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The interaction of butyrate with TNF-α during differentiation and apoptosis of colon epithelial cells: Role of NF-κB activation

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ABSTRACT

We demonstrated that TNF-α suppressed differentiation and potentiated cell death induced by butyrate (NaBt) in both adenocarcinoma HT-29 and fetal FHC human colon cells *in vitro*. Since TNF-α is a typical activator of NF-κB pathway, we studied the role of NF-κB activation in cell differentiation and death during the TNF-α and NaBt co-treatment. TNF-α induced rapid NF-κB activation in both HT-29 and FHC cell lines and this effect was differently modulated by NaBt in these two cell lines. In HT-29 cells, NaBt potentiated NF-κB activity induced by TNF-α after 4 h treatment. However, this initial potentiation of NF-κB activity was not observed in FHC cells. During additional time of TNF-α and NaBt co-treatment, NaBt decreased the TNF-α-mediated NF-κB activity in both cell types. We also detected a different response of HT-29 and FHC cells after the pre-treatment with the NF-κB inhibitor parthenolide. Our results indicated that NaBt-mediated differentiation and apoptosis of colon epithelial cells can be modulated by TNF-α. Furthermore, we found significant differences in the mechanism of the NaBt and TNF-α co-treatment effects between cells of non-cancer and cancer origin, suggesting that the NF-κB pathway may be more effectively involved in these processes in cancer cells.

1. Introduction

The colon epithelial cells are under the influence of many substances originating either from endogenous sources or from the diet. Above all, the quantity and ratio of lipids and fiber content in the diet are among the most important factors. Mutual interaction of endogenous and dietary substances may influence colorectal epithelium in various ways. They can encourage a rise of infectious and inflammatory diseases or cancer of the intestine and, on the other hand, they may contribute to regeneration of injured tissue or disease therapy [1,2].

Short-chain fatty acid butyrate is formed in the gastrointestinal tract of mammals as a result of anaerobic bacterial fermentation of dietary fiber [3,4]. It modulates proliferation and induces differentiation and apoptosis of colon epithelial cells [5,3], both *in vivo* and *in vitro*, at concentrations of approximately 5 mM [6]. Butyrate can also act as an energy source for the colonocytes. It has paradoxical effects on colonic epithelial proliferation—stimulates proliferation of normal crypt cells and inhibits growth of colon cancer cells [7]. Thus, butyrate contributes significantly to the balance between the cytokinetic processes of epithelial cells in the colon crypts.

TNF-α, an endogenously produced cytokine of the TNF family, belongs to the intensively investigated biologically active substances [8]. It plays a crucial role in immune and inflammatory processes, endotoxic shock, and regulation of cell growth and death [9,10]. TNF-α is synthesized by macrophages and other cells in response to bacterial toxins, inflammatory products and other invasive stimuli. It has been suggested that a gut with active injury contains an increased number of TNF-α-secreting cells [11]. Endogenous TNF-α at levels higher than those in the corresponding normal colorectal tissues have also been detected in extracts of colorectal tumor tissues resected from human patients [12,13].

The proinflammatory cytokines, such as TNF-α, are typical activators of the canonical NF-κB signaling cascade, which is activated in response to injury, infection, inflammation and other stress conditions [14,15]. Butyrate has been shown to reduce inflammation in experimental colitis in rats [16], and to decrease proinflammatory cytokine expression in intestinal biopsies from Crohn's disease patients via NF-κB inactivation [17]. Therefore, it could be used during the therapy of colorectal chronic inflammations. On the other hand, it has been suggested that NF-κB activation has anti-apoptotic effects in many cell lines [18]. Increased expression of IκB family proteins has also been found in differentiated parts of the colon crypts where the cells undergo apoptosis [19].

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Transcription factors of the NF-κB family, including RelA (p65), c-Rel, RelB, NFKB1 (p50/p105), and NFKB2 (p52/p100), regulate hundreds of genes in the context of multiple important physiological and pathological processes [14,20]. In most cells, NF-κB is present as a latent, inactive inhibitor of the κB (IκB)-bound complex in the cytoplasm. There are two main pathways leading to the activation of NF-κB, namely the canonical and non-canonical pathways or the classical and alternative pathways, respectively. In both pathways, activation of an IκB kinase (IKK) complex is the common upstream regulatory step. This results in phosphorylation-induced degradation of the IκB, which enables the NF-κB dimers to enter the nucleus and activate specific target gene expression. During the canonical pathway, heterodimers of RelA (p65) with p50, p52, or p50 homodimers are liberated by degradation of IκBs, particularly IκB-α, while p52/RelB heterodimers are translocated to the nucleus during the non-canonical pathway [21,15,22].

