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Icariin inhibits TNF-α/IFN-γ induced inflammatory response via inhibition of the substance P and p38-MAPK signaling pathway in human keratinocytes

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ABSTRACT

Pro-inflammatory cytokines play a crucial role in the etiology of atopic dermatitis. We demonstrated that Herba Epimedii has anti-inflammatory potential in an atopic dermatitis mouse model; however, limited research has been conducted on the anti-inflammatory effects and mechanism of icariin, the major active ingredient in Herba Epimedii, in human keratinocytes. In this study, we evaluated the anti-inflammatory potential and mechanisms of icariin in the tumor necrosis factor-α (TNF-α)/interferon-γ (IFN-γ)-induced inflammatory response in human keratinocytes (HaCaT cells) by observing these cells in the presence or absence of icariin. We measured IL-6, IL-8, IL-1β, MCP-1 and GRO-α production by ELISA; IL-6, IL-8, IL-1β, intercellular adhesion molecule-1 (ICAM-1) and tachykinin receptor 1 (TACR1) mRNA expression by real-time PCR; and P38-MAPK, P-ERK and P-JNK signaling expression by western blot in TNF-α/IFN-γ-stimulated HaCaT cells before and after icariin treatment. The expression of TNF-α-R1 and IFN-γ-R1 during the stimulation of the cell models was also evaluated before and after icariin treatment. We investigated the effect of icariin on these pro-inflammatory cytokines and detected whether this effect occurred via the mitogen-activated protein kinase (MAPK) signal transduction pathways. We further specifically inhibited the activity of two kinases with 20 μM SB203580 (a p38 kinase inhibitor) and 50 μM PD98059 (an ERK1/2 kinase inhibitor) to determine the roles of the two signal pathways involved in the inflammatory response. We found that icariin inhibited TNF-α/IFN-γ-induced IL-6, IL-8, IL-1β, and MCP-1 production in a dose-dependent manner; meanwhile, the icariin treatment inhibited the gene expression of IL-8, IL-1β, ICAM-1 and TACR1 in HaCaT cells in a time- and dose-dependent manner. Icariin treatment resulted in a reduced expression of p-P38 and p-ERK signal activation induced by TNF-α/IFN-γ; however, only SB203580, the p38 alpha/beta inhibitor, inhibited the secretion of inflammatory cytokines induced by TNF-α/IFN-γ in cultured HaCaT cells. The differential expression of TNF-α-R1 and IFN-γ-R1 was also observed after the stimulation of TNF-α/IFN-γ, which was significantly normalized after the icariin treatment. Collectively, we illustrated the anti-inflammatory property of icariin in human keratinocytes. These effects were mediated, at least partially, via the inhibition of substance P and the p38-MAPK signaling pathway, as well as by the regulation of the TNF-α-R1 and IFN-γ-R1 signals.

1. Introduction

Atopic dermatitis (AD) is a common, chronic inflammatory skin disease that causes significant impairment of the quality of life. In this inflammatory skin disease, keratinocytes participate in the inflammatory responses initiated by various stressors, and the majority of AD pathogenesis is due to an immune reaction in keratinocytes [1]. The up-regulation of pro-inflammatory cytokines plays a crucial role in the etiology of atopic dermatitis [2]. Stimulation of keratinocytes with tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) leads to the expression of pro-inflammatory cytokines, such as IL-6, IL-1, IL-8, and specific chemokines. These cytokines and chemokines contribute to the infiltration of inflammatory cells to inflammation sites in the skin. The capacity to synthesize proinflammatory mediators indicates that keratinocytes play an important role in the initial inflammatory response in the skin [3,4]. As a unique, spontaneously immortalized keratinocyte cell line derived from normal adult human skin, HaCaT cells exhibit keratinocyte-like characteristics [5] and are used for in vitro testing of anti-inflammatory compounds.

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Monocytes and neutrophil-specific chemokines, monocyte chemotactic protein (MCP)-1, IL-8 and growth-related oncogene-α (GRO-α)/CXCL1 are presumed to play pivotal roles in the recruitment and accumulation of inflammatory cells in cutaneous inflammatory diseases [6,7]. Then, adhesion molecules, such as ICAM-1, promote the attachment of these immune cells to the inflamed skin [8]. In addition, atopic dermatitis is a type of neurogenic-mediated inflammatory skin disease, and the interaction between the skin and the nervous system plays an important role in cutaneous diseases. Therefore, the production of neuropeptides, such as substance P, from sensory fibers can cause further deterioration of the inflammation. These effects are mediated via tachykinin NK1 receptors (TACR1) [9]. TACR1, also known as neurokinin 1 receptor (NK1R) or substance P receptor (SPR), is a G protein coupled receptor that is found in the central and peripheral nervous system [10]. The binding of SP to the NK_1 receptor has been associated with the activation of the NF-Kb signaling pathway and the secretion of inflammatory products from keratinocytes [11,12]. Therefore, the down-regulation of TACR1 can relieve the skin's inflammatory response.

In this research, we determined whether MAPK signaling can be activated by TNF-α/IFN-γ and whether these pathways are responsible for the expression of various pro-inflammatory genes. The MAPKs belong to a group of serine/threonine protein kinases that include three main subfamilies: the p38 mitogen-activated protein kinase (p38 MAPK), the p42/p44 extracellular regulated kinases (ERKs) and the c-Jun N-terminal kinases (JNKs). A major consequence of MAPK phosphorylation is the activation of inflammatory transcription factors that serve as downstream substrates of kinase to promote the secretion of inflammatory cytokines, such as IL-6, IL-8 and IL-1β. MAPKs regulate key pro-inflammatory pathways following stimulation with cytokines; therefore, we examined the anti-inflammatory function via the mitogen-activated protein kinase (MAPK) signal transduction pathway, particularly, p38 MAPK, extracellular signal-regulated protein kinase 1/2 and c-Jun N-terminal kinase [13,14].

