

Dimethyl itaconate inhibits TNF- α induced NF- κ B signaling pathway in human epithelial cells

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Abstract

Background: Dimethyl itaconate (DMI), a membrane-permeable derivative of itaconate, was found to moderate IL-17-I κ B ζ -induced skin pathology including psoriasis in mouse experiments. TNF- α induced NF- κ B pathway, which controls a variety of immune and inflammatory responses, was also proven to play a crucial role as mediator in psoriasis. However, whether DMI interacts with the TNF- α induced NF- κ B pathway remains unclear. **Results:** Here we show that DMI inhibits TNF- α induced NF- κ B transcriptional activities in dose-dependent manner in several human cell lines using dual luciferase assay and blocks the NF- κ B nuclear entry. Moreover, DMI potently inhibits IKK β dependent phosphorylation and degradation of I κ B α in TNF- α induced activation of NF- κ B pathway. We also demonstrate that DMI covalently binds to cysteine residue in IKK β , a key regulator in NF- κ B pathway, to suppress IKK β activation and inhibit the canonical NF- κ B pathway. **Conclusion** Our study presents a new mechanism for DMI as an anti-inflammatory agent that may have therapeutic potentials in treating NF- κ B related human inflammatory diseases. Our results also suggest that itaconate produced by endogenous IRG1 may regulate NF- κ B at post translation modification level, and the IRG1-itaconate-NF- κ B axis could be targeted as a novel strategy for the treatment of IRG1-NF- κ B mediated diseases.

Background

Nuclear factor- κ B (NF- κ B) signaling pathway plays a critical role in immune and inflammatory responses, cell survival/proliferation, and cell/tissue homeostasis maintenance. It controls the expression of hundreds of genes, including cytokines, chemokines, adhesion molecules and anti-apoptosis proteins¹. Its key element, NF- κ B, is a transcriptional factor consisting of a family of five proteins, including p50, p52, p65 (RelA), c-Rel and RelB. Each of them can form homodimers or heterodimers with another protein of a different type within the protein family². In resting cell, NF- κ B is arrested by I κ B inhibitory proteins (for example, I κ B α , I κ B β , I κ B) in cytoplasm. Upon stimulation by various pro-inflammatory stimuli such as TNF- α , microbial infection, and some of chemotherapeutic agents, I κ B kinase complex (IKK) is activated. This complex is composed of IKK α and IKK β catalytic subunits and a regulatory subunit, IKK γ /NEMO. IKK β is considered as the major subunit responsible for phosphorylation of I κ Bs. The activated IKK then phosphorylates I κ Bs and leads to its ubiquitination and degradation by 26S proteasome. NF- κ B is then liberated and translocates to the nucleus and activates gene transcription³. Hence, the activation of IKK β and phosphorylation of I κ Bs are the central points in NF- κ B pathway. Under normal condition, NF- κ B pathway is activated whenever needed and is terminated properly. However, under abnormal condition, it could be constantly activated, leading to a wide range of chronic inflammatory and autoimmune diseases, such as psoriasis, rheumatoid arthritis and Crohn's disease⁴⁻⁶.

The NF- κ B pathway is considered as a crucial mediator in psoriasis, an inflammatory skin disease featured by cell hyperproliferation and differentiation. The level of active NF- κ B is significantly elevated in psoriasis⁷. Many chemokines and cytokines involved in psoriasis, such as IL-17, TNF- α and IL-12/23, depend on NF- κ B signaling. Therefore, several anti-psoriasis therapies, including TNF- α blockers like

infliximab, etanercept, adalimumab and IL-12/23 inhibitors like ustekinumab are used to reduce the activity of NF- κ B and related downstream elements⁸. However, the use of these antibody drugs is limited by treatment resistance, potential risk for adverse events and high cost. These limitations emphasize the need for additional treatment options for psoriasis. Small molecule drug always represents a more cost-effective way for treatment of many diseases.

Recently, Bambouskova *et al.* discovered that dimethyl itaconate (DMI), a derivative of itaconate which is a newly discovered metabolite with significantly upregulated concentration under the inflammatory condition and has been reported to have anti-inflammatory property, has a significant effect on psoriasis by inhibiting the production of I κ B ζ induced by IL-17⁹. This indicates the potential therapeutic application of DMI on psoriasis. Recent studies have shown that itaconate and its derivatives can inhibit inflammation through NRF2 and also act via ATF3 to block inflammation driven by the IL-17-I κ B ζ axis^{2, 13, 17}. More recently, they were found to attenuate the inflammatory response by impairing glycolysis^{21, 22}. Nevertheless, none of them focused on the anti-inflammatory mechanism of itaconate and its derivatives on NF- κ B signaling pathway which is important in inflammatory diseases including psoriasis.

DMI was firstly used as a membrane-permeable non-ionic form of itaconate to probe itaconate metabolism¹⁰⁻¹¹. Treatment of macrophages with DMI has an inhibitory effect on inflammatory responses induced by LPS or LPS plus IFN γ ¹¹. Nevertheless, ElAzzouny *et al.* found that itaconate produced from DMI was not detectable in cells¹², and the itaconate-mediated inflammatory inhibition effects may be directly induced by DMI. These results demonstrated the potential role of DMI in inflammatory diseases, especially in psoriasis. However, currently DMI was only shown to function on IL-17-I κ B ζ -mediated psoriasis⁹, whether it has effect on other model of psoriasis driven by cytokines, such as TNF- α remains unclear. As NF- κ B pathway is the key element in pathogenesis of psoriasis, we hypothesize that it may also inhibit NF- κ B pathway.

