Magnesium ascorbyl phosphate and coenzyme Q_{10} protect keratinocytes against UVA irradiation by suppressing glutathione depletion

TSANN-LONG HWANG[1,2], CHIN-JU TSAI[3], JHIH-LONG CHEN[3], TZU-TSUNG CHANGCHIEN[3], CHEE-CHAN WANG[3] and CHI-MING WU[3]

[1]Department of Surgery, Chang Gung Memorial Hospital, Tao-Yuan; [2]Department of Surgery, School of Medicine, Chang Gung University, Tao-Yuan; [3]Department of Cosmetic Science, Vanung University, Tao-Yuan, Taiwan, R.O.C.

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Abstract. The aim of this study was to investigate whether magnesium ascorbyl phosphate (MAP) and coenzyme Q_{10} (CoQ_{10}) can protect keratinocytes against ultraviolet (UV)A irradiation by increasing the levels of glutathione (GSH). The cell survival fraction was 89.9% when the keratinocytes were irradiated with UVA at a dose of 4 J/cm^2. The cell survival fractions were 48.4, 9.1 and 4.8%, at doses of 8, 16 and 32 J/cm^2, respectively. MAP was added to the cells prior to UVA irradiation at a dose of 8 J/cm^2 and then the cell viability was assayed. The cell survival fractions were 51.6, 55.5, 64.8 and 76.7%, when MAP was added at concentrations of 125, 250, 500 μM and 1 mM, respectively. The results showed that MAP is capable of protecting keratinocytes against UVA irradiation. The cell survival fractions were 77.2, 89.4 and 90.1%, when CoQ_{10} was added at concentrations of 2.5, 5 and 10 μM, respectively. The results revealed that CoQ_{10} is capable of protecting keratinocytes against UVA irradiation. At the same time, the levels of GSH within cells were detected. The level of GSH within cells was 0.3 mmol/g protein when the keratinocytes were irradiated with UVA at a dose of 8 J/cm^2. We measured the levels of GSH within the cells after MAP or CoQ_{10} was added prior to UVA irradiation at a dose of 8 J/cm^2. The levels of GSH within the cells were 0.344, 0.388, 0.456 and 0.5 mmol/g protein, when MAP was added at concentrations of 125, 250, 500 μM and 1 mM, respectively. The levels of GSH within the cells were 0.328, 0.35 and 0.394 mmol/g protein, when CoQ_{10} was added at concentrations of 2.5, 5 and 10 μM, respectively. These results imply that MAP and CoQ_{10} can protect the keratinocytes against UVA irradiation, possibly via increasing the levels of GSH.

Correspondence to: Professor Chi-Ming Wu, Department of Cosmetic Science, Vanung University, No. 1 Van-Nung Rd, Tao-Yuan 320, Taiwan, R.O.C.
E-mail: chimingwu@mail.vnu.edu.tw

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Introduction

Ultraviolet (UV) irradiation (200-400 nm) causes a number of acute and chronic skin effects, which can result in inflammation, immunosuppression, premature skin aging and the development of skin malignancies (1). UVA irradiation (320-400 nm), which is not absorbed in the ozone layer, comprises more than 95% of the UV light that reaches the earth. UVA penetrates the epidermis and affects the epidermal and dermal layers of the skin. At the cellular level, UVA exposure causes significant oxidative stress via generation of reactive oxygen species (ROS), such as singlet oxygen, hydroxyl radical, superoxide anion and hydrogen peroxide (2). ROS are rapidly removed by non-enzymatic, particularly glutathione (GSH), and enzymatic antioxidants (catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase), and that maintains the pro-oxidant/antioxidant balance, resulting in cell and tissue stabilization. However, a surplus of ROS may overwhelm the skin anti-oxidant defense mechanisms causing pro-oxidant/antioxidant disequilibrium. Overproduction of ROS induces oxidation of nucleic acids, proteins and membrane lipids, which also lead to intracellular GSH and NADH/NADPH depletion, and therefore energy loss from the cell. UV-generated ROS also affect the regulation of the gene expression of signaling molecules/cascades such as mitogen-activated protein kinases and interrelated inflammatory cytokines, as well as NF-κB and activator protein-1 (3).

