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α-Tocopherol Increases the Intracellular Glutathione Level in HaCaT Keratinocytes

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α-Tocopherol is a lipophilic vitamin that exhibits an antioxidative activity. The purpose of this study was to clarify the roles of α-tocopherol in the regulation of intracellular glutathione (GSH) levels in HaCaT keratinocytes. When HaCaT keratinocytes were cultivated with α-tocopherol for 24 h, the intracellular GSH was increased at every concentration of α-tocopherol tested. Furthermore, the HaCaT keratinocytes cultured with α-tocopherol at 50 μM for 24 h exhibited resistance against H_2O_2. However, a short exposure of HaCaT keratinocytes to α-tocopherol for 1 h did not influence either the GSH level or the resistance to H_2O_2. These findings suggest that GSH, which is inductively synthesized by α-tocopherol, effectively reduces exogenous oxidative stress. To evaluate the effect of α-tocopherol on the GSH level, BSO, which is a typical inhibitor of γ-glutamylcysteine synthetase (γ-GCS), was used. When BSO was added to HaCaT keratinocytes, no action of α-tocopherol on the GSH level was observed. On the other hand, α-tocopherol resulted in the up-regulation of γ-GCS-HS (heavy subunit) mRNA. In addition, water soluble α-tocopherol derivatives (α-tocopherol phosphate and trolox) caused no changes in GSH level. From these results, it was concluded that α-tocopherol increases the intracellular GSH level of HaCaT keratinocytes through the up-regulation of γ-GCS-HS mRNA.

Keywords: GSH; α-tocopherol; γ-glutamylcysteine synthetase; HaCaT keratinocyte

INTRODUCTION

Skin is an organ exposed to severe environmental stimuli such as ultraviolet rays and chemicals. In particular, UV irradiation to the skin causes acute kinds of damage including erythema, pigmentation and reduction of skin immunosuppression.[1] Furthermore, chronic UV exposure leads to carcinogenesis[2] due to the reduction of immune reactivity and the acceleration of skin aging resulting in changes such as wrinkles, pigment spots and sagging.[3] Many reports have demonstrated that endogenous or exogenous reactive oxygen species (ROS), which are produced by UV irradiation[4] and mitochondrial respiratory chain reaction,[5,6] are responsible for the skin damage involved in carcinogenesis.

In cells, there are endogenous antioxidative systems including superoxide dismutase, catalase, glutathione peroxidase (GPx) and glutathione (GSH).[7] In keratinocytes exposed to UV lights, such antioxidative systems play a pivotal role on removing cell damage.[8] It is well known that GSH in particular plays a critical role in keeping an intracellular redox potential by removing excess ROS.[9] GSH possesses extensive abilities to scavenge ROS and lipid peroxides in a direct manner.[10] The synthesis of GSH from its constituent amino acids involves two ATP-requiring enzymatic steps: the

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formation of γ-glutamylcysteine from glutamate and cysteine, and the formation of GSH from γ-glutamylcysteine and glycine. The first step of GSH synthesis is rate-limiting and is catalyzed by γ-glutamylcysteine synthetase (γ-GCS) which is composed of a heavy (73 kDa) (γ-GCS-HS) and a light (30 kDa) subunit (γ-GCS-LS).[11,12] The heavy subunit exhibits all of the catalytic activity.[13] On the other hand, the light subunit is enzymatically inactive but performs an important regulatory function by lowering the km value of γ-GCS for glutamate.[12,13]

UVB irradiation to the skin has been known to reduce the amounts of intracellular GSH.[14] The decrease in GSH has been prevented by topical application of α-tocopherol,[15] which is one of the Vitamin E homologues with the highest *in vivo* biological activity and acts as an antioxidant in many biological systems.[16] The effects of α-tocopherol on antiaging are generally accepted as results of its antioxidative properties. However, the possibility that α-tocopherol exerts an effect on the regulation of intracellular redox potential has not yet been demonstrated. Thus, it is particularly interesting to examine the effects of α-tocopherol on intracellular GSH levels.

The purpose of this study was to clarify the effect of α-tocopherol, as an exogenous antioxidant, on the intracellular GSH levels in HaCaT keratinocytes.

