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, cyclin D1, MMP-9, VEGF, IL-6 and Bcl-x_L 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-x_L, 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

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

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-x_L 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% CO_2 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, cyclin D1, 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 Strategene (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 $1x10^5$ 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.

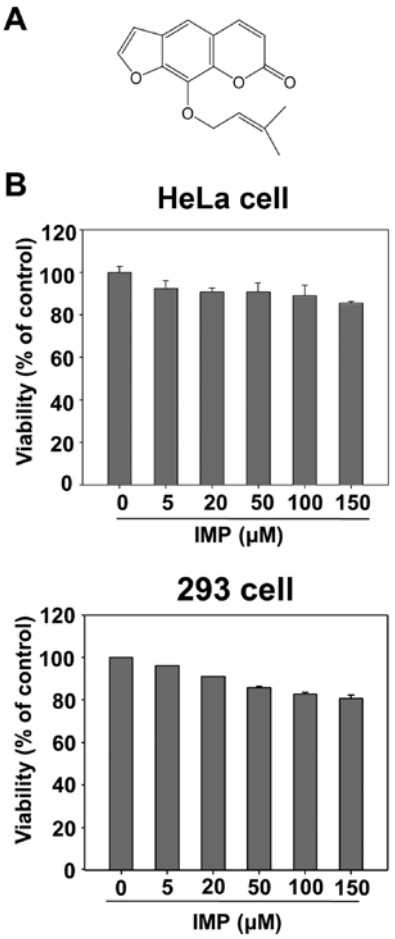

Figure 1. Effect of imperatorin (IMP) on the viability of HeLa and 293 cells. (A) Structure of imperatorin (IMP). (B) HeLa cells and 293 cells were treated with the indicated concentrations of imperatorin (IMP). After 24 h incubation, cell viability was determined by MTT assays. Data are presented as mean ± standard deviation of three independent experiments.

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.