Based on the differential gene expression analysis of bone marrow-derived macrophages (BMDMs) pretreated with DMI or vehicle and then stimulated with LPS from Lampropoulou *et al.*'s work¹¹, we found that DMI-pretreatment modulated the expression of several NF- κ B-regulated genes, including *IL-6*, *IL-1 β* and *Cxcl11*. This provides a strong evidence that DMI can affect NF- κ B pathway. Moreover, recently, DMI was reported to ameliorate LPS-induced mastitis by activating MAPKs and Nrf2 and inhibiting NF- κ B signaling pathways¹³. These indicate that DMI has strong relationship with NF- κ B signaling pathway. Here we show that DMI inhibits TNF- α -induced NF- κ B signaling pathway which is crucial in the pathogenesis of psoriasis in several epithelial cells. The inhibitory effect of DMI on NF- κ B signaling is exerted via targeting IKK β by covalent binding to C412 in IKK β both *in vitro* and in cells and inhibiting the activation process of IKK β . This discovery not only provides the evidence that DMI can inhibit TNF α -induced NF- κ B pathway, but also provides a novel probe to investigate the molecular mechanism of IKK β . Furthermore, we found that itaconate can regulate NF- κ B pathway at post translational modification level. We confirmed that itaconate could covalently modify several cysteines in IKK β . Moreover, we demonstrated that itaconate produced in the cell can inhibit the NF- κ B signaling pathway by

overexpressing IRG1 that can produce itaconate. Therefore, our study provides new insights of itaconate and its derivative DMI's anti-inflammatory function.

Results

Dimethyl itaconate inhibits TNF- α induced NF- κ B signaling pathway in epithelial cells

DMI has been successfully used to moderate IL-17-I κ B ζ -driven skin inflammation in mouse model of psoriasis. As NF- κ B plays crucial role in psoriasis, we investigated if DMI also acts as an anti-inflammatory agent in psoriasis by inhibiting NF- κ B signaling pathway. We first used NF- κ B luciferase assay to evaluate the effects of DMI on NF- κ B-dependent transcriptional activity upon TNF- α stimulation. NF- κ B-luciferase reporter plasmid (pGL4.32) and Renilla luciferase plasmid (pGL4.74) were transiently transfected in several epithelial cell types. As shown in Figure 1, DMI potently inhibited TNF- α -induced NF- κ B-dependent transcriptional activity in several epithelial cells, including human kidney epithelial HEK293T cell, breast epithelial MCF-7 cells, neuroblast epithelial SK-N-AS cells and cervix epithelial Hela cells in a dose-dependent manner. Dose-response analysis revealed that DMI inhibited TNF- α -stimulated NF- κ B signaling pathway with an approximate IC₅₀ value of 0.25 mM. Cell viability analysis showed that DMI did not have significant effect on cell viability at this concentration, eliminating the possible interference from cytotoxicity (Supplementary Figure 2). The result of dimethyl fumarate (DMF) as positive control on cell luciferase assay was shown in Supplementary Figure 1.

Dimethyl itaconate inhibits TNF- α -induced nuclear translocation of NF- κ B

We then tested whether DMI can abolish the TNF- α -induced nuclear translocation of p65. In this experiment, we used a SK-N-AS monoclonal stable cell line transfected with NF- κ B/p65 labeled by red fluorescent protein and nuclear H₂B labeled by green fluorescent were pre-incubated with 0.25 mM DMI for 1 h before stimulation with 10 ng mL⁻¹ TNF- α at 37°C (5% CO₂). The movement of NF- κ B/p65 was captured every 5 min for 4 h as described before¹⁴. We measured the nuclear amounts of fluorescent protein-RelA by time-lapse imaging adding DMI or not. About 120 live cells were quantified for each condition. We found that addition of DMI significantly reduced the percentage of activated cells (Supplementary Figure 3) and nuclear NF- κ B intensity (Figure 2A, B). Collectively, these data suggested that DMI inhibited TNF- α -induced nuclear translocation of p65.

DMI inhibits TNF- α -induced NF- κ B Activation by Targeting IKK

From NF- κ B luciferase assay and time-lapse confocal imaging experiment, we showed that DMI inhibited the NF- κ B signaling pathway. We next identified the molecular target of DMI. Because IKK β plays central role in NF- κ B signaling pathway, we first evaluated the effect of DMI on IKK β -dependent phosphorylation and degradation of I κ B α in TNF- α -induced activation of NF- κ B in Hela cells. As shown in Figure 3, phosphorylated I κ B α and I κ B α were detected in Hela cells pretreated with or without DMI for 12 h and then stimulated with TNF- α for indicated time by Western blot analysis using antiphospho-Ser32/Ser36

I κ B α antibody and no phosphorylated I κ B α antibody. As shown in Figure 3A-C, TNF- α induced phosphorylation and degradation of I κ B α were in time-dependent manner as described before¹⁵. For the control group, in Figure 3A and 3B, it is clear that when the cells were stimulated with TNF α , the level of I κ B α rapidly decreased, while the level of pI κ B α increased to the highest level at 5 minutes and then slowly decreased along with time due to the low level of I κ B α . We further calculated the ratio of pI κ B α /I κ B α (shown in Figure 3D) and the control group showed a tendency of continuing increase with time. For the DMI group, when the cells were stimulated with TNF α , the level of I κ B α almost kept constant (shown in Figure 3A, 3B), while the levels of pI κ B α were significantly lower compared with the control group at corresponding time points. For the ratio of pI κ B α /I κ B α (shown in Figure 3D), the ratio of the DMI group kept at a low level at all time points. These results suggest that DMI inhibits TNF- α -induced NF- κ B activation by preventing I κ B α phosphorylation and degradation by targeting IKK β .

