A mechanistic study of the proapoptotic effect of tolfenamic acid: involvement of NF-κB activation

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Recent studies demonstrate that tolfenamic acid (TA) induces apoptosis and suppresses the development and progression of several types of cancer. However, the underlying mechanisms are complex and remain to be fully elucidated. Nuclear factor-kappaB (NF-κB) plays a critical role in inflammation, cancer development and progression. Although non-steroidal anti-inflammatory drugs modulate NF-κB signaling pathway in different ways, the link between NF-κB and TA-induced apoptosis of colorectal cancer has yet to be thoroughly investigated. In this study, we examined the effects of TA on the NF-κB pathway and apoptosis. TA activated NF-κB transcriptional activity and binding affinity of NF-κB to DNA. TA-induced NF-κB activation was mediated by an increased phosphorylation and proteosomal degradation of IκB-α and subsequent p65 nuclear translocation. We also observed that TA stabilized p65 and increased nuclear accumulation via an increased p65 phosphorylation at Ser276 residue, which was mediated by p38 mitogen-activated protein kinase and extracellular signal-regulated kinase. The knockout of p33 blocked TA-induced transcriptional activation of NF-κB, but not the p65 nuclear accumulation. TA increased transcriptional activity of p53 and the binding affinity of p53 with p65, which are mediated by p38 mitogen-activated protein kinase and extracellular signal-regulated kinase-stimulated Ser276 phosphorylation. TA-induced apoptosis was ameliorated by the knockout of p65 and p53 and the point mutation of p65 at Ser276 residue. We demonstrate a novel molecular mechanism by which TA induced the NF-κB and apoptosis in human colorectal cancer cells.

Introduction

Colorectal cancer is the third leading cause of cancer-related morbidity and mortality in both males and females in the USA m (1). Chemoprevention is an inherently attractive approach to treat this disease, and epidemiological studies have revealed that there is an inverse correlation between the long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) and colorectal cancer incidence and mortality (2). It is estimated that there is a 40–50% reduction in the relative risk of colorectal cancer in subjects taking NSAIDs (3). Thus, the use of NSAIDs has been regarded as an effective chemopreventive strategy for colorectal cancer (4). The proposed mechanisms of NSAIDs’ anticancer activity include increased cell-cycle arrest and apoptosis (5–7), and inhibition of angiogenesis, invasion and metastasis (8,9), and these studies would provide criteria for rationally designing novel agents for chemoprevention and therapy.

Tolfenamic acid (TA), one of the fenamic acid-derived NSAIDs, has been used for the treatment of migraines (10). Recent evidences show that TA possesses anticancer activity in several cancers such as pancreatic cancer (11), head and neck cancer (12) and colorectal cancer (13,14). Like other NSAIDs, TA inhibits cyclooxygenase (COX) and prostaglandin biosynthesis, which contributes to the anticancer activity of NSAIDs (15). However, anticancer activity of NSAIDs is associated with COX-independent mechanisms and other molecular targets (16–19).

One of the potential molecular targets for the anticancer activity of NSAIDs is nuclear factor-kappaB (NF-κB) signal transduction (20–24). In the absence of stimuli, the NF-κB transcription factor formed by p50 and p65 is normally sequestered in the cytoplasm by binding to an inhibitory protein, IκB-α. In response to the cellular stimulation of NF-κB pathway, IκB-α is phosphorylated by the IκB-α kinase, ubiquitinated and then degraded by the 26S proteasome. Dissociation from IκB-α results in the release of NF-κB from the cytoplasmic NF-κB–IκBα complex and allows NF-κB to translocate to the nucleus. The translocated NF-κB contributes to the coordinated transcription of several genes involved in inflammation, cell proliferation and apoptosis (25).

The roles of NF-κB in cancer progression and anticancer therapeutics are complex, as there are evidences to suggest that the NF-κB activation is associated with the increased survival of cancer cells and resistance to chemotherapy, showing that inhibition of NF-κB activity is regarded as the target for anticancer therapy (26,27). However, NF-κB activation promotes a proapoptotic response under different circumstances (28,29). For example, the retinoid-related compounds, 3-CI-AHPC and CD437 induce apoptosis by activation of NF-κB transcriptional activity in prostate cancer cells (30,31). Another evidences supporting a proapoptotic role for NF-κB activation in cancer chemotheraphy is that aspirin and betulinic acid require activation of NF-κB to induce apoptosis in cancer cells (32,33). Therefore, it is suggested that NF-κB activation may be a promising cancer preventive and therapeutic target.

In this study, we aimed to elucidate the role of NF-κB in TA-mediated apoptosis and we demonstrated that TA induces nuclear accumulation of NF-κB p65 and subsequent activation of NF-κB transcriptional activity in p53-dependent manner in colorectal cancer cells. Furthermore, NF-κB activation is required for TA-induced apoptosis. These studies identify an intrinsic activity of TA that is highly relevant to colon cancer chemoprevention.

