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Esculetin Prevents the Induction of Matrix Metalloproteinase-1 by Hydrogen Peroxide in Skin Keratinocytes

ORIGINAL ARTICLE

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Background: Reactive oxygen species (ROS) are involved in various cellular diseases. Excessive ROS can cause intracellular oxidative stress, resulting in a calcium imbalance and even aging. In this study, we evaluated the protective effect of esculetin on oxidative stress-induced aging in human HaCaT keratinocytes.
Methods: Human keratinocytes were pretreated with esculetin for 30 minutes and treated with H_2O_2. Then, the protective effects on oxidative stress-induced matrix metalloproteinase (MMP)-1 were detected by Flou-4-AM staining, reverse transcription-PCR, Western blotting, and quantitative fluorescence assay.
Results: Esculetin prevented H_2O_2-induced aging by inhibiting MMP-1 mRNA, protein, and activity levels. In addition, esculetin decreased abnormal levels of phospho-MEK1, phospho-ERK1/2, phospho-SEK1, phospho-JNK1/2, c-Fos, and phospho-c-Jun and inhibited activator protein 1 binding activity.
Conclusions: Esculetin prevented excessive levels of intracellular calcium and reduced the expression levels of aging-related proteins.
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Key Words: Esculetin, Reactive oxygen species, Matrix metalloproteinase-1, Aging

INTRODUCTION

Many stimuli could contribute to aging in various types of tissues, including the skin [1]. Skin cell aging, in particular, is promoted by complex mechanisms, such as mitochondrial dysfunction [2,3]. Epithelial cells are the outer most protective barrier for humans and have the ability to constantly self-regenerate [4]. Reactive oxygen species (ROS) in skin cells induce cell damage, resulting in skin disorders (e.g., cancer, aging, and inflammation) [5].

Enzymes of matrix metalloproteinase (MMP) family, especially MMP-1, play an important role in maintaining extracellular matrix, which is responsible for skin aging [6]. MMP-1 was reported to be upregulated and involved in lysis of dermal collagen [7]. H_2O_2 has been reported to induce ROS in cells, which

caused oxidative stress [8]. Oxidative stress promotes skin aging by increasing MMP-1 expression levels [9]; it could activate the mitogen-activated protein kinase (MAPK) signaling pathway and the transcription factor activator protein 1 (AP-1) [10].

Plants contain numerous antioxidant compounds. Esculetin, a natural coumarin, has many bioactivities, including antioxidant, antiviral, and anticancer [11]. It is extracted from plants, including *Fraxinus rhynchophylla* Hance, *Fraxinus chinensis* Roxb, and *Fraxinus aboana* Lingelsh [12]. We previously found that esculetin inhibits H_2O_2-induced oxidative stress by blocking ROS generation in Chinese hamster lung fibroblasts (V79-4) [13]. Thus, it protects against H_2O_2-induced cell damage. In vivo studies have also shown that esculetin has neuroprotective, anti-anxiety, and hepatoprotective effects [14-16].

Few studies have evaluated the protective effect of esculetin

against aging in skin cells. Therefore, we evaluated the effects of esculetin on aging induced by oxidative stress in human HaCaT keratinocytes.

MATERIALS AND METHODS

1. Materials

Esculetin (6,7-dihydroxycoumarin; Fig. 1A) was obtained from Wako Pure Chemicals (Tokyo, Japan) and was dissolved in dimethyl sulfoxide (DMSO).

2. Cell culture conditions

Human HaCaT keratinocytes were purchased from Cell Lines Service (Heidelberg, Germany). Keratinocytes were cultured in Dulbecco's modified Eagle's medium (Life Technologies Co., Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (Life Technologies Co.) and antibiotic-antimycotic (Life Technologies Co.) at $37^{\circ}C$ in 5% CO_2.

3. Cell viability assay

Human HaCaT keratinocytes (1.5×10^5 cells/mL) were seeded in 24 well plates and incubated for 16 hours. Subsequently, the cells were treated with esculetin (0, 1, 5, 10 µg/mL) for 30 minutes and then stimulated with H_2O_2 (1 mM) for 24 hours. Next, MTT

A

B

Figure 1. (A) Chemical structure of esculetin (6,7-dihydroxycoumarin). (B) Cell viability was measured using MTT assay. [a]$P < 0.05$ vs. control cells, [b]$P < 0.05$ vs. H_2O_2-treated cells.

was added into each well at 500 µg/mL and plates were incubated for 4 hours. Finally, the formazan crystals formed were dissolved in DMSO, and absorbance was measured by a scanning multi-well spectrophotometer at 540 nm.

