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Full paper

4′,6-Dihydroxy-4-methoxyisoaurone inhibits TNF-α-induced NF-κB activation and expressions of NF-κB-regulated target gene products

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ABSTRACT

The nuclear factor-κB (NF-κB) transcription factors control many physiological processes including inflammation, apoptosis, and angiogenesis. In our search for NF-κB inhibitors from natural resources, we identified 4′,6-dihydroxy-4-methoxyisoaurone (ISOA) as an inhibitor of NF-κB activation from the seeds of *Trichosanthes kirilowii*. However, the mechanism by which ISOA inhibits NF-κB activation is not fully understood. In the present study, we demonstrated the effect of ISOA on NF-κB activation in TNF-α-stimulated HeLa cells. This compound suppressed NF-κB activation through the inhibition of IκB kinase (IKK) activation. ISOA also has an influence on upstream signaling of IKK through the inhibition of expression of adaptor proteins, TNF receptor-associated factor 2 (TRAF2) and receptor interacting protein 1 (RIP1). Consequently, ISOA blocked the phosphorylation and degradation of the inhibitor of NF-κB alpha (IκBα), and subsequent phosphorylation and nuclear translocation of p65. The suppression of NF-κB activation by ISOA led to the down-regulation of target genes involved in inflammation, proliferation, as well as potentiation of TNF-α-induced apoptosis. Taken together, this study extends our understanding on the mechanisms underlying the anti-inflammatory and anti-cancer activities of ISOA. Our findings provide new insight into the molecular mechanisms and a potential application of ISOA for inflammatory diseases as well as certain cancers.

1. Introduction

Nuclear factor κB (NF-κB) is a crucial pleiotropic transcription factor which regulates inflammation, the innate and adaptive immune response, apoptosis, and tumor invasion (1–3). Five members of this transcription factor family have been identified: RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50) and NF-κB2 (p100/p52) (2). All members of the NF-κB family harbor an N-terminal Rel homology domain (RHD), which interacts with DNA elements and mediates homo- and hetero-dimerization. The complex p50/p65 is the most abundant form of heterodimer of the NF-κB family and it keeps in an inactive state in the cytoplasm bound to proteins of the IκB family, which are inhibitors of NF-κB (4). NF-κB is activated by a sequence involving the phosphorylation, ubiquitination, and degradation of IκBα, and the phosphorylation of p65, which in turn lead to the translocation of NF-κB to the nucleus, where it binds to specific response elements in the DNA (5). The phosphorylation of IκBα is catalyzed by IκB kinase (IKK), which is essential for NF-κB activation by most stimuli. Several lines of evidence also support a role for TNF receptor-associated factor 2 (TRAF2) and receptor interacting protein 1 (RIP1) in TNF signaling leading to NF-κB activation (6).

It is reported that NF-κB regulates more than 150 genes, including those involved in immunity and inflammation, anti-apoptosis, cell proliferation, tumorigenesis, and the negative

Abbreviations: NF-κB, nuclear factor-κB; ISOA, 4′,6-dihydroxy-4-methoxyisoaurone; IκBα, inhibitor of NF-κB alpha; IKK, IκB kinase; TRAF2, TNF receptor-associated factor 2; RIP1, receptor interacting protein 1; Topo-I, topoisomerase-I; IL-8, interleukin 8; TNF-α, tumor necrosis factor alpha; MCP1, monocyte chemotactic protein 1; iNOS, inducible nitric oxide synthase; cIAP1, cellular inhibitor of apoptosis 1; Bcl2, B-cell lymphoma 2; FLIP, FLICE inhibitory protein; COX-2, cyclooxygenase-2; ICAM-1, inter-cellular adhesion molecule 1; VEGF, vascular endothelial growth factor; TNFR, TNF receptor; TRADD, TNFR1-associated death domain protein; FADD, Fas-associated protein with death domain; TAK1, transforming growth factor-β-activating kinase 1.

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feedback of the NF-κB signal (7). NF-κB regulates the transcription of various inflammatory cytokines, including interleukin 8 (IL-8), tumor necrosis factor alpha (TNF-α), and monocyte chemotactic protein 1 (MCP1) as well as genes encoding inducible nitric oxide synthase (iNOS), immunoreceptors, cell adhesion molecules, hematopoietic growth factors, and growth factor receptors (8–10). In addition to regulating the transcription of genes important for immune and inflammatory responses, NF-κB also controls the expression of genes that are critical in the early and late stages of aggressive cancers, including antiapoptotic proteins cellular inhibitor of apoptosis 1 (cIAP1), cIAP2, TNF receptor-associated factor 1 (TRAF1), B-cell lymphoma 2 (Bcl2), cellular FLICE inhibitory protein (FLIP); gene required for proliferation such as cyclooxygenase-2 (COX-2); and genes required for invasion and angiogenesis such as inter-cellular adhesion molecule 1 (ICAM-1) and vascular endothelial growth factor (VEGF) (11, 12).

4′,6-Dihydroxy-4-methoxyisoaurone (ISOA) is a natural product isolated from the *Trichosanthes kirilowii* seeds, ISOA showed potent anticancer activity through inhibition of hypoxia-inducible factor-1α (13). ISOA also was a potent inhibitor of nuclear factor κB (NF-κB) activation; however, the molecular mechanism has not been sufficiently explained (14). In present study, we investigated the effects of ISOA on the NF-κB activation pathway and on the expression of NF-κB target genes induced by TNF-α. We here describe molecular mechanism by which ISOA inhibits NF-κB activation, which in turn down-regulates the expression of target genes involved in inflammation (IL-8, TNF-α, and MCP1), cell survival (cIAP1, cIAP2, TRAF1, and FLIP), proliferation (COX-2), invasion (ICAM-1), and angiogenesis (VEGF), and potentiates TNF-α-induced apoptosis significantly via activation of caspase-8. Our finding may expand the application of ISOA to a valuable candidate for the intervention of NF-κB-dependent pathological conditions such as inflammation and cancer.