Materials and methods

Cell culture media, Dulbecco’s modified Eagle medium, RPMI1640, Ham’s F-12 and McCoy’s 5A were purchased from Lonza (Walkersville, MD), and TA was purchased from Cayman Chemical (Ann Arbor, MI). SB203580 and PD98059 were purchased from Calbiochem (San Diego, CA) and recombinant human tumor necrosis factor (TNF)-α was purchased from R&D Systems (Minneapolis, MN). Antibodies against p65 (D14E12, 8242), IκB-α (L35A5, no. 4814), phospho-IκB-α (Ser32; 14D4, no. 2859), poly (ADP ribose) polymerase (PARP, no. 9542), TATA-binding protein (no. 8515), Fas ligand (FasL; no. 4273) and β-actin (no. 5125) were purchased from Cell Signaling (Danvers, MA). p53 (Bp53-12, sc-263) and p-p38 (Ser198/203, no. 4872; ab132523) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Abcam (Cambridge, MA). Control (no. 6201) and p65 siRNA (no. 6261) were purchased from Cell Signaling. p53-siRNA (sc-29453) and p65-siRNA (sc-29455) was purchased from Santa Cruz Biotechnology. All chemicals were purchased from Fisher Scientific, unless otherwise specified.

Cell culture and treatment

Human colorectal cancer cell lines, HCT116, SW480, LoVo, and Caco-2 cells were purchased from American Type Culture Collection (Manassas, VA) and...
grown in McCoy’s 5A (for HCT116 cells), RPMI1640 (for SW480 cells),
Ham’s F-12 (for LoVo cells) and Dulbecco’s modified Eagle medium (for
Caco-2 cells) supplemented with 10% fetal bovine serum, 100 U/ml penicillin
and 100 μg/ml streptomycin. The cells were maintained at 37°C under
a humidified atmosphere of 5% CO₂, TA was dissolved in dimethyl sulfoxide
(DMSO) and then treated to cells. DMSO was used as a vehicle and the final
DMSO concentration was not exceeded 0.1% (v/v).

Expression vectors
Wild-type enhanced green fluorescent protein (EGFP)-p65 expression vec-
tor (WT) was kindly provided from Dr Warner Greene (J David Gladstone
Institute). Point mutation of (Ser276Gly) of EGFP-p65 (MT) was con-
structed from wild-type EGFP-p65 using the QuikChange II mutagenesis kits
(Stratagene, La Jolla, CA) with primers as follows: forward 5′-ctgcggtcggc-
tgctgccaggg-3′ and reverse 5′-cctgccggtcggccgagcag-3′.

Isolation of cytosol and nucleus fraction
Cytosol and nuclear fractions were prepared according to the manufacturer’s
protocols of a nuclear extract kit (Active Motif, Carlsbad, CA). Briefly, after
washing with ice-cold phosphate-buffered saline (PBS) containing phos-
phatase inhibitors, cells were harvested at 1× hypotonic buffer containing
detergent and then incubated at 4°C for 15 min. The supernatants (cytoplasmic
fraction) were collected after centrifugation at 14 000 g for 1 min at 4°C and
stored at -80°C. Nuclear fractions were collected by suspending nuclear pel-
let with lysis buffer at 4°C for 30 min and centrifugation at 14 000 g for 10
min at 4°C.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western
blot
Cells were washed with 1× PBS, centrifuged at 12 000 g for 10 min at 4°C
and cell pellets were resuspended in radioimmunoprecipitation assay buffer
(Boston Bio Products, Ashland, MA) supplemented with protease inhibitor
cocktail (Sigma–Aldrich, St Louis, MO) and phosphatase inhibitor cocktail
(Sigma–Aldrich). The cell suspension was centrifuged at 12 000 g for 10
min at 4°C. Protein content was measured by the bicinchoninic acid protein assay
(Pierce, Rockford, IL). The proteins were separated on sodium dodecyl sul-
fate–polyacrylamide gel electrophoresis, transferred to nitrocellulose mem-
branes and blocked in 5% non-fat dry milk in Tris-buffered saline containing
0.05% Tween 20 for 1 h at room temperature. Membranes were probed with
specific primary antibodies in 5% non-fat dry milk at 4°C overnight and
then with horse radish peroxidase-conjugated immunoglobulin G (IgG) for
1 h at room temperature. Chemiluminescence was detected with Pierce ECL
Western blotting substrate (Thermo Scientific, Rockford, IL) and visualized by
ChemiDoc MP Imaging system (Bio-Rad, Hercules, CA).

Immunoprecipitation
HCT116 cells were lysed in immunoprecipitation lysis buffer (50 mM Tris–
HCl, 150 mM NaCl, 5 mM ethylenedinitrilotetraacetic acid, 0.5% NP-40)
supplemented with protease inhibitor cocktail (Sigma–Aldrich) and phe-
nymethanesulfonylfluoride (Sigma–Aldrich). The cell suspension was cen-
trifuged at 12 000 g for 20 min at 4°C. Protein content was measured by the
bicinchoninic acid protein assay (Pierce). A total of 500 μg of precleared cell
lysates were incubated with anti-p65 monoclonal antibody, normal rab-
bit immunoglobulin G (Santa Cruz Biotechnology) and Protein A/G PLUS-
Aagarose (Santa Cruz Biotechnology) and rotated at 4°C overnight. The pellets
were washed five times with lysis buffer and boiled in 2× loading buffer and
subjected to western blot using an anti-p65 or anti-p53 antibody.

