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Full Paper

Effects of 2'-3'-dihydroxy-4',6'-dimethoxychalcone derived from green perilla on auricle thickness in chronic contact dermatitis model mice

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ABSTRACT

Oxidative stress has been implicated in the pathogenesis of allergic contact dermatitis. The nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway, an *in vivo* antioxidant system, induces antioxidant enzymes. In our previous studies, we isolated 2',3'-dihydroxy-4',6'-dimethoxychalcone (DDC) from green perilla and identified it as a novel activator of the Nrf2-ARE pathway. We also discovered that it exerted cytoprotective effects against oxidative stress in PC12 cells. However, its effects on skin disease model animals *in vivo* remain unclear. In the present study, auricular thickness time-dependently increased with the repeated application of picryl chloride, and significant increases were observed from Day 2 in chronic contact hypersensitivity (cCHS) model mice. Histological changes, such as higher numbers of cells in the epidermis, were observed with increases in auricular thickness. The administration of DDC every two days from Day 6 suppressed the increases in auricular thickness and the number of scratching events in a dose-dependent manner. The expression levels of antioxidant enzymes increased in the mouse auricle 24 h after the administration of DDC. These results presume that DDC inhibits increases in auricular thickness in cCHS mice by up-regulating the expression of antioxidant enzymes through the activation of the Nrf2-ARE pathway.

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Introduction

Contact dermatitis (CD) is an eczematous inflammatory reaction that occurs when foreign irritants and antigens (haptens) contact the skin. There are two types of CD: irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD).¹ ICD is inflammation induced from the activation/disorder of various cells by a physical/chemical stimulation by the contacted substance, and ACD is an immune disease that is classified as a type IV allergic reaction. Chronic exposure of the skin to the causative agent of CD results in chronic CD, which is characterized by lichenification and thickening of

the skin, and a condition in which acute skin inflammation is also involved.

Oxidative stress is one of the pathogenic causes of ACD.^{2,3} Oxidative stress can occur in skin cells for a number of reasons, such as ATP production, which is always performed in the mitochondria and the infiltration of inflammatory cells to physically damaged sites.⁴ Regarding the mechanisms contributing to reactive oxygen species (ROS) production in ACD, when a chemical substance reaches the epidermis through the stratum corneum and binds to the Toll-like receptors (TLRs) of keratinocytes, tumor necrosis factor receptor-associated factor 6 (TRAF 6), an adapter for TLR signaling, is transferred to mitochondria and interacts with evolutionarily conserved signaling intermediates involved in the assembly of the respiratory chain complex protein, ECSIT, to undergo ubiquitination, which generates ROS.² ROS play an important

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role in regulating intracellular protein complex NLR family-pyrin domain-containing 3 protein (NLRP3) inflammasomes.² NLRP3 inflammasomes consist of the pattern recognition receptor, NLRP3, and the signal transduction factor, an apoptosis-associated speck-like protein that contains a caspase recruitment domain (ASC).⁵ ROS activate caspase-1 via NLRP3 inflammasomes to induce maturation and production by cleavage of the inflammatory cytokines interleukin (IL)-1 β and IL-18.

The pathway of the nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) is an *in vivo* defensive mechanism against oxidative stress that induces the expression of antioxidant enzymes.⁶ The transcription factor Nrf2 normally binds to Kelch-like ECH-associated protein 1 (Keap1) and remains in the cytoplasm; however, when an organism is exposed to oxidative stress, Nrf2 translocates into the nucleus and exists upstream of the gene. By binding to the ARE sequence, the expression of antioxidant enzymes, such as γ -glutamyl-cysteine synthetase (γ -GCS), heme oxygenase-1 (HO-1), and NAD(P)H: quinone oxidoreductase 1 (NQO1), is induced.^{7–9} Nrf2 has been implicated in this inflammatory response based on previous findings showing that an Nrf2 deficiency exacerbated inflammatory responses^{3,10} and the removal of ROS in Nrf2-deficient mice suppressed these responses.¹¹ A recent study reported that Nrf2 suppressed inflammatory responses by directly inhibiting the gene expression of IL-1 β and IL-6 without mediating the removal of ROS.¹²

In our previous studies, we have searched for food-derived compounds that activate the Nrf2-ARE pathway and successfully isolated and identified 2', 3'-dihydroxy-4', 6'-dimethoxychalcone (DDC).¹³ Furthermore, we revealed that DDC induced the antioxidant enzymes γ -GCS, HO-1, and NQO1 in rat adrenal pheochromocytoma-derived PC12 cells by activating the Nrf2-ARE pathway, which is cytoprotective against oxidative stress.¹³ However, the effects of DDC in skin disease model animals currently remain unclear.

