lower than that in wild-type cells, suggesting that gp91phox played a role in the UVA-induced production of oxidative stress and was partially responsible for the intracellular ROS formation.

Although gp91phox is predominantly expressed in phagocytes, its mRNA has been detected in human HaCaT keratinocytes (Chamulitrat *et al*, 2003). The protein level in keratinocytes, however, has not yet been determined. In primary mouse keratinocytes, gp91phox protein was present and expressed as detected by immunoblotting, whereas no protein expression was seen in gp91phox-deficient keratinocytes (Fig 2*C*). Although the expression of gp91phox in keratinocytes was much lower that that in mouse macrophages (Fig 2*C*), its presence in keratinocytes may be responsible for the differential ROS production upon UVA exposure (Fig 2*B*).

Role of gp91phox in glutathione oxidation Glutathione, the most abundant redox pool in cells, is responsible for the buffering of redox status in the cells, especially under stress conditions (Schafer and Buettner, 2001). In non-irradiated cells, glutathione existed predominantly in its reduced form

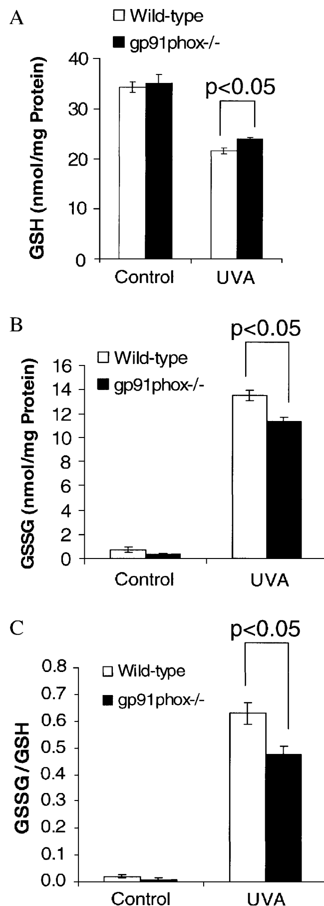

Figure 3
Gp91 deficiency reduced GSH (reduced glutathione) oxidation to GSSG (oxidized glutathione). After washing with phosphate-buffered saline, cell were exposed to ultraviolet A (315–400 nm) (UVA) (16 J per cm^2) or kept in the dark. GSH (*A*) and GSSG (*B*) were determined as described in the "Materials and Methods". The ratio of GSSG to GSH (GSSG/GSH) was calculated (*C*). The data are representative of four independent experiments (n = 4). Values are means ± SEM.

(GSH), whereas the GSSG (oxidized gluthatione) level was low (Figs 3*A* and *B*). The GSH level in wild-type keratinocytes was similar to that in gp91$^{phox-/-}$ cells, whereas GSSG levels and the ratio of GSSG to GSH (GSSG/GSH) in wild-type cells were slightly lower that that in gp91$^{phox-/-}$ cells (Figs 3*A*, *B* and *C*). After UVA exposure, however, approximately 39% and 33% of the GSH was oxidized to GSSG in wild-type and gp91$^{phox-/-}$ cells, respectively (Figs 3*A* and *B*). The GSSG/GSH ratio increased dramatically as compared with control cells (Fig 3*C*). A lower GSSG level and GSSG/GSH ratio were detected in gp91$^{phox-/-}$ cells than in wild-type cells (Figs 3*B* and 3*C*). These data suggest that gp91 was partially involved in the change of oxidative status in cells.

Effect of DPI on UVA-induced apoptosis in keratinocytes To determine the role of NADPH oxidase in apoptosis induced by UVA exposure, cells were preincubated with or without DPI. As shown in Fig 4, when cells were exposed to 16 J per cm^2 UVA, approximately 44% of the cells underwent apoptosis as detected by annexin V staining followed by flow cytometry. The pretreatment of cells with DPI at 5 μM had little effect on UVA-induced apoptosis in keratinocytes. Pre-incubation with 10 μM DPI before UVA exposure, however, resulted in approximately 74% apoptosis in the cells (Fig 4). Vehicle alone (0.1% DMSO) had no effect (data not shown). These data suggest that DPI not only failed to protect cells from apoptosis, but instead enhanced apoptosis after UVA exposure.