Immunofluorescence
HCT116 cells grown on sterile coverslips were transfected with expression
vectors containing wild-type and Ser276Gly mutated-p65 constructs. After
24 h, cells were treated with TA for the additional 24 h. Cells were fixed
with 4% paraformaldehyde for 15 min, permeabilized with PBS containing
0.5% Triton X-100 for 5 min and washed three times with PBS. After several
washes, Cells were mounted with ProLong Gold antifade reagent containing
DAPI (Invitrogen, Grand Island, NY) and images were acquired on fluorescent
microscopy (Nikon Eclipse Ti-U, Nikon Instruments, Melville, NY) at 20×
total magnification.

Electrophoretic mobility shift assay
Electrophoretic mobility shift assay (EMSA) was performed accord-
ing to the manufacturer’s protocol of LightShift Chemiluminescent
EMSA kit (Thermo Scientific). Biotin-labeled NF-κB-specific oligo-
nucleotides 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (sense) and
5′-GCTCGGGAAATGCCCTCAACT-3′ (antisense) were used for EMSA.
Binding reactions were performed for 30 min at the room temperature in 20 μl
buffer (1× binding buffer, 2.5% glycerol, 5 mM MgCl₂, 0.05% NP-40) containing
5 μg nuclear extract protein, 50 ng poly(dI:dC) and 1 μM biotin-labeled NF-κB-
specific oligonucleotides. Binding complexes were resolved by electrophoresis
in vertical non-denaturing 6% polyacrylamide gels using 0.5x Tris–borate–
ethylenediaminetetraacetic acid as running buffer and then transferred to nylon
membrane. After ultraviolet cross-linking, the membrane was blocked with
blocking buffer for 15 min and then incubated with conjugated/blocking buffer
solution containing horseradish peroxidase conjugate for 15 min. After washing, the membrane was incubated with substrate equilibration buffer for 5 min. Chemiluminescence was detected with working solution contain-
ing luminol/enhancer solution and stable peroxide solution and visualized by
ChemiDoc MP Imaging system (Bio-Rad, Hercules, CA).

Transient transfection and luciferase assay
Transient transfection for luciferase plasmids was performed using PolyJet DNA
transfection reagent (SignaGen Laboratories, Ijamsville, MD) according to the
manufacturer’s instruction. Briefly, the cells were plated in 12-well plates at
the concentration of 2×10⁵ cells/well and incubated overnight. Then, plasmid mixtures containing 1 μg of lucase plasmid and 0.1 μg of pRL-null vector were trans-
fected for 24 h at 37°C under a humidified atmosphere of 5% CO₂. The trans-
formed cells were exposed to TA or TNF-α. The cells were harvested in 1× lucase lysis
buffer, and luciferase activity was measured and normalized to the pRL-null lucif-
erase activity using a dual-luciferase assay kit (Promega, Madison, WI).

Transfection of small interference RNA
The cells were plated in six-well plates and incubated overnight. HCT116 cells
were transfected with control small interference RNA (siRNA) and p65 siRNA
for 48 h at a concentration of 100 nM using TransIT-TKO transfection reagent
(Mirus, Madison, WI). LoVo cells were transfected with control siRNA, p53
siRNA and p65 siRNA for 48 h at a concentration of 100 nM using LoVo
Transfection Kit (Allogen Biosystems, Las Vegas, NV) according to the manu-
ufacturer’s instruction. Then the cells were treated with 50 μM of TA for 24 h.

Cell death assay
Cell death was performed using Cell Death Detection ELISA PLUS kit (Roche
Diagnostics, Indianapolis, IN) according to the manufacturer’s instruction.
Briefly, LoVo cells were plated in 12-well plate. After 24 h, cells were trans-
fected with control, p53 and p65 siRNA for 48 h and then treated with TA
for the additional 24 h. After TA treatment, the cytosol was prepared using
Nuclear Extract Kit (Active Motif). Cytosolic extracts, immunoreagent contain-
ing anti-histone-horseradish and anti-DNA-POL were added to microplate well
and incubated for 2 h. Antibody conjugates were added and incubated for 1 h. The
solution containing stabilized streptavidin–horseradish peroxidase conjugate
was added to each well for 20 min. Chemiluminescence was detected with
p-nitrophenyl-4-methylumbelliferyl-6-sulfonic acid stop solution was added. The absorbance was recorded at 405 nm and 490 nm in an
enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments).

Statistical analysis
A statistical analysis was performed with the Student’s unpaired t-test, with
statistical significance set at *P < 0.05.

Results
Effect of NSAIDs and dietary phytochemicals on cell growth and
apoptosis
There are a number of evidences indicating that NSAIDs and dietary
phytochemicals show anticancer activity in human colorectal cancer
(4,34). To observe which NSAIDs and dietary phytochemicals effec-
tively induce cell growth arrest and apoptosis, HCT116 cells were
treated with 50 μM of various NSAIDs (TA, aspirin, diclofenac, SC-560 and celecoxib) and dietary phytochemicals [epigallocate-
chin-3-gallate (EGCG) resveratrol and genistein]. As shown in
Figure 1A, a significant cell growth arrest was observed in the cells
treated with TA, diclofenac, celecoxib, EGCG, resveratrol and gen-
istein. Apoptosis was also induced by TA, EGCG (epigallocatechin-
3-gallate), resveratrol and genistein (Figure 1B). Among NSAIDs and
dietary phytochemicals tested, TA showed the strongest growth arrest
and proapoptotic activity among all other NSAIDs and phytochemi-
cals tested. Thus, we chose TA for further studies.

