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Tolfenamic Acid Suppresses Inflammatory Stimuli-Mediated Activation of NF-κB Signaling

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Abstract

Tolfenamic acid (TA) is a traditional non-steroid anti-inflammatory drug (NSAID) and has been broadly used for the treatment of migraines. Nuclear factor kappa B (NF-κB) is a sequence-specific transcription factor and plays a key role in the development and progression of inflammation and cancer. We performed the current study to investigate the underlying mechanisms by which TA suppresses inflammation focusing on NF-κB pathway in TNF-α stimulated human normal and cancer cell lines and lipopolysaccharide (LPS)-stimulated mouse macrophages. Different types of human cells (HCT116, HT-29 and HEK293) and mouse macrophages (RAW264.7) were pre-treated with different concentrations of TA and then exposed to inflammatory stimuli such as TNF-α and LPS. Transcriptional activity of NF-κB, IκB-α-degradation, p65 translocation and mitogen-activated protein kinase (MAPK) activations were measured using luciferase assay and Western blots. Pre-treatment of TA repressed TNF-α- or LPS-stimulated NF-κB transactivation in a dose-dependent manner. TA treatment reduced degradation of IκB-α and subsequent translocation of p65 into nucleus. TA significantly down-regulated the phosphorylation of c-Jun N-terminal kinase (JNK). However, TA had no effect on NF-κB signaling and JNK phosphorylation in HT-29 human colorectal cancer cells. TA possesses anti-inflammatory activities through suppression of JNK/NF-κB pathway in different types of cells.

Key Words: Tolfenamic acid, NF-kappa B, Inflammation

INTRODUCTION

Many non-steroid anti-inflammatory drugs (NSAIDs) including aspirin, indomethacin, and sulindac exert antipyretic, antirheumatoid, and anti-inflammatory as well as anticancer activities. The major anti-inflammatory efficacy of NSAIDs is partly attributed to the selective inhibitory effect on cyclooxygenase (COX) activity and thereby synthesis of prostaglandin (PG) which act as inflammatory mediators (Proudman and McMillan, 1991).

Tolfenamic acid (TA) is a widely used NSAID and structurally resembles other fenamates (Corell, 1994). TA showed anti-inflammatory activity through inhibition of leukotriene B4 (LTB4)-induced chemotaxis (Kankaanranta *et al*., 1991). However, its anti-inflammatory mechanisms are not fully understood.

Nuclear factor κB (NF-κB) signaling pathway is a primary target for the anti-inflammatory effects of many NSAIDs (Kopp and Ghosh, 1994; Yin *et al*., 1998). NF-κB is a pleiotropic transcription factor that regulates a wide range of genes involved in inflammation, autoimmune, apoptosis and tumorigenesis. In unstimulated cells, NF-κB is generally retained in the cytoplasm through non-covalent interactions with the inhibitory IκB proteins. Upon the phosphorylation and degradation of IκB by various inflammatory stimuli, the activated NF-κB is then translocated into nucleus where it can bind to the promoter regions of target genes and regulate the expression of these specific genes. In previous study, we reported that the treatment of TA stimulated NF-κB transcriptional activity through p65 nuclear accumulation and induced apoptosis in human colorectal cancer cells (Jeong *et al*., 2013c). However, the effects of TA on NF-κB pathway are not yet studied in inflammatory conditions.

In the current study, we hence evaluated whether TA can influence NF-κB pathway in TNF-α stimulated cancer cell lines (HCT116, HT-29 and HEK293) and LPS-stimulated RAW264.7

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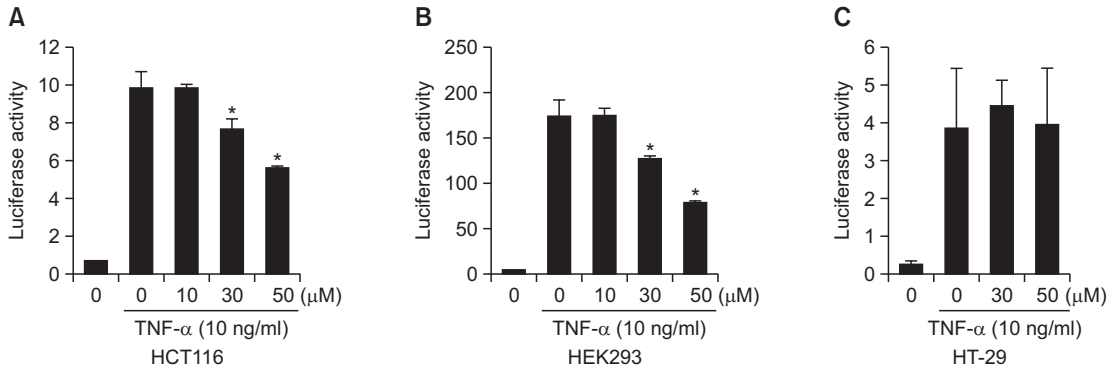