Therefore, we herein examined the suppressive effects of DDC on auricular thickening *in vivo* in chronic contact hypersensitivity (cCHS) model mice.

Materials and methods

Materials

The drugs used in this study and their sources were as follows: picryl chloride (PCI), dexamethasone and DMSO from Nacalai Tesque (Kyoto, Japan); acetone from Junsei Chemical (Tokyo, Japan); olive oil, isoflurane, Mayer's hematoxylin solution and eosin Y solution from Fujifilm Wako (Osaka, Japan). 2', 3'-dihydroxy-4', 6'-dimethoxychalcone (DDC) was synthesized and provided by Pharmaeight (Kyoto, Japan). Anti-HO-1 antibody (Code #ADI-SPA-895) was purchased from Enzo Biochem Inc (Stressgen, Victoria, Canada). Anti- γ -GCS (Code #H-300) antibody was purchased from Santa Cruz Biotechnology (CA, USA). Anti-NQO1 (Code #ab34173) antibody was purchased from Abcam (Cambridge, UK), and anti- β -actin (Code #A1978; Clone AC-15) antibody was purchased from Sigma (St. Louis, MO, USA). Anti-rabbit (Code #NA934) or anti-mouse (Code #NA931) immunoglobulin [IgG] horseradish peroxidase-linked whole antibodies were purchased from GE Healthcare (Buckinghamshire, UK).

Experimental animals

The animal study was carried out in accordance with the guidelines of the Doshisha Women's College Animal Experimentation Committee and the Japanese Pharmacological Society. This

study was approved by Doshisha Women's College Animal Experimentation Committee.

Male ICR mice four weeks old (body weight 17–19 g) were purchased from Japan SLC (Shizuoka, Japan).

Induction of chronic contact hypersensitivity

As a hapten, 10 μ l of 1% picryl chloride (PCI) dissolved in a mixed solvent of acetone: olive oil = 4: 1 (AOO), which was applied to the front and back sides of the right auricle of the mice. DDC was dissolved in dimethyl sulfoxide (DMSO) and then dissolved in AOO. Dexamethasone was dissolved in AOO. Ten microliters of the drug was applied to both the front and back sides of the mouse right ear 2 h after the PCI stimulation.

Administration schedule

The protocol for PCI-induced mice can be seen in Fig. 1. Sensitization was performed by applying PCI to both the front and back sides of the mouse right auricle (Day –7). After 7 days (Day 0), elicitation was conducted by applying PCI every 2 days for 12 days. DDC administration was initiated from Day 6 (Fig. 2A and Figs. 4–7), Day 0 (Fig. 2B), and Day –7 (Fig. 2C).

Assessment of auricular thickness

The thickness of the mouse auricle was measured using a thickness gauge (SM-528, TECLOCK).

Assessment of scratching behavior

Scratching behavior was evaluated by counting the number of incidents of scratching of the auricle, back, abdomen, or face in mice for 10 min assessment periods. Scratching behavior tests were conducted on Day 6 and Day 14.

Western blotting

The mouse auricle was cut on dry ice and homogenized in a lysis buffer containing 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. Next, we sonicated the lysates and centrifuged them at 15,000 rpm at 4 °C for 30 min. After normalisation of protein concentrations, we mixed the supernatants in equal amounts with a sample loading buffer. Protein was denatured by boiling for 5 min. Then, the samples were loaded onto a SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). To block non-specific protein binding, the PVDF membrane was incubated with 10 mM Tris-buffered saline containing 0.1% Tween-20 and 5% dehydrated skim milk. Then, the PVDF membrane was reincubated with primary antibodies (anti-HO-1, anti- γ -GCS, anti-NQO1, or anti- β -actin) and secondary antibodies (anti-rabbit or anti-mouse immunoglobulin [IgG] horseradish peroxidase-linked whole antibodies). 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, Maryland, USA).

