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Fisetin inhibits TNF-α-induced inflammatory action and hydrogen peroxide-induced oxidative damage in human keratinocyte HaCaT cells through PI3K/AKT/Nrf-2-mediated heme oxygenase-1 expression

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ABSTRACT

Oxidative skin damage and skin inflammation play key roles in the pathogenesis of skin-related diseases. Fisetin is a naturally occurring flavonoid abundantly found in several vegetables and fruits. Fisetin has been shown to exert various positive biological effects, such as anti-cancer, anti-proliferative, neuroprotective and anti-oxidative effects. In this study, we investigate the skin protective effects and anti-inflammatory properties of fisetin in hydrogen peroxide- and TNF-α-challenged human keratinocyte HaCaT cells. When HaCaT cells were treated with non-cytotoxic concentrations of fisetin (1–20 μM), heme oxygenase (HO)-1 mRNA and protein expression increased in a dose-dependent manner. Furthermore, fisetin dose-dependently increased cell viability and reduced ROS production in hydrogen peroxide-treated HaCaT cells. Fisetin also inhibited the production of NO, PGE_2 IL-1β, IL-6, expression of iNOS and COX-2, and activation of NF-κB in HaCaT cells treated with TNF-α. Fisetin induced Nrf2 translocation to the nuclei. HO-1 siRNA transient transfection reversed the effects of fisetin on cytoprotection, ROS reduction, NO, PGE_2, IL-1β, IL-6, and TNF-α production, and NF-κB DNA-binding activity. Moreover, fisetin increased Akt phosphorylation and a PI3K pathway inhibitor (LY294002) abolished fisetin-induced cytoprotection and NO inhibition. Taken together, these results provide evidence for a beneficial role of fisetin in skin therapy.

1. Introduction

The skin acts as a barrier between the body and external stimuli. It performs a critical role in homeostasis of the whole body, and is crucial for providing a protective covering [1]. The skin is subject to environmental damage and suffers oxidative cellular death from the effects of various reactive oxygen species (ROS). The skin also encounters multiple stimuli that cause inflammatory and immune responses [2]. Oxidative skin damage and skin inflammation play key roles in the pathogenesis of skin-related diseases and allergic reactions. Keratinocytes function mainly to form a barrier against environmental damage including UV radiation, heat, bacteria, viruses, parasites, and fungi. ROS and reactive nitrogen species (RNS) production in keratinocytes are stimulated by solar radiation. When pathogens start to invade the upper layers of the epidermis, keratinocytes react by producing pro-inflammatory mediators including interleukin (IL)-6 and tumor necrosis factor (TNF)-α, which attract inflammatory cells to the site of invasion [3]. Therefore, recent studies have considered keratinocytes, the predominant epidermal cells, as a target for innate and acquired skin immune responses [4].

Nuclear transcription factor erythroid-2-like factor 2 (Nrf2)-related heme oxygenase-1 (HO-1) has raised interest as a candidate for skin protection from oxidative damage and inflammation as it is involved in cellular antioxidant and anti-inflammatory defenses [5]. The transcription factor Nrf2 is known to trigger antioxidant response element (ARE)-related expression of phase 2 detoxifying genes. HO-1 is included in the family of ARE-containing genes and is regulated by Nrf2 [6]. HO-1, which is the rate-limiting enzyme in heme catabolism under a variety of conditions including those pertinent to this study, is induced in a number of cell types by a range of stimuli including pro-inflammatory conditions, UV light, and hypoxia. A previous study demonstrated that HO-1 blocks neutrophil trafficking in acute inflammation by suppressing neutrophil rolling, adhesion, and migration, suggesting that the HO-1 pathway may regulate immunomodulatory functions [7,8].

Fisetin, 3,7,3′,4′-tetrahydroxyflavone, is a polyphenol and naturally occurring flavonoid that is abundantly found in several fruits and vegetables [9]. Fisetin has anticancer, anti-proliferative, neuroprotective, and antioxidant activities [10–13]. It also suppresses microphthalmia-associated transcription factor (MITF) in melanoma cells and inhibits melanoma cell invasion via regulation of the MAPK and NF-κB pathways

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[14,15]. However, previous studies have not addressed the biological effects or mechanisms of action of fisetin in human keratinocytes.

During the ongoing search for natural bioactive compounds, fisetin has become well-known as a natural anti-inflammatory molecule. In the present study, we detail the skin protective and anti-inflammatory effects of fisetin in hydrogen peroxide- and TNF-α-challenged human keratinocyte HaCaT cells. In addition, we explore the molecular mechanisms by which fisetin exerts its protective effects.

