Original Article

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Nrf2-mediated protection against UVA radiation in human skin keratinocytes

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Summary **Ultraviolet A (UVA, 320-400 nm) radiation is an oxidizing agent that causes significant damage to cellular components and that leads to photoaging and cancer. It strongly induces NF-E2-related factor 2 (Nrf2) expressions in cultured FEK4 human skin fibroblasts but weakly induces it in transformed HaCaT skin keratinocytes. Nrf2 silencing increases cell damage at a moderate dose of UVA irradiation (250 kJ•m^{-2}) in FEK4 fibroblasts, but whether a decrease in Nrf2 sensitizes HaCaT keratinocytes to a moderate to high dose (250-500 kJ•m^{-2}) of UVA irradiation (*i.e.,* 400 kJ•m^{-2}, peak emission 365 nm) is currently unknown. A moderate to high dose of UVA irradiation only slightly increased Nrf2 expression in HaCaT skin keratinocytes. Knockdown of Nrf2 by specific silencing of Nrf2 (siNrf2) strongly increased cell damage as gauged by membrane damage (LDH) and cell viability (MTT assay) following this dose of UVA irradiation. These results suggest that decreased Nrf2 significantly increased UVA irradiation-induced cell damage in skin keratinocytes. Nrf2 may play a role in protecting human skin keratinocytes from UVA radiation-induced damage.**

Keywords: UVA, Nrf2, HaCaT skin keratinocytes

1. Introduction

The major source of human exposure to ultraviolet (UV) radiation is *via* sunlight or artificial sources such as cosmetic tanning spas that use artificial UV lamps (*1*,*2*). UV light that reaches the surface of the earth primarily consists of (> 90%) ultraviolet A (UVA) radiation (320-400 nm), which produces reactive oxygen species (ROS) and has been linked to lipid, protein, and nucleic acid damage (*3*,*4*). The skin acts as a physiological barrier to protect an organism against environmental UV radiation, chemical pollutants, and physical injury. Skin is equipped with an elaborate system of antioxidants and enzymes that maintain the balance between oxidative stress and anti-oxidant defense (*1*,*5*). The most significant enzymes, *e.g.* hemeoxygenase 1 (HO-1) and the phase II detoxification enzymes peroxiredoxin 1 (Prx I), NAD(P)H quinone oxidoreductase 1 (NQO1), glutamate-cysteine ligase, aldo-keto reductase, and thioredoxin reductase, are crucial to protect skin cells from exogenous toxicity, oxidative stress, and carcinogenesis (*1*,*5*). NF-E2-related factor 2 (Nrf2) is a member of the "cap 'n' collar" family of transcription factors, which also include Nrf1 and Nrf3. Upon heterodimerization with leucine zipper proteins such as small Maf proteins, Nrf2 binds to antioxidant response elements (AREs) or Maf recognition elements of its target genes and coordinates transcriptional activation of various antioxidant enzymes (*6*,*7*). During normal cellular quiescence, Nrf2 is held in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) but is released under stress conditions, although the mechanism of its activation may vary depending on the type of cells or tissues (*6*,*7*).

Recently, the role of Nrf2 in protecting against oxidative stress has been studied and reviewed (*7*). Nrf2 is involved in wound healing in mouse skin and Nrf2-deficient mice have an impaired oxidative stress defense (*7*,*8*). A study by Hirota and a previous study by the current authors also showed that UVA irradiation causes nuclear accumulation of Nrf2 in human and

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mouse fibroblasts, respectively, and a deficiency of Nrf2 in these skin fibroblasts significantly increases cell damage caused by low (50-100 kJ•m^{-2}) to moderate (250 kJ•m^{-2}) doses of UVA irradiation (*9*,*10*). Nrf2 may be involved in protection of HaCaT human skin keratinocytes from UVA irradiation by quercetin, an antioxidant (*11*). Therefore, increased cellular antioxidant or detoxification capacity as a result of increased Nrf2 levels may help with cellular defense.