MATERIALS AND METHODS

Materials

α-Tocopherol, α-tocopherol phosphate disodium salt, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), glutathione disulfide (GSSG), DL-buthionine-[S,R]-sulfoximine (BSO), and GSH reductase were purchased from Sigma (St. Louis, USA). β-Nicotinamide adenine dinucleotide 3′-phosphate (β-NADPH), *t*-butylhydroperoxide (*t*-BHP) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), ethydium bromide, neutral red, phenylmethylsulfonyl fluoride (PMSF), and agarose were obtained from Nacalai Tesque (Kyoto, Japan). TRIzol reagent and Taq polymerase were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Dulbecco's modified eagle medium (DMEM) and fetal calf serum (FCS) were obtained from Invitrogen Japan K.K. (Tokyo, Japan).

Total GSH

Total GSH was measured by DTNB-GSH reductase recycling assay.[17] HaCaT keratinocytes were placed in a 96-well plate at a density of 3×10^4 cells per well. After cultivation with DMEM supplemented with 5% FCS and α-tocopherol (100 μM) for 1 or 24 h, HaCaT keratinocytes were sonicated with a handy-type sonicator (TOMY SEIKO Co. LTD., Tokyo, Japan) in 100 μl of 100 mM phosphate buffer (pH 7.5) containing 1 mM PMSF. A 25 μl of the suspension was mixed with 0.25 mM β-NADPH and 0.5 units per ml GSH reductase, and reacted in the dark at room temperature for 10 min. After addition of 25 μl of 10 mM DTNB, the kinetics at 405 nm were monitored for 10 min with a Spectra Max 96-well plate reading spectrophotometer (Molecular Devices, CA, USA). Concentrations were calculated from the standard curve prepared with GSSG and are expressed as nmole GSH per mg of protein.

Resistance to H_2O_2 or *t*-BHP

HaCaT keratinocytes were placed in a 96-well plate at a density of 3×10^4 cells per well. The cells were cultured with DMEM supplemented with 5% FCS and α-tocopherol for 1 or 24 h and exposed to 1 mM H_2O_2 or 50 mM *t*-BHP for adequate periods (1 h for H_2O_2, 4 h for *t*-BHP).[18,19] The survival of the cells was estimated by the neutral red method, which is a rapid colorimetric assay used to estimate the number of living cells.[20] The cells were incubated with DMEM containing 5% FCS and 70 μM neutral red for 2 h, and then washed with phosphate buffered saline. The neutral red incorporated in the living cells was extracted with 30% EtOH in 0.1 M HCI. The absorbance at 550 nm was measured with a Spectra Max 96-well plate reading spectrophotometer. Survivals are expressed as percentage of the amount of neutral red incorporated into the control cells.

RT-PCR

γ-GCS-HS gene expression in HaCaT keratinocytes was assessed using reverse-transcription and polymerase chain reaction (RT-PCR). RNA from HaCaT keratinocytes cultivated in the medium containing α-tocopherol for 6 h was prepared using TRIzol reagent according to the manufacturer's protocol. Total RNA (1 μg from each sample) was used for the first-strand cDNA synthesis using oligo-dT and M-MLV RT, according to the manufacturer's instruction.[21] To ensure that similar amounts of cDNA were used for PCR, samples were assessed for expression of glycerolaldehyde-3-phosphate dehydrogenase (G3PDH) as a housekeeping gene. The cDNA was amplified in the presence of 10 units Taq polymerase per μg cDNA and 10 pmole of both the sense and antisense primers or G3PDH oligonucleotides in PCR buffer (50 mM KCI, 1.5 mM $MgCI_2$, 10 mM Tris–HCI). All PCR procedures were conducted using 45 amplification cycles, which were within the linear amplification range for all cDNAs (denaturation for 1 min at 94°C, annealing for 2 min at 60°C, and extension for 2 min at 72°C). The PCR products were

fractionated by 1% agarose electrophoresis in a volume of 10 μl and visualized by ethydium bromide staining and UV illumination. Fragments of the expected sizes were obtained (452 bp for G3PDH, 1197 bp for γ-GCS-HS). The following primer sets for γ-GCS and G3PDH were used (5′–3′): G3PDH sense: ACCACAGTCCATGCCATCAC, G3PDH antisense: TCCACCACCCTGTTGCTGTA, γ-GCS-HS sense: GGATGATGAAGCTTCAAGGG, γ-GCS-HS antisense: GCTGATCCAAGTAACTCTGG.