We further tested whether DMI can directly interact with IKK β . We generated N-10xHis-tagged IKK β recombinant protein from HEK293T cells transfected with a pCMV-N-His-IKK β expression plasmid for further analysis. The N-10xHis-tagged IKK β was captured by NTA agarose beads. The purified recombinant IKK β protein was incubated with or without DMI for 12 h at 4 °C, and then the mixtures were resolved by SDS-PAGE. As shown in Supplementary Figure 5, one clear band was observed at around 88 kDa. We used western blotting experiment and LC-MS/MS experiment to identify IKK β (shown in Supplementary Figure 5). After that, in-gel digestion of IKK β was performed with trypsin and then analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Notably, we identified several tryptic peptides having mass shift, as shown in Supplementary Figure 4. Tandem mass spectrometry revealed that treatment of recombinant human IKK- β with 0.25 mM DMI led to a combination of dimethyl and monomethyl itaconate at cysteines 412, 370 and 716 (Supplementary Table 1). Neither of these modifications was observed at any cysteine in vehicle-treated IKK- β .

To test whether the DMI can modify IKK- β in cell, we treated HEK293T cells overexpressing recombinant IKK- β with 0.25 mM DMI for 3h. After incubation, we extracted whole-cell lysate using NTA agarose beads and digested the protein with trypsin. As shown in Figure 4, LC-MS/MS analysis identified a peptide with calculated mass of 2876.46 Da, which is 132.34 Da larger than the C412-containing peptide ITYETQISPRQPESVSCILQEPK that has a calculated mass of 2744.12 Da. The mass difference of 132.34 Da matches the molecular weight of itaconate. LC-MS/MS spectra demonstrated that a 132.34 Da mass shift occurred starting from y₅ to the y₁₂ fragment, indicating that the C412 residue was covalently modified by DMI. However, the modified molecule was itaconate rather than DMI. It was shown that DMI was rapidly degraded in cells without releasing itaconate¹², hence it was possible that DMI covalently binds to IKK β first and the complex undergoes further esterase digestion. As the MMI modified IKK β was also observed in vitro, the other possibility is the fragmentation may happen in the mass spectrometry detection. In addition to this, we also hypothesized that the inhibitory effect of NF- κ B signaling pathway from DMI treatment was comprehensive results of both DMI and itaconate. We supposed that only a few DMI may convert to itaconate, and this part of itaconate alkylate cysteine residues on proteins rapidly. It has been discovered that IRG1, an enzyme that produces itaconate in cells,

can suppress NF- κ B signaling pathway by decreasing IKK α / β activation upon LPS stimulation¹⁶. We then tested whether itaconate can bind to IKK β covalently in vitro. As shown in Supplementary Table 2, itaconate can modify several cysteines in IKK β . Next, to determine whether IRG1-mediated itaconate production can affect NF- κ B signaling pathway upon TNF- α stimulation in cells, we overexpressed *IRG1* in HEK293T cells using a pCMV3-N-Flag-IRG1. IRG1 and itaconate was only detected in pCMV3-N-Flag-IRG1-overexpressing cells (for detail, see Supplementary Figure 7A, B). Not surprisingly, itaconate produced by IRG1 inhibited TNF- α -induced NF- κ B signaling pathway (Supplementary Figure 7B), which was consistent with the results before¹⁶. To eliminate the effect of IRG1 itself, we also used 4-octyl itaconate (OI), a recently described cell-permeable derivative of itaconate which can release itaconate in cells¹⁷, to treat cells and then detected the activation of NF- κ B pathway. Cell luciferase assay and Western blot analysis showed that OI can inhibit TNF- α -stimulated NF- κ B signaling pathway (for detail, see the Supplementary Figure 6 and Supplementary Figure 8). However, the inhibitory effect of OI on NF- κ B signaling pathway was weaker than that of DMI on NF- κ B signaling pathway. These results indicated that the inhibitory effect of DMI on NF- κ B signaling pathway may from both DMI and itaconate. Despite of these possibilities, our results suggested that DMI treatment indeed modified IKK β in cells.

Having identified that DMI can directly modify recombinant IKK β in cells, we next explored how this modification affected IKK β structure and activity. IKK β protein exists as a dimer in solution. Each IKK β monomer has a trimodular linear architecture: the N-terminal kinase domain (KD, 1-309), the central ubiquitin-like domain (ULD, 310-404), and the C-terminal dimerization domain (SDD, 408-664)¹⁸. IKK β activity can be described as a kinase cycle of three states: poised, active, and inactivated. In the absence of stimulation, IKK β was in the poised state¹⁹. After stimulation by TNF- α , the poised IKK β is activated by phosphorylation on Ser177 and Ser181 in its activation loop. Then the activated IKK β phosphorylates its substrate I κ B α . In addition to this, the activated IKK β also phosphorylates its own C terminus, thereby inducing conformation change that results in the decrease of kinase activity. Then the inactive hyper-phosphorylated IKK β becomes available by dephosphorylation. Among all these steps in the IKK kinase cycle, activation of IKK β is the most important one. Furthermore, IKK β autophosphorylation seems to be the key step in activation of IKK β . Thus, we tested whether DMI can directly inhibit the activation of IKK β . We monitored the level of active IKK β in Hela cells stimulated with TNF- α pre-treated with or without DMI using Western blot analysis. As shown in Figure 5, DMI dose-dependently and time-dependently inhibited phosphorylation of Ser177/181 on IKK β , the activated state of IKK β . This result indicated that DMI indeed inhibits the activation of IKK β .

