J. Cosmet. Sci., 64, 207–217 (May/June 2013)

Kinetics of ultraviolet B irradiation-mediated reactive oxygen species generation in human keratinocytes

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Accepted for publication September 6, 2012.

Synopsis

Ultraviolet B (UVB)-mediated oxidative stress in keratinocytes has been accepted as an important factor contributing to skin damage. The present study revealed the kinetics of reactive oxygen species (ROS) production and identified the main specific ROS generated in human keratinocytes exposed to UVB. Keratinocytes were exposed to various doses of UVB, and intracellular ROS kinetics were evaluated by specific oxidant probes, namely, 2',7'-dichlorofluorescein diacetate, dihydroethidium, 10-acetyl-3,7-dihydroxyphenoxazine, and 3'-(p-hydroxyphenyl) fluorescein. Results revealed that UVB-irradiated cells exhibited significantly higher rate of ROS production in the early time period (0–2 h) compared to the nontreated control cells; however, the rate of ROS generation afterward (2–6 h) was similar to that of control cells. Specific ROS, including superoxide anion, hydrogen peroxide, and hydroxyl radical were enhanced in keratinocytes treated with UVB. Results regarding kinetics of specific ROS production revealed that superoxide anion and hydroxyl radical were the main ROS contributing to oxidative stress in the early phase (0–2 h) after UVB treatment in these cells. Further, this study showed the effect of known antioxidant, vitamin C, on ROS kinetics in UVB-exposed keratinocytes.

INTRODUCTION

Damaging of cellular components, including keratinocytes and fibroblasts, results in skin complications such as aging and cancer (1,2). Keratinocyte, a principle cellular component of the outmost layer of skin, has been shown to be the most affected cells to the ultraviolet (UV) radiation (3,4). Indeed, reactive oxygen species (ROS) like superoxide anion ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) are continuously generated in human cells along with cellular energy production via mitochondrial electron transport reaction (5–7). Electrons leaking out of transport chain react with oxygen to form superoxide anion (8,9). Then, superoxide anion is transformed by a catalytic activity of superoxide dismutase enzyme to hydrogen peroxide and in the presence of metal, hydrogen peroxide is converted to hydroxyl radical via the Fenton reaction (8,10). Also, glutathione peroxidase and catalase are able to detoxify hydrogen peroxide to nonreactive water. Basically, approximately 1–3% of oxygen consumed by the cells will be converted to superoxide

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anion. However, in response to several stimuli including UV irradiation, increased cellular ROS production frequently occurs (11). An increased cellular ROS overwhelms the cellular antioxidant mechanisms; and increases ROS interaction with cellular DNA, proteins, and lipids; and causes significant impact on cell signaling, cell function, and survival (12,13).

There are three types of solar UV radiation, UVA (315–400 nm), UVB (280–315 nm), and UVC (wavelength < 280 nm). UVB is recognized as the most severely damaging to the epidermal layer of skin (14). In particular, UVB-mediated DNA damage through an oxidative stress-dependent mechanism in keratinocytes was shown in many studies (15,16). Although attempts have been made to evaluate the cytotoxic effects caused by UVB exposure, the information involving kinetics of ROS production, and in particular specific oxidative species induction after UVB exposure, has not been evaluated. To understand the kinetics and species of induced ROS may help to understand the mechanism of UVB-induced cell damage and related cellular responses. The data may facilitate the development of effective strategies in attenuating undesirable effects on UV-exposed skin and help in developing methods for evaluation of cosmeceutical products claiming oxidative stress and UV-relief approaches.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Human immortal keratinocyte (HaCaT) cells were obtained from the Cell Lines Service (Heidelberg, Germany). HaCaT cells were cultured in Dulbecco's Modified Eagle Medium Invitrogen (Carlsbad, CA) containing 10% fetal bovine serum, 2 mM L-glutamine, and 100 units /mL penicillin/streptomycin in a 5% CO_2 environment at 37 °C. Vitamin C and 2',7'-dichlorofluorescein diacetate ($DCFH_2$-DA) Sigma (St. Louis, MO). Dihydroethidium (DHE), 3'-(p-hydroxyphenyl) fluorescein (HPF), and 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) were obtained from Molecular Probes Inc. (Eugene, OR). Vitamin C was freshly prepared by diluting with phosphate buffered saline to obtain the desired concentrations.

