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Abstract This study aims at exploring the oxidative stress in keratinocytes induced by UVB irradiation and the protective effect of nutritional antioxidants. Cultured Colo-16 cells were exposed to UVB *in vitro* followed by measurement of reactive oxygen species (ROS), endogenous antioxidant enzyme activity, as well as cell death in the presence or absence of supplementation with vitamin C, vitamin E, or Ginsenoside Panoxatriol. Intracellular ROS content was found significantly reduced 1 h after exposure, but increased at later time points. After exposure to 150–600 J m⁻² UVB, reduction of ROS content was accompanied by increased activity of catalase and CuZn-superoxide dismutase at early time points. Vitamins C and E, and Ginsenoside Panoxatriol counteracted the increase of ROS in the Colo-16 cells induced by acute UVB irradiation. At the same time, Ginsenoside Panoxatriol protected the activity of CuZn-superoxide dismutase, while vitamin E showed only a moderate protective role. Vitamins C and E, and Ginsenoside Panoxatriol in combination protected the Colo-16 cells from UVB-induced apoptosis, but not necrosis. These findings suggest that vitamins C and E as well as Ginsenoside Panoxatriol are promising protective agents against UVB-induced damage in skin cells.

G.-H. Jin · Y. Liu · S.-Z. Jin · X.-D. Liu (✉) · S.-Z. Liu (✉)
MH Radiobiology Research Unit,
Jilin University School of Public Health, 1163 Xinmin Street,
Changchun, Jilin, 130021, People's Republic of China
e-mail: drliusz@yahoo.com

X.-D. Liu (✉)
e-mail: liuxd2002@yahoo.com

G.-H. Jin
Department of Basic Medical Sciences, Medical College,
Xiamen University, Xiamen, People's Republic of China

Introduction

Free radicals are generated in normal physiologic processes, including aerobic metabolism and inflammatory response, but they may inflict cellular damage at increased generation if antioxidant defense mechanisms are overwhelmed [1, 2]. Solar ultraviolet radiation (UVR) is composed of UVA (320–400 nm), UVB (290–320 nm) and UVC (100–290 nm), but only UVA and UVB reach the earth's surface and are of pathophysiological significance [3]. So far, most studies have been concerned with the effects of UVA in generating free radicals and ROS in skin cells [4, 5], but some data also indicate that cutaneous tissue, both *in vitro* and *in vivo*, generates free radicals and other ROS following exposure to UVB [6, 7]. UVR is considered to be a major carcinogenic factor, but the relationship between dose, timing, and nature of exposure to tumor development is still unclear [2]. Recent research findings supporting the free radical hypothesis in skin carcinogenesis are: (1) ROS are generated in UVR-irradiated skin at excessive doses, and free radicals are involved in all steps of carcinogenesis [8–10]; (2) the natural cutaneous antioxidant defense is impaired upon UV-exposure, e.g., high doses of UVR decrease cellular glutathione content and suppress the activity of protective enzymes, such as superoxide dismutase and catalase in normal cells [11, 12]. In radiation biology, time-course and dose–effect relationship studies are important aspects [13] since different doses of UVR may give rise to different manifestations at different time intervals in the cellular oxidative stress [14, 15].

In the present study a detailed examination of the time-course and dose–effect relationship of changes in

markers of oxidative stress and of cell death (including apoptosis and necrosis) was undertaken in Colo-16 cells (an epithelial cell line established from a human squamous carcinoma) following exposure to UVB *in vitro*. In addition, the possible protective effect of common nutritional antioxidants (vitamin C, vitamin E) and traditional Chinese medicine (Ginsenoside Panoxatriol) in the defense against UVB-induced oxidative stress was studied.

M. Materials and Methods

Cell culture

Colo-16 cells were cultured at 37°C in IMEM (GIBCO, USA) containing 10% fetal calf serum (FCS, HyClone), 1% penicillin-streptomycin (Invitrogen) in a humidified atmosphere with 5% CO₂. Vitamin C and vitamin E (Jilin Chemical Reagents, Ltd, Changchun, China) were dissolved in DMSO and diluted with phosphate buffered saline (PBS) to appropriate concentrations. Ginsenoside Panoxatriol (from Department of Organic Chemistry, School of Basic Medical Sciences, Jilin University) was dissolved in PBS.