TA increases nuclear p65 level and transcriptional activity of NF-κB in human colorectal cancer cells
There is a growing body of evidence that NF-κB pathway could be a
target of several chemotherapeutic agents. To test if TA affects NF-κB
pathway, we measured transcriptional activity of NF-κB in HCT116

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cells transfected with NF-κB Luc plasmid. As shown in Figure 2A, TA treatment resulted in a dose-dependent increase of NF-κB transcriptional activity by 1.8- and 3.4-fold at 30 and 50 µM, respectively. We also used TNF-α as a control, showing activated NF-kB transcriptional activity (Figure 2B). Because NF-kB transcriptional activity is associated with nuclear translocation of NF-kB p65 and subsequent DNA binding of NF-kB, we performed western blot for p65 in HCT116 cells treated with TA or TNF-α. Nuclear translocation of p65 was induced by TA and TNF-α in HCT116 (Figure 2C and D). Time-course experiment showed that induction of p65 nuclear translocation by TA occurred after 6 h stimulation (Figure 2E). In contrast to TA, TNF-α-mediated nuclear translocation of p65 occurred rapidly (Figure 2F). To test whether TA- or TNF-α-induced p65 nuclear translocation correlates to NF-kB DNA-binding activity, we performed the EMSA using nuclear extracts of HCT116 cells treated with TA or TNF-α. TA and TNF-α resulted in an increase of NF-kB DNA binding (Figure 2G). Overall, these data indicate that TA enhances nuclear translocation of p65, binding of p65 to DNA and transcriptional activity of NF-kB in human colorectal cancer cells.

TA-induced NF-κB activation requires IκB-α-dependent nuclear translocation of p65

IκB-κB degradation is essential for nuclear translocation and subsequent transcriptional activation of NF-kB. Thus, we investigated whether NF-kB activation by TA is associated with IκB-κB degradation. As shown in Figure 3A, TA treatment decreased the IκB-κB level in HCT116 cells treated with 30 and 50 µM of TA and downregulation of IκB-κB level was also observed in LoVo cells treated with TA (data not shown). A time-course experiment further showed that IκB-κB began to decrease markedly at 24 h after TA treatment in HCT116 cells. However, TNF-α-mediated decrease of IκB-κB level is very rapid in HCT116 cells (Figure 3B), suggesting that the mechanisms of TA- or TNF-α-mediated IκB-κB degradation might not be same.

IκB-κB is rapidly degraded by 26S proteasome after phosphorylation at serine 32 and serine 36 by IκB kinase complex (25). Thus, we tested whether the decrease of IκB-κB protein by TA was mediated by proteosomal degradation. Pretreatment with MG-132 diminished TA-mediated IκB-κB phosphorylation (Figure 3C) and ameliorated TA-induced p65 nuclear translocation mediated by TA (Figure 3D). In conclusion, these data indicate that TA-induced NF-kB activation might be associated with proteosomal degradation of IκB-κB and subsequent nuclear translocation of p65.

TA stabilizes and increases nuclear p65 via p38 mitogen-activated protein kinase- and extracellular signal-regulated kinase-dependent phosphorylation at Ser276 residue

Interestingly, our findings indicated that TA-stimulated nuclear translocation of p65 is much more prominent than the reduction of cytoplasmic p65 by TA (Figure 2C and D). Moreover, the time...
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Fig. 2. TA increases transcriptional activity of NF-κB. Nuclear p65 and binding affinity of p65 to DNA. (A and B) The pNF-κB-Luc plasmid-transfected cells were treated with 0, 10, 30 and 50 μM of TA for 24 h or 0 and 10 ng/ml of TNF-α for 8 h. The cells were harvested, and luciferase activity was measured using a dual-luciferase assay kit. *P < 0.05 compared with cells without TA or TNF-α treatment. (C and D) HCT116 cells were treated with 0 and 50 μM of TA for 24 h or 0 and 10 ng/ml TNF-α for 30 min. Cytosol (CE) and nucleus extracts (NE) were prepared and subsequently western blot analysis was performed for p65, TATA-binding protein (TBP) and actin. TBP was used nuclear loading control. (E and F) HCT116 cells were treated with 50 μM of TA or 10 ng/ml TNF-α for the indicated time points. CE and NE were prepared and subsequently western blot analysis was performed for p65, TBP and actin. (G) HCT116 cells were treated with 50 μM of TA for 24 h or 10 ng/ml TNF-α for 30 min. Nuclear extracts (5 μg) were prepared and incubated with biotin-labeled NF-κB oligonucleotide as described in Materials and methods. DNA–protein complexes were resolved by 6% non-denaturing polyacrylamide gel and developed using the protocol of LightShift Chemiluminescent EMSA kit.

Point to increase nuclear p65 (6 h after TA treatment) is earlier than that to start IκB-α degradation (24 h after TA treatment) (Figures 2E and Figure 3B). Thus, we hypothesize that TA-induced increase of NF-κB in nucleus could be consequence of p65 nuclear accumulation. To address this question, we firstly investigated the effect of TA on degradation of exogenously expressed EGFP-p65 in HCT116 cells. As shown in Figure 4A, TA treatment increased both cytoplasmic and nucleus level of EGFP-p65 in a time-dependent manner. This result raised the possibility that TA may increase nuclear p65 protein by preventing p65 degradation.