UV IRRADIATION

A UVB irradiator IL-1700 International Light (Newburyport, MA) was used as a source of UVB, with the signal of UVB at the basement level of $1.750E^{-03}$ W/cm^2. HaCaT cells were plated into 96-well plates in growth medium for 6 h until cells were completely attached. After that, the growth medium was removed and immediately replaced with fresh serum-free medium. HaCaT cells were incubated in serum-free conditions overnight. Cells were then exposed to several doses of UVB from the range irradiation of 40–90 J/m^2. Nonirradiated cells were used as control.

ROS DETECTION

Intracellular ROS was determined by using a specific fluorescent probe for ROS detection, dichlorofluoresceindiacetate ($DCFH_2$-DA) (17,18); superoxide anion was determined by

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DHE (19–21); hydroxyl radical was determined by HPF (22); and hydrogen peroxide was determined by Amplex Red (23,24). After irradiation, the mixture of fresh serum-free and $DCFH_2$-DA, DHE, HPF, and Amplex Red were immediately added to the cell and further incubated for specified time intervals. Then, fluorescence intensity was determined using fluorescence microplate reader (Beckton Dickinson, Rutherford, NJ), using a 488-nm excitation beam and a 520-nm band-pass filter for detecting $DCFH_2$-DA-;, using a 490-nm excitation beam and a 515-nm band-pass filter for HPF; using a 488-nm excitation beam and a 610-nm band-pass filter for DHE; and using a 530–nm excitation beam and a 590–nm band-pass filter for Amplex Red. The cell morphology was also visualized under fluorescence microscope. Nonirradiated cells were used as control.

STATISTICAL ANALYSIS

Data were shown as means ± SD from at least three or more independent experiments. Statistical analysis was performed using an analysis of variance (one-way analysis of variance) and a post hoc test. A p value of less than 0.05 would be considered as statistically significant.

RESULTS

KINETICS OF INTRACELLULAR ROS PRODUCTION IN HUMAN KERATINOCYTE CELLS IN RESPONSE TO UVB IRRADIATION

Oxidative stress induced by UVB irradiation has been shown to be involved with many skin complications. However, the kinetics of ROS production in response to such radiation is poorly understood. To investigate the effect of UVB on the oxidative status of human keratinocytes, HaCaT cells were exposed to several doses of UVB (0–100 J/m^2), and time-dependent elevation of intracellular ROS (0–6 h) was performed using ROS-specific probe. Figure 1A shows a time-dependent accumulative ROS signal in the cells. Importantly, UVB irradiation caused a dramatic increase in the rate of ROS accumulation in a dose-dependent manner with approximately 1.75-fold ROS induction detected in the 90 J/m^2-treated cells compared to the nontreated control at 6 h. In response to UVB irritation, the induction of ROS signal was detected as early as 1 h after treatment. Such an increase of oxidative stress in keratinocytes was found to be in a dose-dependent manner up to 90 J/m^2 of UVB, whereas a further increase of UVB doses resulted in the reduction of ROS production (data not shown). Also, the ROS signal represented by an increase of intracellular DCF fluorescence signal is shown in fluorescence microphotographs in Figure 1B.

It was very interesting that the rate of ROS production in the UVB-treated cells appeared to be accelerated in the first 2-h period in comparison to that of control cells and then decreased to the level of basal ROS generation afterward (2–6 h). These kinetics data suggested that UVB-mediated intracellular oxidative stress in an early phase (~2 h) after an exposure to the UVB. We further evaluated the rate of ROS production in these cells in these two distinguished phases and found that the pattern of ROS induction rate was fit to the linear trend line with $R^2 > 0.97$ (Figures 2A and B). The rate constant (k) of each

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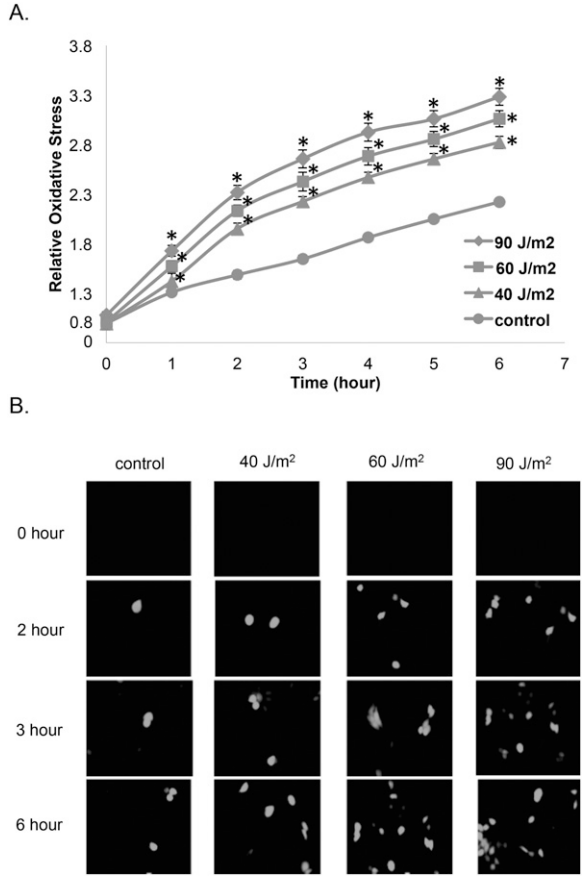


