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A Molecular Mechanism for the Anti-Inflammatory Effect of Taurine-Conjugated 5-Aminosalicylic Acid in Inflamed Colon

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ABSTRACT

In previous reports, a novel colon-specific prodrug, 5-aminosalicyltaurine (5-ASA-Tau) administered orally, is successfully delivered to and liberates 5-aminosalicylic acid (5-ASA) and taurine in the inflamed large intestine of rats. Furthermore, the prodrug ameliorates the 2,4,6-trinitrobenzene-sulfonic acid-induced colitis, and taurine acts not only as a carrier but also as an active therapeutic agent. In this study, we investigated the anti-inflammatory properties of the prodrug at a molecular level. After rectal administration of taurine, formation of taurine chloramine (TauCl) in the inflamed colonic tissue was examined using high-performance liquid chromatography. In human colon epithelial cell lines, nuclear factor-κB (NF-κB) activity was accessed using an NF-κB-dependent luciferase reporter gene. Protein levels were monitored by Western blotting. DNA binding activity of the NF-κB subunit p65 was determined using a DNA binding assay kit. A millimolar level of TauCl was formed in the inflamed tissue. TauCl inhibited tumor necrosis factor (TNF)-dependent NF-κB activation by modifying thiol(s) on p65 and blocking DNA binding. In addition, 5-ASA inhibited phosphorylation of p65 at serine 536, which is critical for transcriptional activity of NF-κB. Furthermore, combined TauCl/5-ASA treatment additively inhibited TNF-dependent NF-κB activation. Together, our data suggest that the colon-specific carrier taurine contributes to the clinical effect of the prodrug by potentiating the inhibitory effect of the active ingredient 5-ASA on a major proinflammatory signal, TNF-dependent NF-κB activation in the inflamed large intestine.

NF-κB is an important transcription factor that regulates genes involved in immunity and inflammation (Li and Verma, 2002). The functional NF-κB protein is a heterodimer composed of two subunits, p65 and p50 (Urban et al., 1991). Under normal conditions, NF-κB is present in the cytoplasm in an inactive state, bound to IκB. Stimulation with proinflammatory cytokines such as TNF-α initiates an intracellular signaling cascade, resulting in the phosphorylation and subsequent degradation of IκB by the 26S-proteasome (Tanaka et al., 2001). The degradation of IκBα releases NF-κB, allowing it to translocate into the nucleus, and activates cyclooxygenase-2 (COX-2), cytokines, chemokines, cell surface receptors, and adhesion molecules that are pivotal mediators of the immune and inflammatory responses (Li and Verma, 2002).

NF-κB activity and levels of the proinflammatory cytokine TNF-α have been shown to be increased in the colon epithelial cells and mucosa of patients with inflammatory bowel disease, ulcerative colitis, and Crohn's disease (Neurath et al., 1998). It is known that expression of TNF-α, which strongly activates NF-κB, is itself up-regulated by NF-κB. This provides a positive autoregulatory loop that amplifies the inflammatory response and perpetuates chronic intestinal inflammation (Neurath et al., 1998). For this reason, therapeutic intervention against TNF-α or NF-κB activation has been used for treatment of inflammatory bowel disease (IBD) (Yamamoto and Gaynor, 2001). In fact, inhibition of NF-κB activity has been suggested to be a major component of the anti-inflammatory activity of glucocorticoid and 5-aminosalicylic acid (5-ASA), both of which are frequently used for treatment of chronic intestinal inflammation (Auphan et al.,

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ABBREVIATIONS: NF-κB, nuclear factor-κB; TNF, tumor necrosis factor; COX, cyclooxygenase; IBD, inflammatory bowel disease; 5-ASA, 5-aminosalicylic acid; TauCl, taurine chloramine; MPO, myeloperoxidase; 5-ASA-Tau, 5-aminosalicyltaurine; TNBS, 2,4,6-trinotrobenzene-sulfonic acid; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; CMV, cytomegalovirus; HIF-1α, hypoxia-inducible factor-1α; NFDL, nuclear factor-dependent luciferase; IKK, IκB kinase complex.

1995; Yan and Polk, 1999). Moreover, new strategies that specifically regulate NF-κB activity using p65 (RelA) antisense oligonucleotides, proteasome inhibitors, or adenoviral IκB expression vector show beneficial therapeutic effects in experimental colitis (Neurath et al., 1996; Conner et al., 1997).

Taurine chloramine (TauCl) is a mild oxidant. It is formed by the reaction of taurine (2-aminoethanesulfonic acid), a free amino acid not incorporated into protein, with the strong oxidant HOCl/OCl^- produced by myeloperoxidase (MPO). MPO is up-regulated under certain physiological conditions such as inflammation (Marquez and Dunford, 1994). The reaction occurs physiologically around or in activated immune cells that contain a large amount of taurine to protect tissues from the highly toxic effect of HOCl/OCl^- (Schuller-Levis et al., 1994). An expanding body of evidence suggests that taurine chloramine plays an important role in inflammatory progression by modulating NF-κB activity and attenuating production of proinflammatory mediators such as prostaglandins, nitric oxide, and cytokines (Marcinkiewicz et al., 1998; Barua et al., 2001).

5-ASA is an important anti-inflammatory agent for long-term treatment of IBD (Biddle and Miner, 1990). In most cases, 5-ASA is absorbed rapidly and extensively in the upper intestine and does not reach the colon (Crotty and Jewell, 1992). Systemically absorbed 5-ASA can cause nephrotic syndrome (Novis et al., 1988). Thus, a number of prodrugs and pharmaceutical formulations have been developed to allow colon-specific delivery of 5-ASA, reducing the side effects and increasing the therapeutic effect of 5-ASA (Yang et al., 2002).