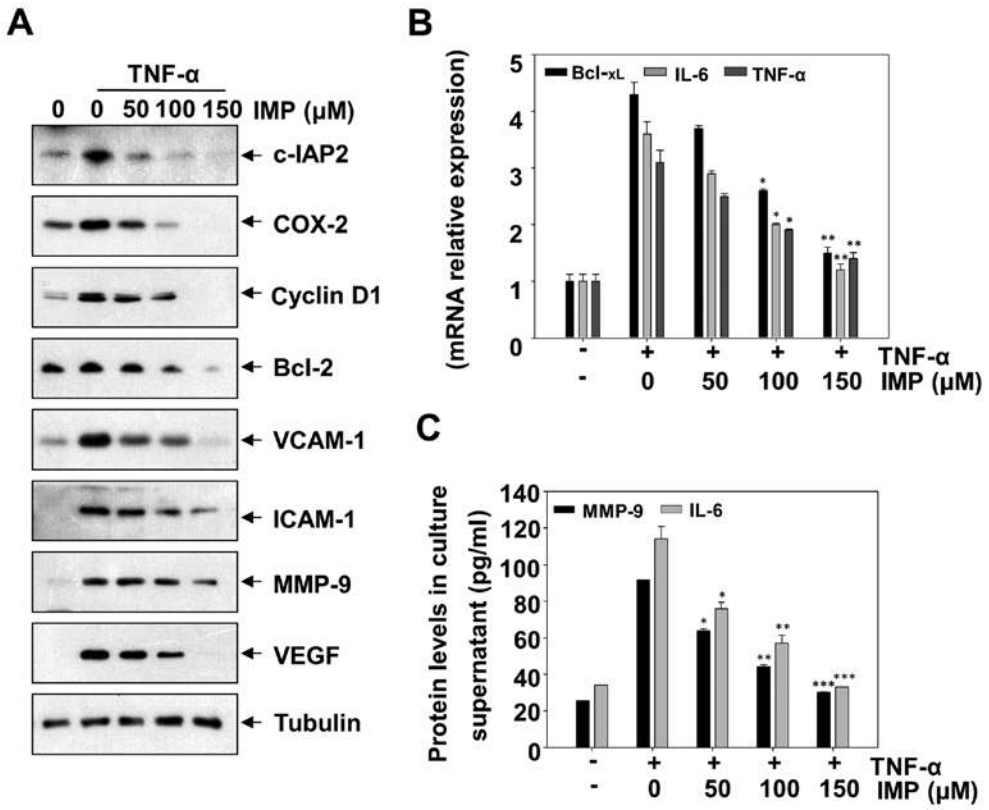

Figure 2. Effect of imperatorin (IMP) on the expression of TNF-α-induced NF-κB-regulated target genes. (A) HeLa cells were incubated with indicated concentrations of imperatorin (IMP) for 12 h and then incubated with TNF-α (10 ng/ml) for 12 h. Whole cell extracts were analyzed by western blotting using indicated antibodies for c-IAP2, COX-2, cyclin D1, Bcl-2, VCAM-1, ICAM-1, MMP-9, VEGF, and tubulin. (B) HeLa cells were incubated with indicated concentrations of imperatorin (IMP) for 12 h and then incubated with TNF-α (10 ng/ml) for 12 h. The mRNA expression of Bcl-X_L, IL-6, and TNF-α was measured by real-time PCR as described in Materials and methods. (C) IL-6, and MMP-9 proteins expression were evaluated by ELISA in culture supernatant of HeLa cells after exposure to TNF-α (10 ng/ml) for 24 h in the presence or absence of the indicated concentrations of imperatorin (IMP). Data presented as mean ± standard deviation of three independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, significant with respect to control.

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 AAGCCAGAGTCCTTCAGAGA-3' and 5'-CTGTTAGGA GAGCATTGGAAATTG-3'; human TNF-α, 5'-CTGCCC CAATCCCTTTATT-3' and 5'-CCCAATCTCTTTTTGA GCC-3'; Bcl-x_L, 5'- GTAAACTGGGGTCGCATTGT-3' and 5'-TGCTGCATTGTTCCCATAGA-3'; GAPDH, 5'-ACC ACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGT 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 $^{\Delta\Delta}$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