UVB irradiation

An UVB irradiator (Guangming Co., Shanghai, China) was used as a source of UVB, which emits UV in the range of 290–320 nm, with a peak at 315 nm. Colo-16 cells were plated into 24-well or 10 cm culture dishes in growth medium and exposed to several doses of UVB from the irradiator at a dose rate of 6 J m⁻² min⁻¹. Unirradiated cells were used as control. After irradiation, the cells were cultured in fresh medium and further incubated for specified time intervals.

Detection of cell death

The Colo-16 cells were harvested at different time points, washed with PBS, centrifuged at 800 rpm for 5 min, and cleared of supernatant. Cell pellets were re-suspended in 100 μl PBS and then incubated with 10 μl Hoechst 33342 (100 μg ml⁻¹, Sigma) at 37°C for 20 min. After centrifuging and washing once more, cells were re-suspended in 100 μl PBS and incubated with 10 μl propidium iodide (PI, 50 μg ml⁻¹) at 4°C for 10 min. The cells were then centrifuged at 800 rpm for 5 min and suspended in 300 μl PBS before being analyzed with flow cytometry (FACScan, BD, USA) using Cellquest software [16, 17].

Measurement of intracellular ROS

For the analysis of intracellular ROS by flow cytometry, the oxidation-sensitive fluorescence dye DCFH-DA (Sigma, USA) was used [18]. Cells were detached by trypsinization after incubation in the presence or absence of the different factors. The cellular fluorescence intensity was measured with flow cytometry after 30 min incubation with 5 mol l⁻¹ DCFH-DA, and CellQuest software was used for data analysis. PI (0.005%) was used to detect dead cells in the case of DCFH-DA analysis. For each analysis, 10,000 events were recorded. ROS production was calculated as percentage of DCFH fluorescence intensity with respect to untreated control cells.

Antioxidant enzyme activities

induced by UVB in the Colo-16 cells was observed (Fig. 1a). The intracellular content of ROS began to increase at 3 h after exposure of Colo-16 cells to 150 and 450 J m^{-2} UVB, being statistically significant at 6 h, reaching its peak at 12 h and returning to the basal level at 24 h. Surprisingly, DCFH fluorescence in the Colo-16 cells decreased significantly to 50% of the control at 1 h following exposure to UVB, and the pattern of change of the intracellular ROS after exposure to 150 J m^{-2} UVB was similar to that after exposure to 450 J m^{-2} UVB. The dose–effect relationship of the intracellular ROS at 1 and 12 h following irradiation of UVB is shown in Fig. 1b. At all doses tested, the intracellular content of ROS decreased

markedly 1 h after exposure, but increased significantly at 12 h.

Changes in antioxidant enzyme activities in Colo-16 cells after exposure to UVB

The time-course of the changes in the CuZn-SOD activity after exposure to UVB is displayed in Fig. 2a. The CuZn-SOD activity was significantly inhibited at 6 and 12 h after exposure to 450 J m^{-2} UVB, with an obvious enhancement at 1 h. There was no inhibition of CuZn-SOD activity after irradiation with 150 J m^{-2} UVB, and the activity was increased at 24 h. The dose–effect study demonstrated that after exposure to different doses of UVB, CuZn-SOD activity was decreased at 12 h in a dose-dependent manner, with the most significant inhibition following 600 J m^{-2} UVB, whereas the activity was increased at 1 h, being statistically significant at doses of 450 and 600 J m^{-2} UVB (Fig. 2b).

The changes in catalase activity were similar to those of CuZn-SOD. After exposure to 450 J m^{-2} UVB, the catalase activity was transiently increased at 1 h and significantly suppressed at 6 and 12 h, returning to the normal level at 24 h. However, in the case of GSH-Px the transient increase of activity was not observed and the suppression of its activity sustained for a longer period of time (Fig. 3a). At 12 h after exposure to different doses of UVB the activity of both catalase and GSH-Px was suppressed in a dose-dependent manner (Fig. 3b).