Role of gp91phox in UVA-induced apoptosis To determine whether gp91phox played a role in UVA-induced apoptosis, primary keratinocytes from newborn mice were irradiated with UVA and apoptosis was determined by Annexin-V/propidium iodide (PI) followed by flow cytometry, caspase activity, western blotting, and DNA fragmentation.

As shown in Fig 5, exposure to UVA (16 J per cm^2) resulted in 44% apoptotic cell death in wild-type mouse keratinocytes, whereas a significantly lower apoptotic cell death (31%) was observed in gp91$^{phox-/-}$ keratinocytes as detected by Annexin V staining followed by flow cytometry ($p < 0.05$), which determined the phosphatidylserine (PS) translocation during cell apoptosis (Fig 5*A*). Similar difference was seen with 24 J per cm^2, but not 8 J per cm^2 of UVA radiation (Fig 5*B*). These data demonstrate that gp91 deficiency provided a partial and limited protection for keratinocytes against UVA-induced PS translocation during apoptosis of keratinocytes.

In addition to PS translocation determined by Annexin-V/PI measurements after UVA exposure, caspase-3 activity was also determined as another hallmark of apoptosis. UVA (16 J per cm^2) was found to induce activation of caspase-3 in a time-dependent manner (Fig 6*A*). A significant activation of caspase-3 was seen at 3 h after UVA irradiation. No significant difference, however, was detected between wild-type and gp91$^{phox-/-}$ keratinocytes. These data indicate that gp91phox has no effect on UVA-induced activation of caspase-3 during apoptosis of keratinocytes.

DNA fragmentation was also performed to compare UVA-induced apoptosis in wild-type and gp91$^{phox-/-}$ cells. As shown in Fig 6*B*, UVA resulted in DNA fragmentation in wild-type cells. No difference, however, was observed between wild-type and gp91$^{phox-/-}$ cells after UVA exposure. This suggests that gp91 has no effect on UVA-induced DNA fragmentation.

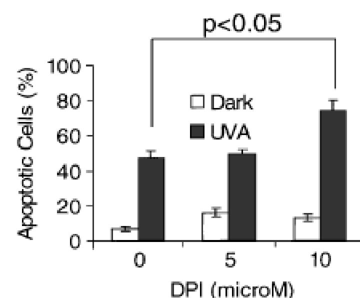

Figure 4
The presence of diphenylene iodonium (DPI) enhanced ultraviolet A (315–400 nm) (UVA)-induced apoptosis. Cells were pretreated with or without DPI (5 and 10 μM) irradiated with 16 J per cm^2 UVA or kept in the dark and then incubated for 15 h. Then cells were stained with Annexin V/propidium iodide and analyzed by flow cytometry. The cell percentages positive for Annexin V were shown. The data are representative of three independent experiments (n = 3). Values are means ± SEM.

