Reactive Oxygen Species in HaCaT Keratinocytes After UVB Irradiation Are Triggered by Intracellular Ca^{2+} Levels

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It is recognized that reactive oxygen species (ROS) are responsible for skin damage due to UVB-radiation (UVB-R). However, the triggering substance(s) for ROS generation after UVB-R is uncertain with respect to the activation of NADPH oxidase (Nox), xanthine oxidase (XOD), and respiratory chain–chain reactions in mitochondria. As a first step in identifying the trigger(s) for UVB-induced ROS generation, we examined the relationship between Ca^{2+} levels and ROS generation in HaCaT keratinocytes. UVB-R exposure of HaCaT keratinocytes resulted in an immediate elevation of ROS that recurred 7 hours later. This was accompanied by immediately elevated intracellular Ca^{2+} . A Ca^{2+} chelating agent, BAPTA, abolished the elevation of ROS after UVB-R completely. In addition, exogenous H_2O_2 did not increase intracellular Ca^{2+} levels. This suggests that intracellular Ca^{2+} is the first trigger for UVB-induced ROS generation.

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INTRODUCTION

UVB-R has high energy, which damages skin cells and contributes strongly to skin tumor generation. In addition, UVB-R causes alterations in skin appearance, such as pigmented spots and wrinkles, and it accelerates skin aging (which is defined as photoaging). UVB-R damages DNA molecules through photo-chemical reactions, and many studies performed in the past two decades have demonstrated the importance of reactive oxygen species (ROS) to the acceleration of skin aging. However, there are no reports to date that elucidate how ROS generated after UVB-R are triggered. To prevent skin cell damage caused by ROS, characterizing its first trigger and its time-dependency process should be an important weapon. This study was conducted as a first approach to understanding the process by which skin cell damage is caused by ROS.

RESULTS

UVB-induced ROS in HaCaT keratinocytes are generated in a distinct manner over time

HaCaT keratinocytes showed a significant elevation in ROS immediately after UVB-R exposure at a dose of 25 mJ cm^{-2} (Figure 1a and b). In addition, ROS generation immediately after exposure exhibited dose-dependency (data not shown). The elevation in ROS then gradually declined up to 5 hours later, after which further elevation was observed at 7 hours (Figure 1). This double-phasic curve indicates the existence of different stimuli for UVB-induced ROS generation.

UVB-R increases intracellular Ca^{2+} levels in HaCaT keratinocytes and ROS elevation caused by UVB-R is abolished by a Ca^{2+}-chelating agent

UVB-R caused an immediate elevation in intracellular Ca^{2+}, which was dose-dependent (Figure 2a). The elevation of Ca^{2+} correlated closely with ROS generation. To address the contribution of Ca^{2+} to ROS generation, we examined the effect of BAPTA, a specific chelating agent for Ca^{2+}, (Chiancone *et al.*, 1986) on ROS generation immediately after UVB-R exposure. BAPTA (25 μM) effectively suppressed ROS elevation (Figure 2b). In addition, a Ca^{2+} ionophor, A23187, elevated ROS levels in HaCaT keratinocytes, which was associated with the increase in intracellular Ca^{2+} levels (data not shown). These results support the concept that changes in intracellular Ca^{2+} concentration contribute to UVB-induced generation of ROS.

H_2O_2 and exogenous ROS do not increase intracellular Ca^{2+} concentrations in HaCaT keratinocytes

To help confirm the contribution of Ca^{2+} to UVB-induced generation of ROS, we examined the effect of exogenous ROS on intracellular Ca^{2+} concentrations. H_2O_2 did not have any effect on intracellular Ca^{2+} levels (Figure 3). These results indicate that elevated intracellular Ca^{2+} concentration in response to UVB-R occurs in advance of ROS generation.

