Regular Article

Protective Effects of 2',3'-Dihydroxy-4',6'-dimethoxychalcone Derived from Green Perilla Leaves against UV Radiation-Induced Cell Injury in Human Cultured Keratinocytes

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Skin exposure to UV rays causes the production of reactive oxygen species (ROS), and it is a major risk factor for various skin disorders and diseases. In particular, exposure to UV-A is a major cause of photoaging. We have previously isolated 2',3'-dihydroxy-4',6'-dimethoxychalcone (DDC) from green perilla leaves as an activator of the nuclear factor erythroid 2-related factor-2 (Nrf2)–antioxidant response element (ARE) and demonstrated the protective effects of DDC both *in vitro* and *in vivo* in PC12 cells and Parkinson's disease models, respectively. In this study, we used HaCaT cells to examine the effects of DDC on ROS production and cell damage induced by UV-A. Our results indicated that UV-A irradiation in HaCaT cells increased ROS production in an energy-dependent manner. In addition, cell viability decreased in an energy-dependent manner 24 h after UV-A irradiation. However, treatment with DDC 24 h prior to UV-A irradiation significantly suppressed UV-A radiation-induced ROS production. In addition, DDC showed cytoprotective effects when used 24 h before and after UV-A irradiation. Treatment with DDC for 24 h also increased the expression levels of heme oxygenase-1 (HO-1) in a concentration-dependent manner. Pretreatment with the HO-1 inhibitor followed by DDC treatment before UV-A irradiation for 24 h reduced ROS production and the cytoprotective effect. These results suggest that DDC increases the expression levels of HO-1 and protects HaCaT cells through the suppression of UV radiation-induced ROS production.

Key words antioxidant enzyme; oxidative stress; HaCaT cell; UV-A irradiation; inflammation; nuclear factor-erythroid 2-related factor-2–antioxidant response element pathway

INTRODUCTION

UV rays are divided into three regions: short-wave UV-C (200–280 nm), mid-wave UV-B (280–320 nm), and long-wave UV-A (320–400 nm). In particular, exposure to UV-B and UV-A can result in various deleterious effects.¹⁾ While UV-B only reaches the skin from the epidermis to the upper dermis, it can reach the deep dermis with long-wave UV-A. In the IR region, UV-B is known to further penetrate subcutaneous tissues. UV-C does not reach the ground because it is absorbed by the ozone layer; however, it is almost absorbed in the upper layer of the epidermis and does not reach basal cells.¹⁾ Sunburn is a phenomenon in which the skin becomes red and inflamed due to UV-B irradiation. The amount of UV-B reaching the surface is approximately one-tenth of the amount of UV-A, but the intensity of UV-B energy is 100–1000 times that of UV-A, which causes not only sunburn but also DNA damage, immunosuppression, and skin cancer. Conversely, UV-A penetrates deeply into the dermis and not only causes a blackening phenomenon (suntan) immediately after irradiation but also generates free radicals inside the skin, thus causing indirect DNA damage and photoaging and reducing skin firmness and elasticity.²⁾

Skin exposure to UV induces the production of reactive oxygen species (ROS); moreover, it is a major risk factor for skin damage associated with various disorders and diseases. In particular, UV-A accounts for more than 90% of the sun's UV radiation that reaches the ground and is known to be one of the leading causes of skin diseases such as photoaging (stains and wrinkles) and skin cancer.^{3–5)} Exposure to UV-A increases ROS production, which damages proteins, lipids, and DNA in cells and subsequently induces cellular apoptosis.^{6,7)} This indicates that UV-A-induced cell injury can be suppressed in the skin by suppressing ROS production.

The nuclear factor erythroid 2-related factor-2 (Nrf2)–antioxidant response element (ARE) pathway is one of the important cellular defense mechanism against oxidative stress. Indeed, activation of this Nrf2–ARE pathway causes an increase in heme oxygenase-1 (HO-1) as well as induces antioxidant enzymes such as γ -glutamylcysteine synthetase (γ -GCS) and reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H): quinone oxidoreductase-1 (NQO1).^{8,9)} As a result of exploring and screening food-derived components that activate the Nrf2–ARE pathway, we successfully isolated 2',3'-dihydroxy-4',6'-dimethoxychalcone (DDC) from green perilla leaves.¹⁰⁾

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DDC activates the Nrf2–ARE pathway in PC12 cells and exhibits cytoprotection against oxidative stress by inducing the antioxidant enzymes HO-1, γ -GCS, and NQO1.¹⁰⁾ Moreover, DDC upregulates the expression of HO-1 in the astrocytes and microglia of the substantia nigra region of C57BL/6N mice as well as of primary mesencephalic cultures.¹¹⁾ However, to the best of our knowledge, the effects of DDC on skin cells have not been studied. Therefore, the present study aimed to examine the effects of DDC on UV-A-induced cytotoxicity and intracellular ROS production and examine its mechanism of action in HaCaT cells, which is a cultured human epidermal cell line.