4. Reverse transcription-PCR

Cells (1.5×10^5 cells/mL) were cultured in a 60-mm culture dish and sequentially treated with esculetin for 30 minutes and then with H_2O_2 (1 mM) for 24 hours. Total RNA was isolated using the Easy-BLUE™ Total RNA Extraction Kit (iNtRON Biotechnology Inc., Seongnam, Korea). Reverse transcription reaction buffer, primers, dNTPs, and Taq DNA polymerase were used to amplify cDNA. The harvested products and ×6 blue/orange loading dye were mixed. Proteins were resolved by electrophoresis on 1% agarose gels. Finally, the gels were stained with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology Inc.). Images were obtained under a UV light and analyzed using Image Quant™ TL analysis software (Amersham Biosciences, Uppsala, Sweden). The PCR conditions were as follows: initial denaturation at $94^{\circ}C$ for 5 minutes, followed by 30 cycles at $94^{\circ}C$ for 30 seconds, $55^{\circ}C$ for 30 seconds, and $72^{\circ}C$ for 1 minute. The primer sequences were as follows: human *MMP-1* forward (5′-GGAGGAAATCTTGCTCAT-3′) and reverse (5′-CTCAGAAAGAGCAGCATC-3′); human *GAPDH* forward (5′-TCAAGTGGGGCGATGCTGGC-3′) and reverse (5-TGCC AGCCCCAGCGTCAAAG-3′).

5. Western blot analysis

Cells were sequentially treated with esculetin and H_2O_2 as described above. Cells were harvested and lysed using a lysis buffer (120 mM NaCl, 40 mM Tris [pH 8], and 0.1% NP 40) on ice, and the protein concentration was detected using the Protein Assay Reagent Kit (Bio-Rad, Hercules, CA, USA). Aliquots of the protein solutions were electrophoresed on a 10% SDS PAGE and transferred onto nitrocellulose membranes. Subsequently, the membranes were shaken with primary and secondary antibodies (Invitrogen, Carlsbad, CA, USA). Protein bands were examined using an Enhanced Chemiluminescence Western Blotting Detection Kit (Amersham, Little Chalfont, UK). The primary MMP-1 (CSB-PA07009A0Rb) antibody was purchased from Cusabio Technology (Houston, TX, USA). Primary antibodies against SAPK/ERK kinase (SEK)1 (#9152), phospho-SEK1 (#9156), MAPK kinase (MEK)1 (#9124), phospho-MEK1 (#98195), c-Jun N-terminal kinase (JNK1/2) (#9252), phospho-JNK1/2 (#9251), c-Fos (#2250), and phospho-c-Jun (#9261) were purchased from Cell Signaling Technology (Danvers, MA, USA). The primary extracellular signal-regulated kinase (ERK) 2 (sc-1647) and
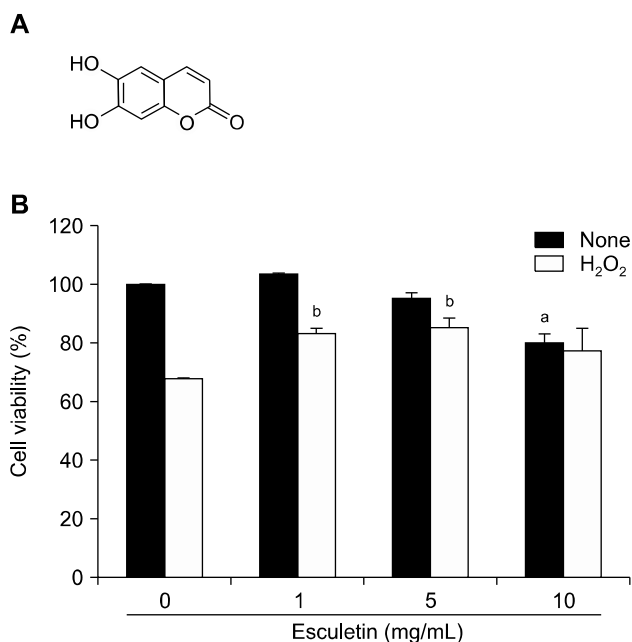
phospho-ERK1/2 (sc-7383) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary Actin (A2066) antibody was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) [17].

6. Matrix metalloproteinase-1 activity

MMP-1 activity was detected by a Fluorokine® E Human Active MMP-1 Fluorescent Assay Kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions [18].