Recent crystal structure studies revealed that IKK β formed higher order homo-oligomers to phosphorylate its dimer partners. The oligomerization surface was a “V shaped” interface including the N-terminal KD-ULD portions and the proximal SDD from the two promoters. The double mutant I413A/L414A within the V-shaped interface was found to disrupt IKK β activation in cells²⁰. We found the modified residue, C412, in IKK β was right in this “V-shaped” interface. Moreover, mutation of Cys-412 (C412A) in IKK β could inhibit its activation. And then DMI had no obvious effect on C412A mutant comparing with wild type IKK β (Supplementary Figure 9). These evidences all support that DMI inhibits the autophosphorylation of

IKK β by modifying C412 to disrupt the oligomerization surface of IKK β . Our results also suggest that C412 in IKK β provides a new and potent druggable binding site for IKK β based drug design.

Discussion

The roles of metabolic regulation in diseases and human health-related processes have received increasing attention. In immune system, extracellular and intracellular signals regulate the activity of metabolic pathways, thus allowing cells to synthesize metabolites to facilitate their growth and to enable their functions. Different metabolic pathways, which correspond to different cellular functions, produce a large number of small molecule metabolites. These metabolites not only provide material and energy supplies for cells, but also serve as signal molecules to transmit information. In addition, because of their endogenous characteristics, they could be potentially applied in drug discovery. For example, dimethyl fumarate (DMF), a derivative of fumarate, is an U.S. Food and Drug Administration and European Medicines Agency approved immunomodulatory drug used to treat multiple sclerosis and psoriasis.

DMI is a derivative of itaconate which is discovered recently as an important metabolite. It is firstly used as the permeable itaconate surrogate to study the function of itaconate in cells. DMI was reported to reduce the inflammatory responses in LPS or LPS plus IFN γ stimulated macrophages¹¹ and to moderate the IL-17-Ik ζ -driven psoriasis⁹. This highlights the potentials of DMI as a novel anti-inflammation agent. Recent research found that DMI, like DMF with electrophilic α , β -unsaturated moieties, could induce electrophilic stress by covalent conjugation with GSH and subsequently induce Nrf2 responses to inhibit the production of cytokines associated with inflammation⁹. It can also block the IkB ζ expression through ATF3⁹. More recently, DMI has been reported to inhibit NF- κ B signaling pathway in LPS-induced mastitis but without precise mechanism¹³. Most recently, another itaconate derivate OI was reported to exert anti-inflammatory effects by limiting aerobic glycolysis²¹. In this study, we demonstrated that DMI blocks the nuclear translocation and transcriptional activity of NF- κ B upon TNF- α stimulation. Particularly, DMI inhibited NF- κ B-dependent responses by directly modifying Cys412 of IKK β . This modification suppressed the activation of IKK β , which demonstrated the essential role of Cys412 in IKK β function. Accumulating evidences show that the anti-inflammatory effect of DMI is complex progress integrating the metabolism and signaling pathway. In the present work, we studied the effect of DMI on IKK β , a hub in the NF- κ B pathway. Whether DMI also bind to other proteins related to NF- κ B pathway need further study.

The molecular forms that DMI function in the cells are still controversial. Synthesis of methyl or ethyl ester analogues of polar carboxylate metabolites is a common approach used to deliver these metabolites intracellularly. These methyl or ethyl ester would be hydrolyzed by esterases in cells. This pro-drug strategy has been applied in glutamate and fumarate intracellular delivery. Therefore, DMI was supposed to presumably deliver intracellular itaconate. But ElAzzouny *et al.* found that DMI was not metabolized into itaconate intracellularly¹². This indicated the inflammatory inhibition effect may be directly induced by DMI. But no matter what form of DMI in cells to exert anti-inflammatory effect, it is

true that DMI indeed functions. In our study, we demonstrated that DMI may be converted to itaconate to some extent. We have shown that another itaconate derivative OI which can release itaconate in cells also has the inhibitory effect of NF- κ B signaling pathway. In addition, overexpression of IRG1 has the similar effect. So we demonstrated that the inhibitory effect of DMI on NF- κ B signaling pathway was a comprehensive results of both DMI and itaconate.

Conclusion

In summary, we identified a new mechanism for the anti-inflammatory effect of dimethyl itaconate. We showed that DMI inhibited TNF- α -induced NF- κ B activation in several epithelial cells. And we confirmed this result using Western blotting experiment to monitor the I κ B α phosphorylation and degradation and time-lapse confocal imaging experiment to monitor the TNF- α -induced nuclear translocation of p65. Moreover, we showed that DMI inhibits NF- κ B signaling pathway by blocking IKK β activation via covalent binding to its Cys412 residue, which may be further explored and used to develop IKK β covalent inhibitor. These findings may lead to the development of DMI as a new anti-inflammatory agent for treatment of various autoimmune diseases.

Methods

Cell lines and chemical compounds

HEK293T, Hela, MCF-7 and SK-N-AS cells were purchased from ATCC. HEK293T, Hela, MCF-7 were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum(FBS, Gibco) and SK-N-AS cells were cultured in DMEM supplemented with 10% FBS and 1% Non-essential amino acids. Dimethyl itaconate was purchased from J&K Scientific.