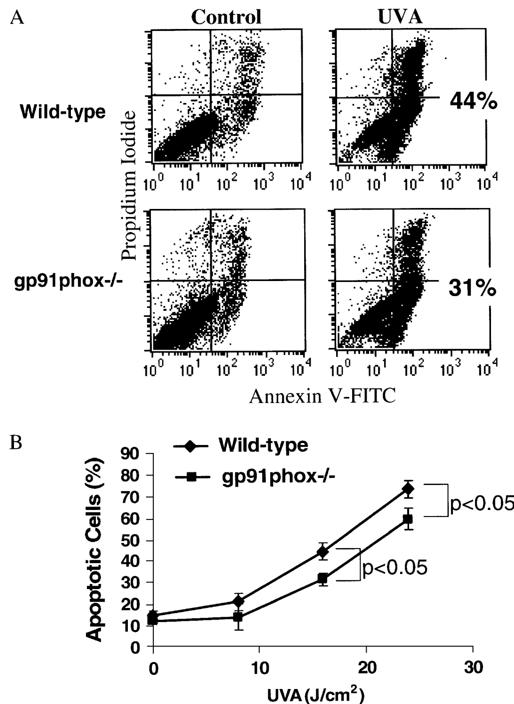

Figure 5
Gp91 deficiency reduced phosphatidylserine translocation. (*A*) After wild-type or gp91$^{phox-/-}$ cells were exposed to ultraviolet A (315–400 nm) (UVA) (16 J per cm^2) and then incubated for 15 h, cells were analyzed as in Fig 4. (*B*) As in *A*, but cells were irradiated with different doses of UVA and then analyzed as in Fig 4. The data are representative of five independent experiments (n = 5). Values in *B* are means ± SEM.

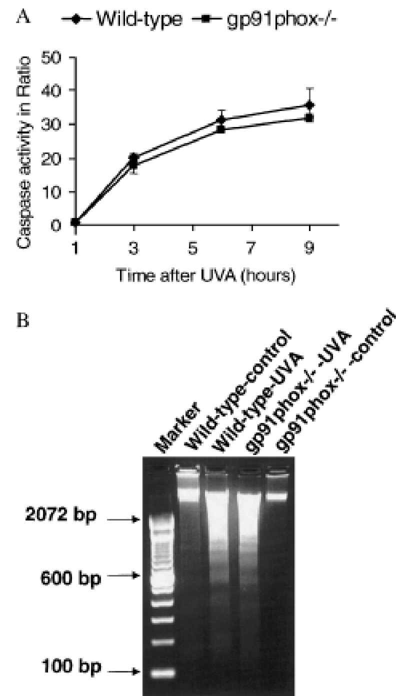

Figure 6
Gp91 deficiency had no effect on the ultraviolet A (315–400 nm) (UVA)-induced increase of caspase activity and DNA fragmentation. (*A*) Wild-type or gp91$^{phox-/-}$ cells were left intact or exposed to UVA (16 J per cm^2) and then incubated for different time periods. Caspase activity was determined as described in the "Materials and Methods". The ratio of the fluorescence of the UVA-irradiated samples to that of the samples kept in the dark was used. (*B*) Cells were treated as in *A* and then incubated for 18 h. DNA was extracted and separated on 1.5% agarose gel. The data are representative of six independent experiments (n = 6) for caspase-3 activity and three independent experiments (n = 3) for DNA fragmentation. Values in *A* are means ± SEM.

These observations were also confirmed by western blotting (Fig 7). There was no activation of caspase-3 at 1 h after UVA exposure, which is consistent with the results of caspase-3 activity (Fig 6), whereas dramatic activation of caspase-3 was observed at 6 h after UVA exposure in wild-type keratinocytes (Fig 7*A*). A similar time course was seen with caspase-7 activation, another member of executor caspases in apoptosis. The cleavage of poly (ADP-ribose) polymerase (PARP), the well-known substrate of caspase-3 (Earnshaw *et al*, 1999), was detected at 6 h but not at 1 h after UVA exposure. A similar response was observed with the cleavage of focal adhesion kinase (FAK), another substrate of activated caspases (Earnshaw *et al*, 1999; van de Water *et al*, 1999). As compared with wild-type cells, there was no difference in the activation of caspase-3 or -7, the cleavage of PARP or FAK in gp91$^{phox-/-}$ cells (Fig 7*B*). These results indicate that gp91 was not involved in UVA-induced caspase activation and its enzymatic cleavage of substrates such as PARP and FAK.

