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Icariin inhibits LPS-induced cell inflammatory response by promoting GRα nuclear translocation and upregulating GRα expression

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ABSTRACT

Aims: Icariin (ICA) is a flavonoid isolated from certain plant species in the genus *Epimedium*, especially *Epimedium brevicornum*. Previous studies indicated that ICA has certain regulatory effects on some inflammatory diseases, and that ICA regulates the activity of glucocorticoid receptor (GR) and NF-κB. But the causal link between GR and NF-κB and other downstream pathways in effects of ICA remained elusive, therefore here we have investigated whether ICA could promote GR function, in turn, to regulate NF-κB and/or other factors to achieve its anti-inflammatory effect.

Main methods: Inflammatory cell models were induced by lipopolysaccharide (LPS) in RAW 264.7 and HeLa cell line. Observation of GRα nuclear translocation by confocal laser scanning microscopy. GRα and inflammatory cytokines expression was detected by RT-qPCR, Western Blotting and ELISA. Co-immunoprecipitation technique was used to detect the binding of GRα to downstream transcription factors. GRα activity was blocked by GRα antagonist RU486, and GR downstream transcription factors including NF-κB, c-Jun, and Stat3 were silenced by corresponding RNA interference.

Key findings: In both inflammatory cell models, ICA decreased LPS-induced production of inflammatory cytokines (IL-6 and TNF-α). While ICA up-regulated the amount of GRα and promoted its nucleus translocation. The increased GRα in the nucleus by ICA bound more NF-κB, c-Jun, and Stat3. Blockade GRα and silence of NF-κB, c-Jun, and Stat3 expression partially abolished the anti-inflammatory effects of ICA.

Significance: Promoted GR function and the consequent inhibition of pro-inflammatory transcription factors contribute a main mechanism by which ICA exerts its anti-inflammatory effect.

1. Introduction

Epimedii Herba is a well-known traditional Chinese herbal medicine for treating osteoporosis, sexual and neurological dysfunctions in oriental countries [1]. The active compounds in Herba Epimedii are generally attributed to various flavones. Studies found that total flavones of Epimedii have neuroprotective and anti-inflammatory effects [2,3]. ICA (C33H40O15; molecular weight: 676.67; Fig. 1) represents an important active component in total flavones of Epimedii and exhibits extensive pharmacological effects, including anti-neuronal injury, promotion of synaptic growth and osteogenesis, anti-inflammation, anti-tumor, improvement of sexual function, and anti-depression effect [4]. In treatment of inflammation-related diseases and/or status, ICA had been shown to slow down the progress of dextran sulfate sodium-induced intestinal inflammation and improve the pathological changes of colonic inflammation in rats [5]. In animal model of depression and psychological stress, ICA simultaneously down-regulated the expression of proinflammatory cytokine genes and up-regulated the expression of anti-inflammatory cytokine genes [6]. Moreover, ICA attenuated cigarette smoke induced inflammatory responses [7] and LPS-induced acute inflammatory responses [8] in rodents. These studies indicated that ICA perhaps has anti-inflammatory effect, either for inflammation-related diseases or status. But, apart from a few studies showing glucocorticoid receptor (GR) and NF-κB are involved [7,8], the anti-inflammatory mechanism of ICA is not clear.

In the present study, we intended to depict the detailed causal link between GR and pro-inflammatory transcription factors including NF-κB, Stat3, and c-Jun (the main component of AP-1), that is, whether ICA

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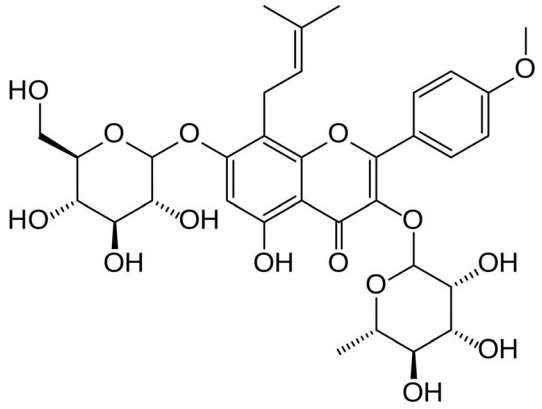

Fig. 1. Molecular structure of Icariin (ICA).

could promote GRα (a major sub-type of GR) nuclear translocation, GRα expression, and/or enhance the inhibitory effect of GRα on these inflammatory transcription factors, as a result, achieve anti-inflammation effect.

2. Materials and methods

2.1. Materials

ICA, LPS, and Mifepristone (RU486) were purchased from Sigma-Aldrich Co. LLC. USA. Dulbecco's modified eagle medium (DMEM) and Roswell Park Memorial Institute 1640 (RPMI 1640) culture medium were purchased from HyClone Laboratories (Logan, UT, USA). Fetal calf serum (FBS), albumin from bovine serum (BSA), 1% double antibiotics (penicillin and streptomycin mixture) and 0.25% trypsin ethylenediaminetetraacetic acid (TRYPSIN-EDTA) were purchased from Gibco Company. Phosphate buffer saline (PBS) and color prestained protein molecular weight standard were purchased from Beyotime Biotechnology Company. Trizol, bicinchoninic acid (BCA) protein assay reagent, SuperSignal West Pico chemiluminescent Substrate, and GRα were provided by Thermo Company. IκBα, NF-κBp65, Stat3, c-Jun, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were obtained from Cell Signaling Technology, Inc. USA. NFκB p65 siRNA (h, m), Stat3 siRNA (h, m), c-Jun siRNA (h, m), and Control siRNA-A assay kit were obtained from Santa Cruz Biotechnology Inc. HeLa and RAW 264.7 cell lines were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences.