According to a recent study (35), phosphorylation of p65 at Ser276 prevents degradation of p65 from the ubiquitin-proteasome machinery and subsequently induces transactivation of p65. To determine whether TA-mediated inhibition of p65 degradation is a result of Ser276 phosphorylation in p65, we investigated the effect of TA on Ser276 phosphorylation in HCT116 cells. As a result, p65 phosphorylation (Ser276) increased in both total cell lysate and nuclear fraction in the cells treated with TA (Figure 4B). We also observed that point mutation of serine to glycine (Ser276Gly) diminished TA-mediated nuclear accumulation of p65 compared with HCT116 cells transfected wild-type p65 (Figure 4F). To verify the western blot data, we used immunofluorescence imaging to directly visualize localization of EGFP after TA treatment in cells transfected with an expression vector encoding EGFP-wild-type p65 (WT) or EGFP-Ser276Gly p65 (MT). As shown in Figure 4D, TA treatment increased the nuclear staining of GFP in wild-type p65-transfected cells; however, it was partially ameliorated in the Ser276Gly p65-transfected cells.

Because p65 phosphorylation is associated with p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathways (36,37), we tested if selective inhibitors of these pathways diminish TA-induced p65 phosphorylation and nuclear translocation. As shown in Figure 4E, pretreatment of SB203580 and PD98059 diminished TA-mediated phosphorylation of p65 (Ser276). In addition, to test the possibility that p38 MAPK and ERK-mediated p65 phosphorylation is associated with IκB-α-mediated NF-κB translocation, we tested if presence of selective inhibitors for p38 MAPK and ERK affects TA-induced IκB-α degradation (Figure 4F). The result showed that TA-induced p65 phosphorylation and protein stability is independent with IκB-α degradation by IκB-α phosphorylation and NF-κB translocation.

In conclusion, these results demonstrate that TA increased nuclear stability of p65 through p38 and ERK-dependent Ser276 phosphorylation, which contributes at least in part to increase of p65 accumulation in nucleus and transcriptional activation.

TA-induced activation of NF-κB is dependent on p53

A growing body of evidence suggests that p53 induces NF-κB activation (38,39). Therefore, to determine whether p53 is associated with TA-stimulated activation of NF-κB, we compared the effects of TA on NF-κB transcriptional activity and nuclear accumulation of p65 in HCT116 and HCT116 p53 null (HCT116p53−/−) cells. As shown in Figure 5A (upper panel), TA treatment increased luciferase activity of NF-κB in dose-dependent manner in p53 wild-type HCT116 but not in HCT116p53−/− cells, indicating that TA-mediated activation of NF-κB is dependent on p53. However, the knockout of p53 did not result in a 100% decrease in NF-κB activity, suggesting that other factors also contribute to the activation of NF-κB.

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Affect the amount of nuclear p65 (Figure 5A, lower panel), implying that TA-induced nuclear accumulation of p65 is not mediated by p53 status.

To further confirm the p53 dependency on TA-stimulated NF-κB transcriptional activity and nuclear accumulation of p65, we also observed the effect of TA on p65 nuclear accumulation and NF-κB transcriptional activity in different human colorectal cancer cells including LoVo (p53 wild type), SW480 (p53 mutant) and CaCo-2 (p53 null) cells. As a result, TA increased nuclear accumulation of p65 in all other colorectal cancer cell lines (Figure 5B–D, lower panel), whereas TA-induced NF-κB transcriptional activation was observed only in p53-wild-type cell line (LoVo) but not in p53-mutant (SW480) or p53-null (CaCO-2) cell lines (Figure 5–D, upper panel). To verify p53-dependent transcriptional activation of NF-κB, LoVo cells (p53 wild type) were knocked down using p53 siRNA and transcriptional activity of NF-κB was measured. As shown in Figure 5B (upper panel), p53 knockdown decreased TA-stimulated NF-κB transcriptional activity. These results strongly indicate that p53 is necessary for TA-stimulated transcriptional activation of NF-κB but not for TA-mediated p65 accumulation. On the other hand, we observed that p53 did not affect TNFα-induced transcriptional activity of NF-κB and p65 accumulation (data not shown), confirming that mechanisms of TA- or TNF-α-mediated NF-κB activation are different.

TA increases p53 transcriptional activity and binding affinity of p65 with p53

Another interesting finding is that TA stabilized p53 in the nucleus of HCT116 and LoVo cells (Figure 5A and B). Because nuclear p53 degradation is an important mechanism of DNA damage and cell stress by anticancer compounds, we tested if TA affects transcriptional activity of p53 in HCT116 cells transfected with p53-MDM-luciferase plasmid. As shown in Figure 6A, TA increased the transcriptional activity of p53 by 1.9- and 2.5-fold at 30 and 50 μM, respectively.