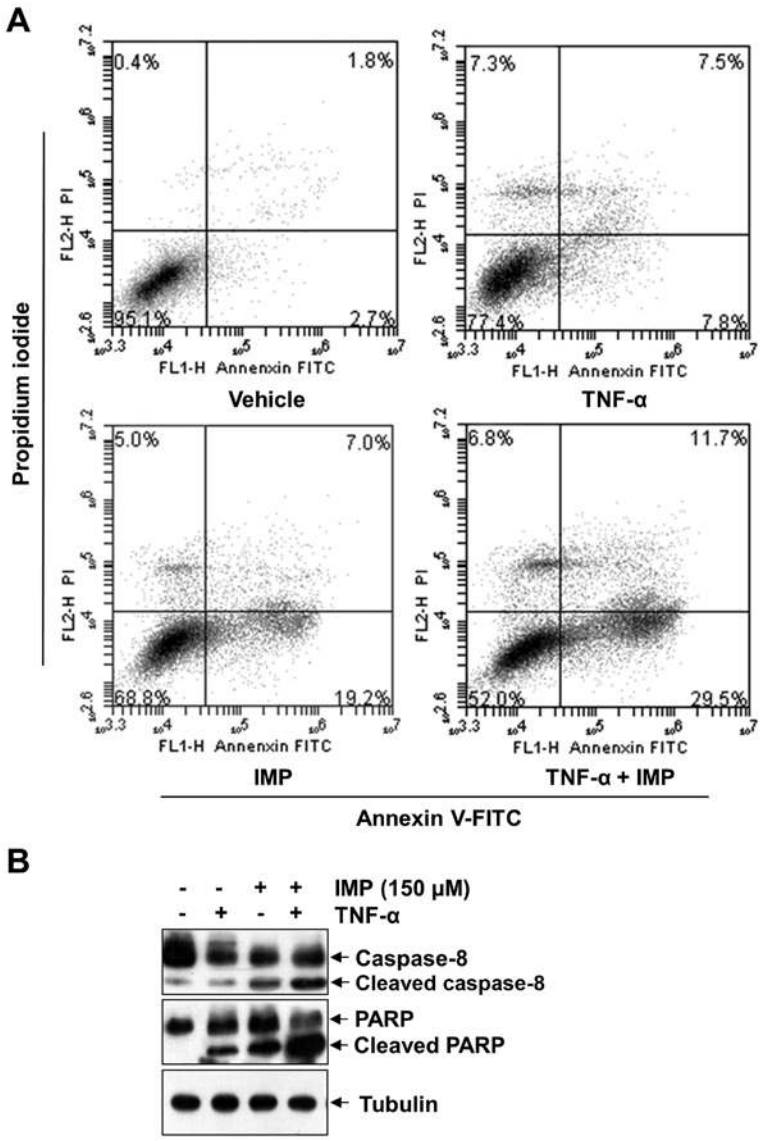

Figure 3. Effect of imperatorin (IMP) on the TNF-α-induced apoptosis. (A) HeLa cells were pretreated with 150 μM imperatorin (IMP) for 12 h and then incubated with TNF-α (10 ng/ml) for 12 h, and subsequently stained with Annexin V-FITC and propidium iodide, followed by analysis using a flow cytometer. Representative plots of one set of triplicate experiments. Early apoptotic cells (Annexin V^+ and PI^-) are displayed in the lower right quadrant and late apoptotic cells (Annexin V^+ and PI^+) are shown in the upper right quadrant. (B) HeLa cells were pretreated with 150 μM imperatorin (IMP) for 12 h and then incubated with TNF-α (10 ng/ml) for 12 h. Whole cell extracts were analyzed by western bloting using indicated antibodies for cleaved capase-8, cleaved PARP and tubulin.

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-x_L 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-x_L 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).