Cytokine and Reagents

Recombinant human TNF- α was expressed by E.coli using the pUC-TNF- α plasmid. The following antibodies were used for Western blotting and all of them were from Cell signaling Technology: Phospho-IKK α / β (Ser176/180)(#2697), I κ B α (#4814), β -actin(#3700) and HRP-conjugated secondary antibodies (#7074,#7076). pGL4.32 [*Luc2p*/NF- κ B-RE/Hygro] and pGL4.74 [*hRluc*/TK] (Promega) were purchased from Promega. pCMV-N-Histag-IKK β were purchased from Sino Biological.

NF- κ B transcriptional activity assays

NF- κ B transcriptional activity assay was performed using Dual-Glo luciferase assay system (Promega). Cells were seeded in six-well plates (70×10^4 cells per well) for 12h and transiently transfected with pGL4.32[*Luc2p*/NF- κ B-RE/Hygro] plasmid (Promega)and pGL4.74[*hRluc*/TK] (Promega). Next day, cells were plated in 96-well plates at a cell density of 2×10^4 per well; 12h later, cells were treated with DMI for indicated time and then activated with TNF- α (10 ng mL^{-1}) for 6 hours. After stimulation, the luciferase

assays were conducted using Dual-Glo Luciferase Assay System (Promega) with BioTek synergy 4 Multi-Mode Microplate Reader.

NF- κ B/p65 nuclear translocation fluorescence

SK-N-AS cells with p65 labeled with mcherry and H₂B labeled with EGFP which was constructed as previously described¹⁴ were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% Non-Essential AminoAcid (NEAA, Gibco) in a humidified atmosphere with 5%CO₂ at 37°C. 1.5x10⁵ SK-N-AS cells were seeded on 35-mm glass-bottom dishes (Invitro Scientific Products, Inc.) in 3 mL medium. The day of experiment, the medium was changed to fresh DMEM containing indicated concentration of DMI. After 3 h incubation at 37°C in cell culture incubator, cells were stimulated by TNF- α (10 ng mL⁻¹). All the images were captured by Volocity software (PerkinElmer) using an inverted TiE microscope (Nikon). All channels were collected every 5min for 4h. For image quantification, we used the method described before.

Western blots

Hela cells lysed in RIPA lysis buffer system(CWBIO, CW2334) and heat-denatured at 95°C for 5 min in reducing sample buffer(CWBIO). Proteins were separated on 10% polyacrylamide gradient gels and transferred onto PVDF membranes (0.45 μ m pore size, Millipore). No-specific binding was blocked with 5% BSA, and membranes were incubated with primary antibody followed by incubation with anti-rabbit-HRP (1:5000; #7074) or anti-mouse-HRP (1:5000; #7076) from Cell Signaling Technology and Western ECL substrate (Millipore). Membranes were imaged using the Fusion FX7 (Spectra) system (VILBER). β -actin run on the same blot was used as a loading control.

Protein Expression and Purification

The HEK293T cells were grown to 80% confluency in 15-cm dished (Corning) at 37°C in DMEM supplemented with 10% FBS. Before transfection, cells were changed to fresh medium. Viafect reagent and purified pCMV-N-10xHis-IKK β plasmid were added to cells in a ratio of 3:1 for transfection. Cells were harvested and lysed in NP40 Cell Lysis buffer (Invitrogen) containing 1mM PMSF and 1xprotease inhibitor cocktail (Roche) for 30 minutes on ice with vortexing at 10 minute intervals. The lysate was clarified by centrifugation at 14000rpm for 30min at 4°C. 10⁸ cells lysate was incubated with 100 μ L of 50% slurry Ni Sepharose beads (GE Healthcare) for 1h at 4°C. The beads were washed three times with NTA-20 (20 mM HEPES pH 7.3, 300 mM NaCl, 20 mM imidazole) and once with NTA-150 (20 mM HEPES pH 7.3, 300 mM NaCl, 150 mM imidazole). The his-tagged IKK β was eluted with elution buffer, NTA-250(20 mM HEPES pH 7.3, 300 mM NaCl, 250 mM imidazole). The purification of IKK β was confirmed by SDS-PAGE, and the concentrations were measured by Nanodrop 2000 (Thermo Scientific, United States).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LS-MS/MS was performed by the Analytical Instrumentation Center of Peking University. The proteins were analysed by LTQ Orbitrap Elite System (ThermoFisher).

Abbreviations

DMI Dimethyl itaconate

TNF- α Tumor necrosis factor alpha

NF- κ B Nuclear factor- κ B

IKK β κ B kinase β

IRG1 Immune responsive gene 1

LPS Lipopolysaccharides

BMDMs bone marrow-derived macrophages

OI 4-octyl itaconate

IC₅₀: Half maximal inhibitory concentration

Declarations

Availability of data and materials

Not applicable.

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Contributions

LHL designed the project; YLZ, KNL performed the experiments; XBD, HL, ANG, QZ, HBL contributed reagents/material/analysis tools; YLZ drafted the manuscript. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