Histology

Pieces of mouse ear were dissected and embedded in Tissue Tek OCT Compound (Sakura Finetek, Tokyo, Japan), rapidly frozen with

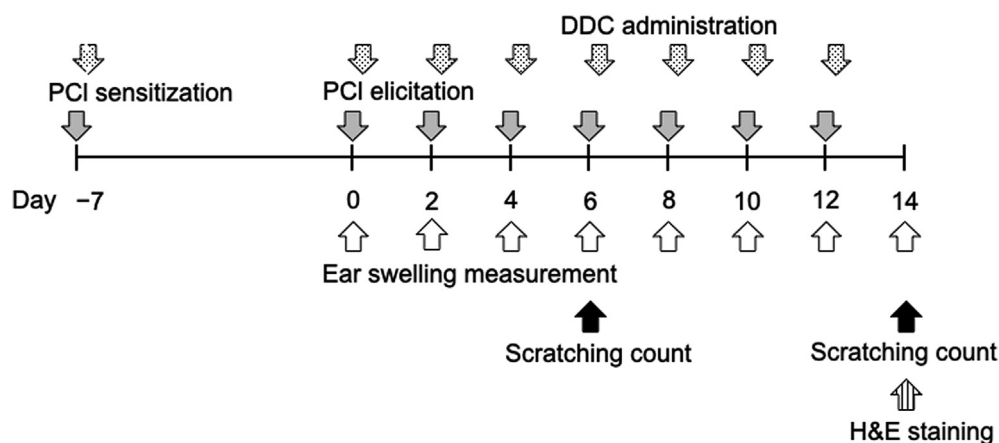


Fig. 1. Protocol for PCI-induced cHS mice. The right ear of mice was sensitized with 1% PCI (Day -7). Seven days after sensitization (Day 0), the same ear was exposed to PCI every 2 days for 12 days. The drug (DDC and Dexamethasone) was administrated 2 h after the PCI treatment.

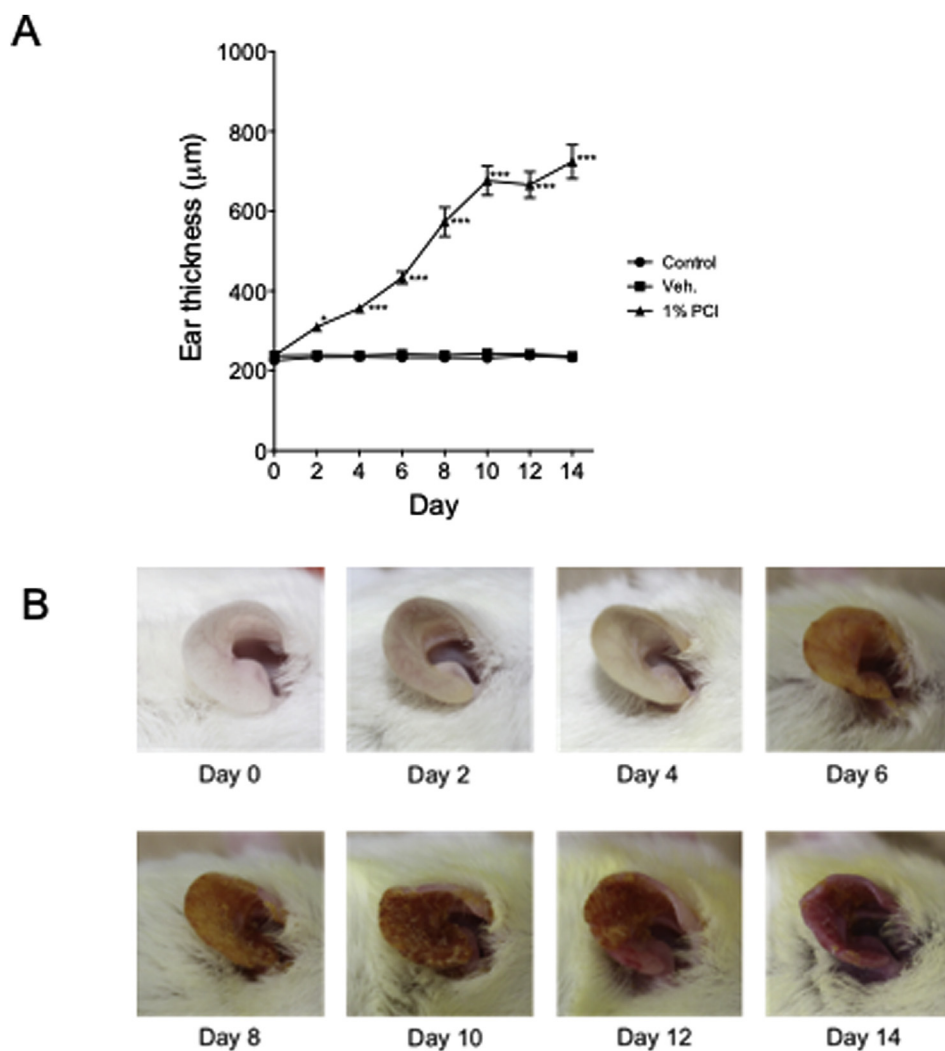
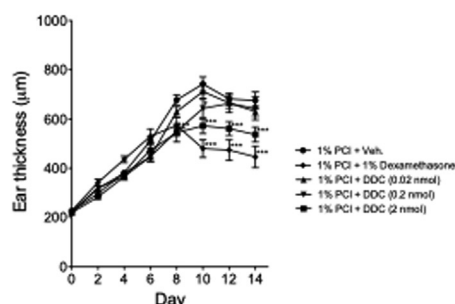
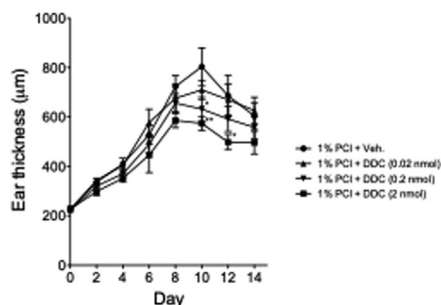