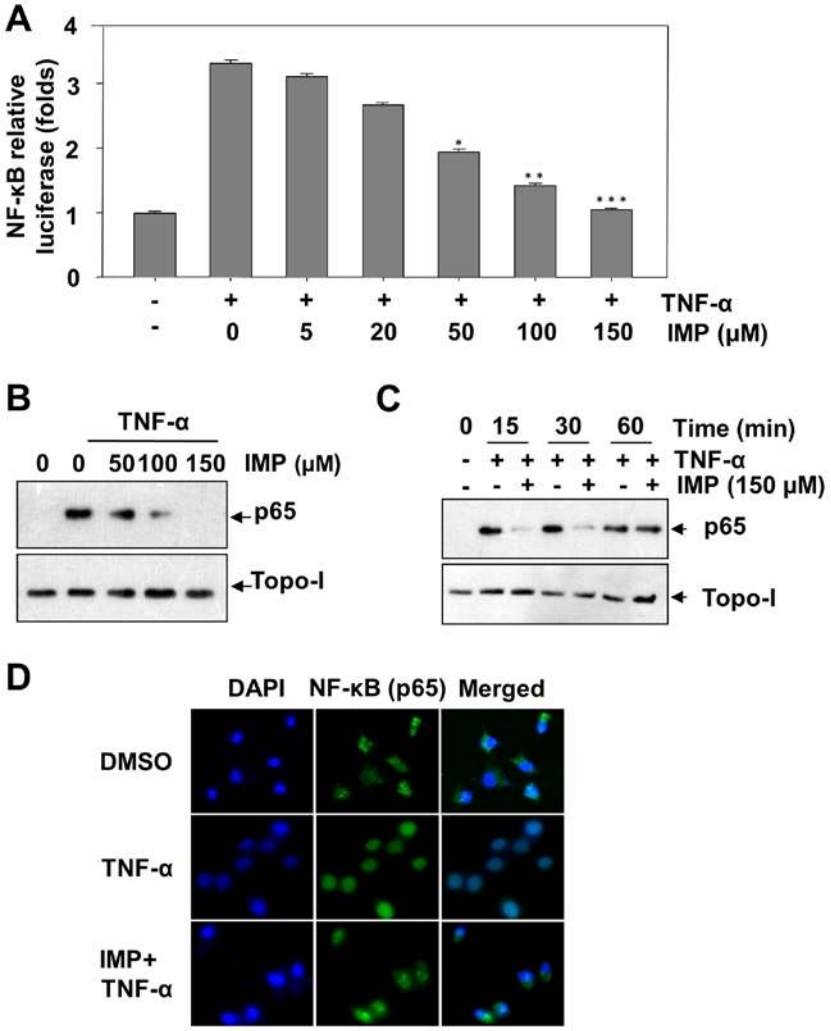

Figure 4. Effect of imperatorin (IMP) on the TNF-α-induced NF-κB activation. (A) HeLa cells were transiently transfected with an NF-κB reporter gene for 48 h and then pretreated for 8 h with the indicated concentrations of imperatorin (IMP) followed by stimulation for 8 h with TNF-α (10 ng/ml), and the luciferase activity was determined as described in Materials and Methods. Data represented as mean ± standard deviation of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, significantly different 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 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 treated with TNF-α and imperatorin, whereas no treatment (4.5%), treatment with TNF-α alone (15.3%) or imperatorin alone (26.2%) had only slight influence on cell apoptosis, as shown in Fig. 3A. In addition, cleavage of caspase-8 and PARP are considered hallmarks of apoptosis. Thus, we examined TNF-α-induced caspase-8 and PARP levels after imperatorin treatment in HeLa cells by western blotting. As shown in Fig. 3B, imperatorin alone had little effect on caspase-8 and PARP cleavage. However, combined treatment of TNF-α with imperatorin resulted in potentiated activation. These results together indicate that imperatorin enhances the apoptotic effect of HeLa cells by TNF-α.

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 NF-κB 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,

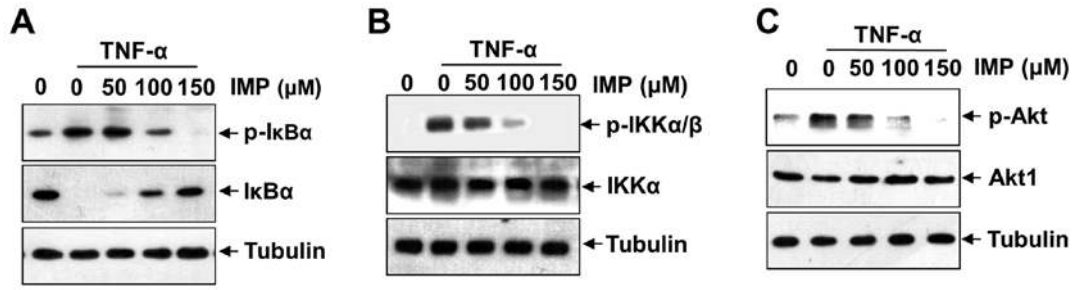

Figure 5. Effect of imperatorin (IMP) on the TNF-α-induced NF-κB activation by modulating phosphorylation and degradation of IκB, phosphorylation of IKKα/β and activation of PI3K/Akt. (A-C) HeLa cells were pre-incubated with indicated concentrations of imperatorin (IMP) for 24 h and then treated with TNF-α (10 ng/ml) for 30 min. Whole cell extracts were analyzed by western blotting using indicated antibodies for p-IκBα, IκBα, p-IKKα/β, IKKα, p-Akt, Akt1 and tubulin.

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 tranlocation 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 tranlocation 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 IKKα/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 convictively repressed the phosphorylation and degradation of IκBα by incubation with TNF-α in a concentration-dependent manner. IKKα/β can regulate IκB 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 IKKα/β. As shown in Fig. 5B, imperatorin can inhibit TNF-α induced phosphorylation of IKKα/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 endocellular 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, IKKα/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-x_L, 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.