Additional information

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Figures

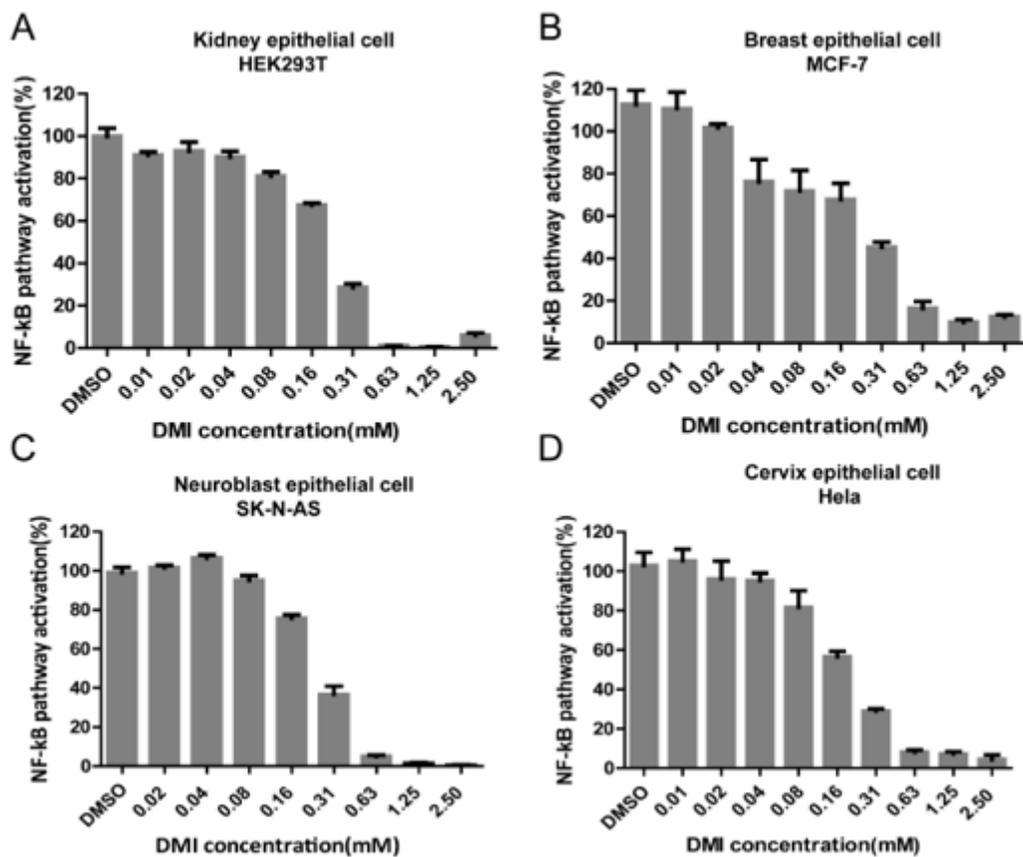


Figure 1

DMI inhibited TNF- α -induced NF- κ B-dependent transcriptional activity in several epithelial cells. Human HEK293T (A) or MCF-7 (B), SK-N-AS (C) and Hela cells (D) transfected with plasmids were pretreated with DMI for 12 h before treatment with TNF- α (10 ng mL⁻¹). Data represent the mean \pm SD of at least three independent experiments, and each experiment was performed in triplicate.