Figure 1. UVB-induced intracellular ROS generation in keratinocytes. (A) Cells were exposed to various doses of UVB (0–90 J/m^2) for 0–6 h, and intracellular ROS was detected by $DCFH_2$-DA probe using fluorescence microplate reader. Data point represent the mean ± standard deviation (SD) (n = 4), *$p < 0.05$ versus nontreated control cells. (B) Cellular ROS signal were determined by $DCFH_2$-DA fluorescence measurements.

UVB dose was then verified by the equation of $y = k_0t + y_0$. Figure 2A revealed that 90 J/m^2 of UVB exhibited the highest k value which was 0.6376, compared to that of 60, 40, and 0 J/m^2, which were 0.5484, 0.5054, and 0.2695, respectively. As the exposure time increased, the difference of ROS production rate in these cells seems to be minimal at the time of 2–6 h (Figure 2B). These data suggested that the oxidative stress in keratinocyte in response to UVB exposure was mainly caused by the induction of cellular ROS within 2 h after irritation.

THE SPECIFIC ROS GENERATED BY UVB EXPOSURE IN HUMAN KERATINOCYTE CELL

The present study further provided the information of specific ROS generated after UVB exposure using DHE, Amplex Red, and HPF as specific probes as described in Materials and Methods. Cells were seeded in a 96-well plate and incubated in a serum-free medium

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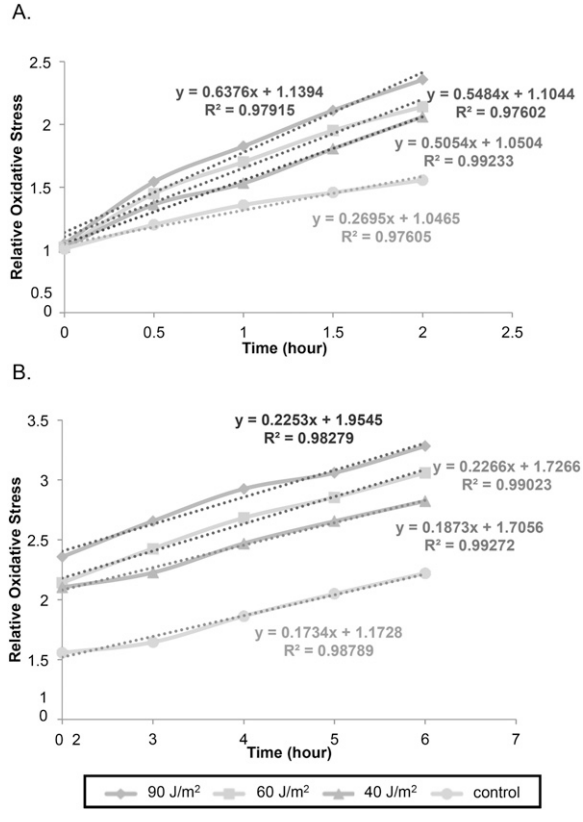


Figure 2. Kinetics of intracellular ROS production. (A) Kinetics of intracellular ROS in response to UVB (0–90 J/m^2) at 0–2 h after UVB irradiation. (B) Kinetics of intracellular ROS in response to UVB (0–90 J/m^2) at 2–6 h after UVB irradiation. Accumulative intracellular ROS content was evaluated by fluorescence microplate reader, $n = 4$.

overnight before being exposed to several doses of UVB. The specific probes were added after cells were exposed, and the results of fluorescence intensity were collected in the time-dependent manner. Figure 3 reveals that superoxide anion, hydrogen peroxide, and hydroxyl radical increased in response to UVB in time and dose-dependent manners. However, the rate of each ROS production was significantly distinguishable. Figure 3A shows that HPF signal, indicating hydroxyl radical level, was found to increase as early as 15 min after UVB irradiation and that the rate of hydroxyl radical increase was significantly higher in the UVB-treated cells relative to the control in the early phase (0–1 h). Also, DHE intensity representing superoxide anion ($^{\bullet}O_2^-$) level indicated that the dramatic increase of superoxide anion in response to UVB was observed in the early 2 h after UVB exposure (Figure 3B). However, Amplex Red data showed that H_2O_2 was gradually generated in the cells in a time-dependent manner, and the effect of UVB on H_2O_2 production could be detected only after 2 h of UVB exposure (Figure 3C).