We previously reported that a novel colon-specific prodrug of 5-ASA, 5-aminosalicyltaurine (5-ASA-Tau) has good colon targetability (Jung et al., 2003) and effectively ameliorates 2,4,6-trinitrobenzene-sulfonic acid (TNBS)-induced colitis in rats and furthermore, taurine is shown to potentiate the clinical effect of 5-ASA upon rectal administration of combined 5-ASA/taurine (Y. Jung, H.-H. Kim, H. Kim, H. Kong, B. Choi, Y. Yang, and Y. Kim, unpublished data). In this study, we investigated at a molecular level how taurine itself contributes to the anti-inflammatory properties of the prodrug 5-ASA-Tau using human colon epithelial cells. Colon epithelial cells are active participants in chronic intestinal inflammation such as IBD by producing and responding to proinflammatory cytokines (Fiocchi, 1997). Our data show that a millimolar level of TauCl was formed from taurine administered rectally in the inflamed large intestine of rats. We also show that either TauCl or 5-ASA inhibits TNF-mediated NF-κB activation and that combined TauCl/5-ASA treatment elicits an additive effect on inhibition of NF-κB activity. Furthermore, we provide a potential molecular mechanism underlying the additive effect on inhibition of NF-κB activation by revealing novel mechanisms by which either 5-ASA or taurine chloramine inhibit TNF-dependent NF-κB activation.

Materials and Methods

Drugs and Chemicals. Dithiothreitol (DTT), hexadecyl-trimethylammonium bromide, TNBS, 5-ASA, taurine, and NaOCl were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of Taurine Chloramine. Taurine chloramine (50 mM) was prepared in our laboratory by adding NaOCl dropwise to 250 mM taurine dissolved in phosphate-buffered saline, pH 7.4. The concentration of TauCl was determined by measuring its absorption at 252 nm (molar extinction coefficient = 415).

Induction of Inflammation. Inflammation was induced by the method of Yano et al. (2002). In brief, before induction of colitis, rats were starved for 24 h but had free access to water. The rats were lightly anesthetized with ether. A rubber cannula (2 mm o.d.) was inserted rectally into the colon such that the tip was 8 cm proximal to the anus, approximately at the splenic flexure. TNBS dissolved in 50% (v/v) aqueous ethanol was instilled into the colon via the rubber cannula (15 mg/0.3 ml/rat).

High-Performance Liquid Chromatography Analysis of TauCl in the Inflamed Colonic Tissue. Male Sprague-Dawley rats were starved for 24 h before use for the experiments but had free access to water. Taurine (30 mM) was administered rectally 6 days after induction of inflammation by TNBS. The colitic rats were ether-anesthetized and sacrificed 5 and 25 min after the rectal administration, and the inflamed colon was cut out and weighed. The inflamed colonic tissue (0.2 g) was mixed with 600 μl of PBS, pH 7.4, and homogenized using a Polytron PT 3100 homogenizer (Kinematica, Basel, Switzerland) after gently removing the colonic contents. The homogenates were centrifuged at 14,000 rpm at 4°C and the supernatants were 4-fold diluted with methanol. The concentration of TauCl in the methanol-diluted supernatants was determined by a reversed-phase high-performance liquid chromatography (Gilson, Villier Le Bel, France) using a μBondapak C18 (300 × 3.9 mm; 10 μm) column (Waters, Milford, MA) with a guard column (20 × 3.9 mm, 5 μm; Waters). The mobile phase consisted of 7.5% acetonitrile in 15 mM phosphate buffer, pH 4.0, containing 0.5 mM tetrabutylammonium chloride, which was filtered through 0.45-μm membrane filter before use. Samples (50 μl) were injected and eluted with the mobile phase at a flow rate of 1.0 ml/min. The eluate was monitored at 252 nm at sensitivity of AUFS 0.001. The retention time of TauCl was 7.6 min.

Cells and Transient Transfection. Human colon epithelial cell lines HT-29, HCT116, and SW620 (American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin/streptomycin (Biofluids, Rockville, MD). Recombinant human-TNF-α was purchased from R&D Systems (Minneapolis, MN). Cell viability was determined by the trypan blue exclusion method. Cell viability was unchanged in each experimental condition. For transient transfection of NF-κB-dependent luciferase plasmid, cells were plated in 12-well plates to be 50 to 60% confluent on the day of transfection with NF-κB-dependent luciferase plasmid (0.4 μg; a gift from Dr. M. Birrer, National Cancer Institute, Bethesda, MD) and CMV *Renilla reniformis* luciferase plasmid (4 ng; Promega, Madison, WI). FuGENE (Roche, South San Francisco, CA) or Lipofectamine 2000 (Invitrogen) was used as a transfection reagent. Sixteen hours after transfection, cells were treated with TNF-α in the presence of each reagent at the indicated concentrations in the figure legends. Cells were lysed 6 h later, and luciferase activities were measured and normalized to CMV *R. reniformis* luciferase activities using a Promega dual-luciferase assay kit (Promega).

Western Blot. Cells were lysed and nuclear and cytosolic extracts were prepared as described previously (Andrews and Faller, 1991). Cell lysates were electrophoretically separated using 4 to 20% gels (Bio-Rad, Hercules, CA). Proteins were transferred to nitrocellulose membranes (Protran; Whatman Schleicher & Schuell, Keene, NH), and COX-2 and IκBα proteins were detected in cytosolic extracts using a monoclonal anti-COX-2 antibody (BD Transduction Laboratories, Lexington, KY) or polyclonal anti-IκBα antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and p65 was detected in nuclear extracts using polyclonal anti-p65 antibody (Santa Cruz Biotechnology, Inc.). Peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was used at a dilution of 1:1000. Signals were visualized

using the SuperSignal chemiluminescence substrate (Pierce Chemical, Rockford, IL). Membranes were reprobed with α-tubulin (Santa Cruz Biotechnology, Inc.) or stained with ponceau S solution to verify equivalent loading.