Fig. 2. Ear swelling reaction in PCI-induced cHS mice. A, B: The right ear of mice was sensitized with PCI (Day -7) and repeatedly challenged with PCI every 2 days from Day 0 to Day 12. A: Right ear thickness was measured with a thickness gauge. B: Representative clinical features of the right ear from Day 0 to Day 14. * $P < 0.05$, *** $P < 0.001$ compared with vehicle (Veh.) alone. Data are expressed as mean $\pm$ SEM of $n = 17$ –18 independent observations. The statistical significance of between-group differences was determined by Bonferroni posttest after two-way repeated measures analysis of variance.

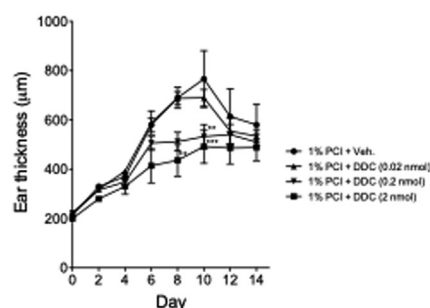
A DDC administration started on Day 6



B DDC administration started on Day 0



C DDC administration started on Day -7



D

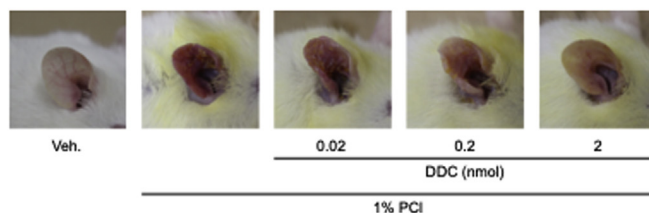


Fig. 3. Effects of DDC on ear swelling in PCI-induced cHS mice. A–D: The right ear of mice was repeatedly challenged with PCI every 2 days from Day 0 to Day 12. DDC (0.02–2 nmol) and 1% dexamethasone were administered every 2 days from Day 6 (A, D), Day 0 (B), and Day -7 (C). A, B, C: Right ear thickness was measured with a thickness gauge. D: Representative clinical features of the right ear on Day 14. The right ear of mice was repeatedly challenged with PCI every 2 days from Day 0 to Day 12. DDC (0.02–2 nmol) were administered every 2 days from Day 6. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ significantly different from vehicle (Veh.) alone. Data are expressed as mean $\pm$ SEM of $n = 13$ –20 (A) or $n = 5$ (B,C) independent observations. The statistical significance of between-group differences was determined by Bonferroni posttest after two-way repeated measures analysis of variance.

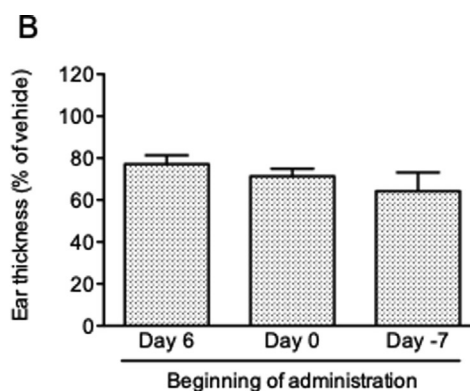
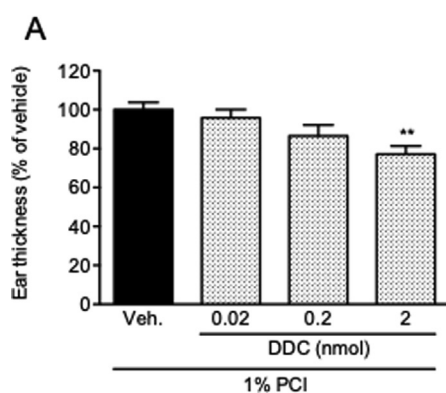