It is worthy to note herein that superoxide anion and hydroxyl radical were found to be specific ROS affected by a UVB exposure in the early phase and that superoxide anion, which was the most affected with approximately 2.5-fold induction, could be detected in response to 90 J/m^2 of UVB. Thus, superoxide anion and hydroxyl radical could be the

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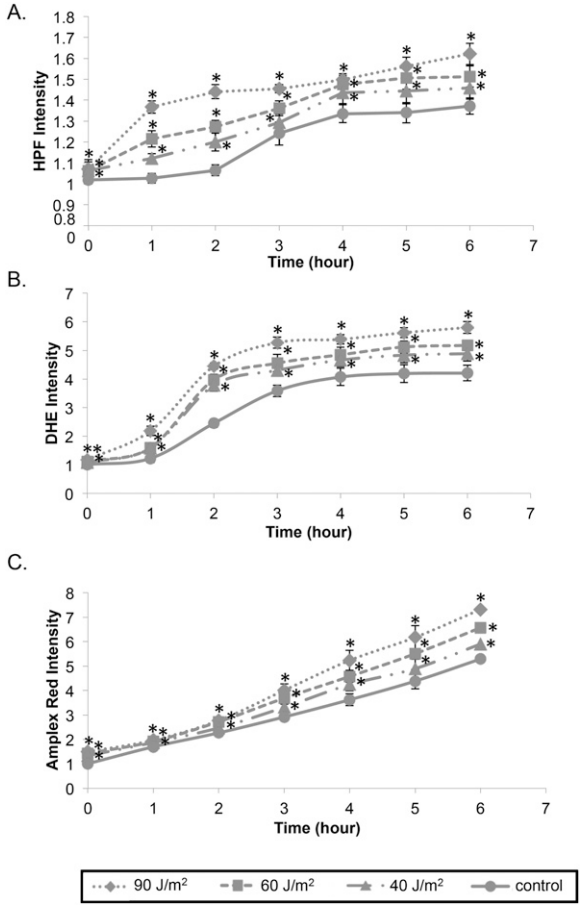


Figure 3. Identification of specific ROS generation in response to UVB irradiation. Cells were exposed to various doses of UVB, and accumulation of specific ROS was determined by specific ROS probes. (A) HPF probe was used for the detection of hydroxyl radical accumulation. (B) DHE fluorescence probe was used for the detection of superoxide anion level. (C) Amplex Red was used as a specific probe to detect hydrogen peroxide level. Data point represent the mean ± SD ($n = 4$), *$p < 0.05$ versus nontreated control cells.

principle specific ROS produced in keratinocytes and responsible for the oxidative stress mediated by UVB irradiation.

THE KINETIC OF SPECIFIC ROS INDUCED BY UVB

Using the data of 90 J/m^2 UVB, the plots representing each specific ROS generation were generated. Figure 4A shows the graph plotting signals of DHE, Amplex Red, and HPF in the keratinocytes exposure to UVB against time. The results revealed that DHE induction was fitted to the polynomial trend line with the k value = 0.3993 and R^2 = 0.9955. Amplex Red induction was aligned with the exponential equation to obtain the k value of 0.3109 and R^2 = 0.99344, whereas the rate of HPF increase fitted with linear equation exhibited the lowest k value = 0.1697 and R^2 = 0.98706. This information led to the understanding that specific ROS generated in response to UVB exposure in human