RESULTS

Effect of α-Tocopherol on Intracellular GSH Levels in HaCaT Keratinocytes

The amount of GSH in HaCaT keratinocytes was 5.32 ± 1.39 nmole/mg protein. When HaCaT keratinocytes were cultured with α-tocopherol at 50 and 100 μM for 24 h, the intracellular GSH increased 18.28 ± 7.67 and 25.70 ± 4.11 nmole/mg protein, respectively. However, the exposure of the HaCaT keratinocytes to α-tocopherol for a short period of 1 h resulted in no changes in the intracellular GSH level (Fig. 1).

To evaluate the effect of the increase in GSH, viabilities of the cells exposed to H_2O_2 were examined. Under normal cultivation conditions, the HaCaT keratinocytes in the presence of 1 mM H_2O_2 for 1 h survived at a rate of 39.3 ± 1.7%. On the other hand, survival of the cells cultured with α-tocopherol at 50 μM for 24 h was increased to 69.6 ± 1.7% (Fig. 2b). Although the cells exhibited resistance to *t*-BHP with α-tocopherol, exposure of the cells to α-tocopherol for 1 h did not result in resistance to H_2O_2 (Fig. 2a). It was believed that the resistance to *t*-BHP after a brief exposure originated from the chemical anti-oxidative property of α-tocopherol, while the resistance to H_2O_2 after a long exposure to α-tocopherol was caused by the increased GSH. Thus, the increase in the GSH level caused by α-tocopherol was indicated to be responsible for the enhancement of the resistance to H_2O_2.

Effect of BSO on GSH Level Enhanced by α-Tocopherol

To investigate whether α-tocopherol stimulates the *de novo* synthesis of GSH in HaCaT keratinocytes, the effect of α-tocopherol on the GSH level in the presence of BSO was examined. It is well known that BSO is an inhibitor of γ-GCS.[22] When HaCaT keratinocytes were exposed to BSO at a concentration of 200 μM for 24 h, the GSH level decreased to 3.17 ± 0.64 nmole/mg protein, the GSH level in the control cells being 5.40 ± 0.95 nmole/mg protein. Thus, BSO inhibited the GSH synthesis in HaCaT keratinocytes. Addition of BSO suppressed the GSH level even in the presence of α-tocopherol (Fig. 3). These results indicated that α-tocopherol stimulates the GSH *de novo* synthesis in HaCaT keratinocytes.

mRNA Expression of γ-GCS-HS in HaCaT Keratinocytes

The expression of γ-GCS-HS mRNA in HaCaT keratinocytes cultured with various concentrations of α-tocopherol was examined by using RT-PCR. The expression of γ-GCS-HS mRNA in the HaCaT keratinocytes was up-regulated at every concentration of α-tocopherol examined, indicating that

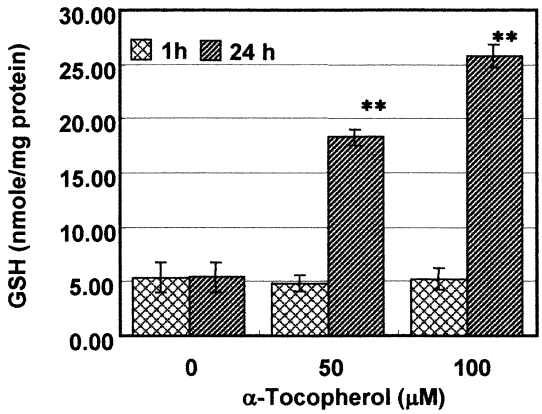

FIGURE 1 Effect of α-tocopherol on intracellular GSH level in HaCaT keratinocytes. HaCaT keratinocytes were placed at a density of 3×10^4 cells, and cultured with DMEM supplemented with 5% FCS and α-tocopherol for 1 h (⊠) or 24 h (▨). Total GSH was measured by DTNB-GSH reductase recycling assay. Data are expressed as means ± standard deviations for five independent experiments. Data were assessed by Student'ts *t*-test ($** p < 0.01$).