According to a literature (40), ERK and p38 MAPK phosphorylate p53 at serine 15 and subsequently induces transactivation of p53. In addition, these two kinases are required for NF-κB activation. To

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Fig. 3. TA increases translocation of p65 via proteosomal degradation of IκB-α. (A) HCT116 cells were treated with 0, 5, 10, 30 and 50 μM of TA for 24 h. Total cell lysates were harvested and subsequently western blot analysis was performed for IκB-α and actin. (B) HCT116 cells were treated with 50 μM of TA or 10 ng/ml TNF-α for the indicated time points. Total cell lysates were harvested and subsequently western blot analysis was performed for IκB-α and actin. (C and D) HCT116 cells were pretreated with 10 μM of MG-132 for 2h and then co-treated with 50 μM of TA for 24h. Whole cell lysates, CE and NE were prepared and subsequently western blot analysis was performed for IκB-α, p-IκB-α, p65, TBP and actin. (E) HCT116 cells were treated with 0, 1 and 5 μM of BAY 11–7085 for 2h and co-treated with 50 μM of TA for 24h. CE and NE were prepared and subsequently western blot analysis was performed for p65, TBP and actin.
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**Fig. 4.** TA stabilizes p65 and increases nuclear accumulation via p38 MAPK- and ERK-dependent phosphorylation of p65 at Ser276 residue. (A) HCT116 cells were transfected with EGFP-p65 for 24h and then treated with 50 μM of TA for the indicated times. CE and NE were prepared and subsequently western blot analysis was performed for EGFP, TBP and actin. (B) HCT116 cells were treated with 50 μM of TA for the indicated times (left panel) or 24 h (right panel). Whole cell lysates (WE), CE and NE were prepared and subsequently western blot analysis was performed for p65, p-p65 (Ser276) and actin. (C) HCT116 cells were transfected with wild type (WT) or mutant (MT) p65 (S276G) and then treated with TA for 24 h. Whole cell lysates (WE), CE and NE were prepared and subsequently western blot analysis was performed for EGFP-p65, TBP and actin. (D) HCT116 cells were transfected with wild type (WT) or mutant (MT) p65 (S276G) and then treated with TA for 24 h. Cells were stained with DAPI and EGFP for confocal microscopy. (E) HCT116 cells were treated with vehicle, SB203580, PD98059 or a combination of both for 24 h and then treated with 50 μM of TA for 24 h and subsequently western blot analysis was performed for p65, p-p65 (Ser276), TBP and actin. (F) HCT116 cells were treated with vehicle, SB203580, PD98059 or a combination of both for 24 h and then treated with 50 μM of TA for 24 h and subsequently western blot analysis was performed for IκB-α, p-IκB-α (Ser32) and actin.
determine whether TA-mediated transactivation of p53 and NF-κB is dependent on p38 MAPK and ERK, HCT116 cells transfected with p53-MDM-luciferase or NF-κB-luciferase plasmids were treated with TA in presence of SB203580 and PD98059. As a result (Figure 6B and C), the selective inhibitors for p38 MAPK and ERK attenuated TA-stimulated transcriptional activities of p53 and NF-κB. On the other hand, p53 binds to p65 and subsequently regulates NF-κB transcriptional activity (41–43). Therefore, we examined the possibility that p53-dependent NF-κB activation by TA is associated with increased binding of p65 and p53 by performing immunoprecipitation. As a result, TA treatment increased the association between p65 and p53 in HCT116 cells (Figure 6D). However, pretreatment of SB203580 and PD98059 blocked the TA-induced binding of p53 to p65 (Figure 6D). We also observed that TA-induced binding of p53 to p65 was diminished in Ser276Gly mutant p65-transfected HCT116 cells compared with cells transfected with wild-type-p65 (Figure 6E). These data imply that TA induces both transcriptional activities of p53 and NF-κB and that p53 plays a significant role in TA-induced NF-κB transcriptional activation, probably via enhancing binding affinity between p53 and p65, which might be associated with p38 MAPK- and ERK-mediated phosphorylation of Ser276 in p65.

Both p65 and p53 mediate TA-induced apoptosis

Having shown previously that TA induces apoptosis in human colorectal cancer cells (13), we investigated whether TA-mediated apoptosis is dependent on p53 and NF-κB activation. As shown in Figure 7A, TA treatment increased PARP cleavage in HCT116 cells expressing wild-type p53, whereas TA-mediated PARP cleavage was ameliorated in HCT116(p53−/−) cells. In addition, knockdown of p65 using siRNA decreased TA-induced PARP cleavage compared with cells transfected with control siRNA in HCT116 (Figure 7B). The double knockdown of p65 and p53 also decreased TA-induced apoptosis (Figure 7C). To confirm that TA-mediated apoptosis is dependent on p53 and p65, cleaved PARP and cell death were investigated in LoVo cells transfected with siRNAs of p65 and p53. As a result, knockdown of p65 and p53 decreased TA-induced PARP cleavage and cell death in LoVo cells (Figure 7D and 7E). Next, to determine if Ser276 phosphorylation of p65 affects TA-induced apoptosis, we transfected HCT116 cells with wild-type p65 (WT) or Ser276Gly (MT) and compared PARP cleavage using western blot. As shown in Figure 7F, TA-induced PARP cleavage was attenuated in the cells transfected with Ser276Gly mutant compared with cells transfected with wild-type p65.

On the other hand, FasL (CD95/APO-1) is a p53- and NF-κB-target protein and induction of FasL is essential for p53- and NF-κB-dependent apoptosis (44–46). So, we also tested if TA affects FasL expression. As shown in Figure 7G and H, TA induced FasL expression in a dose- and time-dependent manner. All together, these results demonstrate that p53 and p65 play a significant role in TA-induced apoptosis through Ser276 phosphorylation-mediated protein interaction and subsequent transcriptional activation of their target gene such as FasL.