Magnesium ascorbyl phosphate (MAP) is a vitamin C derivative and is more stable than vitamin C (4,5). MAP has been used in cosmetic and dermatological products as it has a number of favorable effects on the skin (6). As an antioxidant, MAP can scavenge and destroy aggressive oxidizing agents and radicals. Due to the ability of MAP to suppress the pigmentation of the skin and increase the decomposition of melanin (7), it can be used to whiten the skin.

Coenzyme Q_{10} (CoQ_{10}) is a bioactive, vitamin-like molecule present in all eukaryotic cells containing mitochondria. CoQ_{10} is located in the hydrophobic middle region of the phospholipid bilayer of the mitochondrial membrane and plays a role in the electron transport chain process, where it accepts electrons

from reducing equivalents produced from fatty acid and glucose breakdown and delivers them to electron acceptors (8). CoQ_{10} in its reduced form (ubiquinol) acts as a principal fat-soluble cellular antioxidant that plays an important role in neutralizing free radicals, inhibiting lipid peroxidation of membranes and in protecting mitochondrial membrane proteins and DNA (9).

Among the cutaneous antioxidants, the tripeptide, GSH (γ-glutamylcysteinylglycine), plays a pivotal role in protecting skin cells from oxidative damage by directly scavenging ROS or acting as a co-enzyme in GSH-peroxidase or GSH-S-transferase catalyzed reactions (10,11). Previous studies have shown that GSH is also involved in DNA repair and apoptosis (12,13). Moreover, GSH plays a role in many important biological processes, such as mitochondrial respiration, inflammatory response, signal transduction, regulation of gene expression and cell proliferation (14).

In this study, we investigated the UVA protective activity of MAP or CoQ_{10} on human keratinocytes, using human keratinocyte-derived HaCaT cells as an experimental model. We focused on the effects of MAP or CoQ_{10} on ROS-induced cellular oxidative stress, particularly on intracellular GSH levels.

Materials and methods

Materials. Human keratinocytes (HaCaT cells) were obtained from the Food Industry Research and Development Institute (Taiwan). Dulbecco's modified Eagle's medium (DMEM), heated-inactivated fetal calf serum (FCS), penicillin-streptomycin solution and trypsin-EDTA solution were from Gibco™ Invitrogen Corp. (Carlsbad, CA, USA). Sterile dimethylsulfoxide (DMSO) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), MAP and CoQ_{10} were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. HaCaT cells were grown in DMEM supplemented with heated-inactivated FCS (10%; v/v), streptomycin (100 U/ml) and penicillin (0.1 mg/ml) in a humidified atmosphere with 5% CO_2 at 37˚C. The culture medium was changed three times a week. The cells were subcultured following trypsinization. For the experiment, HaCaT cells were seeded in a 6-well plate at a density of $1x10^5$ cells per cm^2.

UVA irradiation and treatment with MAP or CoQ_{10}. The keratinocytes were pre-treated with MAP (125 μM-1 mM) or CoQ_{10} (2.5-10 μM) at 37˚C for 1 h, and irradiated and incubated in serum-free medium at 37˚C for an additional 24 h. The irradiated and non-irradiated control cells were treated with serum-free medium. Prior to UV irradiation, the cells were washed with phosphate-buffered saline (PBS) and covered with a thin layer of PBS. The dishes with keratinocytes were irradiated (UVA; 4-32 J/cm^2) on ice-cold plates to eliminate UVA thermal stimulation. In parallel, non-irradiated cells were treated similarly and were kept in the dark in an incubator. For irradiation, a solar simulator Bio-Sun (Vilber Lourmat, Marne-la-Vallée, France) with a fixed wavelength (365 nm) was used.

MTT assay. The cell viability was monitored following UVA irradiation and pre-treatment with MAP or CoQ_{10}. MTT was used to quantify the metabolically active living cells. Mitochondrial dehydrogenases metabolize MTT to a purple formazan dye, which is measured photometrically at 570 nm using a spectrophotometer (15).

