Imperatorin efficiently blocks TNF-α-mediated activation of ROS/PI3K/Akt/NF-κB pathway

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Abstract. Inflammation contributes to development and progression in a variety of cancers, including cervical cancer, which is the second leading cause of cancer deaths in women worldwide. In this study, we examined the anti-inflammatory effects of imperatorin, a psoralen-type furanocoumarin from the fruits of Angelica dahurica, in tumor necrosis factor-α (TNF-α)-stimulated HeLa cells by investigating its impact on the production and expression of cytokines and the major signal-transduction pathways. We found this compound significantly inhibited the TNF-α-induced expression of NF-κB target genes, such as COX-2, cycillin D1, MMP-9, VEGF, IL-6 and Bcl-xL in a concentration-dependent manner. Further analysis revealed that imperatorin was a potent inhibitor of NF-κB activation by the suppression of TNF-α-induced IKKα/β phosphorylation, IκB phosphorylation and degradation, and NF-κB p65 nuclear translocation. We also demonstrated that imperatorin downregulated TNF-α-induced activation of PI3K/Akt. Furthermore, our findings show that imperatorin inhibits TNF-α-induced ROS generation. Taken together, imperatorin can blunt inflammation by inhibiting the ROS-mediated activation of the PI3K/Akt/NF-κB pathway.

Introduction

Inflammation is a beneficial host response to foreign challenge or tissue injury that leads ultimately to the restoration of tissue structure and function (1). The response consists of a sequential release of mediators and recruitment of circulating leukocytes which become activated at the inflammatory site and then release further mediators (2). TNF-α is now known more generally as a mediator of inflammatory responses. The pro-inflammatory effects of TNF-α are primarily due to its ability to activate NF-κB (3). NF-κB is responsible for the transcription of the genes encoding many pro-inflammatory cytokines and chemokines (2). In resting cells, NF-κB complexes are inactive, residing predominantly in the cytoplasm in a complex with inhibitory IκBs (1). Upon appropriate stimulation for example by TNF-α, IκB is phosphorylated by IκB kinases (IKKs), polyubiquitinated by a ubiquitin ligase complex, and degraded by the 26S proteasome (4,5). It has been shown that TNF-α can activate the PI3K/Akt pathway, which in turn has been shown to be able to activate the NF-κB signaling pathway through IKKα/β in HeLa cells (6,7).

Reactive oxygen species (ROS) are constantly generated and eliminated in the biological system and are required to drive regulatory pathways (8). Under normal physiologic conditions, cells control ROS levels by balancing the generation of ROS with their elimination by scavenging system. But under oxidative stress conditions, excessive ROS can damage cellular proteins, lipids and DNA, leading to fatal lesions in cells that contribute to carcinogenesis. It has been shown that ROS can mediate the TNF-α-induced inflammation through the Akt-mediated activation of NF-κB (9).

Imperatorin [9-(3-methylbut-2-enyloxy)-7H-furo [3,2-g] chromen-7-one] is a naturally occurring furanocoumarin, which can be found in selected herbal medicines, namely in the roots and fruits of Angelica dahurica and Angelica archangelica (Umbelliferae, Apiaceae) (10). It has multiple effects of imperatorin, a psoralen-type furanocoumarin from the fruits of Angelica dahurica, in tumor necrosis factor-α (TNF-α)-stimulated HeLa cells by investigating its impact on the production and expression of cytokines and the major signal-transduction pathways. We found this compound significantly inhibited the TNF-α-induced expression of NF-κB target genes, such as COX-2, cycillin D1, MMP-9, VEGF, IL-6 and Bcl-xL in a concentration-dependent manner. Further analysis revealed that imperatorin was a potent inhibitor of NF-κB activation by the suppression of TNF-α-induced IKKα/β phosphorylation, IκB phosphorylation and degradation, and NF-κB p65 nuclear translocation. We also demonstrated that imperatorin downregulated TNF-α-induced activation of PI3K/Akt. Furthermore, our findings show that imperatorin inhibits TNF-α-induced ROS generation. Taken together, imperatorin can blunt inflammation by inhibiting the ROS-mediated activation of the PI3K/Akt/NF-κB pathway.