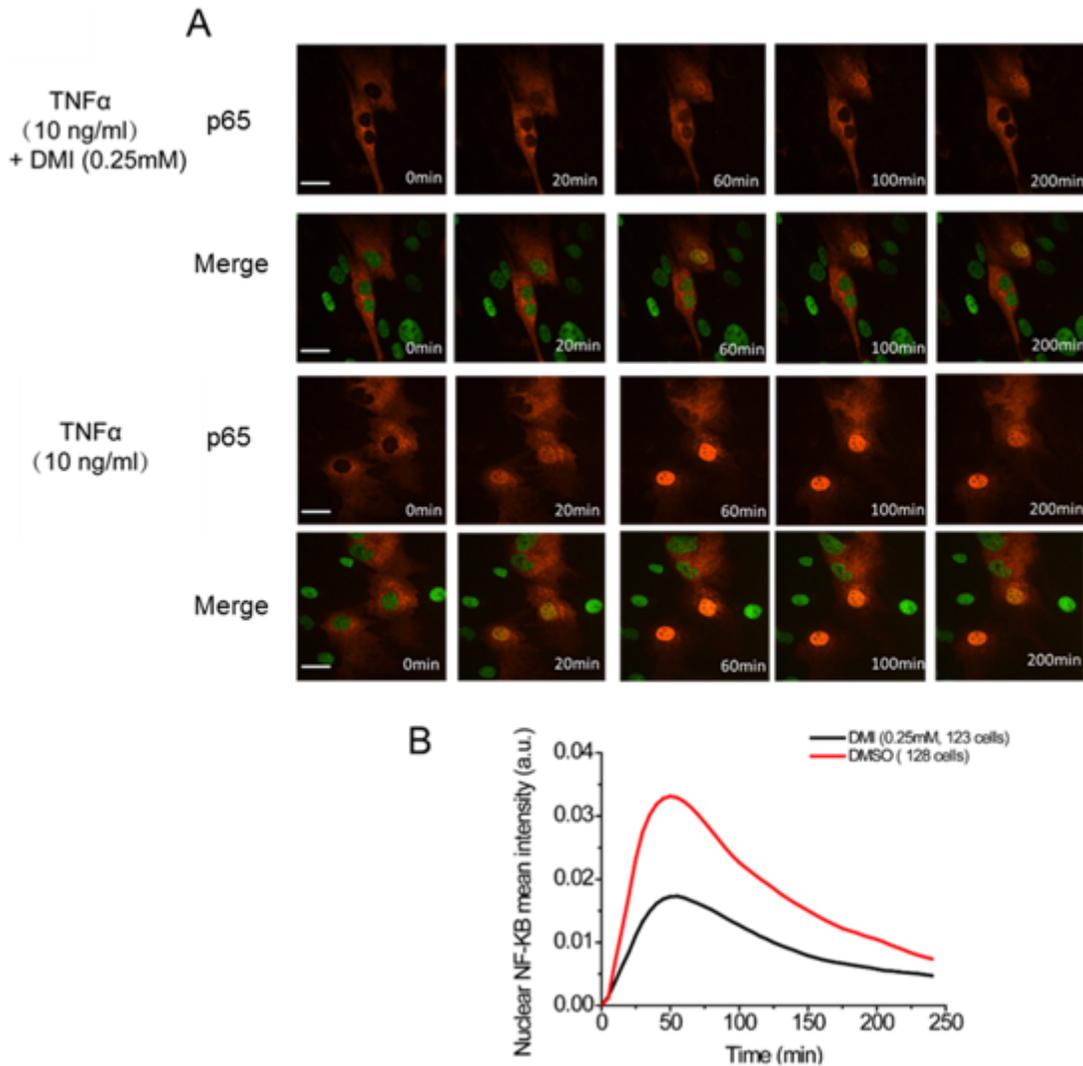
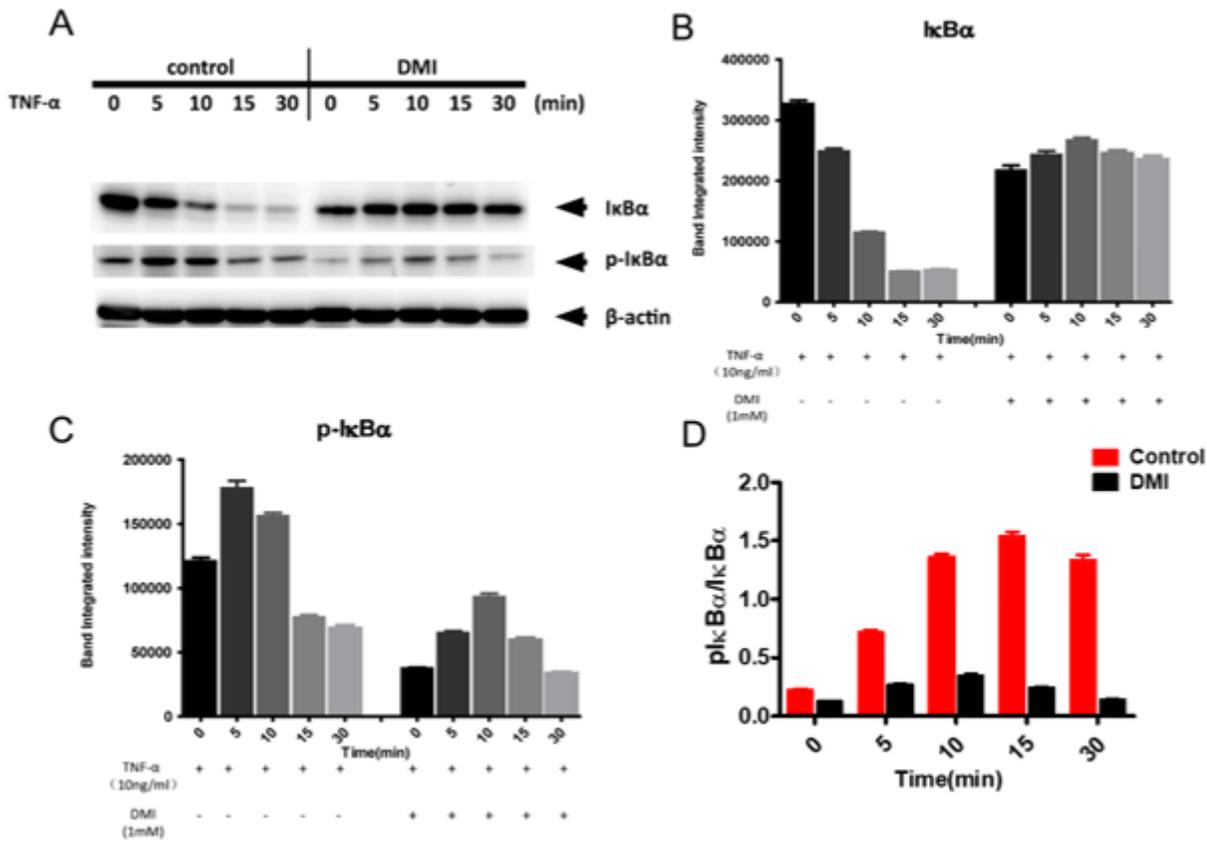


Figure 2

DMI inhibited TNF- α -induced NF- κ B/p65 nuclear translocation in SK-N-AS cells. (A) Time-lapses images of stable transfected SK-N-AS cells with p65-mcherry and H2B-EGFP treated with DMSO or DMI (0.25 mM, 1 h) and then stimulated with 10 ng mL⁻¹ TNF- α . Scale bar, 10 μ m. (B) Average nuclear NF- κ B intensity of cells pretreated with DMSO or DMI and then stimulated with TNF- α (10 ng mL⁻¹).



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Figure 3

DMI inhibited TNF- α -induced NF- κ B signaling pathway by preventing I κ B α phosphorylation and degradation. HeLa cells were incubated in the presence of TNF- α (10 ng mL⁻¹) for indicated time (0 min, 5 min, 10 min, 15 min, 30 min) in the culture medium with or without 1 mM DMI. (A) Western blot imaging of dynamic of phosphorylation and degradation of I κ B α in total lysates. I κ B α and p-I κ B α band integrated intensity were shown in (B) and (C). (D) The ratio of p-I κ B α /I κ B α at indicated time in control group and DMI group.