Fig. 1. TA suppressed inflammatory stimuli-induced NF-κB transcriptional activity. HCT-116 (A), HEK293 (B) and HT-29 (C) cells were transfected with NF-κB-Luc plasmid and pRLNull. The cells were pretreated with indicated concentrations of TA for 6 hours and then co-treated with TNF-α for 7 hours. DMSO was used as a vehicle. Data are mean ± S.D. (n=3).

cell line, and we further explore the underlying mechanism.

MATERIALS AND METHODS

Materials

The human colorectal cancer cell line (HCT116, HT-29), the human embryonic kidney cell line (HEK 293) and the mouse macrophage cell line (RAW264.7) were purchased from American Type Culture Collection (Manassas, VA, USA). Cell culture media was obtained from Invitrogen (Carlsbad, CA, USA). TA was purchased from Cayman Chemical (Ann Arbor, MI, USA). Tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS) were from Sigma Aldrich (St. Lous, MO, USA). *p*NF-κB-Luc cis-Reporter plasmid was purchased from Agilent Technologies (Santa Clara, CA, USA). Antibodies against IκB-α, p65, ERK, p38, JNK, phospho-ERK (Thr^{202}/Tyr^{204}), phospho-p38 (Thr^{180}/Tyr^{182}), phospho-JNK (Thr^{183}/Tyr^{185}) and β-actin were obtained from Cell Signaling (Bervely, MA, USA). All other chemicals were from Fisher Scientific, unless expressly noted otherwise.

Cell culture

All cells were maintained in Dulbecco's Modified Eagle medium (DMEM/F-12) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator at 37°C and 5% CO_2.

Transient transfection

The transient transfection was performed using PolyJet DNA reagent (SignaGen Laboratories, Ijamsville, MD, USA) according to the manufacture's protocol. Briefly, after seeding at 2×10^5 cells/well in 12-well plates and overnight culture, cells were transiently transfected with a plasmid mixture containing 1 μg of *p*NF-κB-Luc plasmid and 0.1 μg of *pRL-null* vector for 24 h. Then, cells were pre-exposed to TA for 6 h and co-treated with TNF-α for additional 7 h, respectively. Cells were finally lysed in 1×luciferase lysis buffer, and the luciferase activity was recorded and normalized to *pRL-null* luciferase activity using a dual luciferase assay system (Promega, Madison, WI, USA).

Western blotting

After the treatment as indicated in the figure legends, cells were harvested and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and phosphatase inhibitor cocktail (Sigma Aldrich) by incubation on ice for 30 min and subsequent centrifugation at 14,000 g for 10 min at 4°C. The supernatant was collected and protein concentration was quantified by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Proteins were resolved on SDS-PAGE gels and transferred onto nitrocellulose membranes (Osmonics, Minnetonka, MN, USA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBS-T) for 1 h at room temperature and then probed with the appropriate primary antibodies in 5% nonfat dry milk in TBS-T at 4°C overnight. After washing three times in TBS-T, the blots were further incubated with the correct secondary antibodies conjugated with horse radish-peroxidase for 1 h at room temperature, and finally chemiluminescence was examined with Pierce ECL Western blotting substrate (Thermo Scientific) and visualized by ChemiDoc MP Imaging system (Bio-Rad, Hercules, CA, USA).

Isolation of cytosolic and nuclear fraction

The cytosolic and nuclear fractions were prepared using a nuclear extract kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instruction.

Statistical analysis

Statistical analysis was performed with student's t-test, and *p*-values <0.05 were considered statistically significant.