Comparing the effects of butyrate and TNF-α co-treatment between cancer and non-cancer cells *in vitro* (using human colon adenocarcinoma HT-29 and fetal colon FHC cell lines) we demonstrated that NaBt-mediated differentiation and apoptosis of colon epithelial cells can be modulated by TNF-α. Furthermore, we investigated involvement of the activation of NF-κB transcription factor in the mechanisms of the butyrate and TNF-α co-treatment effects. It has been demonstrated previously that preincubation of cells with butyrate, which induced differentiation, is able to inhibit TNF-α-activated NF-κB translocation in colon cancer cell lines (HT-29, CaCo-2, SW620) [23,24]. In the present study, we found significant differences in the mechanism of the NaBt and TNF-α co-treatment effects between cells of non-cancer and cancer origin, suggesting that the NF-κB pathway may be more effectively involved in these processes in cancer cells.

2. Materials and methods

2.1. Cell cultures

The human colon adenocarcinoma HT-29 cells and human fetal colon FHC cells (both ATCC, Manassas, VA, USA) were cultured in 25 mm^2 flasks (TPP; Trasadigen, Switzerland) in McCoy's 5A medium and in Dulbecco's modified Eagle medium (DMEM):F12 (1:1) medium, respectively, supplemented with gentamicin (50 μg/ml; all Sigma–Aldrich; Prague, Czech Republic) and 10% fetal bovine serum (PAN Biotech GmbH; Aidenbach, Germany). The cultures were maintained at 37 °C in 5% CO_2 and 95% humidity. The cells were passaged twice a week after exposure to trypsin/EDTA (0.05/0.02%) with a plating density of 1:3. For the experiments, cells were seeded in 40 × 10 mm or 100 × 20 mm Petri dishes, or in 12- or 24-well plates (TPP) at a density of 5×10^4 cells/cm^2. Twenty-four hours after seeding the cells were treated with NaBt (5 mM), TNF-α (15 ng/ml; both Sigma–Aldrich), or both, as indicated in the respective experiments. For the inhibition of NF-κB activation, the cells were pre-treated for 3 h with the inhibitor of IKK phosphorylation parthenolide (Biomol International, LP; PA, USA) dissolved in DMSO in a concentration of 10 (HT-29 cells) or 20 (FHC cells) μM. The cells were harvested 4, 8, 24, 48, or 72 h after the treatments. The culture medium was not replaced during the experiments. Epidermal growth factor (EGF; 25 ng/ml; Invitrogen Corporatiom; Carlsbad, CA, USA) was used as a positive control for MAPK pathway activation.

2.2. Cell number counting

Cell growth was measured by counting the cells with a Coulter Counter (model ZM, Coulter Electronic, Luton, UK). Floating and adherent cells were counted separately, and the number of floating cells was expressed as a percentage of the whole cell number.

2.3. Cell cycle distribution analysis

The cells were harvested by trypsinization, washed twice in phosphate buffered saline solution (PBS), and fixed in 70% ethanol. DNA was stained (37 °C; 30 min) with Vindelov's solution (10 mM Tris buffer, pH 8; 0.7 mg/ml RNAse; 50 μg/ml propidium iodide; 0.1% Triton X-100). The DNA content was analyzed using flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA; argon ion laser, 488 nm for excitation). Cells (15×10^3) were acquired for each sample and the percentage of cells in the individual cell cycle phases was analyzed using ModFit 3.0 software (Verity Software House, Topsham, CA). Single cells were identified and gated by pulse-code processing of the area and the width of the signal. Cell debris was excluded by appropriate rising of the forward scatter threshold.

2.4. MTT assay

The cells were growing on 96-well plates. Ten microliters of the 2.5 mg/ml stock solution of 3-[4,5-dimethylthiasol-2yl]-2,5-diphenyl-tetrasolium bromide (MTT; Sigma) was added to each well. After 4 h of incubation at 37 °C in 5% CO_2 and 95% humidity, the medium was removed, 50 μl of the extraction buffer (10% Triton X-100; 0.1 M HCl) was added and plates were gently shaken for 2 h at room temperature [17]. The optical densities were measured at 570 nm (DigiScan Reader).

2.5. Fluorescence microscopy

The cells were trypsinized and $0.5–2 \times 10^6$ cells (including floating cell population) were incubated with 40 μl of 4,6-diamidino-2-phenyl-indole (DAPI) staining solution (3 μg DAPI/ml of methanol) in room temperature, in the dark for 30 min, and mounted to Mowiol. Apoptotic cells were counted using a fluorescence microscope (OLYMPUS IX70; Olympus; Prague, Czech Republic). The percentage of cells with the characteristic apoptotic nuclear morphology (chromatin condensation and fragmentation) was determined from a total number of 200 cells.