MATERIALS AND METHODS

Materials 2',3'-Dihydroxy-4',6'-dimethoxychalcone (DDC) was synthesized and provided by Pharmedica (Kyoto, Japan). The following materials were used in this study: HaCaT cell lines purchased from Cell Lines Service (Hidelerberg, Germany); Dulbecco's modified eagle medium (DMEM) purchased from Nissui Pharmaceutical (Tokyo, Japan); fetal bovine serum (FBS) purchased from JRH Biosciences (Lenexa, KS, U.S.A.); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) purchased from Nacalai Tesque (Kyoto, Japan); zinc protoporphyrin IX (ZnPP IX) purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.); penicillin–streptomycin purchased from GE Healthcare (Buckinghamshire, U.K.); a Blak-Ray UV Bench Lamp and CM-H₂DCFDA purchased from Thermo Fisher Scientific (Waltham, MA, U.S.A.); anti-HO-1 antibody (Code #ADI-SPA-895) purchased from Enzo Biochem Inc. (Stressgen, Victoria, Canada); anti- γ -GCS (Code #H-300) purchased from Santa Cruz Biotechnology (CA, U.S.A.); anti-NQO1 (Code #ab34173) purchased from Abcam (Cambridge, U.K.); anti- β -actin (Code #A1978; Clone AC-15) antibodies purchased from Sigma (St. Louis, MO, U.S.A.); and anti-rabbit (Code #NA934) and anti-mouse (Code #NA931) immunoglobulin G (IgG) horseradish peroxidase-linked whole antibodies purchased from GE Healthcare (Buckinghamshire, U.K.).

Cell Culture HaCaT cells were cultured in DMEM comprising 10% FBS and 1% penicillin–streptomycin at a density

of 5.0×10^4 cells/cm² in a 5%-CO₂ environment at 37°C in a humidified 5% CO₂ atmosphere. Cells were plated on 96-well plates or 100-mm dishes and cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μ g/mL streptomycin. The media was replaced every 3 d.

UV-A Irradiation For UV-A irradiation, a 365-nm Blak-Ray UV Bench Lamp (XX-15 BLB; Thermo Fisher Scientific, Waltham, MA, U.S.A.) was used and irradiation was performed approximately 5 cm from the cells. The energy emitted from the UV irradiation was measured using a VLX-3W (CSV). The irradiance level was expressed as the energy per unit area (J/cm²); the time to reach 1 J was approximately 12 min.

Evaluation of Cell Viability Cells on the fifth day of culture were used for analysis. The evaluation of cell viability was performed using the MTT assay. The survival rate in each treatment group was calculated assuming that the survival rate in the control group was 100%.

Measurement of Intracellular ROS Production Intracellular ROS levels were measured by microscopic analysis. Cells were incubated for 30 min in a Krebs–Ringer solution comprising 20 μ M CM-H₂DCFDA, followed by UV-A irradiation. Cells were then incubated with a buffer comprising 1% Triton X-100, and fluorescence intensity was measured (Excitation/Emission = 485 nm/535 nm).

Western Blotting Western blotting was performed as previously described.¹¹⁾ Briefly, the culture media was collected, and cells were washed twice with cold Tris-buffered saline, harvested with a cell scraper, and lysed in a buffer comprising 20 mM Tris, 25 mM β -glycerophosphate, 2 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 2 mM dithiothreitol, and 1 mM vanadate on ice. Subsequently, the lysates were sonicated and centrifuged at 15000 rpm at 4°C for 30 min. After normalization of the protein concentrations, the supernatants were mixed in equal amounts with a sample loading buffer. Proteins were denatured by boiling for 5 min. The samples were then loaded onto a sodium dodecyl sulfate (SDS)-polyacrylamide gel, separated electrophoretically, and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, U.S.A.). To

Fig. 1. Effects of UV-A Irradiation on Intracellular ROS Levels (A) and Cell Viability (B) in HaCaT Cells

A: Cells were irradiated with UV-A (0–3 J/cm²) for 0–36 min by UV Lamp. Intracellular ROS levels were measured by microscopic analysis. B: Cells were irradiated with UV-A (0–5 J/cm²) for 0–60 min by UV Lamp. Cell viability was determined by MTT assay ** $p < 0.01$, *** $p < 0.001$, compared with control (Cont). Data are expressed as mean $\pm$ S.E.M. of $n = 4$ independent observations. The statistical significance of between-group differences was determined by Tukey's test after one-way ANOVA.