RESULTS

TA inhibits NF-κB transcriptional activity

Our previous study indicated that the treatment of TA increases transcriptional activity of NF-κB in human colorectal cancer cells (Jeong *et al*., 2013c). In order to investigate whether TA can influence the NF-κB pathway in the presence of inflammatory stimuli, we transfected luciferase reporter

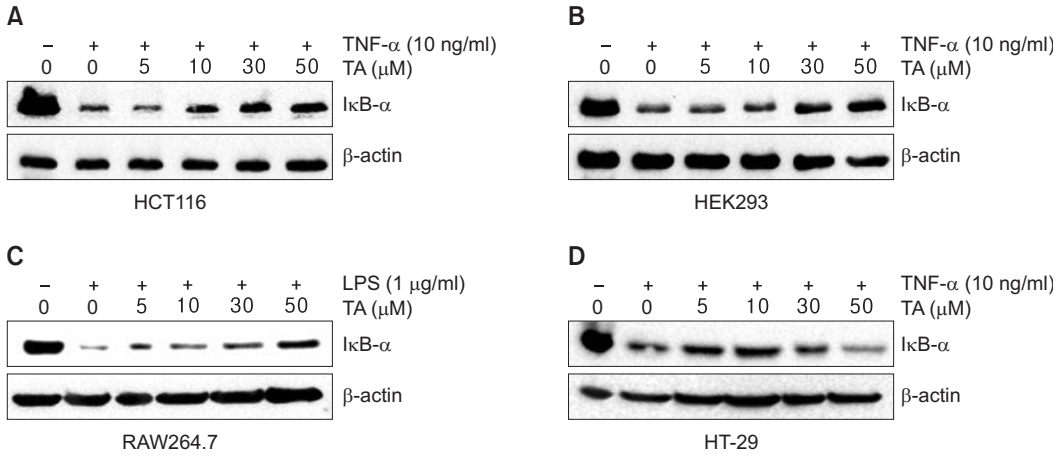

Fig. 2. TA decreased inflammatory stimuli-induced IκB-α degradation in HCT116 (A), HEK293 (B) and RAW264.7 (C) cells, but not in HT-29 (D) cells. Cells were pre-treated with indicated concentration of TA for 6 hours, and then co-treat with TNF-α (10 ng/mL) or LPS (1 μg/mL) for an additional 15 min. DMSO was used as a vehicle. Western blotting was performed for antibodies against IκB-α and β-actin.

constructs (pNF-κB-Luc) and then pre-treated TA for 6 h and then co-treated with different doses of TA and TNF-α for 7 h. As shown in Fig.1A, B, treatment of TNF-α induced transcriptional activity of NF-κB up to 14.3 fold and 41.8 fold in HCT116 and HEK293 cells respectively. However, transcriptional activities of NF-κB were inhibited in dose-dependent manner in the HCT116 (22.2% and 43.3%) and HEK 293 (26.7% and 55.1%) cells pretreated with 30 and 50 μM of TA, respectively. Consistent with these observations, the suppression of LPS-induced NF-κB activation in RAW264.7 cells was also found in our previous study (Jeong *et al*., 2013b). However, treatment of TA did not affect TNF-α-induced transcriptional activity of NF-κB in HT-29 cells (Fig. 1C).

TA down-regulates IκB-α degradation and NF-κB translocation

It is generally accepted that the phosphorylation and degradation of IκB-α is crucial for the nuclear translocation and subsequent activation of NF-κB. Thus, we performed Western blotting to determine if TA affects TNF-α- or LPS-stimulated IκB-α degradation in different cells. Pretreatment with TA dose-dependently ameliorated TNF-α-induced degradation of IκB-α in HCT116 (Fig. 2A) and HEK293 (Fig. 2B) cells, and LPS-induced RAW246.7 (Fig. 2C) cells, respectively. It is noted that, in TNF-α induced HT-29 cells, TA pretreatment showed a biphasic effect which blocked the IκB-α degradation at low doses (5 and 10 μM) but did not change at high doses (30 and 50 μM) (Fig. 2D).

Next, we further examined whether TA can abrogate inflammatory stimuli-mediated NF-κB translocation, which is a consequence of IκB-α degradation. As shown in Fig. 3, TA pretreatment decreased the levels of NF-κB (p65) in nucleus in a dose-dependent manner, whereas TNF-α or LPS only treatment dramatically increased nuclear NF-κB in HCT116, HEK29 and RAW264.7 cells, respectively. However, NF-κB level remained no significant change in HT-29 cells co-treated with TA and TNF-α.