As the top layer of skin, epidermal keratinocytes encounter more UV irradiation and are more resistant to UVA irradiation-mediated cell damage than fibroblasts (*12*,*13*). Nrf2 knockout mice have a prolonged inflammatory response after skin injury (*8*). Previously, a significant increase in Nrf2 was not detected in human HaCaT skin keratinocytes following a low to moderate dose (50-250 kJ•m^{-2}) of UVA irradiation (*14*,*15*). However, down-regulation of Nrf2 expression may suppress cellular functions, such as antioxidant response, so cells may be more susceptible to oxidative damage, including UVA irradiation (*5*,*7*,*8*).

Nrf2 may protect human keratinocytes against UVA exposure and enhanced protection of cells by Nrf2 may prevent radiation damage caused by sunbathing/tanning. The current study investigated the cell response to different doses of UVA irradiation. Earlier studies found that a moderate dose (250 kJ•m^{-2}) of UVA irradiation did not activate Nrf2 in HaCaT human skin keratinocytes while a high dose (> 500 kJ•m^{-2}) of UVA irradiation caused significant damage to these cells (*13*). Therefore, a moderate to high dose (400 kJ•m^{-2}) of UVA irradiation was used to examine Nrf2 accumulation following irradiation. Further, the effect of Nrf2 silencing on cell damage by this dose of UVA irradiation was investigated in HaCaT human skin keratinocytes.

2. Materials and Methods

2.1. *Cell culture and antibodies*

Human immortalized skin keratinocytes (HaCaT cells) (*14*) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). Antibodies against Nrf2 (H300, sc-13032), and β-actin (sc-9104) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-vinculin was purchased from Upstate Biotechnology (Waltham, MA, USA). Anti-mouse and anti-rabbit IgG secondary antibodies were from Sigma-Aldrich China (Shanghai, China) (*10*,*14*). A lactate dehydrogenase (LDH) cytotoxicity assay kit was obtained from Roche Diagnostics (Mannheim, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for a cell viability assay was purchased from Sigma-Aldrich.

2.2. *Irradiation of cells with UVA*

Cells were irradiated with a broad-spectrum UV lamp (ultraviolet B (UVB was blocked with an appropriate filter) with a peak wavelength at 365 nm (MUA-165, Beijing Normal University, Beijing, China). The lamp exposure time was calculated using a radiometer (Beijing Normal University). The irradiation time normally took less than one hour. Prior to UVA irradiation, the growth medium was removed from the cells and retained; each dish was washed twice with phosphate-buffered saline (PBS) and cells were irradiated in PBS. In order to maintain a consistent temperature of 25°C, irradiation of cells with UVA was performed in an air-conditioned room as previously described (*10*,*14*). Non-irradiated cells were covered with foil to prevent exposure and used as a background control (sham = 0 kJ•m^{-2}). After UVA irradiation, PBS was removed and cells were re-incubated in retained conditional medium for a period of time (normally 4-8 h).

2.3. *RNA Interference*

Small interfering RNAs were used to knock down Nrf2 protein levels. All small interfering RNAs (siRNAs) and scrambled control siRNAs (Sb) were obtained from Ambion siRNAs (Ambion, Austin, TX, USA): Sb: Silencer Negative Control siRNA (AM4611), siNrf2: NM_006164. #1: ID 115764, #2: ID 115763.

For siRNA transfection, sub-confluent cells were detached and transfected with siRNAs using siPORT™ NeoFX™ Transfection Agent (AM4511, Ambion). For HaCaT cells, siRNA was diluted in 50 μL OPT medium (A) while 3 μL NeoFX was diluted in 50 μL OPT medium (B); the respective solutions were incubated for 10 min and then A and B were mixed well and incubated further for 10 min to allow the formation of siRNA complex (100 μL). All steps were carried at room temperature (RT). The siRNA complex was then plated into 6-well plates. Then 1×10^5 cells within 1.5 mL normal cell growth medium were added to reach a total volume of 1.6 mL, and cells were then incubated at 37°C and 95% humidity with 5% CO_2. The following day, a half volume (0.8 mL) of fresh medium was added and cells were incubated for a total of 48 h before use (*10*,*14*).