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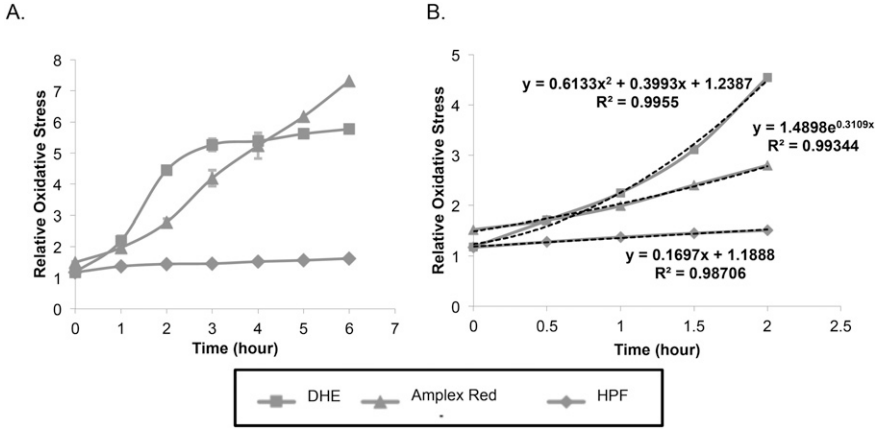


Figure 4. Kinetics of specific ROS generated in response to UVB irradiation. (A) Specific ROS generations in response to 90 J/m^2 UVB were determined by specific probed as described. (B) Kinetics of specific ROS accumulation at 0–2 h after UVB exposure was presented. Data point represent the mean ± SD ($n = 4$).

keratinocytes was found to be in different kinetics (Figure 4B). The scheme of UVB-mediated ROS and oxidative stress induction was presented in Figure 5 explaining that a UVB exposure was able to generate all three major ROS, which were superoxide anion, hydrogen peroxide, and hydroxyl radical. These oxygen species led to oxidative stress in the cells and caused skin damage, such as skin cancer, DNA damage, premature skin aging, and immune suppression.

THE EFFECT OF VITAMIN C ON UVB-MEDIATED ROS PROFILES

We further showed the effect of known antioxidant on UVB-generated ROS in keratinocytes. Human keratinocyte cells were incubated with vitamin C for 1 h before UVB irradiation (0, 40, 60, and 90 J/m^2). After a UVB exposure, intracellular ROS was detected by fluorescence microplate reader as described. Only the nontoxic concentrations of vitamin C (0–5 mM) were used, and Figure 6A indicates that vitamin C at 5 mM caused a significant change in ROS generation induced by UVB. The alteration of ROS generating rate could be detected at 1 h after a UVB exposure and thus suppressed the level of accumulated intracellular ROS. The results were confirmed by fluorescence microscope (Figure 6B). The $DCFH_2$-DA was used to detect the intracellular ROS in the cells. 90 J/m^2 of UVB caused an increase of intracellular DCF signal, and the pretreatment with 5 mM of vitamin C suppressed such ROS induction.

DISCUSSION

Increasing evidence has supported the significant damaging effects of UVB irradiation on human skin, including aging and cancer (25–28). The present study provided information regarding the ROS generating profile in UVB-exposed keratinocytes and reported herein for the first time that superoxide anion and hydroxyl radical are the main specific ROS produced and responsible to oxidative stress in response to UVB.

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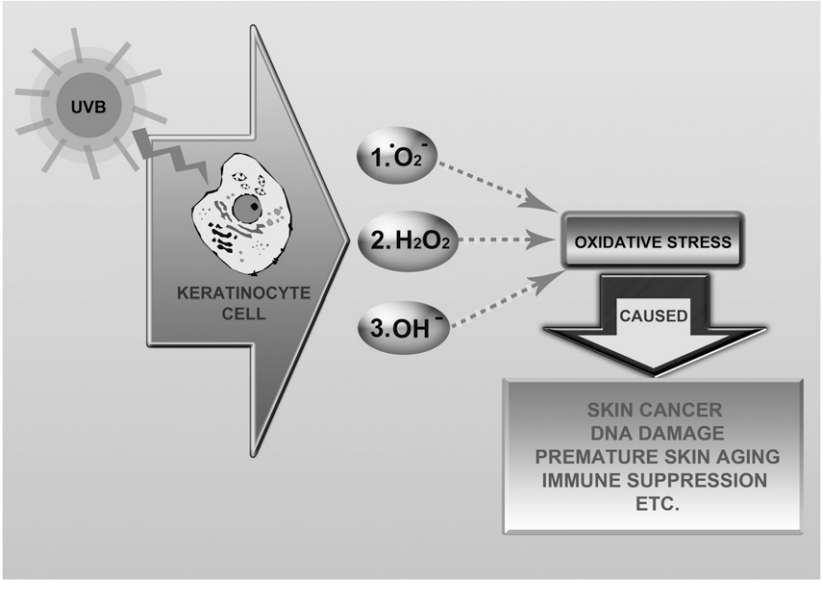


Figure 5. Schematic presentation of the UVB-mediated ROS generation in keratinocytes.