DNA Binding Assay. Cell extracts were obtained using radioimmunoprecipitation assay lysis buffer (1% Nonidet P-40, 0.25% sodium deoxycholate, and 50 mM Tris-HCl, pH 7.4), 150 mM NaCl, 1 mM EDTA, and protease inhibitor cocktail (Roche) or by nuclear extraction as described under *Western Blot*. Reagents were added to the cell extracts as described in the figure legends. DNA binding activity of p65 or HIF-1α was measured with 5 μg of cell lysates using TransAM NF-κB kit or TransAM HIF-1α kit (Active Motif Inc., Carlsbad, CA).

Results

Taurine Chloramine but Not Taurine Inhibits NF-κB Activation by the Proinflammatory Cytokine TNF-α in a Dose-Dependent Manner in Human Colon Epithelial Cells. Our previous data suggest that the colon-specific carrier taurine itself elicits an anti-inflammatory activity and enhances the anti-inflammatory effect of 5-ASA. Thus, we investigated at a molecular level how taurine augmented the ability of 5-ASA to alleviate the colonic inflammation. Because the proinflammatory cytokine TNF-α is up-regulated in gut inflammation (Hibi et al., 2003) and TNF-induced NF-κB activity plays a central role in the production of proinflammatory mediators involved in progression of gut inflammation (Neurath et al., 1998), we examined whether taurine affected NF-κB activation by TNF-α. We transfected human colon epithelial cells HCT116, SW620, and HT-29 with an NF-κB-dependent luciferase (NFDL) reporter gene in combination with an internal standard CMV *R. reniformis* luciferase plasmid. Cells were then treated with TNF-α in the presence of taurine (10–30 mM). TNF-α markedly increased NFDL expression, which represents TNF-mediated-NF-κB activation, in the cell lines as shown in Fig. 1A. Taurine did not at all affect NFDL expression by TNF (data not shown). Because it is known that TauCl is produced at sites of inflammation by the reaction of taurine with HOCl generated from MPO (Stapleton et al., 1998) and plays a role as a negative regulator of inflammation (Marcinkiewicz et al., 1998), it was thought that the taurine effect may be through formation of TauCl in the inflamed colonic tissue. Although MPO activity of inflamed colonic tissue is much greater than that of normal colonic tissue, it is not clear whether TauCl could be formed from exogenous taurine in the inflamed colon. To clarify this, 30 mM taurine was administered rectally to the normal or colitic rats induced by TNBS and formation of TauCl was measured in the normal or inflamed

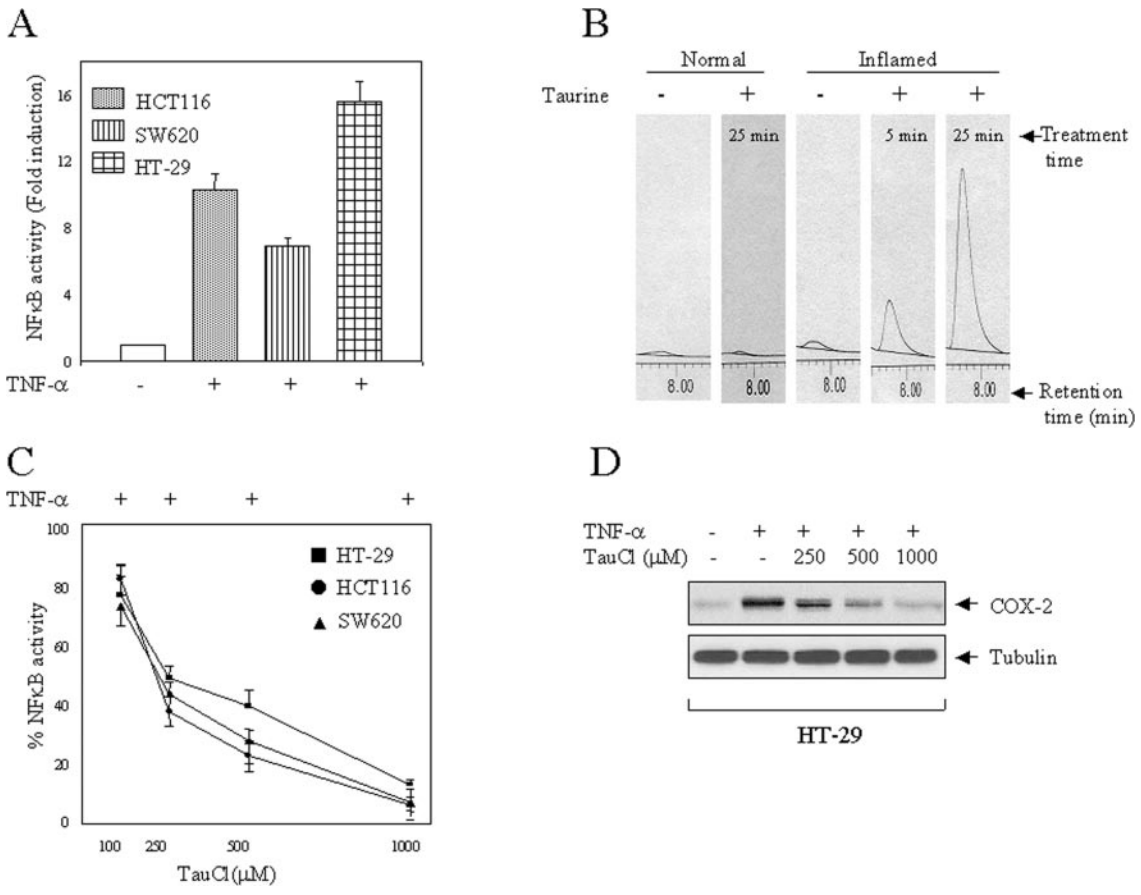

Fig. 1. Taurine chloramine inhibits TNF-dependent NF-κB activation. A, cells were cotransfected with NF-κB-dependent luciferase plasmid (0.4 μg) and CMV *R. reniformis* luciferase plasmid (4 ng) and subsequently treated with TNF-α (10 ng/ml) for 6 h. Reporter activities were measured and normalized to CMV *R. reniformis* luciferase activity. The data are mean ± S.E. (n = 3). B, 300 μl of 30 mM taurine was administered rectally to colitic or normal rats. The colonic tissues were homogenized 5 or 25 min after rectal administration. TauCl in the colonic tissues was analyzed by HPLC as described under *Materials and Methods*. C, same experiment as in A was done in the presence or absence of various concentrations of TauCl. The data are mean ± S.E. (n = 3). D, HT-29 cells were treated with TNF-α (10 ng/ml) in the presence or absence of various concentrations of TauCl. COX-2 protein was detected in 40 μg of whole-cell lysates.