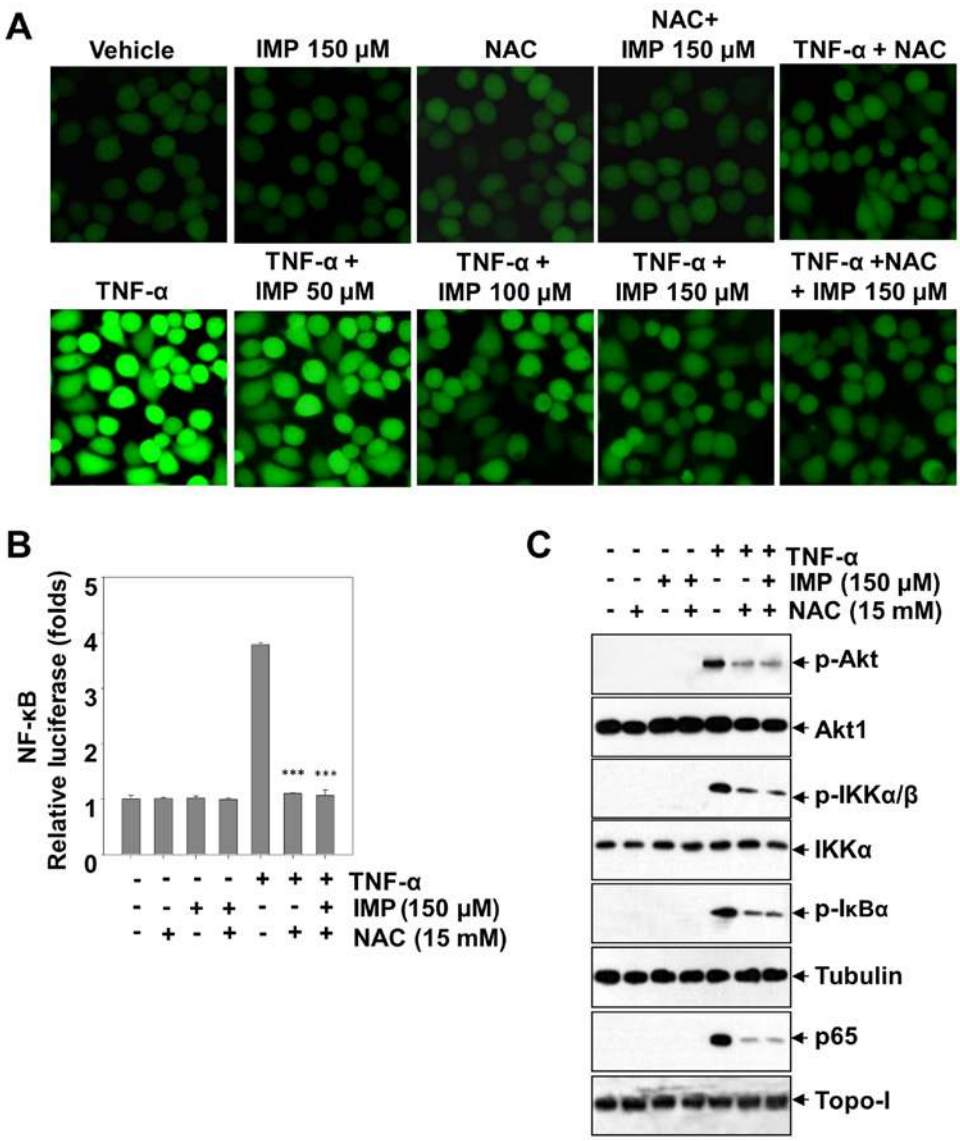

Figure 6. Effect of imperatorin (IMP) on the TNF-α-induced ROS production in HeLa cells. (A) HeLa cells were incubated with NAC (15 mM/l) or/and imperatorin (IMP) for 24 h and then stimulated with TNF-α (10 ng/ml) for 30 min. After stimulation and incubation cells were treated with 10 μM DCFH-DA at 37˚C for 30 min and the fluorescence intensity was measured using ImageJ. Bright green dot-like substances indicate strong ROS expression. (B) HeLa cells were transiently transfected with the NF-κB reporter gene for 48 h, and then stimulated for 8 h with 10 ng/ml TNF-α in the presence of 150 μM imperatorin (IMP) or NAC plus 150 μM imperatorin (IMP) as noted. The level of luciferase activity was determined and presented as the fold of the negative control. (C) HeLa cells were pre-treated with 150 μM imperatorin (IMP) or NAC plus 150 μM imperatorin (IMP) for 24 h, and then stimulated with TNF-α (10 ng/ml) for 30 min. p-IκBα, p-IKKα/β, IKKα, p-Akt, Akt1 and p65 were detected by western blotting.