Discussion

The transcription factor NF-κB is a key mediator of the cellular stress response upon anticancer therapy, and the activation of NF-κB can elicit a prodeath response (29). TA, one of the traditional NSAIDs, induces apoptosis in various cancer cells (11–13). In search for novel approaches to augment the therapeutic efficacy of TA, we investigated the role of NF-κB in TA-mediated apoptosis in colorectal cancer cells. In this study, we for the first time provide evidence that TA induces apoptosis by activating NF-κB transcriptional activity in human colorectal cancer cells.

![Figure 5](image_url)

Fig. 5. TA-stimulated NF-κB activation is dependent on p53. (Upper panel) Different cells (A, HCT116; B, LoVo; C, SW480; D, CaCo-2) were transfected with pNF-κB-Luc plasmid and then treated with TA for 24h. The cells were harvested, and luciferase activity was measured using a dual-luciferase assay kit. *P < 0.05 compared with cells without TA. (Lower panel) Different cells were treated with TA for 24h. CE and NE were prepared and subsequently western blot analysis was performed for p65, p53 and actin.

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were transfected with wild-type p65 (WT) or Ser276Gly-mutated p65 (MT) for 24h and then treated with 50 μM TA for 24h. CE and NE were prepared and subsequently western blot analysis was performed for EGFP, TBP, and actin. (D) After transfection with wild-type p65 (WT) or Ser276Gly-mutated p65 (MT) for 24h and treatment of TA, cells were fixed and mounted with ProLong Gold antifade reagent containing DAPI. DAPI staining was used to visualize the nucleus of the cells. (E and F) HCT116 cells were pretreated with 50 μM of selective inhibitors of p38 MAPK (SB203580) and ERK (PD98059) for 2h and then co-treated with 50 μM of TA for 24h. Whole cell lysates (WE), CE and NE were prepared and subsequently western blot analysis was performed for p65, p-p65 (Ser276), TBP, IκB-α, p-IκB-α (Ser32) and actin.
Tolfenamic acid induces apoptosis via NF-κB

Here, we propose two mechanisms by which TA leads to an increase in nuclear p65 level and transcriptional activation. First, nuclear accumulation of p65 by TA was mediated by the proteosomal degradation of IκB-α. This was accompanied by nuclear translocation of NF-κB subunit p65 and enhanced NF-κB/DNA binding, followed by an increase in NF-κB transcriptional activity, which was similar to NF-κB activation by the typical NF-κB inducer TNF-α. However, p65 translocation by TA and TNF-α markedly differed: TA-stimulated IκB-α degradation occurred between 10 and 24 h, whereas TNF-α rapidly induced p65 translocation within minutes (Figure 3B). Second, in this study, we observed that TA increased p65 nuclear accumulation through phosphorylation of Ser276 residue and subsequent stabilization of p65. TA increased protein level of ectopically expressing IκB-α mediated NF-κB transcriptional activity of both p53 and NF-κB, which is required for p53-induced apoptosis. In our study, activation of NF-κB in response to TA treatment was observed in HCT116 and LoVo cells expressing wild-type p53; however, TA did not affect NF-κB activation in HCT116(S276G) and p53 mutant colorectal cancer cell lines, SW480 (mutant p53/Arg273His) and CaCo-2 (p53 null) (Figure 5A–D, upper panels). We also observed that TA-stimulated NF-κB activation was attenuated by p53 knockdown in LoVo cells.

Fig. 6. TA increases p53 transcriptional activity and binding affinity of p65 with p53. (A) HCT116 cells were transfected with pNF-κB-Luc plasmid and then treated with 0, 30 and 50 μM of TA for 24 h. (B and C) The pNF-κB-Luc plasmid or p53-MDM-Luc plasmid-transfected HCT116 cells were treated with TA for 24 h in absence or presence of selective inhibitors of p38 MAPK (SB203580, 50 μM) and ERK (PD98059, 50 μM). The cells were harvested, and luciferase activity was measured using a dual-luciferase assay kit. *P < 0.05 compared with cells without TA. (D) HCT116 cells were pretreated with 50 μM of SB203580 and PD98059 and then co-treated with TA for 24 h. Immunoprecipitation (IP) was performed by pull down of cellular protein with p65 antibody and immunoblotting with p53 antibody. (E) HCT116 cells were transfected with wild-type p65 (WT) or Ser276Gly-mutated p65 (MT) and then treated with 50 μM of TA for 24 h. IP was performed by pull down of cellular protein with EGFP antibody and immunoblotting with p53 antibody.

In this study, we observed that TA activated p38 MAPK and ERK pathways (14). Recently, several studies demonstrated that degradation of p65 via an ubiquitin-proteasome machinery in the nucleus leads to a negative regulation of NF-κB activation (47–50). Phosphorylation of p65 at Ser276 residue allows increased stability of p65 against ubiquitin-mediated degradation in the nucleus and whereby exerts activation of NF-κB signaling (35). In terms of the anti- or proapoptotic activity of p65, Stark et al. (24) reported that localization of p65 in nucleolus activates apoptosis, whereas location in the nucleoplasm inactivates apoptosis, suggesting that nuclear localization of p65 determines apoptotic activity. Thus, further study is required to investigate if TA treatment affects nuclear localization of p65.