2. Materials and methods

2.1. Cell culture and reagents

HeLa cells were grown in DMEM with penicillin (100 U/ml)-streptomycin (100 U/ml) (Invitrogen, Carlsbad, CA, USA) and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA) at 37 °C with 5% CO_2 atmosphere in a humidified incubator. HeLa cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). TNF-α was obtained from R&D Systems (Minneapolis, MN, USA). ISOA was isolated from *T. kirilowii* seeds and its structure is shown in Fig. 1A. The purity of ISOA was more than 98% in high-performance liquid chromatography analysis.

2.2. Transfections and luciferase reporter assay

A pNF-κB-Luc plasmid for NF-κB luciferase reporter assay was obtained from Strategene (La Jolla, CA, USA). Transfections were performed as described previously (17). NF-κB-dependent luciferase activity was measured using the Dual Luciferase Reporter Assay system. Briefly, HeLa cells (1×10^5 cells/well) were seeded in a well of 96-well plate for 24 h. The cells were then transfected with plasmids for each well and then incubated for a transfection period of 24 h. After that, the cell culture medium was removed and replaced with fresh medium containing various concentrations of ISOA for 12 h, followed by treatment with 10 ng/ml of TNF-α for 12 h. Luciferase activity was determined in Luminoskan™ Ascent Microplate Luminometer (Thermo Scientific, Waltham, MA, USA) by injecting 100 μl of assay buffer containing luciferin and measuring light emission for 10 s. Co-transfection with pRL-CMV (Promega, Madison, WI, USA), which expresses Renilla luciferase, was performed to enable normalization of data for transfection efficiency.

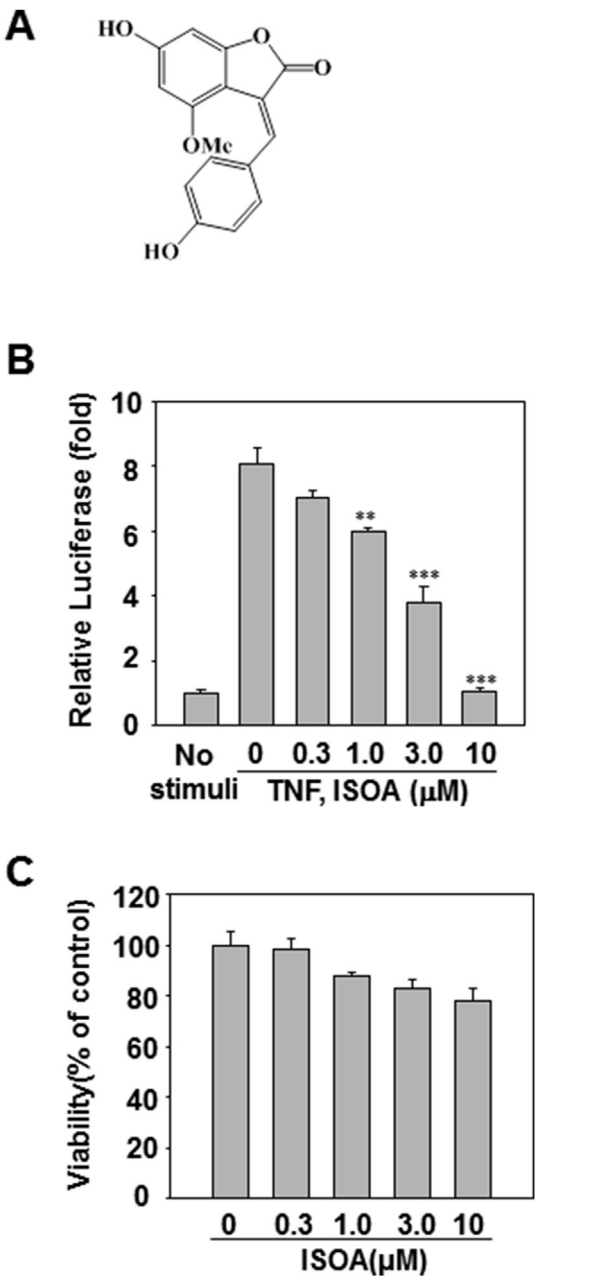

Fig. 1. Effect of ISOA on the TNF-α-induced NF-κB-dependent reporter gene expression. (A) Structure of 4′,6-dihydroxy-4-methoxyisoaurone (ISOA). (B) HeLa cells were transiently transfected with a NF-κB-dependent reporter gene for 48 h and then pretreated for 12 h with the indicated concentrations of ISOA followed by stimulation for 12 h with 10 ng/ml TNF-α, and the luciferase activity was determined as described in "Materials and methods". Data represented as mean ± standard deviation of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$, significantly different when compared with TNF-α-stimulated normal cells. (C) HeLa cells were treated with the indicated concentrations of ISOA. After 24 h incubation, cell viability was determined by MTT assays. Data represented as mean ± standard deviation of three independent experiments.

2.3. Measurement of cell viability by MTT assay

HeLa cells were seeded at 1×10^5 cells/ml in a well of 96-well plate containing 100 μl of DMEM medium with 10% FBS and