colonic tissue 5 and 25 min after rectal administration. As shown in Fig. 1B, although TauCl was not formed from taurine administered rectally in the normal colonic tissue, TauCl was formed in the inflamed colonic tissue and its concentration reached approximately 10 mM, 25 min after rectal administration of taurine. Based on this result, we examined effect of TauCl on NF-κB activation by TNF-α. The same experiment was done as with taurine. As shown in Fig. 1, A and C, although stimulation of cells with TNF-α markedly induced NFDL expression, TauCl inhibited NFDL expression by the cytokine in a dose-dependent manner. The concentration at which TauCl showed 50% inhibitory effect was 150 to 200 μM. To confirm that TauCl inhibited the NF-κB pathway, we examined whether TauCl interfered with expression of a proinflammatory mediator COX-2 in HT-29 cells. TNF-α up-regulates COX-2 through NF-κB in these cells (Jobin et al., 1998). We treated HT-29 cells with TNF-α in the presence or absence of TauCl at various concentrations. As shown in Fig. 1D, in parallel with the above-mentioned result, up-regulation of COX-2 protein by TNF-α was inhibited by TauCl in a dose-dependent manner.

Taurine Chloramine Inhibits TNF-Dependent-NF-κB Activation by Interfering with DNA Binding of the NF-κB p65 (RelA). Because degradation of IκBα in the cytosol and accumulation of p65 in the nucleus is critical for TNF-mediated NF-κB activation (Baeuerle and Baltimore, 1988), we investigated whether TauCl inhibited TNF-mediated NF-κB activation by intervening in these processes. HCT116 cells were incubated with TNF-α in the presence of varying concentrations of TauCl and lysed to obtain cytosolic and nuclear extracts. Western blotting was performed to analyze IκBα levels in the cytosol and p65 levels in the nucleus. As shown in Fig. 2A, stimulation of cells with the cytokine resulted in degradation of IκBα in the cytosol and