2.4. *Western blotting*

After cells were incubated for a period of time (normally 8-12 h) following UVA irradiation, cell lysates were collected as described before (*14*). Equal amounts of proteins were mixed with loading buffer and subjected to electrophoresis using 8% (w/v) SDS-polyacrylamide gels. Separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech, Little Chalfont, UK) and non-

specific bindings were blocked with 5% (w/v) non-fat dried-milk dissolved in tris-buffered saline with Tween (TBS-T) buffer. The membranes were then incubated for 1 h at RT with Nrf2 and actin antibodies, respectively. Subsequently, the membranes were incubated with related horseradish peroxidase conjugated secondary antibodies for 1 h at RT. Protein bands were visualized on X-ray film activated by chemiluminescence using an ECL Western blotting system (Amersham Pharmacia Biotech). The intensity of bands was quantified by digital densitometry using NIH Image J 1.33 software. Data were normalized to actin and expressed as the percentage or fold change compared to the corresponding control, which was set at 1 (*10*,*14*).

2.5. *Immunofluorescence*

HaCaT cells were cultured on glass coverslips. Four hours after irradiation, cells were washed twice with PBS, fixed with 4% (w/v) paraformaldehyde for 10 min at RT, permeabilized with ice-cold methanol for another 10 min, and then washed twice with PBS. They were then blocked with Image-iT™ Fx signal enhancer (Alexa-Fluor system, Invitrogen, Carlsbad, CA, USA) and incubated at RT for 1 h with the primary antibody Nrf2 (1:200) or vinculin (1:50) and washed twice in PBS for 30 min; afterwards, cells were incubated with secondary antibody Alexa Fluor 488-conjugated goat anti-rabbit or anti-mouse IgG or (1:1,000, Invitrogen) for 1 h, washed again in PBS, and mounted onto glass microscope slides using VECTASHIELD® Hard_Set™ Mounting Medium. Cells were analyzed under a Nikon Eclipse TE2000-U epifluorescence microscope. Images were recorded using the software program UltraVIEW (*10*,*14*).

2.6. *LDH assay*

The percentage of extracellular LDH leakage served as an indicator of membrane damage. LDH was determined using a cytotoxicity detection kit (Cat. No. 11644793001, Roche Diagnostics) following the instructions supplied. SiNrf2-treated cells (7,500) were seeded onto 96-well plates for 48 h, irradiated with UVA, and then re-incubated for 4 h. LDH release was then measured as described previously (*8*,*10*). The fraction of extracellular LDH was represented as the percentage of total LDH and expressed as the fold increase relative to the sham-irradiated scrambled siRNA-treated control, which was set at 1.

2.7. *Cell viability assay*

HaCaT cells were transfected with siRNA as described above and seeded onto 96-well plates (200 μL, 7,500 cells/well). Cells were grown for 48 h and then were exposed to 100-500 kJ•m^{-2} of UVA light. After irradiation, the cells were cultured in conditional culture medium again for a further 4 h. Each well then received 50 μL of MTT (0.5 mg/mL) in serum-free medium. The plates were incubated for 2.5 h at 37°C and MTT solution was discarded. Fifty μL of dimethyl sulfoxide was added to each well and the plates were shaken at 200 rpm on an orbital shaker for 5 min before colorimetric analysis. The absorbance at 570 nm was measured in a microplate reader and expressed as the percentage of surviving cells compared to the vehicle control group. The absorbance levels, an indicator of cell viability, were plotted and compared to the sham-irradiated scrambled siRNA-treated control, which was set at 100.

2.8. *Statistical analyses*

Statistical analyses were carried out using a two-tailed *t* test and *p* values below 0.05 were considered statistically significant. The values in the graphs correspond to the mean and the error bars indicate standard error (S.E.).

3. Results

3.1. *Resistance of human skin keratinocytes to UVA-induced morphological changes*

Human primary skin keratinocytes are more resistant to UVA-induced damage, as gauged by membrane damage and morphological changes, than matching fibroblasts from the same biopsy (*12*). To investigate the effect of various doses of UVA irradiation on cell morphology, the HaCaT cells were examined by vinculin staining for focal adhesion following the various doses of UVA irradiation.