Fig. 4. Dose-dependent and administration period-dependent effects of DDC on ear swelling in PCI-induced cHS mice. A: Ear thickness of the DDC (0.02, 0.2, 2 nmol)- and vehicle (Veh.)-treated groups from Day 6. B: Ear thickness of the DDC (2 nmol)-treated groups from Day 6, Day 0, and Day -7. ** $P < 0.01$ compared with Veh. alone. Data are expressed as mean $\pm$ SEM of $n = 13$ –20 (A) or $n = 5$ –20 (B) independent observations. The statistical significance of between-group differences was determined by Tukey's *post-hoc* test after one-way analysis of variance.

dry ice, and stored at -80°C . Then, a $7\text{ }\mu\text{m}$ -thick section was prepared with a cryostat (Leica Instruments, Nussloch, Germany).

The slices were fixed with 10% formalin neutral buffer solution, and stained in hematoxylin solution and eosin solution for about 3 min at room temperature, respectively.

Statistical analysis

All data were expressed as mean $\pm$ standard error of the mean (SEM). The statistical significance of between-group differences was determined by Tukey's *post-hoc* test after one-way analysis of

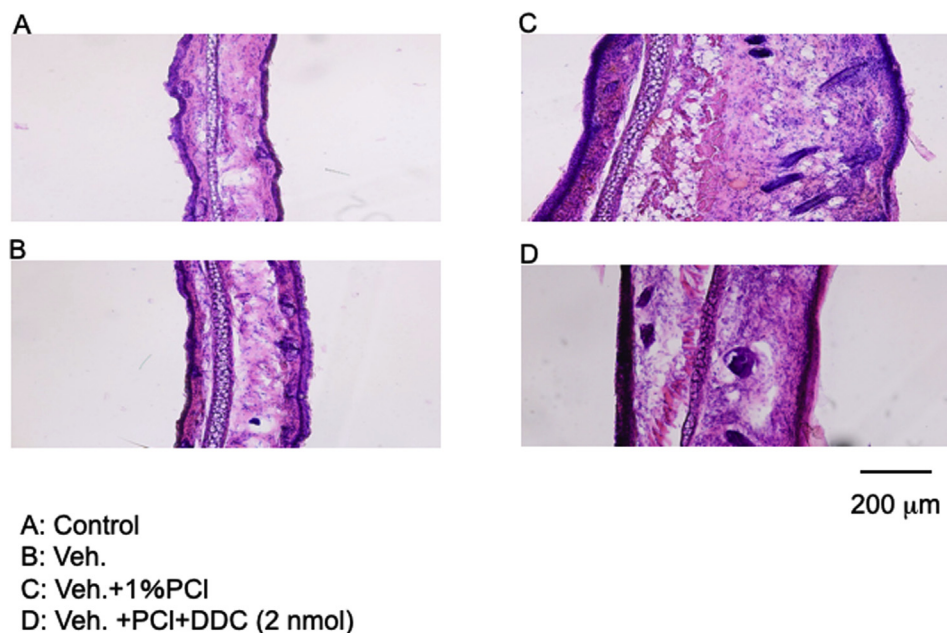


Fig. 5. Representative images of hematoxylin-eosin staining of ear tissue in PCI-induced cCHS mice. The right ear of mice was repeatedly challenged with PCI every 2 days from Day 0 to Day 12. Histopathological changes in slide sections of ear tissue were identified by staining with hematoxylin and eosin followed by observations on Day 14. Scale bar, 200 μm. Veh.: vehicle, DDC: 2', 3'-dihydroxy-4', 6'-dimethoxychalcone.

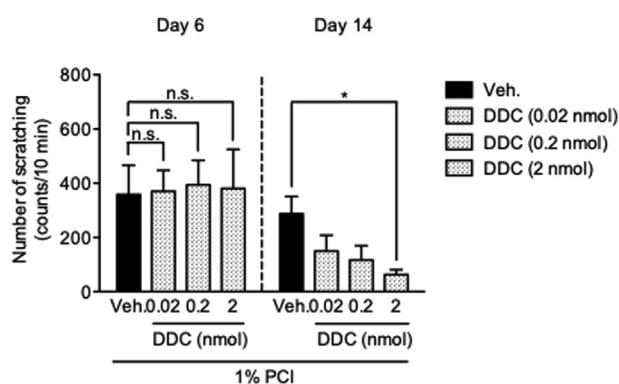


Fig. 6. Effects of DDC on the number of scratching events in PCI-induced cCHS mice. The ear of mice was repeatedly challenged with PCI every 2 days from Day 0 to Day 12. DDC (0.02–2 nmol) was administered every 2 days from Day 6, and the number of scratching events was suppressed in a dose-dependent manner of DDC on Day 14. * $P < 0.05$ compared with vehicle (Veh.) alone. n.s.: not significant. Data are expressed as mean $\pm$ SEM of $n = 6$ independent observations. The statistical significance of between-group differences was determined by Tukey's *post-hoc* test after one-way analysis of variance.

variance or Bonferroni posttest after two-way repeated measures analysis of variance, using Prism software (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered to be statistically significant.