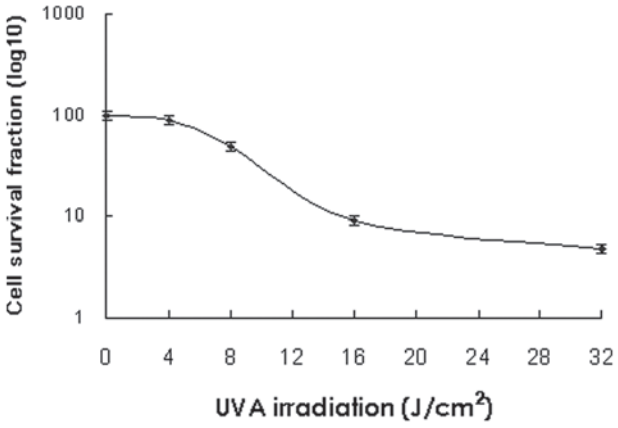

Figure 1. Cell survival fractions following UVA irradiation at various doses. The cell survival fractions were 89.90, 48.40, 9.10 and 4.80% when the keratinocytes were irradiated at the doses of 4, 8, 16 and 32 J/cm^2, respectively. UVA, ultraviolet A.

Intracellular GSH level. Intracellular GSH was estimated using a reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (16). The keratinocytes rinsed with PBS were scraped into cooled perchloric acid (1%; v/v) and sonicated. The aliquots were frozen for protein determination by Bradford assay. The suspension was centrifuged (10 min; 13,000 rpm; 4˚C) and the supernatant was used for estimation of GSH in reaction with the reaction mixture (800 mmol/l Tris/HCl, 20 mmol/l EDTA, pH 8.2; 20 mg/ml DTNB). The absorbance was read on a microplate reader at 412 nm.

Statistical analysis. The mean ± standard error (SE) was calculated from at least three repeated groups in all experiments. A statistical significance between groups was determined by the Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference between the two groups.

Results

Cell survival fractions following UVA irradiation at various doses. A comparison of cell survival fractions in HaCaT cells following irradiation with UVA at various doses from 4-32 J/cm^2 is shown in Fig. 1. The cell survival fraction was 89.9% when the keratinocytes was irradiated with UVA at a dose of 4 J/cm^2. The cell survival fractions were 48.4, 9.1 and 4.8%, at doses of 8, 16 and 32 J/cm^2, respectively. At each dose investigated, a characteristic dose-response curve was observed with decreased survival at increased doses of UVA irradiation.

Modulation of cell viability by MAP in UVA-irradiated cells. Keratinocytes were pre-treated with MAP (125 μM to 1 mM) prior to UVA irradiation (8 J/cm^2). The cell survival fractions were 51.6, 55.5, 64.8 and 76.7%, following the addition of MAP at concentrations of 125, 250, 500 μM and 1 mM, respectively (Fig. 2). MAP pre-treatment suppressed the UVA-induced

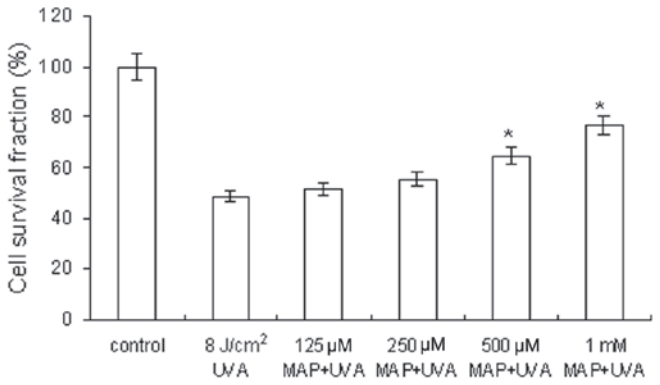

Figure 2. Modulation of cell viability by MAP in UVA-irradiated cells. The cell survival fractions were 51.6, 55.5, 64.8 and 76.7%, when MAP was added at concentrations of 125, 250, 500 μM and 1 mM, respectively, prior to UVA irradiation at a dose of 8 J/cm^2. MAP, magnesium ascorbyl phosphate; UVA, ultraviolet A. *P<0.05, comparison with the group 8 J/cm^2 UVA.