TA inhibits the TNFα- or LPS-stimulated phosphorylation of JNK

Mitogen activated protein-kinases (MAPKs) including ERK-1/2, c-Jun and p38 are implicated in varied signaling cascades wherein various extracellular stimuli induce infiammation. And NF-κB is one of the well-established downstream targets of the MAPK signaling pathway. Therefore, the inhibitory effects of TA on TNFα- or LPS-stimulated expression of phospho-MAPKs were studied. As shown in Fig. 4. TNF-α (HCT116, HEK293 and HT-29 cells) or LPS treatment (RAW264.7) dramatically stimulated phosphorylation of all MAPKs. Pretreatment of TA significantly suppressed the expression of phospho-JNK in HCT116 (Fig. 4A), HEK293 (Fig. 4B), and RAW264.7 cells (Fig. 4C). However, TA did not influence TNF-α-induced JNK phosphorylation in HT-29 cells (Fig. 4D). Overall, phosphorylation of ERK and p38 was not significantly affected by the treatment of TA although decrease of ERK phosphorylation and slight increase of p38 phosphorylation was only observed in 50 μM TA-treated HEK293 cells, HCT116 and HT-29, respectively. Taken together, this result suggests that TA inhibits the activation of NF-κB through phosphorylation of JNK.

TA shows dual function for NF-κB signaling depending on inflammation status

TA activates NF-κB signaling in the absence of inflammatory stimuli (Jeong *et al*., 2013c) and we report that TA down-regulates NF-κB signaling through JNK pathway in response to inflammatory stimuli such as TNF-α and LPS. To clarify this dual function of TA, we compared the each level of IκB-α and p-JNK using HCT116 cells treated with 0 or 50 μM TA in the presence or absence of TNF-α. As shown in Fig. 5A, expression of IκB-α was decreased by TA treatment in the absence of TNF-α. Because TNF-α dramatically decrease IκB-α, we showed same blot with long exposure together. In the presence of TNF-α, TA increased expression of IκB-α.

Next, we tested phosphorylation of JNK. TA treatment did not change phosphorylation of JNK whereas in the presence of TNF-α, TA suppressed phosphor-JNK (Fig. 5B). We also tested phosphorylation of ERK. In the absence of TNF-α, TA induced phosphorylation of ERK, which is consistent with our previous data (Lee *et al*., 2010). However, phosphorylation of ERK was not affected by TA in the presence of TNF-α.