7. Intracellular Ca^{2+} detection

To measure cellular Ca^{2+} levels, Fluo-4-AM (Molecular Probes, Eugene, OR, USA) was used for cell staining. Cells (2.0 × 10^5 cells/mL) were seeded on chamber slides and sequentially treated with esculetin and/or H$_2$O$_2$. The stained cells were imaged using the FV1200 laser scanning confocal microscope (Olympus, Tokyo, Japan).

8. Electrophoretic mobility shift assay

Nuclear proteins were extracted from harvested cells. AP-1-binding double-stranded DNA oligomers were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. DNA–protein complexes were resolved on a 5% polyacrylamide gel, electrophoresed at 200 V for 2 hours, and visualized by autoradiography [19].

9. Statistical analysis

Differences among groups were examined by one-way ANOVA with Tukey's tests. All tests were repeated three times independently and means ± SE were obtained. Statistical analysis was using the SigmaStat ver. 3.5 software (Systat Software Inc., San Jose, CA, USA). A value of $P \leq 0.05$ was considered statistically significant.

RESULTS

1. Protective effect of esculetin against H$_2$O$_2$-induced cell damage

MTT assay showed that treatment with 10 μg/mL esculetin induced death and damage in keratinocytes; however, treatment with 1 and 5 μg/mL esculetin protected cells from H$_2$O$_2$-induced toxicity (83% and 85%, respectively). Therefore, we used the optimal concentration of esculetin 5 μg/mL.

2. Effect of esculetin on reactive oxygen species-induced matrix metalloproteinase-1 expression and activation

H$_2$O$_2$-induced ROS upregulated MMP-1 at the mRNA and protein levels and esculetin inhibited this ROS-induced MMP-1 overexpression (Fig. 2A and 2B). We also found that H$_2$O$_2$ significantly induced the activation of MMP-1; pretreatment with esculetin blocked the high level of MMP-1 activation (Fig. 2C).
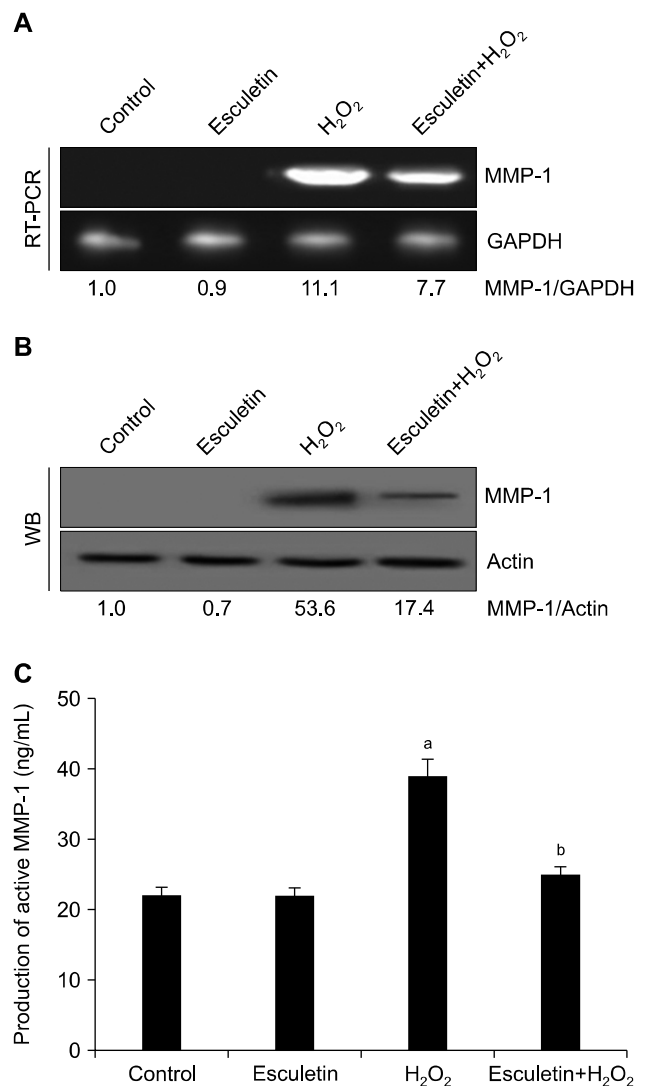
Figure 2. Effect of esculetin on H$_2$O$_2$-induced matrix metalloproteinase (MMP)-1 expression and activation. The mRNA (A) and protein levels of MMP-1 (B) were detected by reverse transcription (RT)-PCR and Western blotting (WB), respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and actin were used as loading controls. (C) MMP-1 activity was examined using a Human Active MMP-1 Fluorescent Assay Kit. [a]$P < 0.05$ vs. control cells, [b]$P < 0.05$ vs. H$_2$O$_2$-treated cells.