HaCaT cells were sham-irradiated and served as the positive control. Staining with vinculin showed that, following a low (100 kJ•m^{-2}) to moderate dose (250 kJ•m^{-2}) of UVA irradiation, cell morphology was maintained without changes and that dot-like focal adhesion of molecules did not alter (Figure 1). The overall cell shape was maintained following the higher dose (500 kJ•m^{-2}) of UVA irradiation, absent some specific and non-specific staining. This indicates that a moderate dose of UVA irradiation did not significantly alter the cell morphology or the staining pattern of focal adhesion molecules, *e.g.* vinculin, while a higher dose of UVA irradiation did alter cell morphology and the focal adhesion molecule vinculin in HaCaT keratinocytes. This result is consistent with previous reports that a moderate dose of UVA irradiation did not significantly alter the morphology of primary skin keratinocytes (*12*).

3.2. *UVA irradiation did not significantly increase Nrf2 protein in skin keratinocytes*

Low and moderate doses (100 and 250 kJ•m^{-2}) of

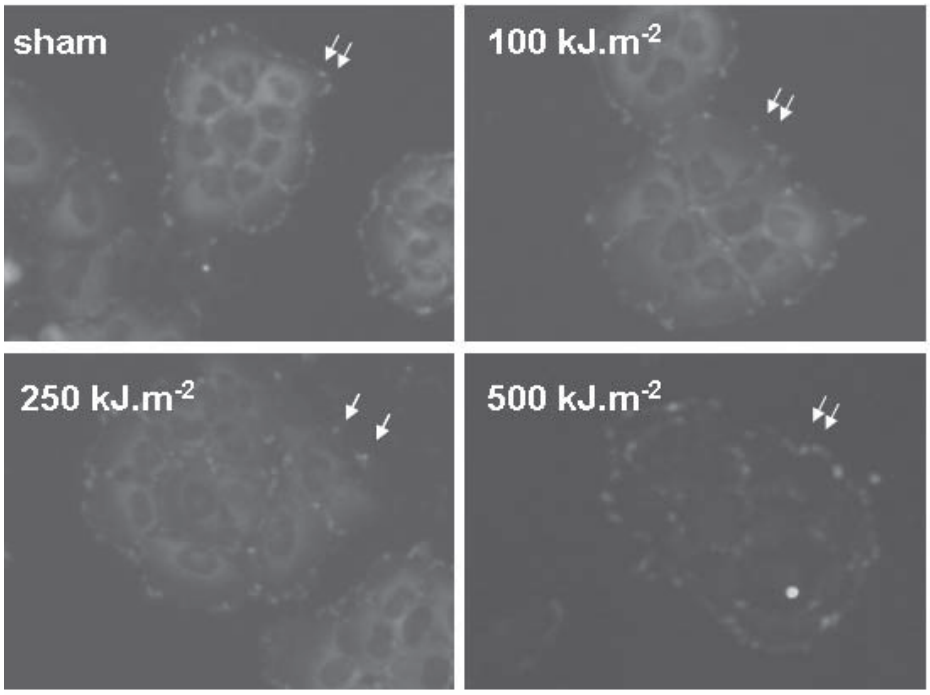

Figure 1. Immunocytochemical analysis of morphology and vinculin pattern following UVA irradiation of HaCaT cells. Cells were non-irradiated (sham) or irradiated with the doses indicated and then fixed and subjected to vinculin immunostaining for 4 h following UVA irradiation, as described in the Materials and Methods.

UVA irradiation are strong inducers of Nrf2 in FEK4 human skin fibroblasts but weak inducers of Nrf2 in human skin keratinocytes (*10*,*14*,*15*). To investigate if a moderate to high dose (400 kJ•m^{-2}) of irradiation altered Nrf2 localization, HaCaT was exposed to this dose of irradiation. Results revealed a less than 2-fold increase in Nrf2 protein levels in HaCaT cells 4 and 8 h after UVA irradiation (Figure 2A). Immunostaining analysis showed that Nrf2 protein was maintained in the nucleus of non-irradiated control cells, and it accumulated slightly in cells 4 and 8 h after this dose of irradiation. This result indicates that despite its activation in skin fibroblasts (*10*,*14*) Nrf2 was not significantly activated in human skin keratinocytes following UVA irradiation.