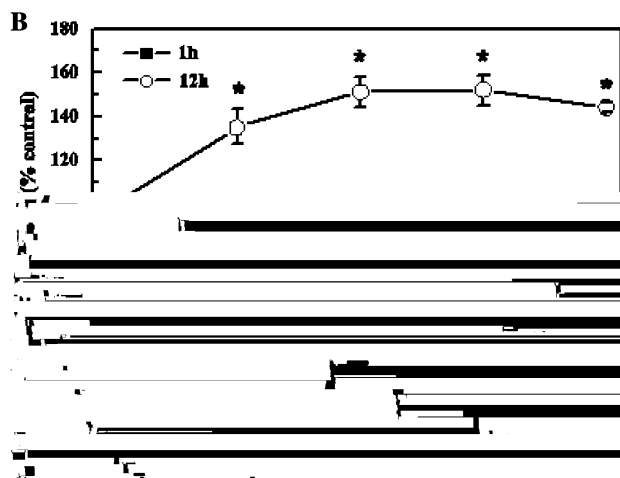
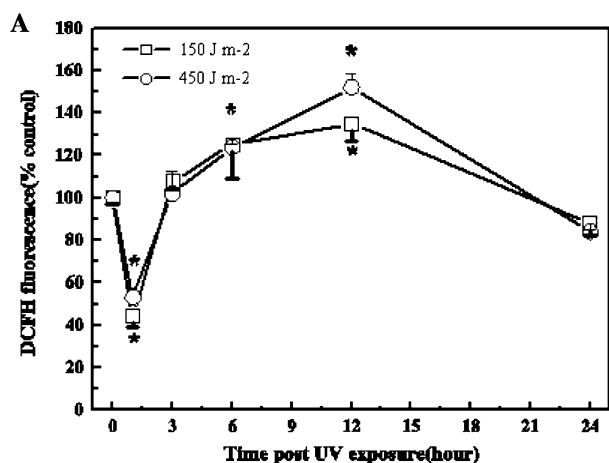


Fig. 1 UVB-induced intracellular ROS production in Colo-16 cells. After exposure of Colo-16 cells to 150 and 450 J m^{-2} UVB, the intracellular content of ROS decreased significantly at 1 h, began to increase at 3 h, reached a peak at 12 h, and returned to normal level at 24 h. After exposure to 150–600 J m^{-2} UVB, DCFH fluorescence in the Colo-16 cells increased markedly at 12 h but decreased significantly at 1 h. Each data point is expressed as the ratio of the irradiated sample to the control. Indicated are mean \pm SD, $n = 6$, * $P < 0.05$ compared to the control group

Protective effect of antioxidants in UVB induced oxidative stress

To test for a protective effect of vitamin C, vitamin E, and Ginsenoside Panoxatriol, Colo-16 cells were exposed to 600 J m^{-2} UVB, where the up-regulation of the ROS content and the inhibition of CuZn-SOD activity are clearly evident (Fig. 4a). Vitamin C (20 g ml^{-1}), vitamin E (20 g ml^{-1}), and Ginsenoside Panoxatriol (2.5 g ml^{-1}) added to the culture medium immediately after exposure, conferred a protective effect on the intracellular up-regulation of the ROS content and the inhibition of CuZn-SOD activity. All three agents markedly reduced the intracellular increase of ROS to the control level 12 h after irradiation (Fig. 4a). While vitamin C did not significantly protect the activity of CuZn-SOD, vitamin E appeared to partially relieve the suppression, although the effect was not statistically significant. Surprisingly, Ginsenoside Panoxatriol significantly protected the activity of CuZn-SOD from the UVB-induced suppression (Fig. 4b).