Results

Daily changes in auricular thickening in cCHS with the application of PCI

To examine the effects of PCI on skin tissue *in vivo*, we created a cCHS model. Sensitization was performed by applying PCI as a hapten to both the front and back sides of the right auricle of mice (Day –7). After seven days (Day 0), the application was repeated by applying PCI every 2 days for 12 days. Auricular thickness increased

in a time-dependent manner, with a significant difference being observed from Day 2 and the greatest change occurring between Days 10 and 14 (Fig. 2A, B).

Effects of DDC on auricular thickening in cCHS

The effects of DDC on auricular thickness were examined. When DDC (0.02–2 nmol) and dexamethasone, a steroidal anti-inflammatory drug, were administered every 2 days from Day 6, the time point at which auricular thickening due to the application of PCI was sufficient, thickening was suppressed (Fig. 3A, D). When DDC (0.02–2 nmol) was administered every two days from Day 0, the time point at which no thickening was observed, and Day 7, the time point at which sensitization was performed, auricular thickening was suppressed in a dose-dependent manner (Fig. 3B, C).

DDC dose and administration time-dependent effects in cCHS

When the administration of DDC (0.02–2 nmol) was initiated on Day 6, auricular thickness peaked on Day 10. We calculated the rate of inhibition of auricular thickening on Day 10. The dose dependency of DDC was observed (Fig. 4A). We also calculated the suppression rate of auricular thickening on Day 10 when the administration of DDC (2 nmol) was initiated on Day 6, Day 0, and Day –7. The results obtained showed that the earlier the initiation of DDC administration, the more strongly auricular thickening was suppressed (Fig. 4B).

Changes in hematoxylin and eosin-stained images of the mouse auricle with the application of DDC in cCHS

To examine changes in the histology of the mouse auricle in cCHS mice with the application of DDC, an analysis was performed based on hematoxylin and eosin staining of auricles on day 14 (Fig. 5). Nuclei stained blue purple, while cell membranes, connective tissue, and fibers stained red. Histological changes were