2.6. Alkaline phosphatase (ALP) activity determination

The cells were trypsinized, 5×10^5 cells were resuspended in 500 μl of substrate buffer (10% diethanolamine; 5 mM $MgCl_2$; pH 9.7), and lysed by sonication for 5 × 10 s using a Branson Sonifier B-12 (Branson Ultrasonics Corp.; Danbury, CT) at a power of 30 watts. The cell lysates or alkaline phosphatase (ALP; Sigma–Aldrich) in several concentrations ($15.6–1000 \times 10^{-6}$ U/well) for a calibration curve were incubated with ALP substrate (4-*p*-nitrophenylphosphate; Fluka) in a 96-well plate (4 parallel wells in each group) at 37 °C for 30 min. The reaction was stopped by adding 3 M NaOH (50 μl/well) and the optical densities were measured at 405 nm by FluostarGalaxy (BMG Labtechnologies GmbH, Offenburg, Germany). The reading values (Units $\times 10^{-6}/5 \times 10^4$ cells) were converted to the percentage of control.

2.7. Nuclear extracts preparation

Cells grown on 100 × 20 mm Petri dishes were washed with ice-cold PBS, scraped into tubes, and centrifuged for 10 min at 0 °C (1000*g*). The pellets were then incubated in a lysis buffer (10 mM Tris base, pH 8.0, 60 mM KCl, 1.2 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 0.05% NP-40) for 10 min on ice and centrifuged for

4 min at 0 °C (750*g*). The resulting "nuclear pellets" were rinsed with the above lysis buffer without PMSF and NP-40. Nuclear extracts were prepared by resuspending nuclear pellets in nuclear extraction buffer (20 mM Tris base, 420 mM NaCl, 0.7 mM $MgCl_2$, 0.25 mM EDTA (chelate II), 25% glycerol), incubating 30 min at 4 °C, and then centrifuged for 15 min at 4 °C (13,000*g*). Protein concentration of the nuclear extracts was determined by the Bradford assay, and stored at −80 °C until used.

2.8. Electrophoretic mobility shift assay (EMSA)

Double stranded NF-κB oligonucleotide (AGTTGAGGGGACTTTC CCAGGC; VBC-Genomics GmbH; Vienna, Austria) was end-labeled with biotin using the Biotin end-labeling kit (Pierce; Rockford, IL). The binding reactions were performed by mixing 8 μg of the nuclear extract (prepared as described above) with 0.3 μg poly-dIdC and 3 μl of the binding buffer 5× (50 mM Tris base, 6 mM EDTA (chelate II), 0.5 mM DTT, 50% (v/v) glycerol) to give a final volume of 17 μl. After 10 min incubation in room temperature, 3 μl of 10 μM oligonucleotide was added to each reaction and the reactions were incubated for an additional 30 min in room temperature. For the supershift, 1 μl of mouse anti-NF-kB p65 antibody (#sc-8008; Santa Cruz Biotechnology, Inc.; Santa Cruz, CA) was then added to the reaction, and the mixture was incubated for 1 h in 4 °C. To control specificity of the shifts, we also used specific competition (addition of the excess of unlabeled NF-κB oligonucleotide), non-specific competition (addition of the excess of unlabeled EBNA DNA), free probe, and the reaction mixture without the nuclear extract. The reaction mixtures were separated in a 6.5% polyacrylamide/TBE gel and then transferred to a nylon membrane (Intergen; Purchase, NY). The visualization by streptavidin–horseradish peroxidase conjugate was performed using the LightShift™ Chemiluminescent EMSA Kit (Pierce).