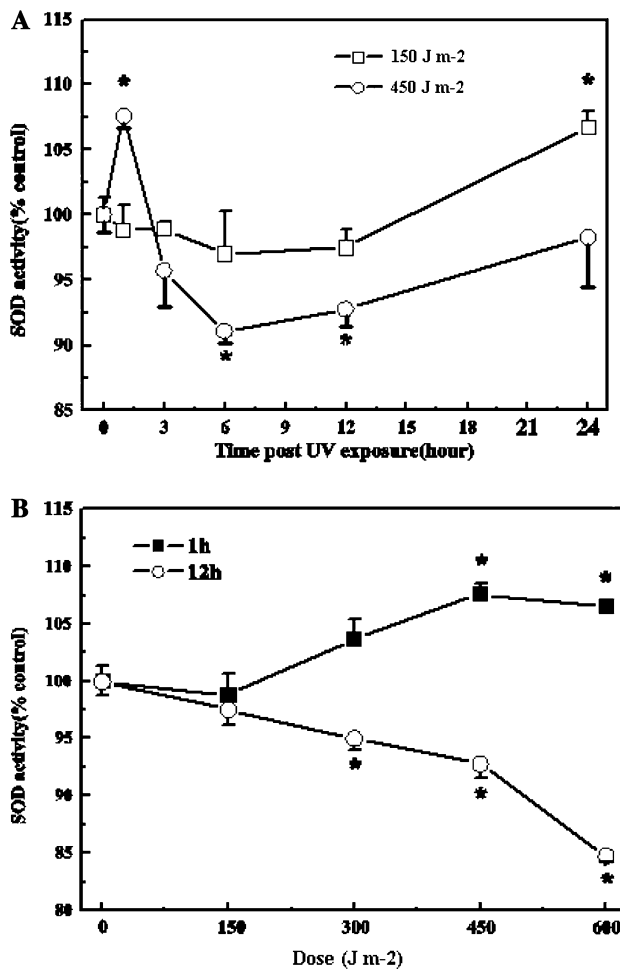


Fig. 2 Changes in CuZn-SOD in Colo-16 cells after UVB. After exposure to 450 J m⁻² UVB the CuZn-SOD activity was stimulated at 1 h and inhibited at 6 and 12 h, and with 150 J m⁻² UVB the activity was increased at 24 h. After exposure to different doses of UVB the CuZn-SOD activity decreased dose-dependently at 12 h and increased at 1 h after 450 and 600 J m⁻². Each data point was expressed as a ratio of the irradiated group to the control group. Indicated are mean \pm SD, $n = 6$, * $P < 0.05$ compared to the control group

The protective role of antioxidants on UVB-induced death of colo-16 cells

Apoptosis and necrosis were detected with flow cytometry, using double staining with PI and Hoechst 33342. The dose–effect relationship of apoptosis and necrosis was studied with cultured Colo-16 cells 24 h after exposure to UVB at doses from 150 to 900 J m⁻². A significant increase in the rate of necrotic cells was observed at all doses tested, while a significant increase in the rate of apoptotic cells was observed at doses of more than 300 J m⁻² (Fig. 5). The time course of apoptosis and necrosis was studied under exposure to UVB at a dose of 450 J m⁻² from 6 to 48 h. The rate of necrotic