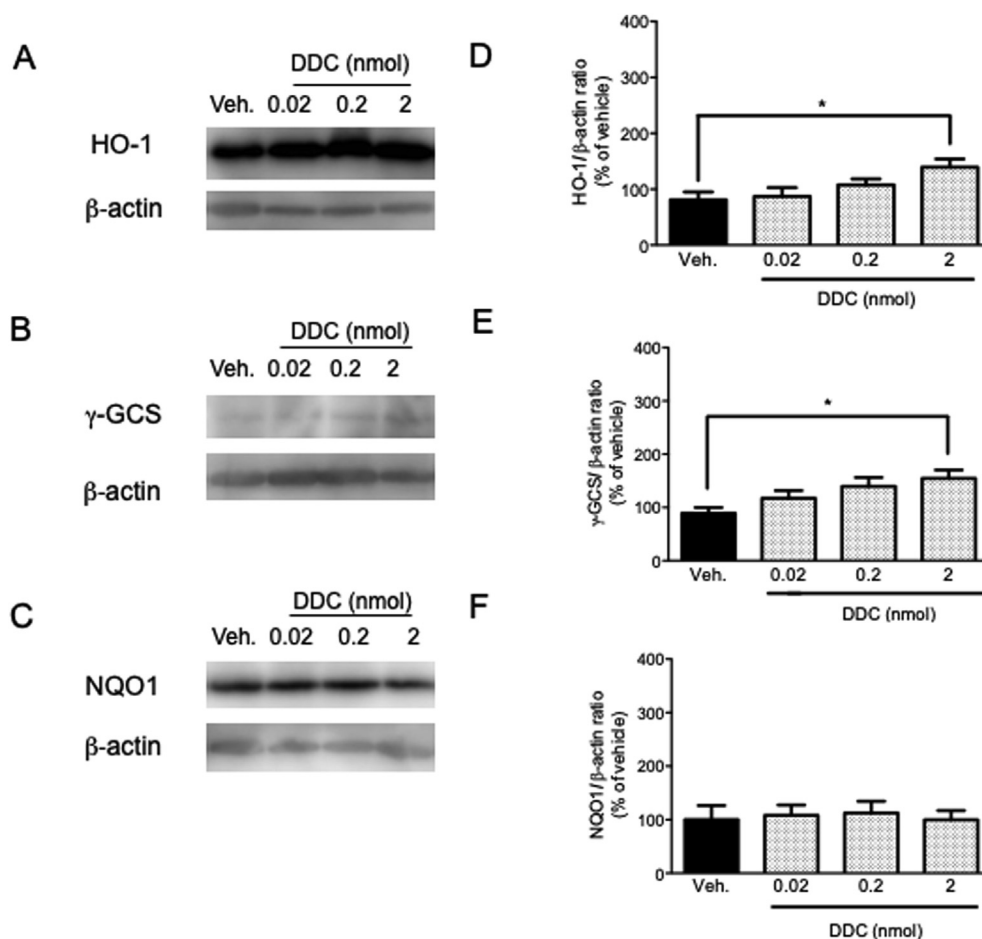


Fig. 7. Effects of DDC on HO-1 and γ -GCS expression in PCI-induced cCHS mice. A, C, E: Representative image of a Western blot analysis. The ear was treated with DDC (0.02–2 nmol) for 24 h. Total ear lysates were analyzed. B: The HO-1/ β -actin signal ratio was quantified and expressed as a fold change from the value of vehicle (Veh.) alone. D: The γ -GCS/ β -actin signal ratio was quantified and expressed as a fold change from the value of Veh. F: The NQO1/ β -actin signal ratio was quantified and expressed as a fold change from the value of Veh. * $P < 0.05$ compared with Veh. Data are expressed as mean $\pm$ SEM of $n = 5$ –8 (B), $n = 9$ (D) or $n = 5$ –7 (F) independent observations. The statistical significance of between-group differences was determined by Tukey's *post-hoc* test after one-way analysis of variance.

observed in the PCI-treated auricle, including thickening of the epidermis and dermis and an increase in the number of cells in the epidermis (Fig. 5C). With the application of DDC (2 nmol), auricular thickening was suppressed (Fig. 5D).

Effects of DDC on scratching behavior

Scratching behavior was evaluated on the first day of DDC (0.02–2 nmol) administration (Day 6) and on the final day (Day 14) of administration. Scratching behavior was suppressed dose-dependently on Day 14 (Fig. 6).

Induction of antioxidative enzyme expression in mouse ear tissue by DDC administration

In order to clarify the mechanisms underlying the suppressive effects of DDC on auricular thickening and scratching behavior in cCHS mice, the expression levels of antioxidant enzymes were examined. The expression levels of the antioxidant enzymes HO-1 and γ -GCS slightly increased in the mouse ear tissue 24 h after the administration of DDC (Fig. 7A–D). However, no changes were observed in the expression level of NQO1 (Fig. 7E, F).

Discussion

In the present study, we revealed that DDC exerted suppressive effects on auricular thickening and scratching behavior in cCHS mice and increased the expression levels of antioxidative enzymes. We used a cCHS model mouse as an evaluation system for the inflammatory responses that cause skin redness, auricular thickening, and scratching behavior *in vivo*. The cCHS mouse model is a standard method using basic techniques that has long been employed by many researchers,^{14,15} and comparisons with conventional data are easy.¹⁶ Preparation of the cCHS model using mice is established for the purpose of analyzing the pathological conditions of chronic CD.¹⁷ Furthermore, in research on pruritus, as the usefulness and evaluation methods of scratching behavior of mice have been established,¹⁸ developmental research of drugs for pruritus using mice are currently being conducted.

To investigate the effects of DDC on skin disease model animals *in vivo*, we attempted to create a cCHS model mouse as an evaluation system for the inflammatory reaction causing redness, auricular thickening, and scratching behavior. In cCHS mice, auricular thickness increased over time with the repeated application of PCI, and significant auricular thickening was observed from Day 2. This is consistent with previous findings that the

showed application of PCI in BALB/c mice resulted in auricular thickening.¹⁹

To examine the effects of DDC on auricular hypertrophy, DDC and the steroidal anti-inflammatory drug dexamethasone as a positive control were administered from Day 6, the time point at which auricular thickening due to the application of PCI was sufficiently observed. The results obtained showed that auricular thickening was suppressed by DDC in a dose-dependent manner. This is consistent with previous findings that showed the administration of dexamethasone suppressed auricular thickening.¹⁹ Furthermore, to clarify the preventive effects of DDC on auricular thickening, DDC was administered from Day 0, the time point at which sensitization was not performed, and Day -7, the time point at which sensitization was initiated. Auricular thickening was slightly suppressed in a manner that depended on the administration time. A previous study demonstrated that auricular thickening was significantly greater in Nrf2-deficient mice than in wild-type mice³; therefore, the Nrf2-ARE-activating effects of DDC may contribute to the suppressive effects observed on auricular thickening.