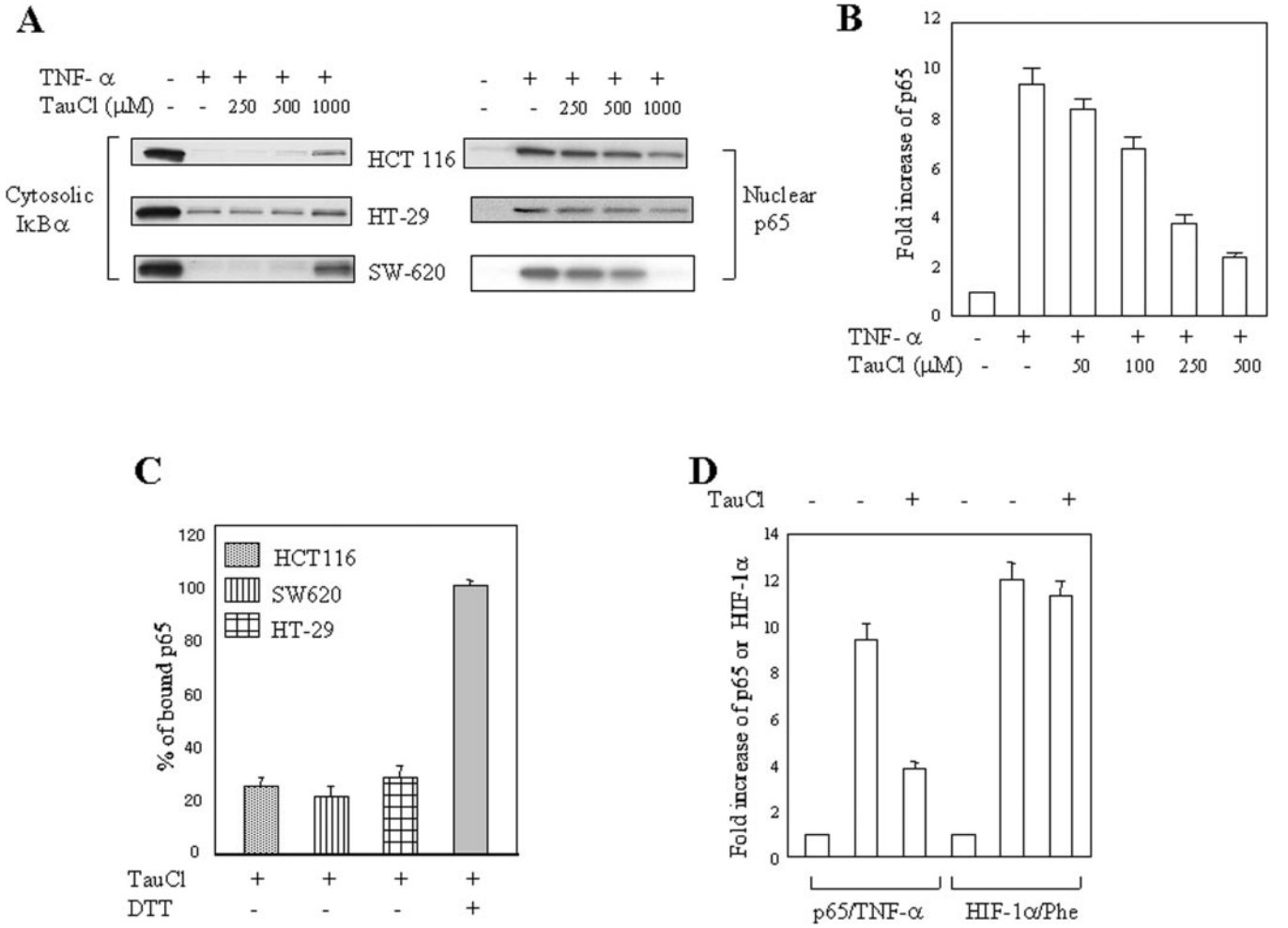

Fig. 2. Taurine chloramine interferes with DNA binding of the NF-κB subunit p65 by modifying thiols in the protein. A, cells were treated with TNF-α (10 ng/ml) in the presence or absence of various concentrations of TauCl, and IκBα or p65 protein levels were monitored in cytosolic or nuclear extracts, respectively. B, HCT116 cells were left untreated or treated with TNF-α for 20 min and lysed to obtain nuclear extracts. TauCl at various concentrations was added to the nuclear extracts obtained from TNF-treated cells and incubated for 30 min at room temperature. DNA binding of p65 was measured using 5 μg of the nuclear extracts. C, colon epithelial cells were treated as in B, and nuclear extracts were obtained. TauCl (500 μM) was added to the nuclear extracts with or without the thiol-reducing reagent DTT (1 mM) and incubated for 30 min at room temperature. DNA binding of p65 was measured using 5 μg of the nuclear extracts. Results are expressed as a percentage of DNA binding of p65 in the TauCl-untreated nuclear extract. The gray bar in the figure represents mean ± S.E calculated from means (n = 3) of percentages of bound p65 (100 × p65 DNA binding of TauCl-treated nuclear extract/p65 DNA binding of TauCl-untreated nuclear extract) for three colon epithelial cells. D, HCT116 cells were treated with either TNF-α for 20 min or an iron chelator phenanthroline (Phe; 100 μM) for 4 h and lysed to obtain nuclear extracts. TauCl (250 μM) was added to the nuclear extracts and incubated for 30 min. DNA binding of p65 and HIF-1α was measured using 5 μg of each nuclear extract. The data in B, C, and D are mean ± S.E. (n = 3).

accumulation of p65 in the nucleus. We were interested to observe that TauCl prevented nuclear accumulation of p65 without inhibiting degradation of IκBα up to 500 μM in HCT116 cells. However, consistent with a previous study (Kanayama et al., 2002), it significantly inhibited degradation of IκBα at 1000 μM. Similar results were observed in the other human colon epithelial cell lines, HT-29 and SW620 (Fig. 2A), suggesting that TauCl inhibits TNF-mediated NF-κB activation via a previously unrecognized mechanism.

Because DNA binding activity of transcription factors is important for their transcriptional activity (Kunsch et al., 1992) and is probably to affect their ability to remain in the nucleus during cytosolic extraction (Chilov et al., 1999), we hypothesized that TauCl might inhibit DNA binding of p65. To test this hypothesis, various concentrations of TauCl were added to nuclear extracts derived from TNF-treated HCT116 cells, and the DNA binding activity of p65 was measured using TransAM p65 transcription assay kit. As shown in Fig. 2B, although stimulation with TNF-α increased the DNA binding activity of p65 up to approximately 9-fold, consistent with our hypothesis, TauCl interfered with binding of p65 to the NF-κB consensus DNA motif in a dose-dependent manner. The concentration of TauCl to show 50% inhibition of p65 DNA binding was 200 to 250 μM for the colon epithelial cells HCT116, HT-29, and SW620 cells (data not shown). Because thiol(s) in p65 plays an important role in its DNA binding activity (Garcia-Pineres et al., 2001) and TauCl readily reacts with and modifies thiols (Peskin and Winterbourn, 2001), we examined possible involvement of thiol modification in TauCl-mediated inhibition of p65 DNA binding. TauCl (500 μM) was added to nuclear extracts from TNF-treated colon epithelial cells with or without 1 mM DTT, and DNA binding activity of p65 was measured. As shown in Fig. 2C, DTT treatment completely neutralized the inhibitory effect of TauCl on DNA binding of p65. To examine whether the effect of TauCl is specific for NF-κB, TauCl (250 μM) was added to the nuclear extract from iron chelator-treated HCT116 cells, and DNA binding activity of HIF-1α in the nuclear extracts was measured using TransAM HIF-1 transcription assay kit. As shown in Fig. 2D, in contrast to p65, DNA binding activity of HIF-1 was not affected significantly by TauCl.

Combined 5-ASA/Taurine Chloramine Treatment Inhibited TNF-Dependent NF-κB Activation in an Additive Manner. Because 5-ASA is also able to inhibit NF-κB activity (Kaiser et al., 1999; Yan and Polk, 1999) and 5-ASA and TauCl should coexist in inflamed gut after oral administration of 5-ASA-Tau, we thought that the better clinical effect of combined 5-ASA/taurine treatment on the colitis than that of single 5-ASA treatment was because of taurine potentiating inhibitory effect of 5-ASA on NF-κB activity. Therefore, we examined the effect of cotreatment with the two compounds on TNF-dependent-NF-κB activity. We stimulated NFDL-transfected HCT116 cells with TNF-α in the presence of 5-ASA (5–20 mM) and/or TauCl (50–150 μM), and NFDL activities were measured. As shown in Fig. 3A, additive inhibitory effects on NFDL expression by TNF-α were observed by cotreatment at the various concentration ranges. To further test this, we examined whether additive inhibitory effect of the cotreatment on TNF-mediated NFDL expression reflected additive down-regulation of a NF-κB target gene. We stimulated NFDL-transfected HT-29 cells with TNF-α in the presence of varying concentrations of TauCl and/or 15 mM 5-ASA. NFDL activities were measured and normalized to the internal control. As shown in Fig. 3B, consistent with the result in HCT116 cells, combined TauCl/5-ASA treatment resulted in an additive inhibitory effect on TNF-dependent NF-κB activation. To see whether additive down-regulation of an NF-κB gene occurred by the cotreatment, we stimulated HT-29 cells with TNF-α in the presence of TauCl and/or 5-ASA and examined TNF-induced-COX-2 protein expression. Consistent with the results obtained using an NFDL reporter gene, combined TauCl/5-ASA treatment interfered with COX-2 expression in an additive manner (Fig. 3C).