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Abbreviations: ROS, reactive oxygen species; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; IκBα, inhibitor of NF-κBα; IL-6, interleukin-6; c-IAP2, cellular inhibitor of apoptosis-2; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; Bcl-2, B-cell lymphoma-2; Bcl-xL, cell lymphoma/leukemia-xl; COX-2, cyclooxygenase-2; MMP-9, matrix metalloproteinase-9; VEGF, vascular endothelial growth factor

Key words: imperatorin, NF-κB, reactive oxygen species, inflammation
therapeutic activities and is used to treat antibacterial activity, cancer, inflammation, and antiviral activity. The anti-inflammatory effects of imperatorin have also been reported (11). However, the effect of imperatorin on PI3K/Akt/NF-κB pathway has not been studied. Herein, we were able to show that imperatorin not only inhibited the activation of NF-κB, which in turn induced the expression of NF-κB target genes, such as angiogenesis (VEGF), invasion (MMP-9), proliferation (cyclin D1 and COX-2) and major inflammatory cytokines (IL-6), but also potentiated TNF-α-induced apoptosis (cIAP-1, Bcl-xL and Bcl-2) the production of reactive oxygen species (ROS) and phosphorylation of Akt, IKKα/β, and IκBα, trans-activity of NF-κB. These findings showed that the suppression of NF-κB activation by imperatorin is a possible strategy to inhibit inflammation.

Materials and methods

Cell culture and reagents. HeLa cells and 293 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HeLa cells and 293 cells were cultured in DMEM containing 10% FBS (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C with 5% CO₂ atmosphere in a humidified incubator. TNF-α was obtained from R&D Systems (Minneapolis, MN, USA). N-acetyl-L-cysteine (NAC) was from Sigma (St. Louis, MO, USA). The primary antibodies for IκBα, phosphor (Ser32)-specific IκBα, (Ser536)-specific p65, PARP, c-Jun, caspase-8, c-IAP2, Akt1, phospho-Akt (Ser473), phosphor-IKKα/β were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for COX-2, MMP-9, VEGF, IKKα and Topo-I were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for α-tubulin was from Sigma. Imperatorin was bought from the National Institutes for Food and Drug Control (NIFDC, Beijing, China) and its structure is shown in Fig. 1A. The purity of imperatorin was over 99% in HPLC analysis.

Measurement of cell viability by MTT assay. Cell viability was measured by a MTT assay (Sigma-Aldrich). MTT assays were performed as previously described (12). Briefly, the cells were treated with imperatorin (5-150 µM) in triplicate wells for 24 h followed by the addition of MTT to the cells. The MTT formazan crystals were dissolved in DMSO. The absorbance at 570 nm was measured by microplate reader.

Plasmids, transfections, and luciferase reporter assay. A pNF-κB-Luc plasmid for NF-κB luciferase reporter assay was obtained from Stratagene (La Jolla, CA, USA). Transfections were performed as previously described (13). In brief, at 50-80% confluence, HeLa cells, were cotransfected with the vectors for pGL3-NF-κB-Luciferase plasmid using Lipofectamine 2000 reagent (Invitrogen). Cells were lysed and luciferase activity was determined using the Dual Luciferase Reporter Assay system.

Enzyme-linked immunosorbent assay (ELISA). HeLa cells were plated in 96-well plate at a density of 1x10⁵ cells per well and treated with various concentrations of imperatorin for 12 h and then incubated with TNF-α for 12 h. The IL-6 and MMP-9 levels in the culture supernatant were determined by ELISA kit (Cusabio Biotech Co., Ltd., Newark, NJ, USA) according to the manufacturer's instructions.

Apoptosis assays. Apoptosis assays were performed as previously described (14). The HeLa cells were stained with Annexin V-FITC using a FITC Annexin V apoptosis detection kit (BD Biosciences, San Jose, CA, USA). The cells were washed twice with PBS (pH 7.4) and re-suspended in binding buffer. The pooled cells were stained with Annexin V-FITC in the dark for 15 min at room temperature. Last, 2 µg/ml Propidium Iodide (PI) was added, and the suspension was incubated in the dark for 5 min at 37°C. After 400 µl of binding buffer was added, the samples were analyzed by flow cytometry. The data were analyzed by Cell Quest software (Becton-Dickinson).