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Abbreviations: ROS, reactive oxygen species; UVB-R, UVB-radiation

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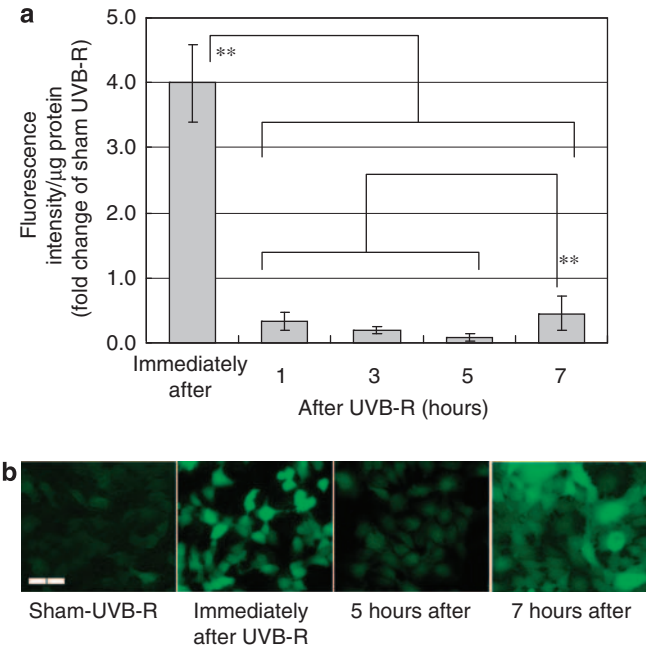

Figure 1. **Time-dependent profile of intracellular ROS levels in HaCaT keratinocytes after UVB-R.** 20 μM H_2DCFDA-loaded cells were exposed to UVB-R at a dose of 25 mJ cm^{-2}. Immediately after exposure, intracellular ROS levels were measured. At 1, 3, 5 and 7 hours after exposure, intracellular ROS levels of the cells were measured again. Exposed cells were loaded with 20 μM H_2DCFDA for 30 minutes and were incubated for 30 minutes. (**a**) The fluorescence intensity (Ex: 485 nm per Em: 530 nm) of each cell lysate was then measured. Intracellular ROS levels are expressed as fold change in fluorescence intensity per μg protein between UVB-R cells and sham-irradiated cells. Significance, $^{**}P<0.01$. (**b**) Photographs show representative fluorescence images of intracellular ROS levels at each point after UVB-R. Scale bar, 20 μm.

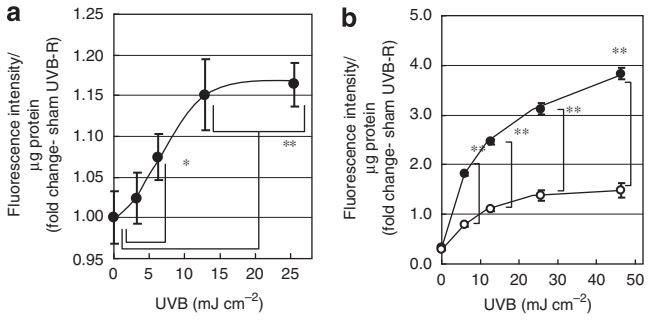

Figure 2. **Changes in intracellular Ca^{2+} levels in HaCaT keratinocytes after UVB-R and the effects of BAPTA on intracellular Ca^{2+}.** (**a**) After pre-loading with 3 μM Fura-2AM for 30 minutes, cells were exposed to various doses of UVB-R. Immediately after exposure, cells were lysed and measured for fluorescence intensity (Ex: 340 nm per Em: 510 nm) per μg protein. Intracellular Ca^{2+} concentrations are expressed as fold change in fluorescence intensities between UVB-R cells and sham-irradiated cells. (**b**) Cells were treated with or without 25 μM BAPTA-AM for 30 minutes, and then loaded with 20 μM H_2DCFDA. Immediately after irradiation at various doses of UVB-R, cells were lysed and the fluorescence intensity (Ex: 485 nm per Em: 530 nm) per μg protein was measured. Intracellular ROS levels are expressed as fold change in fluorescence intensities between exposed cells and sham-irradiated cells. Control: —●—, BAPTA: —○—, Significance; $^{*}P<0.05$, $^{**}P<0.01$.