5-Aminosalicylic Acid inhibited TNF-Stimulated NF-κB Activation by Preventing Phosphorylation of p65 at Serine 536 in Human Colon Epithelial Cell Lines. To explore how the cotreatment inhibits TNF-dependent NF-κB activation in an additive manner, we investigated the mechanism by which 5-ASA inhibited TNF-dependent NF-κB activation in the colon epithelial cells. Cells transfected with NFDL plasmid were treated with TNF-α in the presence of varying concentrations of 5-ASA. Consistent with a previous study (Kaiser et al., 1999), 5-ASA inhibited TNF-dependent NF-κB activation in a dose-dependent manner (Fig. 4A). Then, we stimulated cells with TNF-α in the presence of various concentrations of 5-ASA and monitored cytosolic IκBα level and nuclear p65 level by Western blotting. 5-ASA (10–30 mM) treatment prevented neither degradation of cytosolic IκBα nor accumulation of nuclear p65 (Fig. 4B). Because phosphorylation of p65 has been shown to regulate its transcriptional activity (Vermeulen et al., 2002), we examined whether 5-ASA affected the post-translational modification of p65. Cells were treated with TNF-α in the presence of 5-ASA, and phosphorylation of p65 at serine 536 was detected in nuclear extraction using a 536 phosphoserine-specific p65 antibody. As shown in Fig. 4C, 5-ASA inhibited phosphorylation of p65 at serine 536 in a dose-dependent manner without altering p65 protein levels. To examine whether 5-ASA and TauCl shared molecular mechanisms for inhibition of TNF-mediated NF-κB activation, DNA binding activity of p65 obtained from the nuclear extract of TNF-treated HCT116 and HT-29 cells was measured in the presence of 5-ASA, and phosphorylation of p65 at serine 536 in TNF-treated cells was monitored in the presence of TauCl. 5-ASA (30 mM) did not interfere with DNA binding of p65 (data not shown) and as shown in Fig. 4D, TauCl (250–500 μM) did not seem to prevent TNF-mediated phosphorylation of p65 at serine 536. These results strongly suggest that 5-ASA and TauCl use distinct mechanisms to inhibit TNF-dependent NF-κB activation.

Discussion

In this study, we show that TauCl inhibits TNF-dependent NF-κB activation and potentiates the ability of 5-ASA to inhibit NF-κB activation in human colon epithelial cell lines. Furthermore, we demonstrate molecular mechanisms by which either 5-ASA or TauCl inhibits TNF-dependent NF-κB activation.

In agreement with another study (Barua et al., 2001), we found that TauCl but not taurine inhibits NF-κB activity. We showed this using NF-κB-dependent luciferase reporter gene

and expression of COX-2, a target gene of NF-κB. We observed similar effect of TauCl on NF-κB activity in other colon epithelial cell lines, suggesting that TauCl is generally effective at inhibiting NF-κB in the colon. Although we did not determine how much TauCl was formed from taurine liberated from the prodrug 5-ASA-Tau administered orally, it is very likely that TauCl is generated at a sufficient level to reach a therapeutic concentration in inflamed colon. This is based on our observations that 1) rectal administration of 30 mM taurine (300 μl) to the colitic rats generated millimolar level of TauCl in the inflamed colonic tissue; 2) concentration of taurine in the feces collected for 24 h was between 20 to 30 mM after oral administration of the prodrug 5-ASA-Tau (Y. Jung, H.-H. Kim, H. Kim, H. Kong, B. Choi, Y. Yang, and Y. Kim, unpublished data); and 3) IC_{50} of TauCl for NF-κB inhibition was 150 to 250 μM, which is much lower than the fecal concentration of taurine.

TauCl has been shown to attenuate production of proinflammatory mediators through inhibition of NF-κB (Barua et al., 2001). It has been reported that TauCl makes IκBα resistant to IKK-mediated degradation by oxidizing methionine, resulting in inhibition of NF-κB activation (Kanayama et al., 2002). Although our data show that 1 mM TauCl significantly prevents IκBα degradation in cytosol, which is consistent with the previous observation, we suggest that inhibition of DNA binding of p65 is the predominant mechanism by which TauCl inhibits TNF-mediated NF-κB activation. This is based on our findings that TauCl interferes with DNA binding of p65 in a dose-dependent manner and 0.5 mM TauCl inhibits NF-κB activity up to 60 to 80% without evident prevention of IκBα degradation. Our results also suggest that p65 is a more sensitive target than IκBα in regulation of NF-κB activity by the mild oxidant TauCl. This is not surprising considering that reduction-oxidation of thiols modulates DNA binding activity of NF-κB and subsequent NF-κB transcriptional activity (Cargnoni et al., 2002; Nishi et al., 2002). We provide evidence of involvement of thiol modification (most likely oxidation) in TauCl-mediated NF-κB inhibition. Pretreatment with the thiol reducing agent DTT completely blocks the ability of TauCl to inhibit DNA binding of p65.