3.3. *Reduction of Nrf2 increased cell damage induced by 400 kJ•m^{-2} of UVA in HaCaT cells*

A study demonstrated that a moderate dose (250 kJ•m^{-2}) of UVA irradiation significantly induced the expression of Nrf2 in skin fibroblasts and that Nrf2 protected the cells against this dose of UVA irradiation-mediated membrane damage, which was measured with an LDH assay (*10*). Nrf2 was expected to protect HaCaT skin keratinocytes, though UVA irradiation caused only a slight increase in Nrf2 protein levels and nuclear accumulation in these cells (Figure 2).

To demonstrate the importance of Nrf2 in UVA irradiation-mediated cell damage, the Nrf2 gene was knocked down in HaCaT cells using siRNA. Knock down of Nrf2 protein levels was confirmed by Western blotting analysis, which revealed a significant decrease in Nrf2 protein levels following siRNA transfection (Figure 3).

Cell damage was demonstrated by the loss of the integrity of the membrane, as gauged by LDH release and viability (MTT assay). HaCaT Cells were pretreated with vehicle or scrambled siRNA and siNrf2 during plating and grown to 90% confluence. Cells were then either sham or UVA (400 kJ•m^{-2})-irradiated and further incubated in normal medium for 4 h. As shown in Figure 4, without UVA irradiation siRNA had a negligible effect on the leakage of LDH and viability of HaCaT cells, but 400 kJ•m^{-2} of UVA irradiation caused a 1.7-fold increase in LDH leakage, representing a significant increase (Figure 4A). This dose of UVA irradiation caused a 10% decrease in cell viability (Figure 4B), representing a significant reduction in viability. UVA-induced LDH leakage was further increased to 2.3- and 2.5-fold by pre-treatment with 10 and 50 nM of siNrf2, respectively ($p < 0.05$) (Figure 4A). UVA-induced loss of viability was further increased to 15% and 20% by pre-treatment with 10 and 50 nM of siNrf2, respectively ($p < 0.05$) (Figure 4B). A different exon targeting siNrf2 also provided similar results (data not shown). These results indicate that loss of Nrf2 significantly increased cell damage in human HaCaT keratinocytes irradiated with a moderate to high dose (400 kJ•m^{-2}) of UVA.