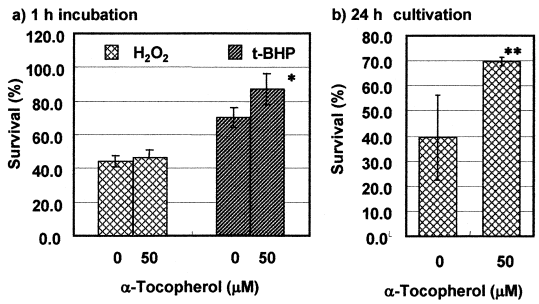

FIGURE 2 Cell viability of HaCaT keratinocytes exposed to H_2O_2 or *t*-BHP. HaCaT keratinocytes were placed at a density of 3×10^4 cells, and cultured with DMEM supplemented with 5% FCS and α-tocopherol for 1 or 24 h. The cells were exposed to 1 mM H_2O_2 (⊠) or 50 mM *t*-BHP (▨) for adequate periods (1 h for H_2O_2, 4 h for *t*-BHP). (a) 1 h exposure, (b) 24 h cultivation. The survivals of the cells were estimated with neutral red method. Data are expressed as means ± standard deviations for six independent experiments. Data were assessed by Student'ts *t*-test ($* p < 0.05$, $** p < 0.01$).

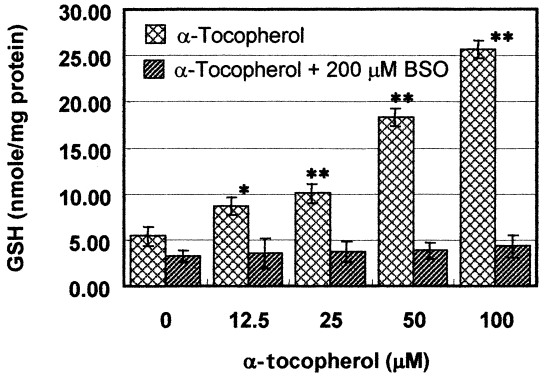

FIGURE 3 Influence of BSO on the increase of GSH induced by α-tocopherol. HaCaT keratinocytes were placed at a density of 3×10^4 cells, and cultured with DMEM supplemented with 5% FCS, α-tocopherol and BSO for 24 h ( ): α-tocopherol, ( ): α-tocopherol plus 200 μM BSO. Total GSH was measured by DTNB-GSH reductase recycling assay. Data are expressed as means ± standard deviations for five independent experiments. Data were assessed by Student's *t*-test ($*p < 0.05$, $**p < 0.01$).

α-tocopherol increased the intracellular GSH level through γ-GCS-HS- mRNA expression (Fig. 4).

Effect of Vitamin E Derivatives on Intracellular GSH Level in HaCaT Keratinocytes

Because α-tocopherol increased the intracellular GSH in HaCaT keratinocytes (Fig. 1), the mechanism by which this occurred was examined by using Vitamin E derivatives such as α-tocopherol phosphate and trolox (Fig. 5). In α-tocopherol phosphate, the OH group at the 6 position of α-tocopherol is modified to phosphate, and in trolox, the alkyl chain at the 2 position of α-tocopherol is converted to a carboxilic acid. The derivatives are chemically modified to enhance the water solubility of α-tocopherol. When such water soluble Vitamin E derivatives were used in place of α-tocopherol, no alterations in GSH level were observed (Fig. 6).

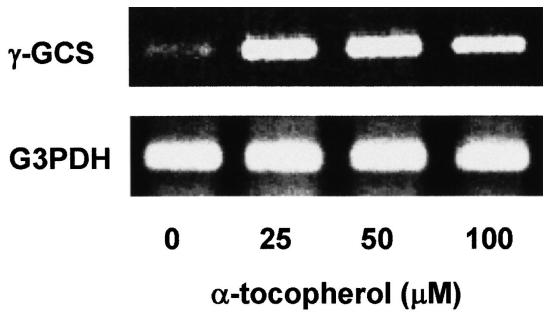

FIGURE 4 Effect of α-tocopherol on the expression of γ-GCS-HS mRNA. γ-GCS-HS gene expression in HaCaT keratinocytes was assessed using RT-PCR. RNA from HaCaT keratinocytes cultivated in the medium containing α-tocopherol for 6 h was prepared. RT-PCR was carried out with primers described in the Materials and Methods section. Data are obtained from three independent experiments.

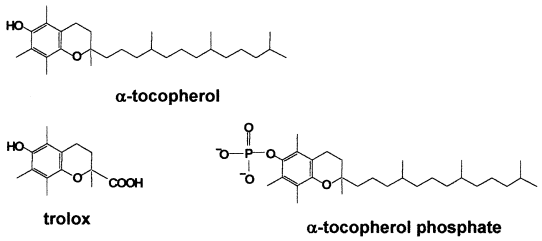

FIGURE 5 Chemical structure of α-tocopherol and Vitamin E derivatives.