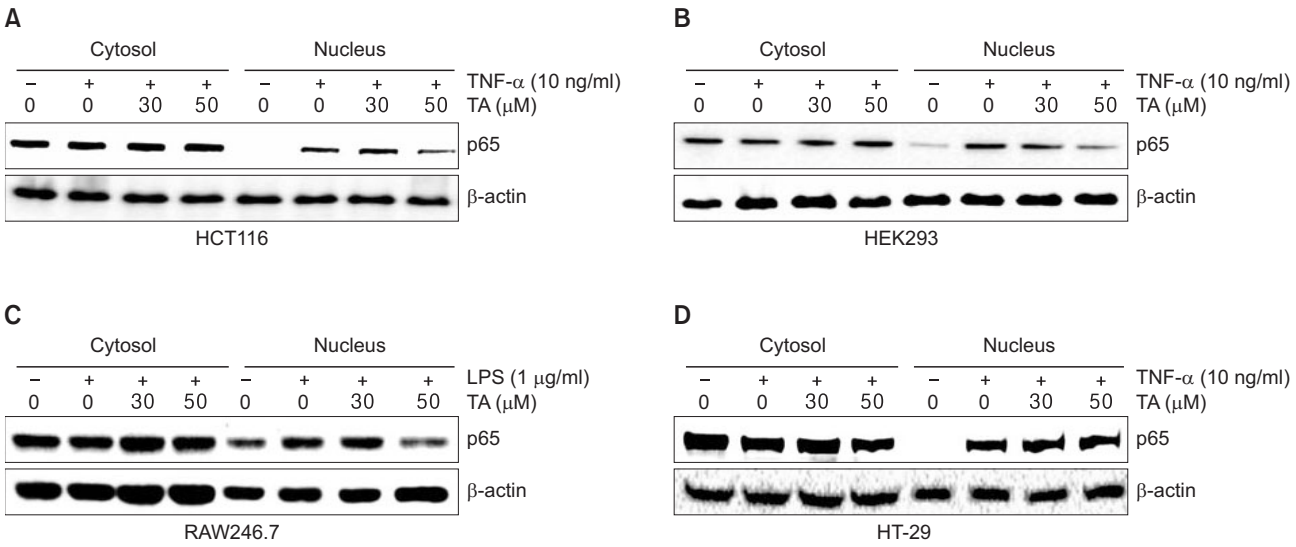

Fig. 3. TA decreased inflammatory stimuli-induced NF-κB translocation into nucleus in HCT116 (A), HEK293 (B) and RAW264.7 (C) cells, but not in HT-29 (D) cells. Cells were pre-treated with indicated concentration of TA for 6 hours, and then co-treat with TNF-α (10 ng/mL) or LPS (1 μg/mL) for an additional 30 min. DMSO was used as a vehicle. Western blotting was performed for antibodies against p65 and β-actin.

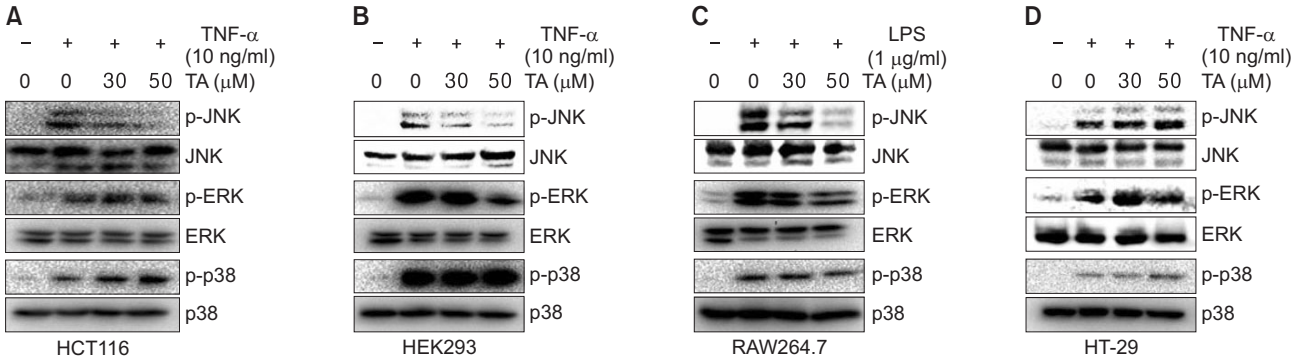

Fig. 4. TA decreased inflammatory stimuli-induced phosphorylation of JNK in HCT116 (A), HEK293 (B) and RAW264.7 (C), but not in HT-29 (D) cells. Cells were pre-treated with indicated concentration of TA for 6 hours, and then co-treat with TNF-α (10 ng/mL) or LPS (1 μg/mL) for an additional 15 min. Western blotting was performed for antibodies against phospho-JNK, phosphor-ERK, phosphor-p38, JNK, ERK, p38 and actin. DMSO was used as a vehicle.

DISCUSSION

During the past a few decades, several NSAIDs have held considerable interest due to their anti-inflammatory and cancer preventive properties. The first anti-cancer activity of TA has been studied by Dr. Safe's group in Texas A&M University (Abdelrahim *et al*., 2006). Since then, our group observed that TA significantly suppresses the growth of human colorectal cancer cells and enhance apoptosis in various types of cancer with diverse mechanisms. They include transcriptional up-regulation of tumor suppressive transcription factors such as EGR-1, ATF3, and CHOP and down-regulation of oncogenic transcription factors Sp1 and β-catenin in cyclooxygenase-independent manner (Lee *et al*., 2010; Kang *et al*., 2012; Eslin *et al*., 2013; Liggett *et al*., 2014; Pathi *et al*., 2014). Of note, our previous data proposed a novel apoptotic mechanism of TA including activation of NF-κB (Jeong *et al*., 2013c).

Interestingly, in the present study, we found that TA blocked the TNFα- or LPS-induced NF-κB activation in several human cells and mouse macrophages. NF-κB activation is mainly regulated through the inhibitory IκB proteins, and the stimulus-induced degradation of IκB lead to the release and subsequent activation of NF-κB. NSAIDs such as aspirin and sulindac have been reported to inhibit NF-κB by inhibiting IκB-α phosphorylation (Kopp and Ghosh, 1994). Flufenamic acid which chemical structure is similar with TA, also suppressed the TNFα- or LPS-induced NF-κB activation (Paik *et al*., 2000). Similarly, our result revealed that TA attenuated the degradation of IκBα induced by TNF-α and LPS (Fig. 2), and then the translocation of the free NF-κB from cytoplasm to nucleus (Fig. 3). These observations were further confirmed by the inhibitory effect of TA on the NF-κB's transcriptional activity (Fig. 1).