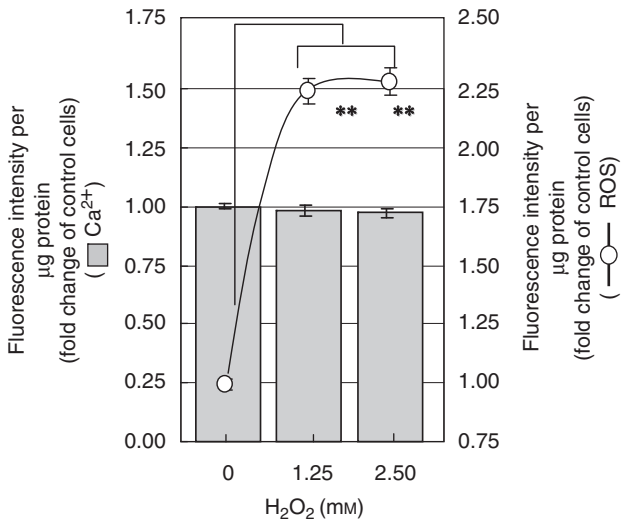

Figure 3. **Influence of exogenous H_2O_2 on intracellular Ca^{2+} levels/ROS in HaCaT keratinocytes.** Cells were loaded with 3 μM Fura-2AM or 20 μM H_2DCFDA for 30 minutes and were then exposed to H_2O_2 (at 1.25 or 2.50 mM). At 10 minutes after exposure, cells were lysed and the fluorescence intensity (Ex: 340 nm per Em: 510 nm, f) per μg protein was measured. Intracellular Ca^{2+} levels are expressed as fold changes in fluorescence intensities between exposed cells and sham-irradiated cells. Intracellular ROS levels are expressed as fold changes in fluorescence intensities between exposed cells and sham-irradiated cells. Significance; $^{**}P<0.01$.

DISCUSSION

It has been established that Ca^{2+} functions as a critical signal for various physiological responses, such as inflammation (Wang *et al.*, 2000). In skin, intracellular Ca^{2+} modulates the terminal differentiation of keratinocytes. In addition, recent studies have reported that Ca^{2+} regulates skin barrier recovery after disruption (Denda *et al.*, 2002). These reports indicate that Ca^{2+} plays critical roles in physiological responses of the epidermis.

On the other hand, UV radiation has adverse effects on the skin, such as the formation of skin tumors and premature skin aging (Emerit, 1992; Moloney *et al.*, 1992). In particular, UVB-R frequently damages skin due to its high energy compared with UVA-R. UVB initiates various alterations in the skin through DNA damage in the form of cyclobutane pyrimidine dimers, 6-4 photoadducts and in the generation of ROS (Wolf *et al.*, 1993; Kim *et al.*, 1995; Masaki *et al.*, 1995). One recent report demonstrated that the generation of ROS from UVA-R is due to NADPH oxidase, which is activated through elevations in intracellular Ca^{2+} concentrations (Valencia and Kochevar, 2008).

In our study, we found that UVB-R increases intracellular ROS levels immediately after irradiation and also 7 hours later. Because BAPTA, a specific Ca^{2+}-chelating agent completely abolished the elevation of ROS that occurs immediately after UVB-R exposure and because exposure to H_2O_2 had no effect on intracellular Ca^{2+}, we conclude that UVB-induced generation of ROS immediately after exposure is triggered by Ca^{2+}. In addition, we hypothesize that the elevation in ROS 7 hours after UVB-R is also

regulated by Ca^{2+}. Several pro-inflammatory cytokines, such as IL-1α and TNF-α, are stored in cells as inactive forms. For instance, IL-1α is converted by calpain, which requires Ca^{2+} for its proteolytic function (Kobayashi *et al.*, 1990). Because it has been reported that IL-1α induces ROS generation (Radeke *et al.*, 1990), we hypothesize the existence of a Ca^{2+}-triggering cascade for the UVB-R-induced generation of ROS.

MATERIALS AND METHODS

Materials

BAPTA-AM (1,2-bis-(*O*-aminophenoxy)-ethane-*N*,*N*,N0,N0-tetra-acetic acid, tetraacetoxymethyl ester), H_2DCFDA (2,7-dichlorodihydrofluorescein diacetate), and Fura-2AM were purchased from Molecular Probes (Eugene, OR). The BCA Protein Assay Reagent kit was purchased from Pierce Chemical Co. (Rockford, IL). Bovine serum albumin was from Sigma (St Louis, MO), DMEM was obtained from Nikken Bio Medical Laboratory (Kyoto, Japan) and fetal bovine serum was obtained from Invitrogen (Carlsbad, CA).

Cell culture

HaCaT keratinocytes, which were a kind gift from Professor Akamatsu (Fujita Health University School of Medicine), were cultured in DMEM with 10% fetal bovine serum.