Because, as demonstrated, TauCl inhibits NF-κB activity by interfering with DNA binding activity of p65, which is the last step of the signal transduction pathway for NF-κB acti-

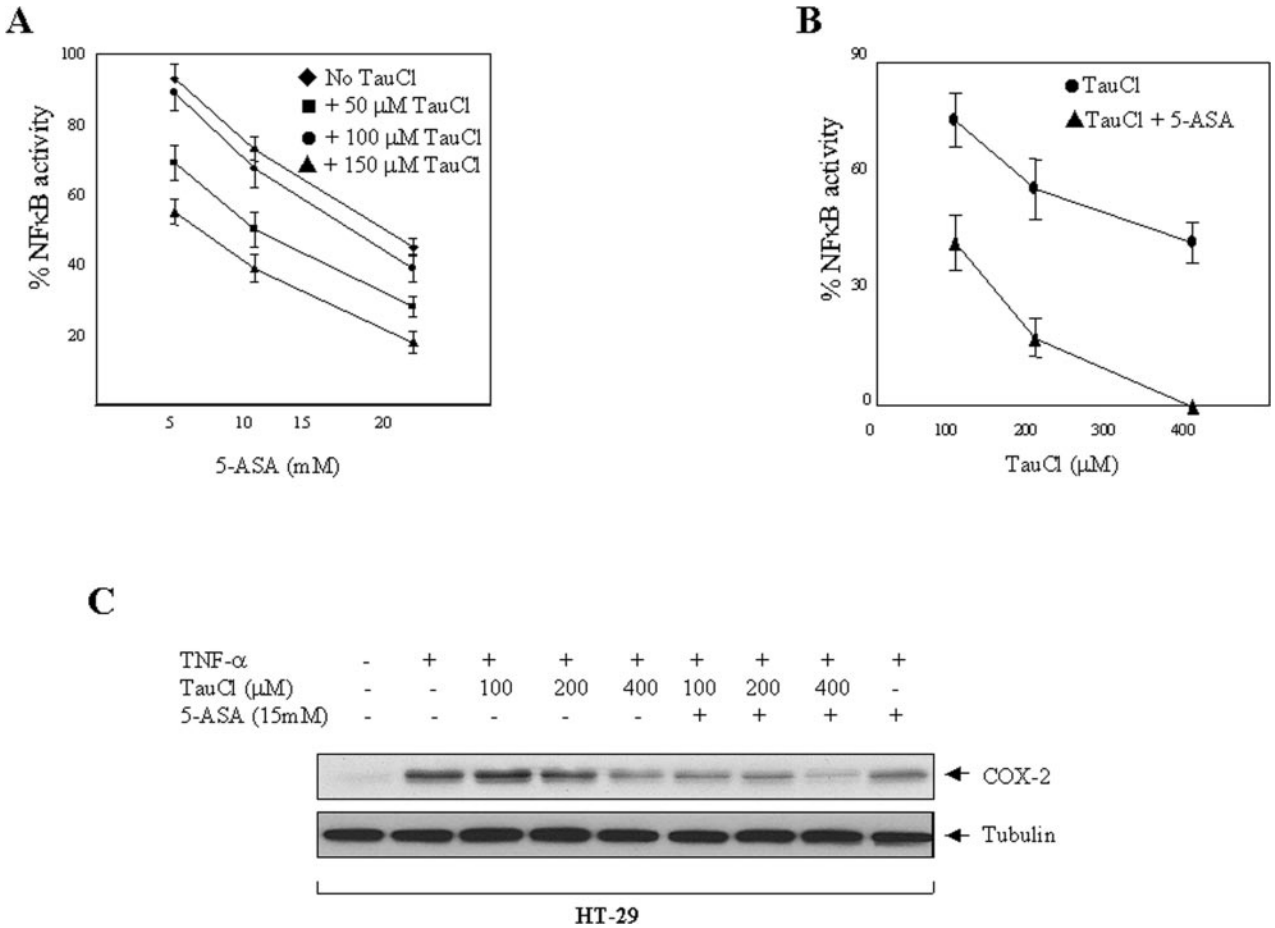

Fig. 3. Taurine chloramine additively inhibits TNF-dependent NF-κB activation upon cotreatment with 5-ASA. A, HCT116 cells were cotransfected with NF-κB-dependent luciferase plasmid (0.4 μg) and CMV *R. reniformis* luciferase plasmid (4 ng) and subsequently treated with TNF-α (10 ng/ml) in the presence of either 5-ASA (5–20 mM) alone or combination of 5-ASA (5–20 mM) and TauCl (50–150 μM) for 6 h. Reporter activities were measured and normalized to CMV *R. reniformis* luciferase activity. Results are expressed as a percentage of NF-κB activity in TauCl-untreated cells. The data are mean ± S.E. (n = 5). B, HT-29 cells were cotransfected with NF-κB-dependent luciferase plasmid (0.4 μg) and CMV *R. reniformis* luciferase plasmid (4 ng) and subsequently treated with TNF-α (10 ng/ml) in the presence of either TauCl (100–400 μM) alone or in combination with 5-ASA (15 mM) for 6 h. Reporter activities were measured and normalized to CMV *R. reniformis* luciferase activity. Results are expressed as a percentage of NF-κB activity in cells treated with only TNF-α. The data are mean ± S.E. (n = 5) C, HT-29 cells were treated for 4 h with TNF-α (10 ng/ml) in the presence of 5-ASA (15 mM) and/or TauCl (100–400 μM). COX-2 protein levels were monitored in the whole-cell lysates.

vation, TauCl is able to prevent NF-κB activation regardless of signaling pathways, culminating in increased NF-κB activity. Considering that under inflammatory condition, NF-κB can be activated by a number of stimulants, including cytokines through several mechanisms (Mercurio and Manning, 1999), and TauCl is an endogenous molecule whose production is induced by inflammation, our data suggest that TauCl is an efficient physiological negative regulator for NF-κB activity, possibly leading to resolution of inflammatory processes.

Our data show that combined 5-ASA/TauCl treatment down-regulates the crucial inflammatory mediators NF-κB activity and COX-2 in an additive manner. This suggests that taurine contributes to the anti-inflammatory effect of the prodrug not only through the effect of TauCl itself but also through potentiation of anti-inflammatory effect of 5-ASA. In addition, taurine delivered to large intestine should exert a beneficial effect in IBD through scavenging reactive oxygen species that damage tissue and aggravate inflammation as reported by Son et al. (1998).