Measurement of ROS production. Intracellular ROS production was measured using 2',7'-dichlorodihydrofluorescein-diacetate (H2DCFDA; Life Technologies). HeLa cells were treated with imperatorin for 24 h and then 10 ng/ml TNF-α for 30 min. Then HeLa cells were incubated with H2DCFDA (10 µM) for 30 min at 37°C. After removal of the medium and washing of the cells, images were obtained using a fluorescence microscopy.
Western blot analysis. Cell lysates were separated by SDS-polyacrylamide gels and transferred to a PVDF (Millipore). The blots were blocked and then incubated with specific antibodies against indicated primary antibodies. Proteins were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Real-time PCR. The total RNA was extracted using RNeasy Mini kits according to the manufacturer’s instructions (Qiagen, CA, USA). RT-PCR was performed with a Qiagen one-step RT-PCR system kit (Qiagen OneStep RT-PCR kit handbook). In brief, 1 µg of total RNA from each sample was added to 50 µl of a reaction mixture containing 0.4 mM dNTP, 0.6 µM sense and antisense specific primers, 5 units of RNase inhibitor, 2 µl of Qiagen One-step RT-PCR Enzyme Mix including Omniscript™ and Sensiscript™ reverse transcriptase. The following primer pairs were used for reverse transcription-PCR amplification: human interleukin-6 (IL-6), 5'-ACA AAGCCAGAGTCTTTCAAGAAG-3' and 5'-CTGTGAGGAAATG-3'; human TNF-α, 5'-CTGCCCA GATCCCTTTATT-3' and 5'-CCCCAATTCTTTTGA GCC-3'; Bel-xL, 5'-GAATCTTGGAAGTGGTATTGTTG-3' and 5'-TGCTGATTGTTCCATAGA-3'; GAPDH, 5'-ACC CAAGTCATGCTGATC-3' and 5'-TCCACCCACCTGT TGCTGTA-3'. The reaction mixture was incubated for 30 min at 50°C for the reverse transcription reaction, for 15 min at 95°C for the inactivation of reverse transcriptase and the activation of HotStarTaq DNA polymerase, and then amplified using a three-temperature PCR system consisting of denaturation at 94°C for 30 sec, primer annealing at 55°C for 30 sec, and extension at 72°C for 1 min. The number of cycles was 25-40. The cycle threshold values (Ct values) were used to calculate the fold differences using the △△Ct method, and GAPDH expression was used as the internal control.

Immunofluorescence assay. HeLa cells were grown directly on cover slips in 24-well plates (1x10^4 cells/well) for 24 h, then cells were pretreated with imperatorin (150 µM) for 24 h, whereafter, treated with 10 ng/ml TNF-α. Cell treated with DMSO was used as negative control and treated with 10 ng/ml TNF-α alone was used as positive control. After treatment, the cells were washed in PBS, fixed at room temperature with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Immunofluorescence staining was performed according to standard procedures. Briefly, the treated cells were first stained with the anti-p65 antibody followed by incubation with FITC conjugated anti-rabbit IgG secondary antibody and co-staining with DAPI.

Statistical analysis. All results are expressed as mean ± SD at least three independent experiments. A comparison of
the results was analyzed with one-way ANOVA followed by Tukey’s multiple comparison tests (Graphpad Software, Inc, San Diego, CA, USA). P-value <0.05 was considered to be statistically significant.

**Results**

**Effect of imperatorin on the viability of HeLa and 293 cells.** In order to assess imperatorin toxicity in cell cultures, preliminary experiments were carried out using MTT test. As shown in Fig. 1B, imperatorin did not adversely affect cell viability on HeLa and 293 cells up to 150 µM.

**Imperatorin downregulates TNF-α-induced NF-κB-regulated gene products.** To address effects of imperatorin on the expression of NF-κB targets genes, we evaluated TNF-α-induced expression of antiapoptotic protein c-IAP2, proliferative proteins COX-2 and cyclin D1, adhesion proteins ICAM-1 and VCAM-1, and metastatic protein MMP-9 and angiogenic protein VEGF by western blotting. Imperatorin inhibited TNF-α-induced expression of all these proteins in a concentration-dependent manner (Fig. 2A). Because NF-κB regulates major inflammatory cytokines, including IL-6, Bcl-2L and TNF-α, many of which are potent activators for NF-κB (15-17), we determined whether imperatorin affected the expression of TNF-α-induced IL-6, Bcl-2L and TNF-α mRNA levels, the results suggest that imperatorin blocked TNF-α-induced expression of anti-inflammatory gene products in a dose-dependent manner (Fig. 2B). Moreover, we measured the release of some cytokines in cell supernatant, such as IL-6 and MMP-9, imperatorin could suppress the levels of these cytokines in a concentration-dependent manner (Fig. 2C).
Imperatorin promotes TNF-α-induced apoptosis. We used Annexin V-FITC/PI double staining to assess the ability of imperatorin (150 µM) to TNF-α-induced apoptosis. The Annexin V-positive cell population (41.2%) significantly increased when compared with TNF-α-stimulated normal cells. (B) HeLa cells were preincubated with indicated concentrations of imperatorin (IMP) for 24 h and then treated with TNF-α (10 ng/ml) for 30 min. Nuclear extracts were analyzed by western blotting using indicated antibodies for p65 and Topo-I. (C) HeLa cells were incubated with 150 µM imperatorin (IMP) for 24 h and then incubated with TNF-α (10 ng/ml) for the indicated times. Cells were harvested at the indicated time points and then nuclear extracts were prepared. Nuclear p65 was detected by western blot analysis. (D) HeLa cells were incubated with 150 µM imperatorin (IMP) for 24 h and followed by TNF-α (10 ng/ml) stimulation for 30 min. After fixation, cells were stained with specific anti-p65 antibody followed by Alex Flour® 488 (green), and the nucleus was counterstained with DAPI (blue) and examined by Fluorescence microscopy. Scale bars: 20 µm. Images were acquired for each fluorescence channel, using suitable filters with x40 objective. The green and blue images were merged using J software.