2. Materials and methods

2.1. Reagents

Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), phosphate-buffered saline (PBS), and other tissue culture reagents were obtained from Gibco BRL Co. (Carlsbad, CA, USA). Tin protoporphyrin IX (SnPP IX) and cobalt protoporphyrin (CoPP) were purchased from Porphyrin Products (Logan, UT, USA). Small interfering RNA (siRNA) for HO-1 and Nrf2, primary antibodies including mouse/goat/rabbit anti-COX-2, iNOS, p50, and p65, and secondary antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany), and HO-1 antibody was purchased from Calbiochem (San Diego, CA, USA). Nrf-2, phosphorylated-p38, and p38 antibodies were obtained from Cell Signaling Technology (Cell Signaling, Danvers, MA, USA). Lipofectamine 2000™ was obtained from Invitrogen Life Technologies (Grand Island, NY, USA). Enzyme-linked immunosorbent assay (ELISA) kits for PGE_2, IL-1β, and IL-6 were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Fisetin (>98% pure) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell culture and viability assay

The HaCaT human keratinocyte cell line (passages 30–40) was cultured in DMEM supplemented with 10% heat-inactivated FBS, penicillin G (100 U/mL), streptomycin (100 mg/L), and L-glutamine (2 mM) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. For measurement of cell viability, cells were maintained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at a final concentration of 0.5 mg/mL (4 h), and the formazan that formed was dissolved in acidic 2-propanol. Optical density was measured at 540 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). The optical density of the formazan formed in control (untreated) cells was used to represent 100% viability.

2.3. Measurement of reactive oxygen species (ROS) production

To measure ROS levels, cells were stained with 10 μM 2′,7′-dichlorofluorescein diacetate (DCFDA) in Hank's balanced salt solution for 30 min in the dark. The cells were then washed twice with phosphate-buffered saline (PBS) and lysed with 1% Triton X-100 in PBS for 10 min at 37 °C. Fluorescence was recorded at an excitation wavelength of 490 nm and an emission wavelength of 525 nm (Spectramax Gemini XS; Molecular Devices, Sunnyvale, CA). Cells were immediately observed under a laser-scanning confocal microscope (Leica TCS SP2). DCF fluorescence was excited at 488 nm with an argon laser, and the evoked emission was filtered with a 515-nm long pass filter.

2.4. Cytoplasmic and nuclear extracts

Cells were homogenized (1:20, w:v) in PER-mammalian protein extraction buffer with freshly added 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail I (EMD Biosciences, San Diego, CA, USA). The cellular cytosolic fraction was prepared by centrifugation at 16,000 ×*g* for 5 min at 4 °C. Nuclear and cytosolic cell extracts were separated with NE-PER nuclear and cytosolic extraction reagents (Pierce Biotechnology, Rockford, IL, USA), respectively.

2.5. Determination of nitrite, PGE_2, IL-1β, and IL-6 production and NF-κB DNA binding activity

Nitrite concentrations in the medium were measured using the Griess reaction. 100 μL of supernatant was mixed with an equal volume of Griess reagent (Solution A: 222488; Solution B: S438081, Sigma), and the absorbance of the mixture was determined at 525 nm using an ELISA plate reader. The levels of TNF-α, IL-1β, IL-6, and IL-12 p40 present in each sample were determined using a commercially available kit from R&D Systems, according to the manufacturer's instructions. In addition, NF-κB DNA-binding activity was measured using the TransAM kit (Active Motif, Carlsbad, CA, USA) in nuclear cell extracts, according to the manufacturer's instructions.

2.6. Western blotting analysis

Cells were harvested and pelleted by centrifugation at 200 ×*g* for 3 min. Subsequently, the cells were washed with PBS and lysed using RIPA lysis buffer containing 25 mmol/L Tris–HCl buffer (pH 7.6), 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS. Protein concentration was determined using Bradford Assay Reagent (Bio-Rad, PA, USA) and the Lowry protein assay kit (P5626; Sigma Chemical Co.). An equal amount of protein for each sample was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% skim milk and then incubated with primary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and horseradish peroxidase–conjugated secondary antibody followed by ECL detection (Amersham Life Science, Arlington Heights, IL, USA).

2.7. siRNA transfections of HO-1 and Nrf2

Cells were transiently transfected with HO-1 and Nrf2 siRNA (Santa Cruz Biotechnology, CA, USA) for 6 h using Lipofectamine 2000™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and then incubated in fresh media containing 10% FBS for 24 h before further manipulation.

2.8. Real-time PCR

RNA was extracted from cells and tissues using an RNeasy Mini Kit (Qiagen). For real-time polymerase chain reaction (PCR), first-strand complementary DNA (cDNA) was synthesized from 4 μg total RNA using an Advantage RT-for-PCR Kit (Takara Korea Biomedical Inc., Seoul, Korea). Relative messenger RNA levels were determined by real-time PCR using a Brilliant II SYBR Green QPCR Master Mix kit (Stratagene) and an Mx3000P thermal cycler (Stratagene). The primer sequences were as follows: HO-1 forward 5′-CTCTTGGCTGGCTTCCTT-3′ and reverse 5′-GGCTCCTTCCTCCTTTCC-3′; GAPDH forward 5′-AGGT CGGTGTGAACG GATTTG-3′ and reverse 5′-TGTAGACCATGTAGTTGAGG TC A-3′.

2.9. Statistical analysis

Data are expressed as the mean ± standard deviation (S.D.) of at least three independent experiments. To compare three or more groups, one-way analysis of variance was used, followed by a Newman–Keuls *post-hoc* test. Statistical analyses were performed with GraphPad Prism software, version 3.03 (GraphPad Software Inc., San Diego, CA, USA).