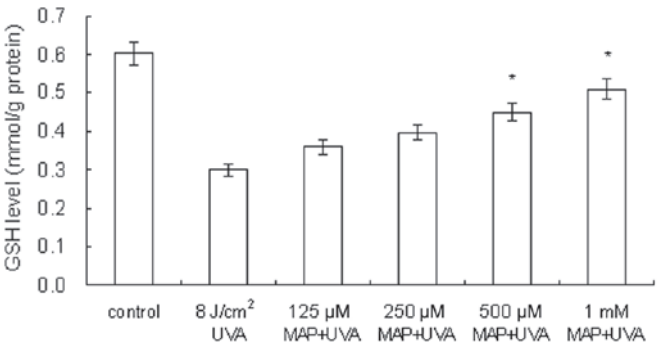

Figure 4. Prevention of UVA-induced GSH depletion by MAP. Keratinocytes were pre-treated with MAP (125 μM-1 mM) and then irradiated (8 J/cm^2). The intracellular GSH levels were assayed. Data were expressed as the means ± SD. P<0.05 indicates a statistically significant difference from irradiated cells. UVA, ultraviolet A; GSH, glutathione; MAP, magnesium ascorbyl phosphate; SD, standard deviation. *P<0.05, comparison with the group 8 J/cm^2 UVA.

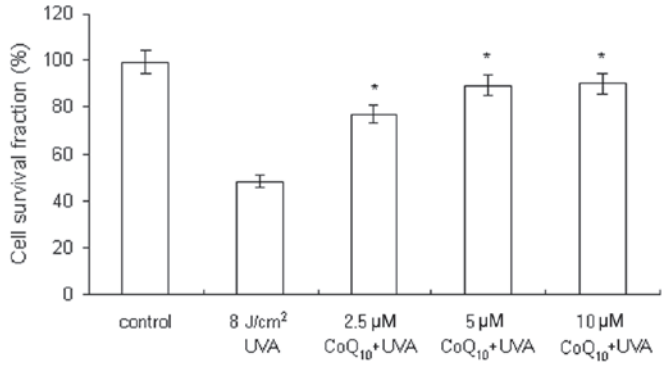

Figure 3. Modulation of cell viability by CoQ_{10} in UVA-irradiated cells. The cell survival fractions were 77.2, 89.4 and 90.1%, following the addition of CoQ_{10} at concentrations of 2.5, 5 and 10 μM, respectively, prior to UVA irradiation at a dose of 8 J/cm^2. CoQ_{10}, coenzyme Q_{10}; UVA, ultraviolet A. *P<0.05, comparison with the group 8 J/cm^2 UVA.

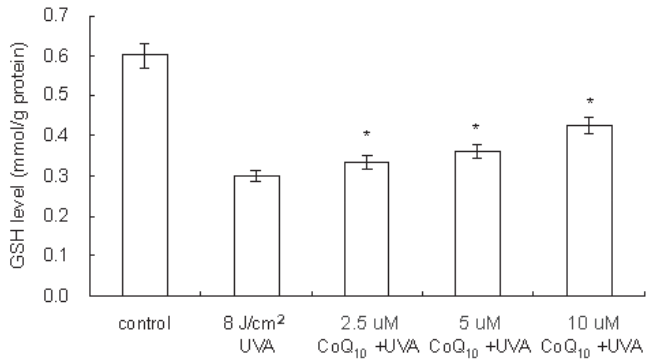

Figure 5. Prevention of UVA-induced GSH depletion by CoQ_{10}. Keratinocytes were pre-treated with CoQ_{10} (2.5 μM-10 μM) and then irradiated (8 J/cm^2). The intracellular GSH levels were assayed. Data were expressed as the means ± SD. P<0.05 indicates a statistically significant difference from irradiated cells. UVA, ultraviolet A; GSH, glutathione; CoQ_{10}, coenzyme Q_{10}; SD, standard deviation. *P<0.05, comparison with the group 8 J/cm^2 UVA.

decrease in cell viability in a concentration-dependent manner. The results showed that MAP is capable of protecting the keratinocytes against UVA irradiation.

Modulation of cell viability by CoQ_{10} in UVA-irradiated cells. Keratinocytes were pre-treated with CoQ_{10} (2.5-10 μM) prior to UVA irradiation (8 J/cm^2). The cell survival fractions were 77.2, 89.4 and 90.1%, following the addition of CoQ_{10} at the concentrations of 2.5, 5 and 10 μM, respectively (Fig. 3). CoQ_{10} pre-treatment suppressed the UVA-induced decrease in cell viability in a concentration-dependent manner. The results revealed that CoQ_{10} is capable of protecting the keratinocytes against UVA irradiation.