Hematoxylin and eosin staining was performed to examine DDC-induced histological changes with auricular thickening. Histological changes were observed in the PCI-treated auricle, including the thickening of the epidermis and dermis and an increase in the number of cells in the epidermis. The application of DDC also suppressed increases in auricular thickness. Although a previous study showed that the application of PCI increased the thicknesses of the epidermis and dermis,²⁰ other studies demonstrated that the thickness of the epidermis increased, whereas that of the dermis decreased.²¹ This issue needs to be examined in more detail in future studies.

To investigate the effects of DDC on scratching behavior, the number of scratching events in mice was counted. Auricular thickening was suppressed in a dose-dependent manner on Day 14 when DDC was administered from Day 6, the time point at which auricular thickening was sufficient. Dexamethasone has been shown to suppress the thickness of the auricle with the application of PCI, similar to DDC, but has also been found to induce scratching behavior.¹⁹ However, the anti-inflammatory effects of the immunosuppressive drug tacrolimus have been found to be weaker than those of dexamethasone, but inhibited scratching behavior.^{22,23} These findings indicate that the induction of scratching behavior involves a mechanism that is not suppressed by dexamethasone. These results suggest that DDC exerts inhibitory effects on swelling and scratching behavior, which are signs of inflammation in cCHS.

To elucidate the mechanism of action of DDC, we examined changes in the expression levels of HO-1, γ -GCS, and NQO1. It was revealed that the expression levels of HO-1 and γ -GCS in the auricle increased 24 h after the administration of DDC, but no changes were observed in the expression level of NQO1. These results suggest the involvement of HO-1 and γ -GCS in the suppressive effects of DDC on auricle thickening and scratching behavior.

DDC has been shown to increase the expression levels of HO-1, γ -GCS, and NQO1 in PC12 cells.¹³ DDC upregulated the expression of HO-1 in astrocytes and microglia of the substantia nigra of C57BL/6N mice brain,²⁴ and DDC also increased the HO-1 expression in astrocytes and microglia in primary mesencephalic cultures. A study on cCHS was performed using the same concentration range as that for C57BL/6N mice brain. It may be challenging to detect the induction of NQO1 expression in addition to HO-1 and γ -GCS due to the difficulties associated with penetrating skin tissue. In addition, the expression patterns of changes in antioxidant enzymes are dependent on age and tissue type.²⁵ As described above, differences in the expression levels of antioxidant enzymes *in vitro* and *in vivo* have been attributed to differences in treatment

densities, dosage concentrations, and the site of samples or cell-type examined. We could not perform experiments to support the concept that the Nrf2-ARE pathway is involved in the action of DDC in this study. Therefore, the relationship between the improvement of behavioral phenotypes and the induction of antioxidant enzymes is not elucidated in the present study. However, DDC is likely to inhibit increases in auricular thickness in cCHS mice by up-regulating the expression of antioxidative enzymes through the activation of the Nrf2-ARE pathway. In this respect, further studies are warranted.

DDC is a compound that has a basic skeleton of chalcone and is found in the leaves of *Uvaria dulcis* Dunal, which is a plant of a perennial family that inhabits the tropics.²⁶ Chalcone is an aromatic enone that belongs to the flavonoid family and is a precursor of flavone. Many chalcone derivatives have been found in plants²⁷ and have been reported to exert anti-inflammatory and antioxidant effects.^{28,29} Since DDC in the present study is a food-derived compound called green perilla, its safety is advantageous. Therefore, DDC is expected to be a useful lead compound in the prevention or treatment of ACD, the pathogenesis of which involves oxidative stress.

In conclusion, the green perilla-derived Nrf2-ARE pathway activator, DDC, exerted suppressive effects against auricular thickening and scratching behavior in cCHS mice. Furthermore, DDC increased the expression levels of antioxidant enzymes in cCHS mouse ear tissues. From these findings, DDC was presumed to suppress inflammatory responses in cCHS mice by activating the Nrf2-ARE pathway and increasing the expression levels of antioxidant enzymes. The present results also indicate that the inflammatory reactions of CD involve the activation of the Nrf2-ARE pathway, which may have potential as a therapeutic target for allergic skin diseases.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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