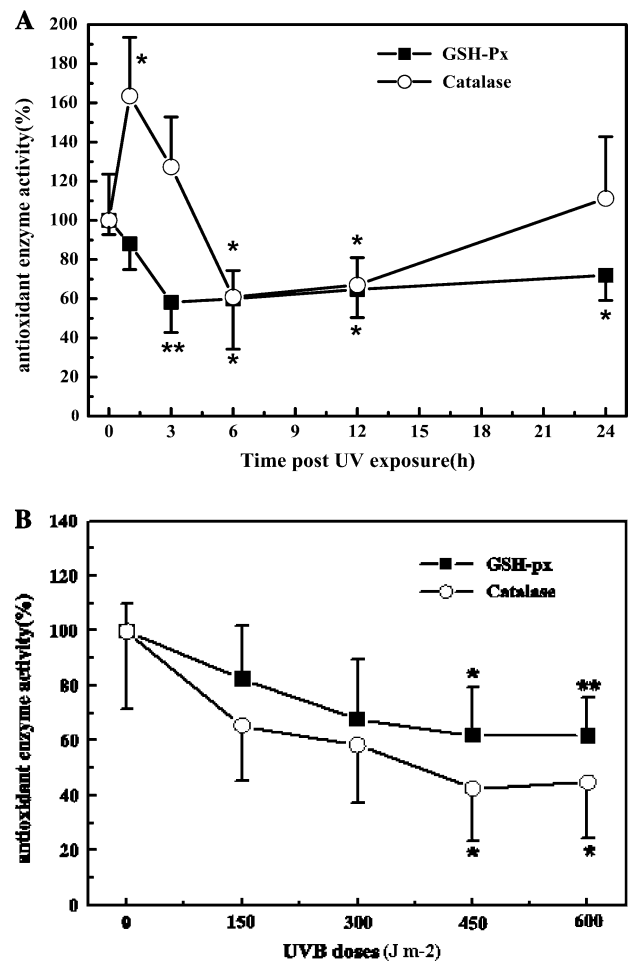


Fig. 3 Changes in GSH-Px and catalase activity by UVB in Colo-16 cells. After exposure to 450 J m⁻² UVB the catalase activity was transiently increased at 1 h, significantly suppressed at 6 and 12 h, returning to normal level at 24 h; and the activity of GSH-Px began to decrease gradually beginning from 1 h, reaching the lowest level at 3 h and remaining still much lower than the control group at 24 h. At 12 h after exposure to different doses of UVB the activity of GSH-Px and catalase was suppressed in a dose-dependent manner. Data was expressed as the ratio of the irradiated group to the control group. Indicated are mean \pm SD, $n = 6$, * $P < 0.05$, ** $P < 0.01$ compared to the control group

cells began to rise significantly after 4.5 h, while the rate of apoptotic cells began to rise after 12 h (Fig. 5).

After exposure to 600 J m⁻² UVB, apoptosis and necrosis were markedly increased at 12 h in the Colo-16 cells. With the addition of vitamin C at 20 g ml⁻¹, vitamin E at 20 g ml⁻¹, or Ginsenoside Panoxatriol at 2.5 g ml⁻¹ after exposure to UVB, the apoptosis rate decreased significantly (Fig. 6). The simultaneous addition of vitamin C at 2.5 g ml⁻¹, vitamin E at 2.5 g ml⁻¹ and Ginsenoside Panoxatriol at 1.0 g ml⁻¹ also led to a significantly decreased apoptosis rate (Fig. 6), while at these concentrations none of the compounds alone was able to protect Colo-16 cells from apoptosis