Discussion

This study demonstrates the role of phagocyte NADPH oxidase (PHOX) in the production of ROS, oxidative stress, and apoptosis induced by UVA radiation by using PHOX-deficient keratinocytes. UV radiation has been shown to be involved in DNA damage and apoptosis in several models, such as cyanobacteria (Dunlap *et al*, 2002; He and Hader, 2002), plant (Green and Fluhr, 1995), and mammalian cells, especially skin cells (Huang *et al*, 2001; Ichihashi *et al*, 2003). With western blotting or a caspase activity assay, no immediate apoptosis was detected in keratinocytes after UVA exposure. UVA-induced apoptosis is time- and concentration-dependent as seen in caspase activation and PS translocation.

Recently phagocyte NADPH oxidase (gp91phox) was found to catalyze the non-mitochondrial production of ROS (Lambeth, 2004) and expressed in non-phagocyte human keratinocytes (Chamulitrat *et al*, 2004). In this study, gp91phox protein is also found to be expressed in primary mouse keratinocytes. We have used gp91phox-deficient primary keratinocytes to investigate the role of gp91phox in the ROS production and apoptosis by UVA exposure. Upon UVA exposure, ROS production and oxidative stress can also be detected, but to a lesser degree in gp91phox-deficient cells as compared to wild-type cells, indicating that gp91 may play a partial but limited role in UVA-induced ROS production. This may be due to the following facts. (1) Other members of NOX family such as NOX1 may also be involved in ROS production in epidermal keratinocytes upon UVA exposure. (2) Damage to mitochondria by UVA may elevate intracellular ROS levels. (3) Photosensitization upon UVA exposure in the presence of unknown intracellular chromophores may also produce ROS in the cells. Wondrak *et al*

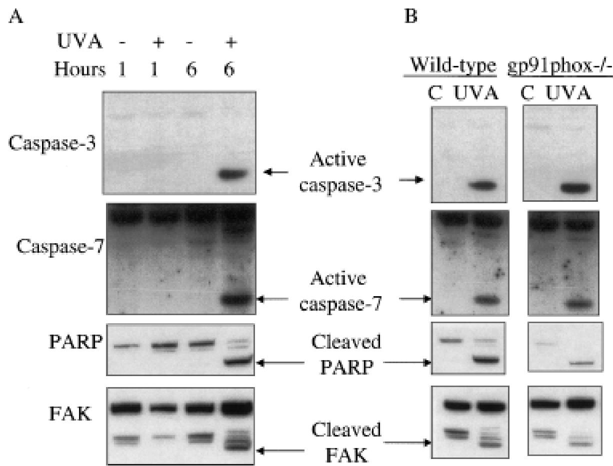

Figure 7
Gp91 deficiency had no effect on the activation of caspase-3/7, the cleavage of poly (ADP-ribose) polymerase (PARP) and focal ahesion kinase (FAK), or the downregulation of epidermal growth factor receptor (EGFR). Wild-type cells were exposed to ultraviolet A (315–400 nm) (UVA) (16 J per cm^2) and then incubated for 1 or 6 h. Cell lysates were subjected to electrophoresis, transferred to membranes, and immunoblotted with detection with anti-caspase-3, caspase-7, PARP, FAK, and EGFR antibodies. (*B*) Same as in *A* except that wild-type or gp91$^{phox-/-}$ cells were exposed to UVA (16 J per cm^2) and then incubated for 6 h. The data are representative of three independent experiments (n = 3).

have suggested 3-hydroxypyridine derivatives (Wondrak *et al*, 2004), advanced glycation end products (Wondrak *et al*, 2002), and extracellular matrix proteins (Wondrak *et al*, 2003) may constitute novel classes of UVA photosensitizers, capable of ROS production and skin photooxidative damage. Further knockout or knockdown studies are needed to identify the *in vivo* and *in situ* UVA photosensitizers.