Fig. 2. Effects of DDC on UV-A-Induced Intracellular ROS Production in HaCaT Cells

Cells were treated with DDC (3 μM) for 24h and then irradiated with UV-A (3 J/cm²) for 36min by UV Lamp. *** $p < 0.001$ compared with control (Cont). # $p < 0.01$, compared with UV-A irradiation. Data are expressed as mean $\pm$ S.E.M. of $n = 12$ independent observations. The statistical significance of between-group differences was determined by Tukey's test after one-way ANOVA.

block nonspecific protein binding, the PVDF membrane was incubated with 10mM Tris-buffered saline comprising 0.1% Tween-20 and 5% dehydrated skim milk. Subsequently, the PVDF membrane was incubated again with primary antibodies (anti-HO-1, anti-γ-GCS, anti-NQO1 or anti-β-actin) and secondary antibodies (anti-rabbit or anti-mouse IgG horseradish peroxidase-linked whole antibody). Finally, we performed immunodetection with an enhanced chemiluminescence detection system (GE Healthcare). Band intensities were measured using the ImageJ software (National Institutes for Health, Bethesda, MD, U.S.A.).

Drug Treatment DDC was administered from day 3 to 48h after cell plating or from day 4 to 24h after plating.

Statistical Analysis All data were expressed as mean $\pm$ standard error of the mean (S.E.M.). The statistical significance of between-group differences was determined by Tukey's test after one-way ANOVA using the Prism software (GraphPad Software, San Diego, CA, U.S.A.). p -Value of < 0.05 was considered statistically significant.

RESULTS

Effects of UV-A Irradiation on ROS Production and Cell Viability

We examined the effects of UV-A irradiation

Fig. 3. Effects of DDC on Cell Viability and UV-A-Induced Cytotoxicity in HaCaT Cells

A: Cells were treated with DDC (1–100 μM) for 48h. B: Cells were treated with DDC (1–3 μM) for 24h and then irradiated with UV-A (3 J/cm²) for 36min by UV Lamp. Thereafter, cells were treated with DDC (1–3 μM) for 24h. C: Cells were irradiated with UV-A (3 J/cm²) with or without pretreatment for 24h and post-treatment with DDC (3 μM) for 24h. *** $p < 0.001$ compared with control (Cont). ### $p < 0.001$ compared with UV-A irradiation. Data are expressed as mean $\pm$ S.E.M. of $n = 4$ (A) and $n = 8$ (B, C) independent observations. The statistical significance of between-group differences was determined by Tukey's test after one-way ANOVA.

on intracellular ROS levels in HaCaT cells. UV-A (1–3 J/cm²) irradiation at 365 nm increased ROS production in an energy-dependent manner, and a significant increase in ROS production was observed after irradiation at 3 J/cm² (Fig. 1A). In addition, we examined cell viability by UV-A irradiation in HaCaT cells. After 24 h of UV-A irradiation (1–5 J/cm²), cell viability decreased in an energy-dependent manner (Fig. 1B).

Effects of DDC on ROS Production by UV-A Irradiation

Because significant ROS production was observed after UV-A irradiation at 3 J/cm², this irradiation level was used for subsequent analysis. When cells were pretreated with DDC (3 μ M)

for 24 h before UV-A irradiation, ROS production by UV-A irradiation was significantly suppressed at a concentration of 3 μ M (Fig. 2).

Cytoprotective Effects of DDC against UV-A-Induced Cell Injury When HaCaT cells were treated with DDC (1–30 μ M) for 48 h, no change in cell viability was observed at a concentration of $\leq$ 30 μ M (Fig. 3A). Therefore, DDC was used at a concentration of $\leq$ 3 μ M in the subsequent analysis. When cells were pretreated with DDC (1–3 μ M) for 24 h before UV-A irradiation (3 J/cm²) and for 24 h after UV-A irradiation, DDC at a concentration of 3 μ M demonstrated a significant cytoprotective effect (Fig. 3B). When cells were pretreated with DDC (3 μ M) for 24 h before UV-A irradiation (3 J/cm²) and the cells were then treated for another 24 h after irradiation or when given alone for 24 h, the same degree of cytoprotection was obtained (Fig. 3C). However, when cells were pretreated with DDC for 24 h only after UV-A irradiation (3 J/cm²), no cytoprotective effect was observed.