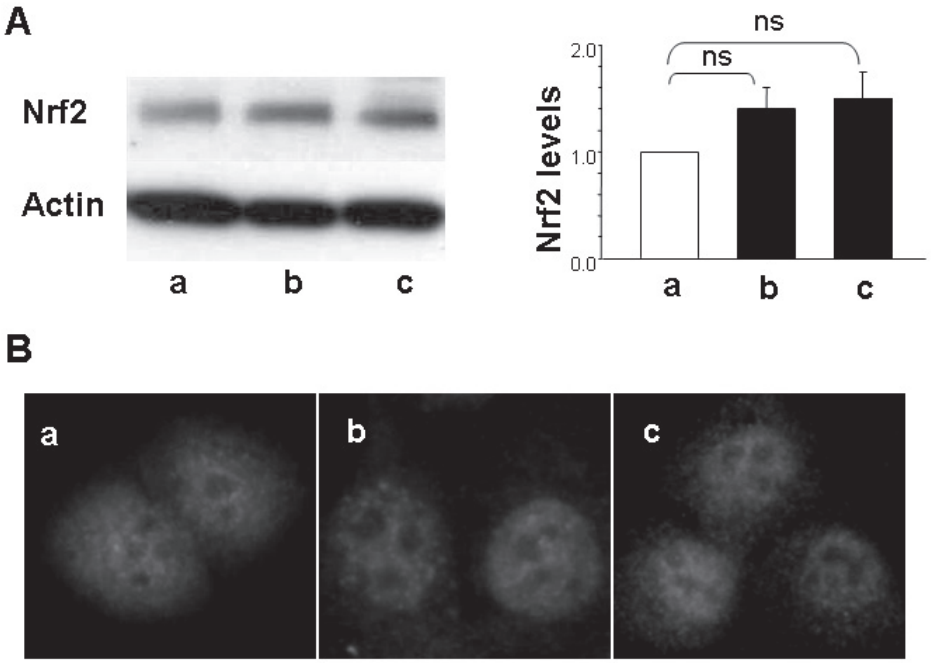

Figure 2. UVA irradiation slightly induced Nrf2 protein expression and nuclear accumulation in HaCaT. HaCaT cells were subjected to 400 kJ•m^{-2} UVA irradiation and re-incubated for the time indicated and then subjected to Western blotting **(A)** and immunofluorescence **(B)** analysis using Nrf2 antibody, as described in the Materials and Methods. The result shown is representative of 3 independent experiments. In the densitometric analysis of the Western blotting data, Nrf2 expression was normalized to actin and the sham-irradiated control was set to 1. Each bar represents the mean ± S.E.M., $n = 3$. $p > 0.05$. **a**, sham-irradiated; **b**, 4 h after UVA; **c**, 8 h after UVA.

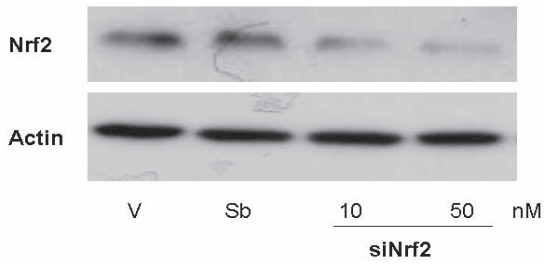

Figure 3. Silencing of Nrf2 reduced Nrf2 protein levels. HaCaT cells were transfected with vehicle control (V), 50 nM of negative scrambled control siRNA (Sb), and siNrf2 using siPORT NeoFX transfection reagent (Ambion), as described in the Materials and Methods. Cells were grown on 6-well plates for 48 h and collected and then Nrf2 levels were analyzed using Western blotting.

4. Discussion

Exposure to excessive UV irradiation in the form of sunlight adversely affects the skin, causing erythema, sunburn, pigmentation, hyperplasia, immunosuppression, skin aging, and cancer (*2*,*3*). In recent years, air pollution has caused ozone layer depletion, leading to increased exposure to UV. As a result, the incidence of skin ailments, such as skin aging and skin cancer, has dramatically increased worldwide (*1*,*3*). Therefore, a natural area of interest would be to identify protective molecules, including those in natural products, that can activate cellular defense, *i.e.*, *via* activation of Nrf2 to protect against UVA-induced oxidative stress.

Earlier work demonstrated that HaCaT keratinocytes are resistant to UVA-mediated membrane damage at lower doses of radiation (*13*,*14*). Study of cell morphology found that low and moderate doses (100 and 250 kJ•m^{-2}) of UVA irradiation did not alter cell focal adhesions and overall cell morphology, which is consistent with results of an earlier study indicating that human primary keratinocytes are generally resistant to UVA-mediated damage as gauged by LDH and cell morphology (*12*).

Nrf2 is ubiquitously expressed in a wide range of tissues and cell types, including keratinocytes (*14*). Nrf2 is involved in wound healing in mouse skin and Nrf2-deficient mice have an impaired oxidative stress defense (*8*). Previous studies have shown that UVA causes nuclear accumulation of Nrf2 in murine fibroblasts (*9*). Nrf2 is activated by UVA irradiation, and silencing of Nrf2 significantly increases UVA-mediated membrane damage in human primary skin fibroblasts (*10*). However, a recent study by the current authors showed that moderate dose of UVA irradiation did not significantly increase Nrf2 protein levels in HaCaT skin cells (*14*). UVA irradiation also has no effect on Nrf2 expression levels in murine skin keratinocytes, though electrophilic chemicals activate Nrf2 and induce Nrf2-mediated gene expression (*15*).