Imperatorin inhibits TNF-α-induced NF-κB activation. To test whether imperatorin inhibits the effect of imperatorin on TNF-α-induced NF-κB activation, we performed NF-κB reporter assay. After cells were transiently transfected with the NF-κB-regulated luciferase reporter vector, the cells were further incubated with TNF-α in the presence of various concentrations of imperatorin. We discovered that imperatorin can substantially block TNF-α-stimulated luciferase reporter activity in a concentration-dependent manner (Fig. 4A). Then we inspected whether imperatorin regulates TNF-α-induced translocation of p65 to the nucleus. HeLa cells were treated with TNF-α (10 ng/ml) and various concentrations of imperatorin. After a 24 h incubation, NF-κB p65 was assessed by western blot assay. The results revealed that imperatorin also suppressed TNF-α-induced translocation of p65 to the nucleus in a concentration-dependent manner (Fig. 4B). In HeLa cells,
TNF-α-induced p65 nucleus translocation was completed in 15 min (Fig. 4C). To validate the suppression of NF-κB nuclear translocation by imperatorin, we implemented immunofluorescence to survey p65 nuclear translocation in HeLa cells. NF-κB p65 was located at the cytoplasm without incubation (Fig. 4D, top panel, control). HeLa cells were treated with TNF-α, p65 was activated and translocated into the nucleus (Fig. 4D, middle panel, TNF-α). TNF-α-stimulated p65 nuclear translocation was inhibited by imperatorin (150 µM) (Fig. 4D bottom panel). These results suggested that imperatorin can suppress the activation of NF-κB, which induced by TNF-α, through inhibiting nuclear translocation of p65.

**Imperatorin inhibits TNF-α-induced NF-κB activation by modulating phosphorylation and degradation of IκB, phosphorylation of IKKa/β and activation of PI3K/Akt.** The translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα (18). To determine whether inhibition of TNF-α-induced NF-κB activation by imperatorin is caused by inhibition of IκBα degradation, we exposed the HeLa cells to imperatorin and then treated with TNF-α. As shown in Fig. 5A, imperatorin convectively repressed the phosphorylation and degradation of IκBα by incubation with TNF-α in a concentration-dependent manner. IKKa/β can regulate IκBα phosphorylation and appears to be vital in NF-κB transcriptional responses. To further investigate the mechanism of imperatorin suppression of NF-κB activation, we examined the effect of imperatorin on the TNF-α-induced phosphorylation of IKKa/β. As shown in Fig. 5B, imperatorin can inhibit TNF-α induced phosphorylation of IKKa/β. These results sufficiently certify that imperatorin can repress activation of NF-κB pathway through the canonical signaling cascade. To ascertain activation of PI3K-Akt in response to TNF-α, we examined the phosphorylation of Akt after stimulation of imperatorin with TNF-α. As shown in Fig. 5C, a dose-dependent phosphorylation of Akt was observed, while non-phosphorylated Akt remained the same.

**Anti-oxidative effects of imperatorin on ROS production.** Reactive oxygen species (ROS) activate various transcription factors such as NF-κB, AP-1, hypoxia-inducible factor-1α and STAT3, leading to expression of proteins that control inflammation. In order to investigate whether this is also true in HeLa cells and can be regulated by imperatorin, we used CM-H2DCF-DA, a fluorescent probe, to detect the level of ROS production. Treatment with TNF-α improves the endo-cellular level of ROS, however, imperatorin could blunt the promotion in a dose-dependent manner. After adding NAC (an inhibitor of ROS), the TNF-α-stimulated ROS production was absolutely blunted. The level of ROS production has not been ulteriorly decreased by co-treatment with NAC and imperatorin, which showing that imperatorin inhibited the level of ROS similarly to NAC. Furthermore, treatment with imperatorin abrogated the TNF-α induced phosphorylation of Akt, IKKa/β and IκBα, and NF-κB activation, and markedly decreased the NF-κB-dependent luciferase expression induced by TNF-α (Fig. 6B and C). Taken together, these results suggest that imperatorin can inhibit the TNF-α-stimulated inflammation in HeLa cells through blunting the ROS-regulated PI3K/Akt and NF-κB activation.