Induction of HO-1 Expression after DDC Administration We examined the cytoprotective mechanism by DDC. To date, studies using PC12 cells have reported that DDC treatment increases the expression levels of HO-1, γ -GCS, and NQO1.^{10,11)} Therefore, with an aim of elucidating the protective mechanism of DDC against UV-A-induced cytotoxicity in HaCaT cells, the influence of DDC on the expression levels of HO-1, γ -GCS, and NQO1 was examined (Figs. 4A, B). When the cells were pretreated with DDC (1–3 μ M) for 24 h, the expression levels of HO-1 increased in a concentration-dependent manner. Conversely, the expression levels of γ -GCS and NQO1 were not changed after DDC treatment (Figs. 4C, D).

Involvement of HO-1 in the Cytoprotective Action of DDC against UV-A-Induced Cell Injury We investigated the involvement of HO-1 in the cytoprotective action of DDC in HaCaT cells. Pretreatment with the HO-1 inhibitor zinc protoporphyrin IX (ZnPP IX: 3 μ M), followed by DDC treatment (3 μ M) before UV-A irradiation (3 J/cm²) for 24 h reduced ROS production (Fig. 5A). However, when cells were

Fig. 4. Effects of DDC on the Expression Levels of HO-1 in HaCaT Cells

A, C, D: Representative image of Western blotting analysis. Cells were treated with DDC (0–3 μ M) for 24 h, and total cell lysates were analyzed. B: HO-1/ β -actin signal ratio was quantified and expressed as the fold change from the value of control (Cont). *** p <0.001 compared with Cont. Data are expressed as mean $\pm$ S.E.M. of n =3 independent observations. The statistical significance of between-group differences was determined by Tukey's test after one-way ANOVA.

Fig. 5. Involvement of HO-1 in the Decreased Intracellular ROS Production and Cytoprotection Induced by DDC in HaCaT Cells

A: Cells were treated with DDC (3 μ M) and zinc protoporphyrin IX (ZnPP IX: 3 μ M) for 24 h and then irradiated with UV-A (3 J/cm²) for 36 min by UV Lamp. B: Cells were treated with DDC (3 μ M) and ZnPP IX (3 μ M) for 24 h and then irradiated with UV-A (3 J/cm²) for 36 min by UV Lamp. Thereafter, cells were treated with DDC (3 μ M) and ZnPP IX (3 μ M) for 24 h. *** p <0.001 compared with control (Cont). ### p <0.001 compared with UV-A irradiation. + p <0.05, +++ p <0.001 compared with Cont. Data are expressed as mean $\pm$ S.E.M. of n =4 independent observations. The statistical significance of between-group differences was determined by Tukey's test after one-way ANOVA.

pretreated with ZnPP IX ($3\mu\text{M}$) and DDC ($3\mu\text{M}$) for 24 h before and after UV-A irradiation (3 J/cm^2), the cytoprotective effect was suppressed (Fig. 5B).

DISCUSSION

It has been previously shown that DDC activates the Nrf2–ARE pathway and this activation can induce HO-1, an important antioxidant enzyme. In addition, our results indicate that DDC has a protective effect against UV-A-induced cytotoxicity in HaCaT cells.

Increased intracellular ROS production is considered to be a major factor in UV-A-induced cell injury in skin cells.^{6,7)} In fact, it has been reported that α -tocopherol, an antioxidant vitamin, suppresses UV-A-induced skin cell injury.¹²⁾ In the present study, a 24-h pretreatment regimen with DDC significantly suppressed ROS production. In addition, a 24-h pretreatment was required to observe the protective effect of DDC against UV-A-induced cytotoxicity. These results suggest that DDC suppresses skin cell injury by suppressing ROS production and changing the intracellular environment, such as the induction of antioxidant enzymes *via* the Nrf2–ARE pathway before UV-A irradiation.