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 VCAM-1, ICAM-1, 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-apoptotic 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-κB-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-κB pathway.

Taken together, our findings indicate that imperatorin can affect activation of each step of the NF-κB signaling pathway and NF-κB-regulated gene products, which are connected with anti-angiogenic, anti-proliferative, anti-invasive, 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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References

1. Lawrence T, Willoughby DA and Gilroy DW: Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nat Rev Immunol 2: 787-795, 2002.
2. Hayden MS and Ghosh S: NF-κB in immunobiology. Cell Res 21: 223-244, 2011.
3. Sethi G, Sung B and Aggarwal BB: TNF: A master switch for inflammation to cancer. Front Biosci 13: 5094-5107, 2008.
4. Karin M and Ben-Neriah Y: Phosphorylation meets ubiquitination: The control of NF-[kappa]B activity. Annu Rev Immunol 18: 621-663, 2000.
5. Chen J and Chen ZJ: Regulation of NF-κB by ubiquitination. Curr Opin Immunol 25: 4-12, 2013.
6. Seo SH and Jeong GS: 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. Int Immunopharmacol 29: 246-253, 2015.
7. Bai C, Yang X, Zou K, He H, Wang J, Qin H, Yu X, Liu C, Zheng J, Cheng F, *et al*: Anti-proliferative effect of RCE-4 from *Reineckia carnea* on human cervical cancer HeLa cells by inhibiting the PI3K/Akt/mTOR signaling pathway and NF-κB activation. Naunyn Schmiedebergs Arch Pharmacol 389: 573-584, 2016.
8. Dickinson BC and Chang CJ: Chemistry and biology of reactive oxygen species in signaling or stress responses. Nat Chem Biol 7: 504-511, 2011.
9. Murillo MM, Carmona-Cuenca I, Del Castillo G, Ortiz C, Roncero C, Sánchez A, Fernández M and Fabregat I: Activation of NADPH oxidase by transforming growth factor-beta in hepatocytes mediates up-regulation of epidermal growth factor receptor ligands through a nuclear factor-kappaB-dependent mechanism. Biochem J 405: 251-259, 2007.
10. Baek NI, Ahn EM, Kim HY and Park YD: Furanocoumarins from the root of *Angelica dahurica*. Arch Pharm Res 23: 467-470, 2000.
11. Guo W, Sun J, Jiang L, Duan L, Huo M, Chen N, Zhong W, Wassy L, Yang Z and Feng H: Imperatorin attenuates LPS-induced inflammation by suppressing NF-κB and MAPKs activation in RAW 264.7 macrophages. Inflammation 35: 1764-1772, 2012.
12. Mi C, Shi H, Ma J, Han LZ, Lee JJ and Jin X: Celastrol induces the apoptosis of breast cancer cells and inhibits their invasion via downregulation of MMP-9. Oncol Rep 32: 2527-2532, 2014.
13. Hwangbo C, Kim J, Lee JJ and Lee JH: Activation of the integrin effector kinase focal adhesion kinase in cancer cells is regulated by crosstalk between protein kinase Calpha and the PDZ adapter protein mda-9/Syntenin. Cancer Res 70: 1645-1655, 2010.
14. Jin HR, Jin SZ, Cai XF, Li D, Wu X, Nan JX, Lee JJ and Jin X: Cryptopleurine targets NF-κB pathway, leading to inhibition of gene products associated with cell survival, proliferation, invasion, and angiogenesis. PLoS One 7: e40355, 2012.
15. Aggarwal BB: Nuclear factor-kappaB: The enemy within. Cancer Cell 6: 203-208, 2004.
16. Baeuerle PA and Henkel T: Function and activation of NF-kappaB in the immune system. Annu Rev Immunol 12: 141-179, 1994.