Our data further demonstrate that 5-ASA attenuates TNF-dependent NF-κB activation by inhibiting phosphorylation of serine 536 in p65. Phosphorylation of this residue is critical for transcriptional activity of NF-κB (Vermeulen et al., 2002). This indicates that NF-κB inhibition by either 5-ASA or TauCl occurs by distinct mechanisms, thus partly explaining how combined 5-ASA/TauCl treatment is able to interfere with NF-κB activation in an additive manner. Serine 536 in p65 is reported to be phosphorylated by an IKK complex composed of IKKα, IKKβ, and IKKγ (Sakurai et al., 1999). This result is in line with a previous report demonstrating that 5-ASA inhibits IKKα and IKKβ activity (Yan and Polk, 1999). However, in that report, the authors observed prevention of IκBα degradation resulting from 5-ASA-mediated inhibition of IKK activity, which is in contrast to our data. This discrepancy seems to exist because of cell type specificity because the study was done using mouse intestinal cells, where IKKα plays a major role in TNF-mediated phosphorylation and degradation, unlike in other cell lines where IKKα/IKKβ has a role for p65 phosphorylation and IKKβ but not IKKα is largely responsible for phosphorylation and degradation (Hu et al., 1999; Sizemore et al., 2002). Therefore, our data suggest that IKKα may be a preferable target of 5-ASA in the colon epithelial cell lines. It should be noted that although interleukin-1β activated NF-κB through IKK-mediated-IκBα degradation, 5-ASA interferes with interleukin-1 mediated NF-κB activation through inhibition of p65 phosphorylation without prevention of IκBα degradation in a colon epithelial cell line (Egan et al., 1999).

Together, our data suggest that a molecular mechanism by which taurine enhanced the clinical effect of 5-ASA in ameliorating TNBS-colitis in rats is that TauCl, formed from

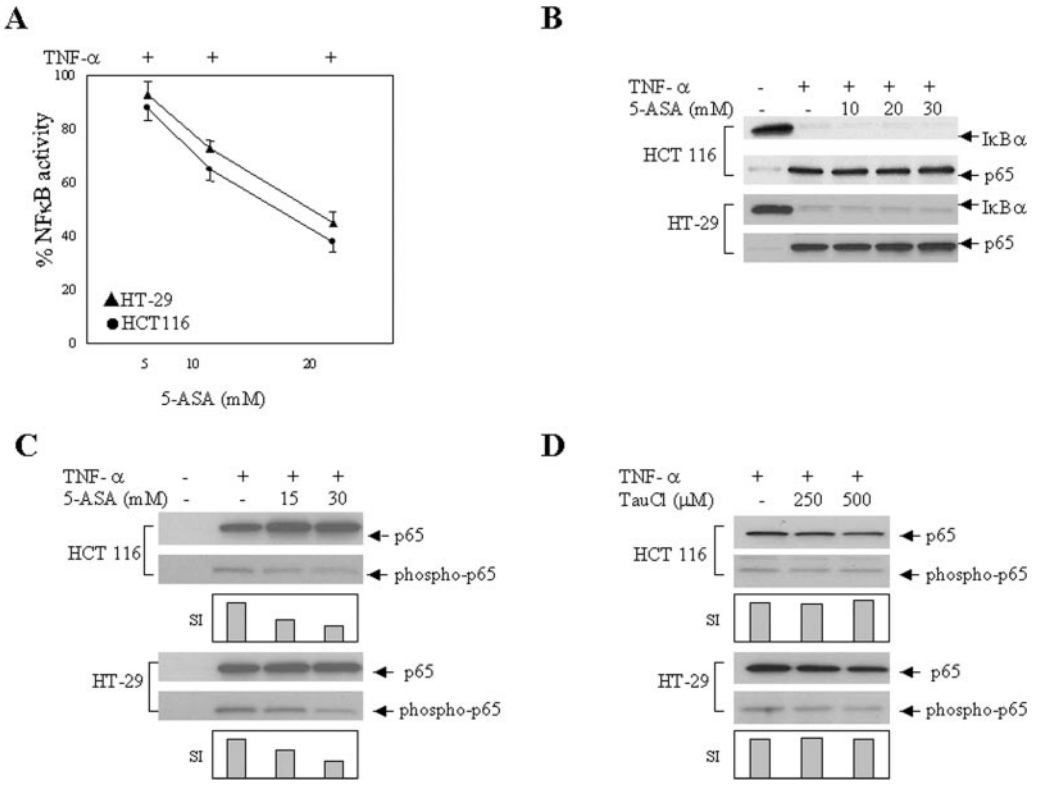

Fig. 4. 5-ASA attenuates TNF-dependent NF-κB activation by inhibiting phosphorylation of serine 536 in p65. A, HCT116 and HT-29 cells were cotransfected with NF-κB-dependent luciferase plasmid (0.4 μg) and CMV *R. reniformis* luciferase plasmid (4 ng) and subsequently treated with TNF-α (10 ng/ml) in the presence or absence of 5-ASA for 6 h. Reporter activities were measured and normalized to CMV *R. reniformis* luciferase activity. Results are expressed as a percentage of NF-κB activity in cells treated with only TNF-α. The data are mean ± S.E. (n = 3). B, cells were treated with TNF-α (10 ng/ml) in the presence or absence of various concentrations of 5-ASA for 20 min. IκBα was detected in cytosolic extracts and p65 was detected in nuclear extracts. C, cells were treated with TNF-α in the presence or absence of various concentrations of 5-ASA for 20 min and serine 536-phosphorylated p65 was detected in nuclear extracts. Top, membranes were reprobed with p65 antibody to detect total p65 protein levels. The signal intensities of phospho-p65 were normalized to those of corresponding total p65. SI, normalized signal intensities of phospho-p65. D, same experiment as in C was done with TauCl (250 and 500 μM). The signal intensities of phospho-p65 were normalized to those of corresponding total p65. SI, normalized signal intensities of phospho-p65.

taurine in the inflamed colonic tissue, not only inhibits TNF-dependent NF-κB activation but also potentiates the ability of 5-ASA (active ingredient of 5-ASA-Tau) to interfere with the NF-κB activation.

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