UVB source and irradiation

The UVB source used was a Toshiba fluorescent sunlamp (FL-20SE, Tokyo, Japan), which has an emission spectrum from 280 to 370 nm, peaking at 305 nm. The UVB irradiance was measured using a UV light meter UV-340 (Lutron, Coopersburg, PA).

Measurement of intracellular ROS

Two assays were used to measure ROS. (1) Cells pre-loaded with 20 μM H_2DCFDA for 30 minutes were exposed to UVB or H_2O_2, and then were lysed with 0.1% Triton X-100. The fluorescence intensity of each lysate was measured with excitation of 485 nm and emission at 530 nm. Intracellular ROS are expressed as fold change of the fluorescence intensity normalized by μg protein against control cells. (2) Cells pre-loaded with 20 μM H_2DCFDA for 30 minutes were exposed to UVB or H_2O_2. Cells were then observed immediately under a fluorescent microscope (Venox AHBT3/Q imaging system, Olympus, Tokyo, Japan) equipped with a No. 15 filter.

Measurement of intracellular Ca^{2+} concentration

Cells loaded with 3 μM Fura-2AM for 30 minutes were exposed to UVB or H_2O_2 and then were lysed with 0.1% Triton X-100. Fluorescence intensity of each lysate was measured with excitation of 340 nm and emission at 510 nm. Intracellular Ca^{2+} is expressed as fold change of fluorescence intensity normalized by μg protein against control cells.

Protein assay

Protein contents were measured using a BCA Protein Assay Reagent kit using bovine serum albumin as the protein concentration standard.

Statistical analysis

Statistical analyses were performed using a two-tail paired *t*-test. Differences are considered significant if $P<0.05$ (indicated by $*P<0.05$, $**P<0.01$).

CONFLICT OF INTEREST

The authors state no conflict of interest.

REFERENCES

Chiancone E, Thulin E, Boffi A, Forsén S, Brunori M (1986) Evidence for the interaction between the calcium indicator 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid and calcium-binding proteins. *J Biol Chem* 261:16306–8

Denda M, Inoue K, Fuziwara S, Denda S (2002) P2X purinergic receptor antagonist accelerates skin barrier repair and prevents epidermal hyperplasia induced by skin barrier disruption. *J Invest Dermatol* 119:1034–40

Emerit I (1992) Free radicals and aging of the skin. *EXS* 62:328–41

Kim JK, Patel D, Choi BS (1995) Contrasting structural impacts induced by cis-syn cyclobutane dimer and (6-4) adduct in DNA duplex decamers: implication in mutagenesis and repair activity. *Photochem Photobiol* 62:44–50

Kobayashi Y, Yamamoto K, Saido T, Kawasaki H, Oppenheim JJ, Matsushima K (1990) Identification of calcium-activated neutral protease as a processing enzyme of human interleukin 1 alpha. *Proc Natl Acad Sci USA* 87:5548–52

Masaki H, Atsumi T, Sakurai H (1995) Detection of hydrogen peroxide and hydroxyl radicals in murine skin fibroblasts under UVB irradiation. *Biochem Biophys Res Commun* 206:474–9

Moloney SJ, Edmonds SH, Giddens LD, Learn DB (1992) The hairless mouse model of photoaging: evaluation of the relationship between dermal elastin, collagen, skin thickness and wrinkles. *Photochem Photobiol* 56:505–11

Radeke HH, Meier B, Topley N, Flöge J, Habermehl GG, Resch K (1990) Interleukin 1-alpha and tumor necrosis factor-alpha induce oxygen radical production in mesangial cells. *Kidney Int* 37:767–75

Valencia A, Kochevar IE (2008) Nox1-based NADPH oxidase is the major source of UVA-induced reactive oxygen species in human keratinocytes. *J Invest Dermatol* 128:214–22

Wang D, McGiff JC, Ferreri NR (2000) Regulation of cyclooxygenase isoforms in the renal thick ascending limb: effects of extracellular calcium. *J Physiol Pharmacol* 51:587–95

Wolf P, Yarosh DB, Kripke ML (1993) Effects of sunscreens and a DNA excision repair enzyme on ultraviolet radiation-induced inflammation, immune suppression, and cyclobutane pyrimidine dimer formation in mice. *J Invest Dermatol* 101:523–7