A slight decrease in PS translocation in gp91phox-deficient cells was observed after UVA exposure as compared with wild-type cells, whereas gp91phox deficiency had no effect on caspase activation. This is consistent with our previous findings that redox status played a role in PS translocation but not in caspase activation during UVA-induced apoptosis (He *et al*, 2003). ROS play a regulatory role in cellular signaling pathways, including apoptosis. Although a large number of signaling pathways are regulated by ROS, the signaling molecules targeted by ROS are far from clear. Redox status change is only one factor in some processes of apoptosis. In this study, this slight difference in ROS production might contribute to the PS translocation process. In addition to oxidative stress, UVA exposure may alter several other processes such as mitochondria and death receptor signaling that lead to the activation of caspase cascade (Earnshaw *et al*, 1999). The transient oxidative stress in this study may only affect PS translocation, but not other processes such as caspase activation. Previously Kagan *et al* (2000) and Shvedova *et al* (2002) reported that oxidative stress regulated PS translocation in a caspase-independent manner. More investigation is needed to identify the role of ROS or oxidative stress in UVA-induced apoptosis.

A greater inhibitory effect was observed with pretreatment with DPI than with gp91phox deficiency. In the past years, DPI has been widely used as an inhibitor for NADPH oxidase, since Cross (1987) and Cross and Jones (1986) reported the inhibitory effect of DPI on NADPH oxidase. It is known that DPI is not a specific inhibitor for gp91phox or other NADPH oxidase family members, but is an uncompetitive inhibitor of flavoenzymes. The presence of DPI, however, is cytotoxic to keratinocytes. It appears that the additive effect of DPI in UVA-induced apoptosis is not connected to its inhibitory effect on the ROS production by UVA exposure. Other unknown DPI targets may be responsible for the enhanced PS translocation, which played a dominant role and overrode the effect of reduced ROS formation by DPI (Riganti *et al*, 2004) observed in this study.

In conclusion, this work has demonstrated that gp91phox plays a partial and limited role in UVA-induced ROS production and PS translocation but has no effect on caspase activation and DNA fragmentation during apoptosis. The molecular targets of UVA radiation and those of the resultant ROS are under investigation in our laboratory.

Materials and Methods

Primary keratinocyte culture Primary keratinocytes were obtained from newborn mice as described previously (Dlugosz *et al*, 1995; Akunda *et al*, 2004). Wild-type and gp91$^{phox-/-}$ cells were obtained from neonates generated from ongoing breeding in the National Institute of Environmental Health Sciences (NIEHS) animal facility. The experiments have been approved by the institute. Keratinocytes from the epidermis of each neonate were collected individually (PHOX-deficient (Pollock *et al*, 1995) and wild-type neonates) and seeded at 2.5–3.0 $\times$ 10^6 cells in 60 mm dishes. Cells were seeded in calcium-free EMEM (BioWhittaker, Walkersville, Maryland) supplemented with 10% fetal bovine serum (FBS), 100 units per mL penicillin, and 100 μg per mL streptomycin (Invitrogen, Carlsbad, California). Five hours after plating, the medium was removed, replaced with Ca^{2+}-free EMEM with 4% chelexed (Bio-Rad Laboratories, Hercules, California) FBS, supplemented with 5 ng per mL recombinant human epidermal growth factor (Invitrogen), antibiotics, and the calcium concentration adjusted to 0.05 mM (referred to as low Ca^{2+} media).

UVA treatment The medium was removed and cells were washed twice with sterile phosphate-buffered saline (PBS) (PBS-CMF, calcium/magnesium-free). After the addition of sterile PBS, the cells were irradiated with fluorescent lamps (Houvalite F20T12BL-HO PUVA, National Biological Corp., Twinsburg, Ohio) with the dish lid on. The UVA dose was monitored with a Goldilux UV meter equipped with a UVA detector (Oriel Instruments, Stratford, Connecticut). Control samples were kept in the dark under the same conditions. At predetermined time points, attached and floating cells were harvested and subjected to analysis. In selected experiments, cells were pre-incubated with DPI at 37°C prior to irradiation.