17. Zhang G and Ghosh S: Toll-like receptor-mediated NF-kappaB activation: A phylogenetically conserved paradigm in innate immunity. J Clin Invest 107: 13-19, 2001.
18. Ghosh S and Karin M: Missing pieces in the NF-kappaB puzzle. Cell 109 (Suppl): S81-S96, 2002.
19. Kang OH, Lee GH, Choi HJ, Park PS, Chae HS, Jeong SI, Kim YC, Sohn DH, Park H, Lee JH, *et al*: Ethyl acetate extract from *Angelica dahuricae* Radix inhibits lipopolysaccharide-induced production of nitric oxide, prostaglandin E2 and tumor necrosis factor-alphavia mitogen-activated protein kinases and nuclear factor-kappaB in macrophages. Pharmacol Res 55: 263-270, 2007.
20. Yang PY, Rui YC, Li K, Huang XH, Jiang JM and Yu L: Expression of intercellular adhesion molecule-1 in U937 foam cells and inhibitory effect of imperatorin. Acta Pharmacol Sin 23: 327-330, 2002.
21. Savill J: Apoptosis. Phagocytic docking without shocking. Nature 392: 442-443, 1998.
22. Savill J, Dransfield I, Gregory C and Haslett C: A blast from the past: Clearance of apoptotic cells regulates immune responses. Nat Rev Immunol 2: 965-975, 2002.
23. Rovere P, Sabbadini MG, Vallinoto C, Fascio U, Recigno M, Crosti M, Ricciardi-Castagnoli P, Balestrieri G, Tincani A and Manfredi AA: Dendritic cell presentation of antigens from apoptotic cells in a proinflammatory context: Role of opsonizing anti-beta2-glycoprotein I antibodies. Arthritis Rheum 42: 1412-1420, 1999.
24. Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E and Ben-Neriah Y: NF-kappaB functions as a tumour promoter in inflammation-associated cancer. Nature 431: 461-466, 2004.
25. Pober JS and Sessa WC: Evolving functions of endothelial cells in inflammation. Nat Rev Immunol 7: 803-815, 2007.
26. Hermida N and Balligand JL: Low-density lipoprotein-cholesterol-induced endothelial dysfunction and oxidative stress: The role of statins. Antioxid Redox Signal 20: 1216-1237, 2014.
27. Yeom M, Kim JH, Min JH, Hwang MK, Jung HS and Sohn Y: Xanthii fructus inhibits inflammatory responses in LPS-stimulated RAW 264.7 macrophages through suppressing NF-κB and JNK/p38 MAPK. J Ethnopharmacol 176: 394-401, 2015.
28. Hoesel B and Schmid JA: The complexity of NF-κB signaling in inflammation and cancer. Mol Cancer 12: 86, 2013.
29. Saito Y, Nishio K, Ogawa Y, Kimata J, Kinumi T, Yoshida Y, Noguchi N and Niki E: Turning point in apoptosis/necrosis induced by hydrogen peroxide. Free Radic Res 40: 619-630, 2006.
30. Osaki M, Oshimura M and Ito H: PI3K-Akt pathway: Its functions and alterations in human cancer. Apoptosis 9: 667-676, 2004.
31. Porta C and Figlin RA: Phosphatidylinositol-3-kinase/Akt signaling pathway and kidney cancer, and the therapeutic potential of phosphatidylinositol-3-kinase/Akt inhibitors. J Urol 182: 2569-2577, 2009.
32. Chen J, Yang H, Wen J, Luo K, Liu Q, Huang Y, Zheng Y, Tan Z, Huang Q and Fu J: NHE9 induces chemoradiotherapy resistance in esophageal squamous cell carcinoma by upregulating the Src/Akt/β-catenin pathway and Bcl-2 expression. Oncotarget 6: 12405-12420, 2015.
33. Kim JH, Na HJ, Kim CK, Kim JY, Ha KS, Lee H, Chung HT, Kwon HJ, Kwon YG and Kim YM: The non-provitamin A carotenoid, lutein, inhibits NF-kappaB-dependent gene expression through redox-based regulation of the phosphatidylinositol 3-kinase/PTEN/Akt and NF-kappaB-inducing kinase pathways: Role of H(2)O(2) in NF-kappaB activation. Free Radic Biol Med 45: 885-896, 2008.