Nrf2 may play an important role in protecting skin

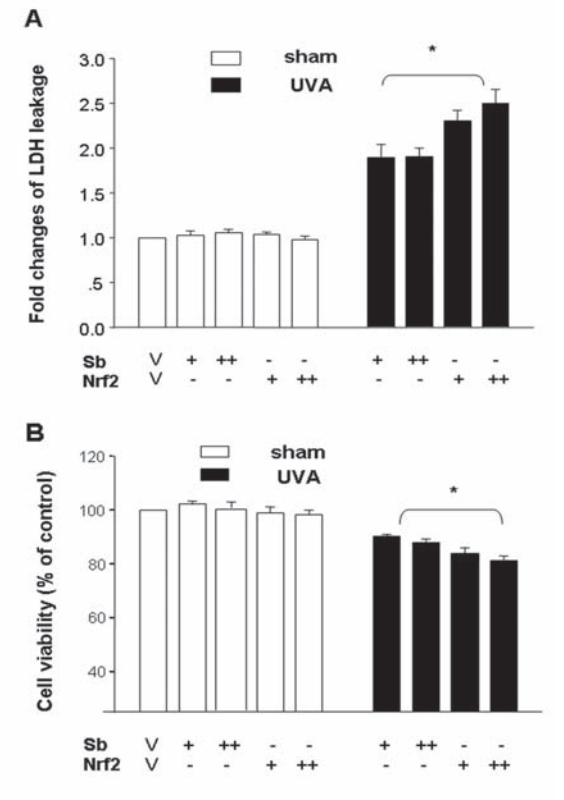

Figure 4. Silencing of Nrf2 increased UVA irradiation-mediated damage. HaCaT cells were transfected as described in Figure 3 and in the Materials and Methods. Cells grown on 96-well plates were sham or UVA-irradiated and re-incubated and assayed for 4 h following irradiation for LDH **(A)** and MTT **(B)**. The control value was set at 1 and 100 for A and B, respectively. Values are means ± S.E. (n = 4). *, $p < 0.05$ *vs.* relevant sample. V, vehicle control; Sb, scrambled control; +, 10 nM siRNA; ++, 50 nM siRNA.

cells against oxidative stress, which includes UVA irradiation-induced damage (*10*,*11*). A moderate to high dose (400 kJ•m^{-2}) of UVA irradiation slightly increased Nrf2 protein levels, although not significantly, and immunostaining also revealed that Nrf2 accumulated only slightly in the nucleus following this dose of UVA irradiation, the same as happens at 250 kJ•m^{-2} (*14*) and 500 kJ•m^{-2} (data not shown). The current results are supported by those of Durchdewald's study that observed no activation of Nrf2 following a low to moderate dose of UVA radiation (*15*). Further, cells sensitized to Nrf2 suffered more UVA irradiation-mediated cell damage, as demonstrated by a decrease in cell viability and increase in the release of LDH. This supports the notion that Nrf2 plays a role in protecting both skin keratinocytes and fibroblasts from exposure to UVA irradiation (*10*,*11*).

Skin is a major target of exposure to UVA irradiation and the chronic toxic and carcinogenic effects of different substances. Oxidative damage plays a role in many age-related chronic degenerative diseases like aging of the skin, which is induced by UVA irradiation, so further understanding of the protection offered by Nrf2 expression may provide clues to new modalities for *in vivo* skin protection and prevention of skin disorders (*7*). A decline in Nrf2 function also sensitizes cells to oxidative stress during aging; increased Nrf2 levels are noted in the cells of long-lived mice and high Nrf2 levels protect these cells against oxidant-induced damage (*7*,*16-20*). Liu demonstrated that Nrf2 levels increased by the suppression of Keap1 can therefore protect human keratinocytes against UVA-induced damage (*21*). Although the mechanisms underlying Nrf2 protection of skin cells must be evaluated further, there is evidence that it may protect skin keratinocytes from damage to the cell membrane caused by UVA irradiation by maintaining membrane integrity and enhancing cell survival (*12*).

In summary, the current study has provided the evidence that Nrf2 protects skin keratinocytes from damage caused by UVA irradiation. Since epidermal keratinocytes are constantly exposed to UVA irradiation, activation of Nrf2 may prove to be critical to the effective protection of human skin cells. Increased Nrf2 levels may be a desirable action of skin care supplements or sun protection products.

Acknowledgements

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