A

B
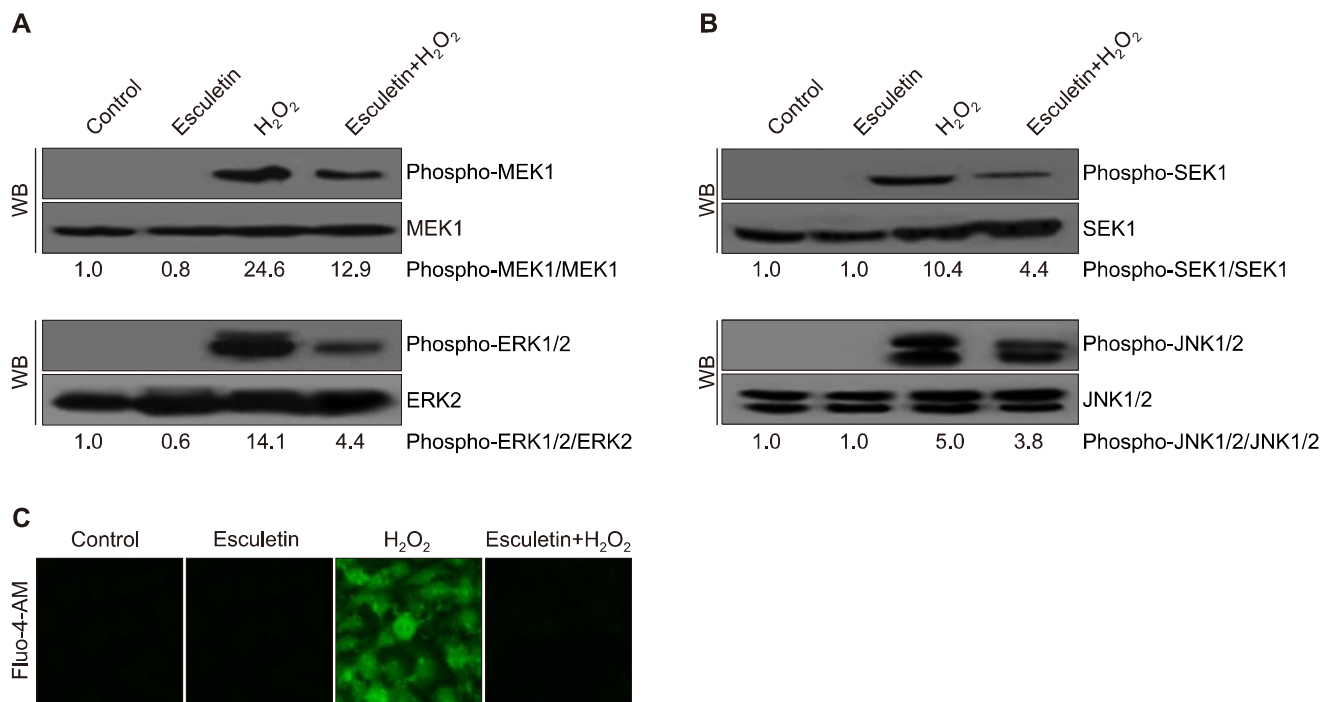
Figure 3. Effect of esculetin on H_2O_2-induced mitogen-activated protein kinases and intracellular Ca^{2+} levels. (A) Protein expression levels of phospho-MEK1 and phospho-ERK1/2 were detected by Western blotting (WB). (B) Expression levels of phospho-SEK1 and phospho-JNK1/2 were determined by WB. (C) Fluorescence indicating the Ca^{2+} level was captured by confocal microscopy using Flou-4-AM staining (×100).