2.2. Cell culture and treatments

HeLa and RAW 264.7 cells were cultured using the adherent culture method in high glucose DMEM and RPMI 1640 medium respectively at 37 ˚C in 5% CO_2 under a constant temperature and humidity condition. Both mediums contained 10% FBS and 1% double antibiotics (penicillin and streptomycin mixture). The culture medium in the flask was discarded when the cells reached 80% of confluence. The cells were then rinsed twice with sterilized PBS before the addition of a fresh medium. 1 μg/ml of LPS (Lipopolysaccharides from *Escherichia coli*) was added to induce the inflammatory cell model, the concentration of LPS is according to the literature [9], and at the same time cells were treated with ICA at three different concentrations (7.5 μM, 15 μM, and 30 μM) respectively, the concentration of ICA is according to previous research [10]. Cells were then collected at different time intervals for subsequent experiments.

2.3. Observation of GRα nuclear translocation by confocal laser scanning microscopy

Fifty to eighty thousand HeLa and RAW264.7 cells were placed in a 15 mm NEST petri dish with glass bottom and respectively cultured with high glucose DMEM and RPMI 1640 medium containing 10% carbon-adsorbed fetal bovine serum (Biolnd, Israel). After the treatment of corresponding groups, the culture medium was removed at each time point and the adherent cells were washed once in PBS. Cells were then fixed in 4% paraformaldehyde at 4 ˚C for 15 min, followed by rinsing with PBS 3 times (2 min each time). Cells were incubated with 0.3% Triton X-100 at room temperature for 10 min to rupture their cell membranes, and they were rinsed again with PBS 3 times (2 min each time). Subsequently, cells were incubated with a primary antibody (GRα, working concentration of 1:100) for 1 h at room temperature. After rinsing with PBS 3 times (2 min each time), cells were incubated with a secondary antibody (cy3 Affinipure goat anti-Rabbit IgG, Jackson Immuno Research Inc., working concentration of 1:200) at 37 ˚C for 60 min. Cells were rinsed with PBS 3 times and counterstained with DAPI (4′,6-diamidino-2-phenylindole; Beyotime Biotechnology Company, China) in a dark place at room temperature for 10 min. Cells were washed with PBS before observation under an LSM880 (Carl Zeiss, Oberkochen, Germany) confocal laser microscope, and the microscope objective for image acquisition was under 40 × oil immersion lens.

2.4. RNA extraction and real time RT-PCR analysis

The total RNA of HeLa and RAW264.7 cells were extracted using Trizol according to the manufacturer's instructions. The extracted total RNA was used for the synthesis of the first strand of cDNA by the reverse transcription kit (Fermentas, Canada). The prepared cDNA was amplified using a SYBR Green real-time PCR Master Mix kit (Thermo, USA) on a real-time detector (ABI, Model ABI-7300). The reaction parameters were as follows: pre-denaturation at 95 ˚C for 10 min, followed by 40 cycles of amplification (95 ˚C for 15 s, 60 ˚C for 45 s), 95 ˚C for 15 s, 60 ˚C for 1 min, 95 ˚C for 15 s, and 60 ˚C for 15 s. The intensity of the fluorescence signal was measured at the end of each cycle, and the specific DNA sequence in the sample to be measured was quantitatively analyzed and normalized against the internal reference (GAPDH). The results were calculated by the $2^{-\Delta\Delta Ct}$ method [11].

The primer sequences are as Table 1:

2.5. Enzyme linked immunosorbentassay (ELISA)

The respective levels of IL-6 and TNF-α in the supernatant of HeLa

Table 1
Primer sequences for RT-PCR.

Name	species	Sequence (5′ to 3′)
GRα_F	Human	5′ ACAGCATCCCTTTCTCAACAG 3′
GRα_R	Human	5′ AGATCCTTGGCACCTATTCCAAT 3′
GRα_F	Mouse	5′ ACAGCATCCCTTTCTCAACAG 3′
GRα_R	Mouse	5′ AGATCCTTGGCACCTATTCCAAT 3′
TNF-α_F	Human	5′ CCTCTCTCTAATCAGCCCTCTG 3′
TNF-α_R	Human	5′ GAGGACCTGGGAGTAGATGAG 3′
TNF-α_F	Mouse	5′ CAGGCGGTGCCTATGTCTC 3′
TNF-α_R	Mouse	5′ CGATCACCCCGAAGTTCAGTAG 3′
IL-6_F	Human	5′ ACTCACCTCTTCAGAACGAATTG 3′
IL-6_R	Human	5′ CCATCTTTGGAAGGTTCAGGTTG 3′
IL-6_F	Mouse	5′ CTGCAAGAGACTTCCATCCAG 3′
IL-6_R	Mouse	5′ AGTGGTATAGACAGGTCTGTTGG 3′
GAPDH_F	Human	5′ CACCCACTCCTCCACCTTTG 3′
GAPDH_R	Human	5′ CCACCACCCTGTTGCTGTAG 3′
GAPDH_F	Mouse	5′ CTGCCCAGAACATCATCC 3′
GAPDH_R	Mouse	5′ CTCAGATGCCTGCTTCAC 3′

F, forward; R, reverse.