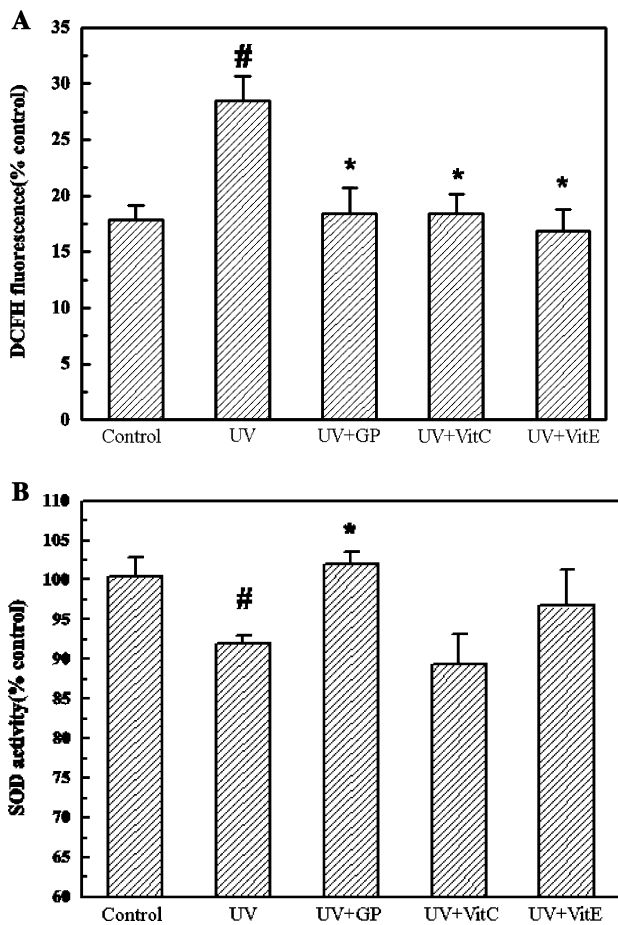


Fig. 4 Protective effect of antioxidants on UVB-induced oxidative stress. After exposure to 600 J m⁻² UVB at 12 h, ROS content in Colo-16 cells was increased. Vitamin C (20 g ml⁻¹), vitamin E (20 g ml⁻¹) or Ginsenoside Panoxatriol (2.5 g ml⁻¹) could prevent the ROS increase induced by UVB. After exposure to 600 J m⁻² UVB at 12 h, CuZn-SOD activity was inhibited. Ginsenoside Panoxatriol at 2.5 g ml⁻¹ could prevent the suppression of SOD activity, but vitamin C did not significantly protect the activity of SOD, and vitamin E only partially reverted the inhibition of SOD activity to the control level. Indicated are mean \pm SD, *n* = 6, [#]*P* < 0.05 compared to control, ^{*}*P* < 0.05 compared to the group with UV alone

induced by UVB (data not shown). None of the compounds could protect the Colo-16 cells from necrosis induced by 600 J m⁻² UVB (Fig. 6).

DNA damage

Induction of DNA damage as a consequence of exposure to UV light has been established as the major cause of skin cancer [21]. UVR-induced cancer develops as a result of a complex cascade of events initiated by damage to DNA [12]. During this process several components in this cascade, including mutation of critical genes, can be mediated by the intracellular genera-

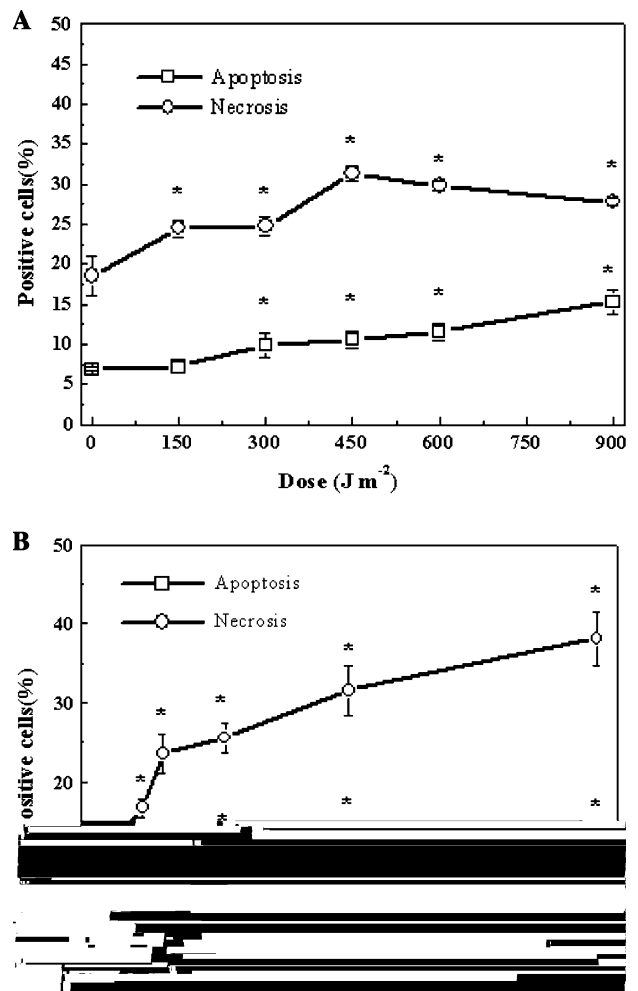


Fig. 5 Dose-effect relationship (upper panel) and time course (lower panel) of apoptosis and necrosis induced by UVB in Colo-16 cells. For the dose-effect study, the assay using double staining with PI and Hoechst 33342 was performed 24 h after exposure to different doses of UVB, and for the time course study the assay was performed at different times after exposure to 450 J m⁻². Indicated are mean \pm SD, *n* = 5, ^{*}*P* < 0.05 compared to control

tion of ROS [10, 22–24] and the UVB photons themselves [15]. Modifications of DNA and other critical cellular macromolecules by the higher energy, shorter wavelength components of UVB spectra (290–320 nm) are the most damaging to the skin [12]. In addition, the radicals and singlet molecular oxygen formed at the same locations in the plasma membrane vary in their efficiency and specificity for membrane damage, but may, in some cases, operate by a common secondary damage mechanism in the presence of oxygen [25].