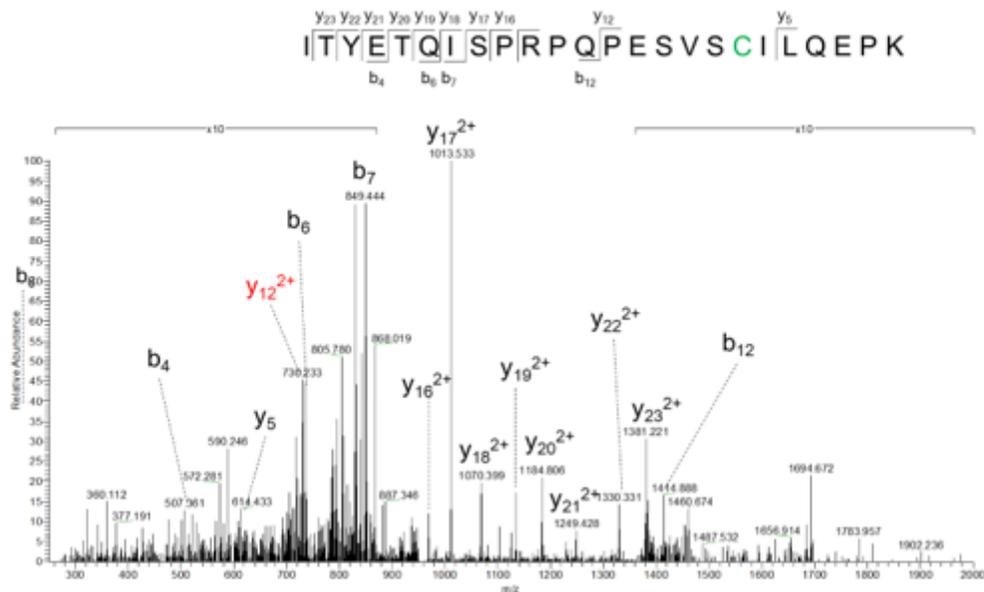


Figure 4

DMI directly modified IKK β in cells. HEK293T cells overexpressing recombinant IKK β were incubated with 0.25 mM DMI for 3 h. LC-MS/MS analysis of DMI-mediated modification of recombinant IKK β in cells.

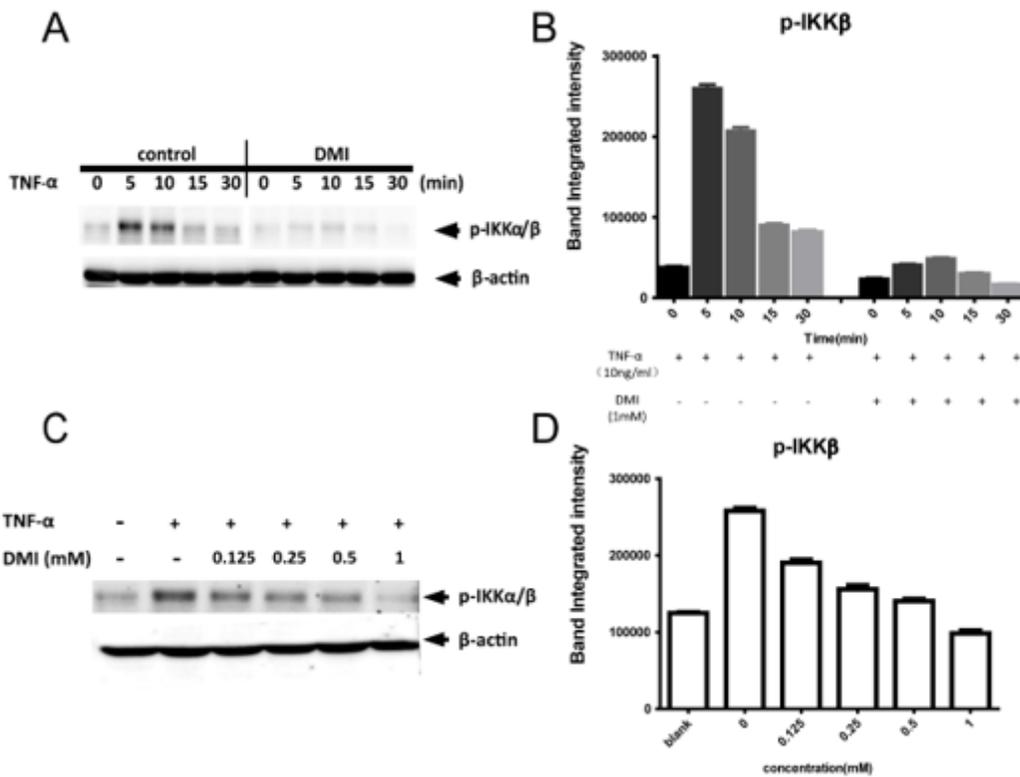


Figure 5

DMI suppressed the activation of IKK β . HeLa cells were incubated in the presence of TNF- α for indicated time (0 min, 5 min, 10 min, 15 min, 30 min) with or without 0.25 mM DMI for 12 h. Cell extracts were separated on a 10% SDS gel, and the level of activated IKK β were determined by Western blotting using phospho-IKK α/β (Ser176/180) antibody. One representative experiment of three was shown (A). Quantitation of the level of phospho-IKK α/β was shown (B). (C) Western blot analysis of phospho-IKK α/β (Ser176/180) in lysates of HeLa cells untreated or different concentrations of DMI-pretreated and then stimulated with TNF- α (10 ng mL⁻¹, 10 min). β -actin was used as loading control. Blot shown is representative of three independent experiments. (D) Histogram of intracellular phospho-IKK α/β (Ser176/180) from (C).

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