Determination of Intracellular ROS production by flow cytometry CM-DCFH-DA (Molecular Probes, Eugene, Oregon) was used to detect production of intracellular ROS or the resultant oxidative stress in keratinocytes after exposure to UVA. After irradiation, cells were incubated with serum-free medium CM-DCFH-DA (2 μM) at 37°C for 30 min, followed by washing with PBS containing and trypsinization. Cells were then washed and resuspended in PBS containing 0.1% BSA. The fluorescence was quantified by flow cytometry using a Becton Dickinson FACSort (Becton Dickinson, Mountain View, California). The results were expressed as the ratio of the fluorescence of UVA-irradiated samples to that of dark control samples.

Determination of intracellular glutathione GSH and GSSG were measured using a modified method for glutathione determination in

microtiter plates as described previously (He *et al*, 2003). Briefly, cells were scraped and sonicated and then centrifuged at 10,000 $\times$ *g* for 10 min at 4°C. The supernatants were collected for the GSH/GSSG and BCA protein assays (Pierce, Rockford, Illinois). The GSH or GSSG levels were expressed as nmol per mg protein. The ratio of GSSG to GSH (GSSG/GSH) was also calculated.

Determination of apoptosis by flow cytometry Annexin V staining was used to determine the translocation of PS in UVA-induced apoptosis as described previously (He *et al*, 2003). After incubation, control or treated cells including attached and floating cells were harvested and collected by centrifugation at 300 g for 5 min at room temperature. Cells were washed with cold PBS and stained with TACS Annexin V Kits according to the manufacturer's instructions (Trevigen, Gaithersburg, Maryland). Cells positive for Annexin V-FITC and/or PI were quantified by flow cytometry using a Becton Dickinson FACSort.

Caspase activity Caspases were assayed using ApoAlert Caspase Fluorescent Assay Kits according to the manufacture's instructions (Clontech, Palo Alto, California). Briefly, cells were extracted in lysis buffer and the cell lysate were incubated for 1 h at 37°C with assay buffer containing the fluorescent caspase substrate Ac-Asp-Glu-Val-Asp-AFC(AFC-7-amino-4-trifluoromethyl-coumarin) (Ac-DEVD-AFC) for caspase-3. The fluorometric detection of cleaved AFC product was performed on a plate reader (ex400/em505 nm). For preparation of the AFC calibration curve, 80 μM free AFC was diluted in the caspase assay buffer without substrate to give 0.5, 1, 2 and 4 μM of free AFC. The results were expressed as the ratio of the treated samples to respective control samples.

Western blotting Equal amounts of protein were subjected to electrophoresis followed by electrophoretic transfer to nitrocellulose membranes. Membranes were blocked followed by incubation with primary antibody and secondary antibody. Proteins were visualized using SuperSignal chemiluminescent substrate (Pierce, Rockford, California). Blotting antibodies used are as follows: anti-caspase-3 caspase-7 and PARP from Cell Signaling (Beverly, Massachusetts); anti-FAK from Santa Cruz Biotechnology (Santa Cruz, California); anti-gp91phox from BD Transduction Laboratories (Suwanaee, Georgia).

DNA fragmentation The pattern of DNA cleavage was analyzed by agarose gel electrophoresis as described previously (He *et al*, 2003). Briefly, after cell pellets were lysed on ice at 4°C overnight, cell lysates were incubated with RNase A (100 μg per mL) and then with proteinase K followed by extraction with phenol/chloroform/isopropyl alcohol (25:24:1, vol/vol). After precipitation with ethanol, DNA samples were separated by horizontal electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV light.

Statistics Data are presented as mean $\pm$ SEM of three to six experiments. The Student's *t* test was used for comparisons between experimental groups ($n = 3$–6). A value of $p < 0.05$ was considered statistically significant.

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