The sensitivity of Colo-16 to oxidative stress induced by UVB observed in our study is similar to that of normal keratinocytes as shown in previous reports [15, 26, 27], for example, with regard to the ini-

tiation of the up-regulated ROS content at 3 h after exposure to UVB. It is interesting to note that in our experiments the cellular content of ROS was initially (1 h after exposure to $150 \gg 600 \text{ J m}^{-2}$ UVB) significantly decreased (Fig. 1). Our previous studies on exposure of fibroblasts to acute UVB have also demonstrated rapid adaptive changes in the intracellular ROS as part of a coordinated response aiming to reduce the subsequent risk of oxidative damage [28]. In that study, ROS were also measured by FCM and showed similar response to UVB as reported here [28]. Since the ROS content in the organism is closely associated with the activity of the antioxidant systems, we speculate that the transient decrease of ROS after exposure to large doses of UVB may be related to corresponding UVB-induced changes in the cellular antioxidant activity. Thus, exposure to UVB may activate the antioxidant system in an early stage. This contention is supported by literature reports in which also in four human cell lines antioxidant defense mechanisms (superoxide dismutase, catalase, and glutathione peroxidase) were observed. These authors found that Cu,Zn-SOD is the most important enzymatic antioxidant to protect cells from UVB damage [15].

Previous data from studies with both normal human and murine skin cells also showed that UVR increased the intracellular ROS content [2, 29] and decreased the

activity of superoxide dismutase, catalase, and glutathione peroxidase activities [30]. McArdle [3] reported that normal human skin exposed to an acute dose of UVR ($1,200 \text{ J m}^{-2}$) could significantly raise the proportion of the oxidized form of cellular glutathione at 6 h after UVR exposure, and rapidly increase the catalase activity at 1 h post-exposure. Katiyar [31] also noted an increase in catalase activity in UVR-exposed human skin, in agreement with the data presented in the present study. The rapidity of the response with significant changes at 1 h post-exposure suggests that the increased activity was due to posttranscriptional mechanisms. This type of regulation of catalase activity has previously been reported [32]. In the study by McArdle et al. [3] a UVR-challenge of 120 mJ cm^{-2} of UVB applied to buttock skin of healthy volunteers caused a rapid and significant rise in activity of skin catalase at 1 h and an increase in the ratio of oxidized to total glutathione at 6 h post-UV irradiation. The results in the present study show that several key antioxidants in the Colo-16 cells differ in their response pattern to acute exposure to UVB at 450 J m^{-2} , i.e., in the early stage the activity of catalase and CuZn-SOD was markedly enhanced, followed by an inhibition from the third hour onward, while the activity of GSH-Px was inhibited from the very beginning after exposure (Figs. 2, 3).

The dose–effect study demonstrated that after acute exposure to UVB the antioxidant activity and intracellular ROS content in the Colo-16 cells changed in a dose-dependent manner. The observation of the above-mentioned early decrease in the cellular ROS content after exposure to UVB may implicate that the Colo-16 cells have a strong mechanism to protect themselves from the oxidative damage induced by UVB. In the early stage after exposure, the activity of cellular antioxidants is most probably mobilized by a posttranscriptional mechanism to resist the ROS damage induced by UVB [32]. When the burden of irradiation surpasses the metabolic capability of the cells, the ROS content is immediately enhanced and the intracellular target molecules may then be damaged.

The potential role of free radicals in UVR-induced skin damage has also led to considerable interest in whether supplementation with nutritional or pharmacological antioxidants could reduce the deleterious effects of UVR on skin [33]. Vitamin C and vitamin E

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