Next, to elucidate the protective mechanism of DDC against UV-A-induced cytotoxicity, changes in the expression levels of HO-1, γ -GCS, and NQO1 induced by the activation of the Nrf2–ARE pathway were investigated. Western blotting analysis revealed a marked increase in the expression level of HO-1 after the 24-h treatment with $3\mu\text{M}$ DDC. Conversely, no increase was observed in the expression levels of γ -GCS and NQO1. Therefore, we investigated the involvement of HO-1 in the cytoprotective mechanism of DDC against UV-A-induced cytotoxicity. The protective action of DDC against UV-A-induced cell injury was significantly suppressed by the HO-1 inhibitor ZnPP IX. Taken together, these results suggest that increased HO-1 greatly contributes to the protective action of DDC against UV-A-induced cytotoxicity. These findings are consistent with those of previous reports stating that DDC upregulates the expression of HO-1 in astrocytes and microglia of the substantia nigra region of C57BL/6N mice and of primary mesencephalic cultures.¹¹⁾ Similar to our findings, a previous study has also reported that increased intracellular HO-1 levels are cytoprotective against oxidative stress *via* the Nrf2–ARE pathway in PC12 cells.¹³⁾ However, a study has also reported that the expression levels of HO-1, γ -GCS, and NQO1 were increased in PC12 cells by DDC.¹⁰⁾ In addition, it has been reported that the expression levels of HO-1, γ -GCS, and NQO1 are increased by sulforaphane, which is known to induce antioxidant enzymes in HaCaT cells by activating the Nrf2–ARE pathway.¹⁴⁾ In this study, the expression level of HO-1 was increased in HaCaT cells but those of γ -GCS and NQO1 remained unchanged. The following two points should be considered regarding these differences noted. The first is the difference in the treatment concentration of DDC. Because HaCaT cells are more susceptible to DDC cytotoxicity than PC12 cells and the concentration examined in HaCaT cells was lower than that in PC12 cells, the induction of the expressions of γ -GCS and NQO1 may not have been detected. The second may be due to the differences in the compounds that induce antioxidant enzymes by activating the Nrf2–ARE pathway. It has been reported that different compounds have

different points of action and they induce different types of antioxidant enzymes.^{15,16)} Furthermore, certain Nrf2 activator was reported to fail to increase the protein levels of γ -GCS and NQO1, although the mRNA levels of them were up-regulated.¹⁷⁾ Sulforaphane is not only a powerful antioxidant phytochemical but also anti-inflammatory phytochemical with great promise in its ability to protect the nervous system from many diseases and toxins and reduce the symptomatic burden of multiple pervasive diseases.¹⁸⁾ There is a possibility that unknown factors should be involved in DDC-induced up-regulation of HO-1 in HaCaT cells in addition to Nrf2–ARE pathway.

HO-1 degrades heme to produce Fe^{2+} , carbon monoxide (CO), and biliverdin.¹⁹⁾ Biliverdin is converted to bilirubin, which has an antioxidant effect on oxidative stress.²⁰⁾ In addition, CO is known to activate the guanylcyclase (GC)–protein kinase (PK) G pathway and exhibits antiapoptotic effects by inducing downstream cellular signals.²¹⁾ Therefore, heme degradation products (CO, and biliverdin) are suggested to be involved in the HO-1-mediated cytoprotective action of DDC.

Recent reports have demonstrated that the Nrf2–ARE pathway is activated *in vivo* to inhibit UV-A-induced cell death and apoptosis through the induction of HO-1²²⁾ as well as compounds with antioxidant and/or UV-A absorption properties. Moreover, UV-A-induced melanogenesis can be mitigated through an indirect regulatory effect on the Nrf2–ARE pathway.²³⁾ The protective effect on 6-hydroxydopamine toxicity is being investigated in Parkinson's disease.²⁴⁾ Skin effects and Nrf2–ARE pathway activation have been studied for the treatment of various other diseases and disorders, including chronic obstructive pulmonary disease, endometritis, and osteoporosis, thus suggesting the efficacy of DDC in the treatment and management of these diseases.^{25–27)}

In recent years, DDC research has advanced for the purpose of exploring new compounds that enhance antioxidant function *in vivo*. Compounds that exhibit protective effects through the activation of the Nrf2–ARE pathway have also been identified in naturally derived plants other than green perilla.^{28–31)} Some of these compounds are derived from food sources and are considered safe and nontoxic^{24,26)}; DDC used in this study is also a food-derived compound and is highly safe and approachable. Therefore, although further study is required in the future, DDC is expected to be useful in the prevention or treatment of skin damage caused by UV-A irradiation.

Taken together, our findings demonstrated that DDC exhibited a protective effect on HaCaT cells against ROS-induced injury caused by UV irradiation by induction of the expression of HO-1. It is speculated that DDC activated the Nrf2–ARE pathway as one of the mechanisms by which HO-1 was upregulated. The results of our study suggest the efficacy of DDC in the prevention or treatment of skin injury caused by UV-A irradiation.

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Conflict of Interest The authors declare no conflict of interest.

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