**Discussion**

In previous study, Kang et al identified imperatorin is a type of coumarin compound with antibacterial and antiviral activities from Angelica dahurica, which has been used to treat headache of common cold, nasal stuffiness, supraorbital neuralgia, painful swelling on the body, leukorrhea and arthralgia due to wind-dampness in Chinese traditional medicine (19). However, the molecular mechanism of anti-inflammatory effect of imperatorin among these pharmacological activities has not been adequately explained. We not only identified imperatorin as an inhibitor of NF-κB and PI3K-Akt activation, but also investigated how this compound works.

It has been reported that imperatorin can attenuate inflammation via weakening LPS-induced macrophages and oxLDL-induced U937 foam cells (11,19,20). In our results, we show that imperatorin suppressed inflammation by block TNF-α-induced PI3K/Akt/NF-κB signaling pathway. First of all, imperatorin can promote TNF-α-induced apoptosis, efficient phagocytosis of apoptotic cells is of great importance in vivo, because the clearance of apoptotic cells prior to lysis is critical to prevent inflammation (21-23). In this study, imperatorin inhibited TNF-α-induced expression of anti-apoptotic proteins such as c-IAP2, Bcl-2 and Bcl-xL, which are known to be regulated by NF-κB, then activated caspase protein family, prompted PARP cleavage, and finally induced apoptosis by mitochondrial pathway. On the other hand, imperatorin promoted TNF-α-induced apoptosis analyzed with Annexin V/PI staining by flow cytometry.
NF-κB also controls the gene expression which is important for the cell cycle, adhesion and proliferation (24). Cyclin D1 takes part in many biological processes and play critical roles in NF-κB mediated tumorigenesis, such as regulating the mitotic cell cycle. ICAM-1 and VCAM-1 participate in the recruitment of the immune cells (25,26). COX-2-induced in HeLa cells by pro-inflammatory cytokines, may be responsible for the edema and vasodilation associated with cellular proliferation and survival. Our results also indicate that imperatorin blocks the expression of VEGF, MMP-9, COX-2 and cyclin D1, which have been shown to be expressed in response to NF-κB activation. Besides, based on the current study, we detected that imperatorin can reduce the TNF-α induced expression of IL-6 and TNF-α, which are typical cytokines and have a wide variety of biological functions in the regulation of immune response, homeostasis, and inflammation (27). As shown in Fig. 2, imperatorin dose-dependently inhibited expression of these genes. The activation of NF-κB requires phosphorylation of IκB, which then targets IκB for ubiquitination and degradation. Inhibition of Akt, which was demonstrated as diminished Akt phosphorylation in the present experiment, caused decreased phosphorylation of IκB and attenuated the degradation of IκB in HeLa cells. This might inhibit translocation of NF-κB to the nucleus, where it normally activates gene transcription.

In addition to adaptor molecules, kinases or ubiquitinases and de-ubiquitinases, other classes of molecules were reported to have an influence on NF-κB activity: These include reactive oxygen species (ROS), which are compounds containing free electrons usually linked to oxygen atoms that are not part of an atomic bond (28). ROS are capable of eliciting a variety
of pathological changes, including apoptotic and peroxidation of lipids, proteins, and DNA. In general, moderate oxidative stress induces apoptosis, whereas anti-oxidative system is triggered when cells have a higher exposure to ROS (29). The high level of ROS can activate PI3K/Akt signaling pathways, so NF-kB-regulated Bcl-2 proteins will be produced, while apoptosis will be inhibited (30-32). Therefore, modulators of ROS production and their signaling pathways could represent potential targets for anti-inflammatory intervention (33). Then, it was proved that imperatorin suppresses the production of TNF-α-induced ROS, indicating its anti-inflammatory function in the ROS/PI3K/Akt/NF-kB pathway. 

Taken together, our findings indicate that imperatorin can affect activation of each step of the NF-kB signaling pathway and NF-kB-regulated gene products, which are connected with anti-angiogenic, anti-proliferative, anti-inflammatory, anti-oxidation pro-apoptotic and anti-inflammatory effects. Based on our results, we have provided preclinical evidence of imperatorin as a potential agent against inflammatory diseases.

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