Another interesting finding of this study is that TA-induced transcriptional activity of NF-κB is dependent on p53. Interestingly, many proapoptotic stimuli such as DNA-damaging agents induce the transcriptional activation of both p53 and NF-κB. Ryan et al. (39) suggested that p53 directly activates transcriptional activity of NF-κB, which is required for p53-induced apoptosis. In our study, activation of NF-κB in response to TA treatment was observed in HCT116 and LoVo cells expressing wild-type p53; however, TA did not affect NF-κB activation in HCT116(S276G) and p53 mutant colorectal cancer cell lines, SW480 (mutant p53/Arg273His) and CaCo-2 (p53 null) (Figure 5A–D, upper panels). We also observed that TA-stimulated NF-κB activation was attenuated by p53 knockdown in LoVo cells.
However, TA increases p65 nuclear accumulation in a p53-independent manner (Figure 5A–D, lower panels). These results imply that p53 do not play a significant role in p65 translocation and nuclear accumulation. Taken together, it is possible that p53 may be essential for TA-induced NF-κB activation but not for p65 stabilization.

We also found that TA increases p53 nuclear accumulation in a p53-independent manner (Figure 5A–D, lower panels) and transcriptional activity (Figure 6A), and that TA-mediated p53 transcriptional activation is p38 MAPK and ERK dependent (Figure 6B). Indeed, p38 MAPK and ERK phosphorylated p53 at Ser15 (40) and Ser15 phosphorylation of p53 suppresses ubiquitination of p53 and subsequent 26S proteasome-mediated degradation by reducing the interaction of p53 with MDM2. As a result, Ser15 phosphorylation of p53 promotes the accumulation and transactivation (51,52). In the line of this notion, we propose that TA-mediated nuclear accumulation and transcriptional activation of p53 may be associated with p38 MAPK- and ERK-mediated Ser15 phosphorylation of p53 by TA.

In terms of p53 involvement in NF-κB activation, we observed that TA treatment induced binding affinity of p65 with p53 and that TA-stimulated protein interactions and NF-κB activation were blocked by selective inhibitors of p38 MAPK and ERK (Figure 6C and D), indicating that Ser276 phosphorylation of p65 may be important in TA-stimulated binding affinity of p65 with p53 and subsequent NF-κB activation. Furthermore, we tested whether Ser276Gly mutation affects TA-mediated binding affinity of p65 with p53 and observed that TA-mediated binding affinity was diminished in cells transfected with Ser276Gly mutant. These results indicate that Ser276 phosphorylation of p65 may be essential for TA-mediated binding affinity of p65 with p53 and subsequent NF-κB activation. However, we do not exclude the possibility that another p38 MAPK- and ERK-mediated p65 modifications are involved in p65–p53 bindings because point mutation of p65 (S276G) did not completely block the protein binding, whereas chemical inhibitors of p38 MAPK and ERK blocked protein interaction completely (Figure 6D and E).
Recent studies indicate that p53-dependent NF-xB activation could contribute to the apoptotic response (38,39), and the loss of p53 could abrogate NF-xB-mediated apoptotic response (53). In addition, p65 has been reported to promote apoptosis by actively repressing transcription of antiapoptotic genes through association of p65 with histone deacetylase-containing complexes acting as corepressor (54). In this study, we found that TA-induced apoptosis was significantly reduced in p53-null and p65-knockdown cells (Figure 7A–E). We also observed that point mutation of p65 at Ser276 residue attenuated TA-induced apoptosis. These results indicate that TA-induced apoptosis may be mediated by Ser276 phosphorylation-mediated p65 stabilization and subsequent p53-dependent transcriptional activation of p65.

According to several studies, it is likely that p53 regulates NF-xB transcriptional activity via different pathways in normal and cancer cells. For example, the binding of p53 to p65 inhibits NF-xB activation in normal fibroblasts (42), whereas an increase of binding affinity stimulates NF-xB activation in cancer cells (43). In the line of this notion, TA-stimulated increase in binding affinity of p65 to p53 may contribute to apoptosis by TA in human colorectal cancer cells.

Both p53 and NF-xB regulate FasL (CD95/APO-1) activating a apoptosis signaling cascade by binding Fas on the target cell triggering cell death and induction of FasL is essential for p53- and NF-xB-dependent apoptosis (44–46). So, FasL could be a good target protein studying an apoptosis associated with p53 and p65. Thus, we test if TA affects FasL expression and observed TA-mediated FasL induction in a dose- and time-dependent manner (Figure 7G and H). In terms of time kinetics, we observed that the time point of p65 accumulation (6 h after TA treatment; Figure 2E) is earlier than that of FasL expression (10 h after TA treatment; Figure 7H). A number of studies indicate that FasL has NF-xB response elements on their promoters and p65 binds to these response elements (55,56). Thus, we claim that TA-stimulated p65 accumulation and transcriptional activation leads to an increase of FasL transcription.

Colorectal tumorigenesis is affected by many signaling pathways including COX. TA activated NF-xB transcriptional activity in HCT116 (COX negative) and LoVo cells (COX negative) but did not affect in Caco-2 (COX negative) cells. It is likely that TA-induced apoptosis and NF-xB activation is COX independent. However, we do not exclude the possibility that inflammatory status may affect anti-cancer effects of TA.

In summary, the data presented here show that TA induces p65 nuclear accumulation through 1bx1-dependent nuclear translocation and increased stability of nuclear p65 through p38- and ERK-dependent Ser276 phosphorylation. Increased nuclear p65 directly interacts with p53 and activates NF-xB transcriptional activity, which is predominantly responsible for the ability of TA to induce apoptosis.

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References