Icariin was isolated from the Chinese medicinal herb *Epimedium brevicornum* (Family Berberidaceae), which has been used in traditional Chinese medicine for thousands of years. Previous studies from our team and other researchers showed that icariin exhibited a wide range of pharmacological and biological properties, including anti-inflammatory, anti-depressant, anti-osteoporosis and anti-aging [15–19]. In previous animal research, we demonstrated that Herba Epimedii had anti-inflammatory potential in an atopic dermatitis mice model [20]; however, there is limited knowledge about the exact role of icariin in skin inflammation, and the potential mechanisms have not been evaluated. We hypothesized that icariin may modulate inflammatory cytokine and chemokine expression in the HaCaT cell line.

2. Materials and methods

2.1. Chemicals and reagents

Icariin was purchased from Shanghai Ronghe (Shanghai, China). The purity (>98%) of the icariin used for in vitro biological assays was determined by high-performance liquid chromatography (HPLC). Penicillin and streptomycin were purchased from Invitrogen/GIBCO BRL (Grand Island, NY). Cell Counting Kit-8 was purchased from Dojindo (Dojindo Laboratory, Japan). Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human recombinant TNF-α and IFN-γ were purchased from R&D systems (Tokyo, Japan). The 6-well, 48-well and 96-well plates were purchased from Corning Inc. (Corning Inc., NY, USA). TRIzol was purchased from Sigma Chemical Co. (St. Louis, MO, USA). PrimerScript RT Enzyme Mix I was obtained from TaKaRa Bio (TaKaRa Bio, Japan). SYBR Green I Master was from Hoffmann-La Roche, Ltd. (Roche, Swiss). IL-6, IL-8, IL-1β, MCP-1 and GRO α enzyme-linked immunosorbent assay (ELISA) kits were purchased from Xitang Biological Pharmaceutical Co. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY, USA). TNF-α and IFN-γ were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Antibodies against β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SB203580 (a p38 MAPK inhibitor) and PD98059 (an ERK1/2 inhibitor) were purchased from Biosource (Camarillo, CA). Antibodies against phospho-p38 MAPK, phosphor-p42/p44 ERK and phospho-JNK1/2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal anti-p44 ERK and rabbit polyclonal anti-p38 were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). PE-TNF-αR1 (CD119) antibody was purchased from R&D Systems Inc. (Minneapolis, MN, USA). FITC-IFN-γR1 antibody and the chemiluminescence kit were purchased from Millipore (Billerica, MA, USA). The primer sequences were synthesized by Generay Biotech (Generay, PRC).

2.2. Cell culture

Immortalized human HaCaT keratinocytes were maintained at 37 °C and 5% carbon dioxide (CO_2) in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. HaCaT cells were digested for 5 to 8 min at 37 °C with 0.25% trypsin and 0.02% EDTA. The cells were then collected by centrifugation at 1000 rpm for 5 min and cultured at a density of 1×10^6 cells/plate at 37 °C in a humid atmosphere of 5% CO_2 and 95% air. The culture medium was changed twice a week.

2.3. Cell viability assay

Cell viability was detected using Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions. Briefly, HaCaT cells were cultured in 96-well plates, with 6 duplicate wells in each group. When the cells achieved 70–80% confluence, the media were changed with FBS-free DMEM, and the cells were treated with conditioned medium as indicated. Then, 10 μl of a CCK-8 solution was added to each well followed by a further 0.5–2 h incubation at 37 °C. Absorbance was measured at 450 nm with a microplate reader (Molecular Devices, Sunnyvale, CA). The mean optical density (OD) of 4 wells in the indicated groups was used to calculate the percentage of cell viability as follows: percentage of cell viability = (OD(treatment group) − OD (blank group)) / (OD (control group) − OD (blank group)) × 100%. The experiment was performed in triplicate.

2.4. ELISA analysis

HaCaT cells (5×10^5 cells/ml in a 6-well plate) were treated with medium alone or with TNF-α/IFN-γ in the presence or absence of icariin (1, 10 or 100 μM) or hydrocortisone (0.01 μM) for 24 h for the detection of IL-6, IL-1β, IL-8, GRO-α and MCP-1. After stimulation for 24 h, the culture supernatants were collected, centrifuged (12,000 rpm, 10 min) and stored at −80 °C until analysis. The concentrations of IL-6, IL-1β, IL-8, GRO-α and MCP-1 in the culture supernatant were measured by an immunoassay kit according to the manufacturer's instructions. Three independent experiments were performed in triplicate.

2.5. Real-time RT-PCR

HaCaT cells (5×10^5 cells/ml in a 6-well plate) were treated with medium alone or pretreated with icariin (1, 10, or 100 μmol/ml) for 1 h, then treated with TNF-α/IFN-γ for 30 min, 60 min, and 120 min, except for the control group. After the treatment, the total RNA was extracted from HaCaT cells using TRIzol according to the manufacturer's instructions. Quantification was performed according to our previous study. The primers used for PCR amplification are listed in Table 1. The expression levels of mRNAs were normalized to β-actin and were calculated using the 2-ΔΔCt method [20].

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