Prevention of UVA-induced GSH depletion by MAP. As demonstrated in Fig. 4, in UVA-irradiated HaCaT cells (8 J/cm^2) the GSH level was decreased to 50% of the level of the control cells (0.6→0.3 mmol/g protein). When MAP was added prior to UVA irradiation, the GSH levels within the cells were 0.344, 0.388, 0.456 and 0.50 mmol/g protein, at MAP concentrations of 125, 250, 500 μM and 1 mM, respectively. The application of MAP to UVA-irradiated keratinocytes led to dose-dependent prevention of GSH depletion.

Prevention of UVA-induced GSH depletion by CoQ_{10}. As demonstrated in Fig. 5, in UVA-irradiated HaCaT cells (8 J/cm^2) the GSH level was decreased to 50% of the level of the control cells (0.6→0.3 mmol/g protein). When CoQ_{10} was added prior to UVA irradiation, the GSH levels within the cells were 0.328, 0.350 and 0.394 mmol/g protein, at CoQ_{10} concentrations of 2.5, 5 and 10 μM, respectively. CoQ_{10} application to UVA-irradiated keratinocytes led to a dose-dependent prevention of GSH depletion.

Discussion

UV irradiation is the principal factor in skin cancers in humans. Several studies have shown that supplementation with antioxidants can decrease UV-induced skin damage *in vitro* and *in vivo* (17). In this study, we demonstrate the ability of MAP and CoQ_{10} to prevent and reduce UVA-related damage at a cellular level in human keratinocytes. In particular, it was shown

that treatment of HaCaT cells with MAP or CoQ_{10} prior to UVA exposure increased cell viability and suppressed intracellular GSH depletion. The cell viability assay showed that MAP and CoQ_{10} protect against UVA-induced cell death in a human keratinocyte cell line. It is well known that during and after UVA irradiation, generation of ROS dramatically increases in exposed cells (18,19). As UVA-induced biological effects are primarily mediated by ROS, their elimination is essential for UVA damage protection. Application of MAP or CoQ_{10} led to a significant increase in cell survival in irradiated HaCaT cells. MAP and CoQ_{10} pre-treatment demonstrated maximal protection at the highest concentration tested.

Pre-treatment of cells with MAP or CoQ_{10} resulted in concentration-dependent reduced GSH depletion. The importance of GSH in protecting the skin from oxidative damage caused by various chemicals and UV exposure is also well documented. Among non-enzymatic antioxidants, GSH is the most important as it also serves as the substrate for two major antioxidant enzymes, GSH peroxidase and GSH transferase, and is involved in vitamin C and vitamin E regeneration (20). The GSH level is directly associated with the degree of lipid peroxidation in the cell membrane (21), since GSH plays a role in eliminating lipid peroxidation products, including 4-hydroxynonenal, by forming a GSH conjugate (22).

The cutaneous antioxidant system is complex and and is not yet completely understood. Our results revealed that MAP and CoQ_{10} can increase intracellular GSH levels. Previously, Kagan *et al* showed that vitamin C can regenerate vitamin E from the α-tocopheroxyl radical (23). α-lipoic acid has been shown to elevate intracellular GSH levels *in vitro* by increasing *de novo* synthesis (24). The effect depends on the metabolic reduction of lipoic acid to dihydrolipoic acid. Dihydrolipoic acid is released into the culture medium where it reduces cystine. Cysteine thus formed is readily taken up by the neutral amino acid transport system and utilized from glutathione synthesis. By this mechanism, lipoic acid enables cysteine to bypass the Xc^- transport system, which is weakly expressed in lymphocytes and inhibited by glutamate. Thereby lipoic acid enables the key enzyme of glutathione synthesis, γ-glutamylcysteine synthetase, which is regulated by uptake-limited cysteine supply, to function at optimum conditions. The mechanisms for the MAP and CoQ_{10} increased intracellular GSH levels are not yet clear. Further studies are required to investigate this mechanism.

However, from the results of the present study, we can conclude that MAP and CoQ_{10} can protect keratinocytes against UVA irradiation by suppressing GSH depletion. Therefore, the protection mechanism is perhaps via increasing the levels of GSH.

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