Oxidative stress caused by the excessive amounts of intracellular ROS is shown to be an important cause of damage to cellular components. Three main types of ROS were identified, and evidence indicated that superoxide anion ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) were generated continuously in the living cells (29,30). Superoxide anion after being generated could be converted to hydrogen peroxide by the function of superoxide dismutase enzyme. Consistent with this notion, our results revealed that hydrogen peroxide induction in UVB-exposured keratinocytes was found to follow the induction of superoxide anion. However, the increasing level of hydroxyl radical was detectable at the very early time after UVB irritation indicates the possibility that this free radical may be generated by the direct effect of UVB. Previous studies indicated that UVB-mediated damage of pyrimidine base of DNA is tightly associated with cellular oxidative stress (31–33). Also, UVB-induced DNA damage through hydroxyl radical formation was previously shown (34).

Although a UVB exposure caused significant ROS upregulation in keratinocytes up to 24 h (data not shown), we found significant induction of ROS accumulation only at 0–2 h after UVB exposure, compared to that of the nonirradiated control. These results suggest that oxidative stress generated in this study was mediated by the induction of specific ROS in the early time period. We found that superoxide anion and hydroxyl radical were increased in UVB-treated cells at a higher rate over that of control cells, whereas a rate of hydrogen peroxide increase is of minimal difference. Therefore, we have provided information that oxidative stress caused by UVB is generated during the 2-h period after exposure by cellular induction of superoxide anion and hydroxyl radical.

Various nonenzymatic dietary antioxidants can protect cells within the body from ROS-induced oxidative damage (35,36). Among them, vitamin C is a powerful antioxidant that was shown to reduce an oxidative damage induced by UVB in mouse keratinocytes *in vitro* (37). In the present study, vitamin C at the concentration of 5 mM could suppress the generation of ROS induced by 90 J/m^2 UVB irritation. The effect of vitamin C on ROS generation

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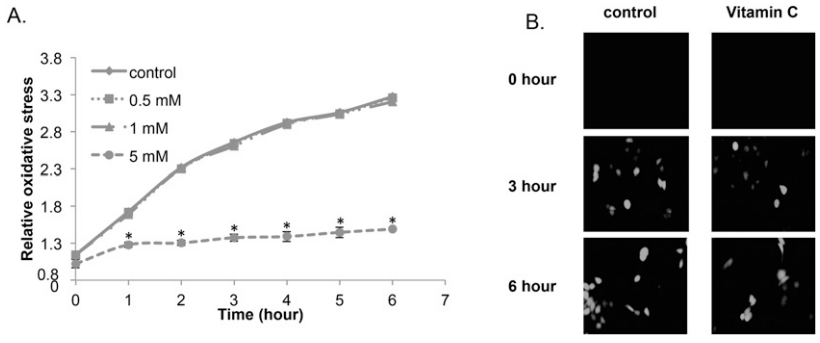


Figure 6. Effect of vitamin C on UVB-mediated oxidative stress in keratinocytes. Cells were treated with 0.5, 1, and 5 mM of vitamin C for 1 h before 90 J/m^2 UVB irradiation. (A) ROS level was determined by $DCFH_2$-DA probe using fluorescence microplate reader. Data point represent the mean ± SD ($n = 4$), *$p < 0.05$ versus UVB-treated cells. (B) Cellular ROS signal were determined by $DCFH_2$-DA fluorescence measurements.

in response to UVB could be detected as early as 15 min (data not shown), and the explanation of such prompt effect may be due to the reducing property of vitamin C. Vitamin C was shown to reduce and maintain function of glutathione in human keratinocytes (38).

Even though further investigations as well as *in vivo* studies are necessary to support these findings in human keratinocytes and results observed in this study may not necessarily translate into effects observed *in vivo* in human skin. The present study provided the initial evaluating method supplanting the use of animal for the evaluation of cosmetic and cosmeceutical constituents. Also, the knowledge gained from the present study might benefit the development of sunscreen products and help to design appropriate active ingredients that effectively block the dominant oxidative species induced by UVB.

CONCLUSIONS

In conclusion, we revealed herein the kinetic of ROS generation and identified specific ROS in UVB-exposed human skin keratinocytes. These data enhance the understanding on skin biology and benefit the development of strategies in protection of UVB-mediated damages.

ACKNOWLEDGMENT

This research was supported by Cell-based Drug and Health Products Development Research Unit, Chulalongkorn University and Agricultural Research Development Agency. The authors thank Mr. Krich Rajprasit for editing the manuscript.

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