DISCUSSION

It is well known that α-tocopherol is a potent chain-breaking type of antioxidant and therefore effectively scavenges lipid peroxides (LOOH). On the other hand, GSH, the most prevalent intracellular non-protein thiol, is critical for reducing oxidative stress and preserving the proper cellular redox balance due to its reactivity with ROS.[9,10] Furthermore, GSH participates in GPx-catalyzed reactions in eliminating H_2O_2 and LOOH and forms conjugates with potentially harmful electrophilic species in the reaction catalyzed by GSH S-transferase. Under physiological conditions, it is well known that ROS are mainly produced by mitochondrial respiratory chain reactions.[5,6] ROS are certainly removed by intracellular antioxidants including GSH, GPx and GSH S-transferase. During such processes, intracellular GSH is consumed. However, when an exogenous antioxidant, α-tocopherol, is supplied under the same conditions, the preservation of GSH in cells may occur.

In this study, we indicated that α-tocopherol increases the intracellular GSH levels in HaCaT keratinocytes (Fig. 1). There are two possible mechanisms to explain this observation. The first is the inhibition of GSH consumption by α-tocopherol. The second is the stimulation of GSH synthesis. Previously, α-tocopherol was proposed to prevent GSH consumption during UVB irradiation.[15] However, our results indicated that total GSH levels in

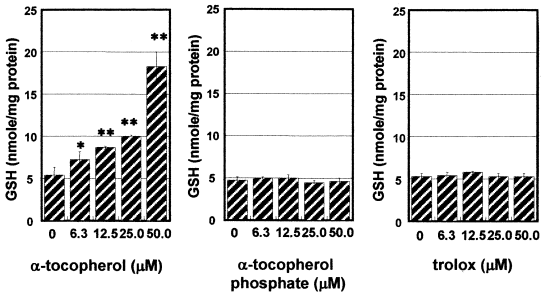

FIGURE 6 Influence of Vitamin E derivatives on intracellular GSH level in HaCaT keratinocytes. HaCaT keratinocytes were placed at a density of 3×10^4 cells, and cultured with DMEM supplemented with 5% FCS and α-tocopherol or Vitamin E derivatives for 24 h. Total GSH was measured by DTNB-GSH reductase recycling assay. Data are expressed as means ± standard deviations for five independent experiments. Data were assessed by Student's *t*-test ($*p < 0.05$, $**p < 0.01$).

HaCaT keratinocytes were increased by α-tocopherol application (Fig. 1). If the action of α-tocopherol is based on the prevention of GSH consumption, the oxidation of GSH in the cells should occur. For this reason, it is not possible that α-tocopherol preserves the GSH level by its antioxidative property.

In our preliminary study, 2,3-*t*-butyl-4-hydroxyanisole (BHA), which is an antioxidant with a similar chemical structure to that of α-tocopherol, increased the GSH level in a similar manner to that of α-tocopherol (data not shown). Because dietary BHA has been reported to enhance the intracellular GSH by up-regulation of γ-GCS-HS mRNA,[23,24] α-tocopherol is also proposed to stimulate the *de novo* synthesis of GSH. BSO is a typical inhibitor of γ-GCS,[22] and thus this compound is usually used as a tool to assess the contribution of γ-GCS to the regulation of intracellular GSH. In the present study, BSO completely suppressed the action of α-tocopherol and decreased the GSH level (Fig. 3). From these results, it was concluded that α-tocopherol up-regulates the expression of γ-GCS-HS mRNA (Fig. 4).

The essentiality of α-tocopherol was examined in terms of which moiety of α-tocopherol contributes to the increase of intracellular GSH. Water soluble Vitamin E derivatives, α-tocopherol phosphate without the OH group and trolox without the alkyl chain of α-tocopherol, did not affect the GSH level (Fig. 6). Although these findings tentatively suggest that both the OH group and alkyl chain in α-tocopherol play important roles in the stimulation of GSH synthesis by up-regulation of γ-GCS-HS mRNA, more information are needed to deduce a structure-activity relationship for true role of α-tocopherol, by using several types of α-tocopherol derivatives. We are continuing this study in order to elucidate the effect of α-tocopherol on GSH biosynthesis in terms of the up-regulation of γ-GCS-HS mRNA.

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