A

B
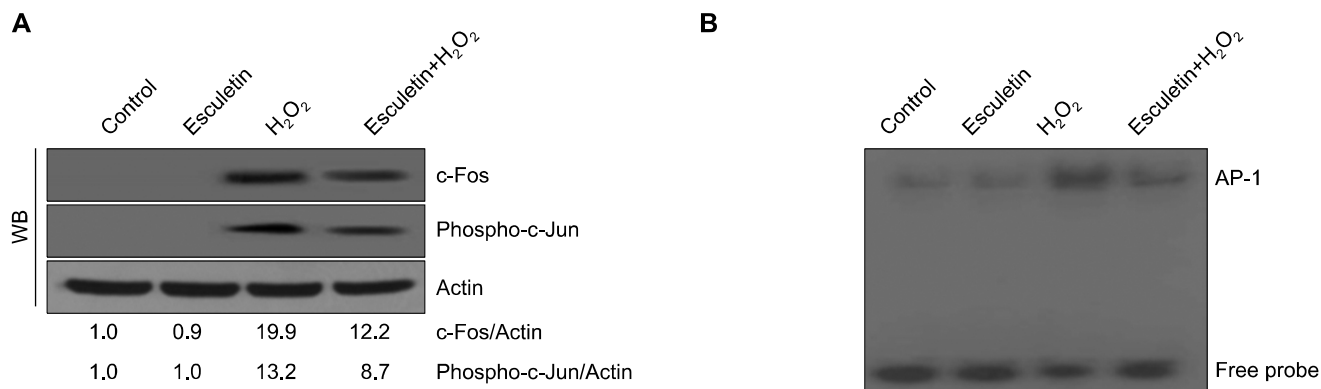
Figure 4. Effect of esculetin on H_2O_2-induced activator protein 1 (AP-1) expression. (A) Expression level of phospho-c-Jun and c-Fos determined by a Western blot (WB) analysis. Actin was used as a loading control. (B) AP-1 binding activity was assessed by electrophoretic mobility shift assay.

3. Effect of esculetin on H_2O_2-induced mitogen-activated protein kinases and intracellular Ca^{2+} accumulation

Our results showed that H_2O_2 enhanced the phosphorylation of MEK1 and ERK1/2 (Fig. 3A) and activated SEK1 and JNK1/2 (Fig. 3B). However, pretreatment with esculetin inhibited the activation of these four proteins (phospho-MEK1, phospho-ERK1/2, phospho-SEK1, and phospho-JNK1/2). A high level of intracellular

Ca^{2+} could activate MAPKs; thus, we evaluated intracellular Ca^{2+} levels [18]. We found that esculetin blocked the increase in intracellular Ca^{2+} levels induced by H_2O_2 (Fig. 3C).

4. Effect of esculetin on H_2O_2-induced activator protein 1 expression

Previous studies have shown that increase in MAPKs could regulate the heterodimerization of c-Jun/c-Fos as well as the nuclear transcription factor AP-1 [18,20,21]. Thus, we detected

these 3 proteins by Western blotting and found that c-Fos and phospho-c-Jun were induced by H_2O_2, and these levels were reduced by esculetin (Fig. 4A). In addition, esculetin inhibited H_2O_2-induced AP-1 binding to the promoter region (Fig. 4B).

DISCUSSION

Hydrogen peroxide (H_2O_2), an unstable ROS, is harmful to cells, influencing cell proliferation and differentiation [22]. It can freely pass through the cell membrane, resulting in damage, such as replicative senescence [23]. Increased ROS production has the potential to impair normal skin function, causing skin aging, cancer, and inflammation. Aging resulting from ROS-induced oxidative stress, affecting macromolecules (DNA, lipid, and protein), is a complex phenomenon [24]. Therefore, a proper exogenous antioxidant agent is necessary for the reconstruction of the skin barrier. Antioxidant effect is one of the main biological activities of esculetin and is involved in its anti-inflammatory and anti-proliferative effects [12]. Furthermore, esculetin has antiphotoaging and anticancer activities [25-28]. In this study, we examined the protective effect of esculetin on ROS-induced aging.

Excessive ROS results in an increase in intracellular Ca^{2+}, which promotes MMPs in the process of aging [29,30], and the long-term secretion of MMPs contributes to the degradation of collagen, especially MMP-1 [31]. Oxidative stress could stimulate MAPK signaling pathways, which regulate many transcription factors, including AP-1 [18]. Moreover, the binding of activated (phosphorylated) c-Jun and c-Fos to AP-1 promotes gene transcription, including *MMP-1* transcription [21,32].

Accordingly, we performed experiments to determine the effects of esculetin on ROS-induced aging. In particular, we found that the induction of MMP-1 mRNA and protein levels by ROS was reversed by esculetin. Similar results were obtained for MMP-1 activity. Furthermore, the excessive Ca^{2+} induced by ROS was decreased by esculetin. In addition, ROS activated the expression of MAPKs, and this activation was blocked by esculetin. Finally, transcription-related protein levels were balanced by esculetin via the inhibition of ROS.

In conclusion, esculetin shows anti-aging effects by inactivating the MAPK/AP-1 pathway. Thus, in vivo studies on anti-senescence activity of esculetin need to be carried out in the future.

ACKNOWLEDGMENTS

CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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