Emerging evidence suggest that mitogen-activated protein

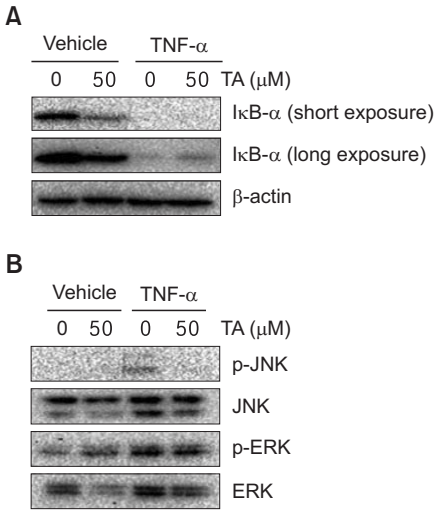

Fig. 5. TA shows dual function for NF-κB signaling depending on inflammatory status HCT116 cells (A, B) were treated with 0 or 50 μM of TA for 6 h or pre-treated for 6 h and then co-treated with TNF-α (10 ng/mL) for an additional 15 min. Western blotting was performed for antibodies against IκB-α, phospho-JNK, phospho-ERK, JNK, ERK and actin.

kinases (MAPKs) participate in the regulation of NF-κB activation. Our early observation showed that the TA-induced ROS generation results in ERK activation and DNA damage and thereby NF-κB and ATF3 activation, and eventually apoptosis in human colorectal cancer cells (Jeong *et al*., 2013a). However, in this study we found that TA specifically blocks the TNF-α- and LPS-induced JNK phosphorylation (Fig. 4), indicating that TA suppresses TNF-α and LPS-induced NF-κB activation by selectively inhibiting the activation of intracellular JNK signaling cascade. Recent study indicates that TNF activated both NF-κB and JNK cascade, leading to increased expression of pro-inflammatory factors in astrocytes (Dvoriantchikova and Ivanov, 2014). Although we do not know how JNK links to NF-κB and regulates inflammatory responses, it seems that NF-κB-mediated survival could require transient activation of JNK because JNK/JunD pathway can collaborate with NF-κB to increase antiapoptotic gene expression (Lamb *et al*., 2003).

One of interesting findings is that TA effects on NF-κB signaling are determined by inflammatory status. For example, TA activates NF-κB signaling in the absence of inflammatory stimuli, whereas it suppressed NF-κB signaling in the presence of inflammatory stimuli. Dual effects of NSAIDs on NF-κB pathway were also observed in other studies. Aspirin activates NF-κB transcriptional activity, resulting in induction of apoptosis in human colorectal cancer cells (Stark *et al*., 2001; Din *et al*., 2005; Stark *et al*., 2007), while TNF-α-induced NF-κB activation is inhibited by aspirin pretreatment prior to a burst of TNF-α by blocking IKK-mediated IκB-α degradation (Kopp and Ghosh, 1994; Yin *et al*., 1998). Although the reason why TA differently regulates NF-κB pathway in the absence or in the presence of TNF-α is not clear, a discrepancy of NF-κB effect may be caused by differences in signaling pathways in the presence or absence of inflammatory stimuli. One speculation we propose here is that TA may use different MAPK signaling depending on inflammatory status because we observed that TA stimulate ERK in the absence of inflammation but suppressed JNK in the presence of TNF-α (Fig. 5B). Further study is needed to clarify this possibility.

Another interesting finding is that anti-NF-κB signaling activity by TA is not observed in HT-29 cells. However, other NSAIDS such as aspirin suppress inflammation in HT-29 cells (Bergman *et al*., 2011). These results indicate that anti-inflammatory activity of TA may be affected by cell context or NSAID specificity.

In summary, the data presented here shows that in absence of inflammatory stimuli, TA induces p65 nuclear accumulation and activates NF-κB transcriptional activity and apoptosis. However, in the presence of inflammatory stimulus, TA prevented inflammatory cytokine-induced NF-κB transcriptional activity. Collectively, our result shed more lights on the complex action mechanism of TA that the anti-inflammatory effects of TA are partially mediated through the blockage of JNK/NF-κB pathway.

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