2.9. Western blot analysis

The cells were scraped and lysed in an SDS lysis buffer (100 mM Tris, pH 6.8; 2% sodium dodecyl sulfate (SDS); 10% glycerol). The extracts of the total proteins were assayed with a DC protein assay kit (Bio-Rad Laboratories, Inc.; Hercules, CA). Equal amounts (20 μg) of whole cell lysates or nuclear extracts with 0.01% bromphenol blue and 1% mercaptoethanol were subjected to SDS–PAGE using 10% polyacrylamide gels. The gels were transferred to polyvinylidene fluoride membranes (Millipore Corp.; Bedford, MA) electrophoretically in a buffer containing 192 mM glycine, 25 mM Tris, and 10% methanol. The membranes were blocked for 1 h in 5% powdered non-fat milk in wash buffer (0.05% Tween-20 in 20 mM Tris; pH 7.6; 140 mM NaCl). Primary antibodies (rabbit anti-NF-κB p65, 1:500, #3034; rabbit anti-phospho-ERK1/2, 1:2000, #9101; rabbit anti-ERK1/2, 1:1000, #9102; rabbit anti-phospho-p38, 1:2000, #9211; rabbit anti-p38, 1:1000, #9212; all Cell Signaling Technology, Inc.; Beverly, MA; rabbit anti-I-κBα, 1:500, #sc-203, Santa Cruz Biotechnology) were incubated with the blots for 1 h at room temperature. After washing the membranes in a wash buffer, secondary antibodies coupled to horseradish peroxidase (anti-mouse IgG, 1:3000; #NA931 and anti-rabbit IgG, 1:5000, #NA934, both Amersham Biosciences; Buckinghamshire, UK) were added for 1 h. The membranes were washed and antibody reactivity was visualized with the enhanced chemiluminescence (ECL^+) reagent (Amersham Biosciences) against X-ray film-CP (Agfa-Gevaert Group; Mortsel, Belgium). The equal loading was verified by β-actin or α-tubulin expression (mouse anti-β-actin, 1:6000, #A5441; mouse anti-α-tubulin, 1:4000, #T6074; both Sigma) and non-specific amidoblack staining of proteins on the membrane after immunoblotting.

2.10. Luciferase reporter construct and stable cell transfection

The reporter construct pBIIX-LUC was kindly donated by Dr. Kalle Saksela (Institute of Molecular Medicine, University of Tampere, Finland). The NF-κB-driven plasmid pBIIX-LUC was constructed by inserting a synthetic fragment with two copies of the sequence ACA GAG GGG ACT TTC CGA GAG separated by four nucleotides (ATCT) in front of the mouse *fos* promoter in plasmid pfLUC [25].

The reporter plasmid was cotransfected with pSV2neo neomycin-resistance plasmid into HT-29 cells using electroporation (Bio-Rad Laboratories, Inc.; Hercules, CA). Briefly, approximately 1×10^7 cells was mixed with 20 μg of reporter plasmid and 2 μg of pSV2neo plasmid, and electroporated (275 V, 950 μF, 2× pulse). Subsequently, the cells were seeded on a 100 × 20 mm dish (TPP) with a feeder of HT-29 cells treated with mitomycin-C (5 μg/ml; Sigma–Aldrich). Neomycin resistant clones were selected in 300 μg/ml of geneticin (G418 sulfate; Alexis; San Diego, CA). The clones containing the reporter plasmid were confirmed by luciferase activity assay.

2.11. Luciferase activity assay

Stably transfected cells were seeded in 12-well plates and treated 24 h later. After 4, 8, or 24 h treatment, the cells were rinsed with PBS and then lysed with 100 μl of reagent (Luciferase Assay System; Promega Corp.; Madison, WI). Fifty microliters of the extracts was mixed with 50 μl of luciferase substrate, and luminescence was quantified by a luminometer.

2.12. Statistical analysis

All the data are expressed as the means ± SD of three independent experiments. Differences between the groups were calculated using one-way ANOVA. With all statistical analyses, an associated probability (*P*-value) of <5% was considered as significant.

3. Results

3.1. Growth, death, and differentiation of colon epithelial cells during TNF-α and NaBt co-treatment

3.1.1. Cell growth

Compared to control, TNF-α in a concentration of 15 ng/ml did not significantly affect cell proliferation of both cell lines used at any time of treatment. On the other hand, NaBt (5 mM) inhibited cell growth in both HT-29 and FHC cell lines (Fig. 1A), but with different dynamics. The NaBt effect was already apparent after 24 h, being most significant in FHC cells after 72 h (approximately 70% decrease of cell number versus control). In HT-29 cells, NaBt inhibited cell growth the most significantly after 24 and 48 h of the treatment (approximately 40% decrease of cell number versus control), and the effect diminished after 72 h (only 20% decrease). No additional changes of cell growth were detected after NaBt and TNF-α co-treatment in both cell lines.

3.1.2. Cell cycle

NaBt arrested the cells in the G_0/G_1-phase of the cell cycle and simultaneously decreased the proportion of cells in the S-phase (Fig. 1B). This effect corresponded with cell growth inhibition shown in Fig. 1A in both HT-29 and FHC cells, and was not significantly affected during co-treatment of NaBt with TNF-α. Moreover, TNF-α alone had no effect on cell cycle distribution.

3.1.3. Cell differentiation

NaBt induced a significant increase in intracellular ALP activity (a marker of colonocyte differentiation) in both HT-

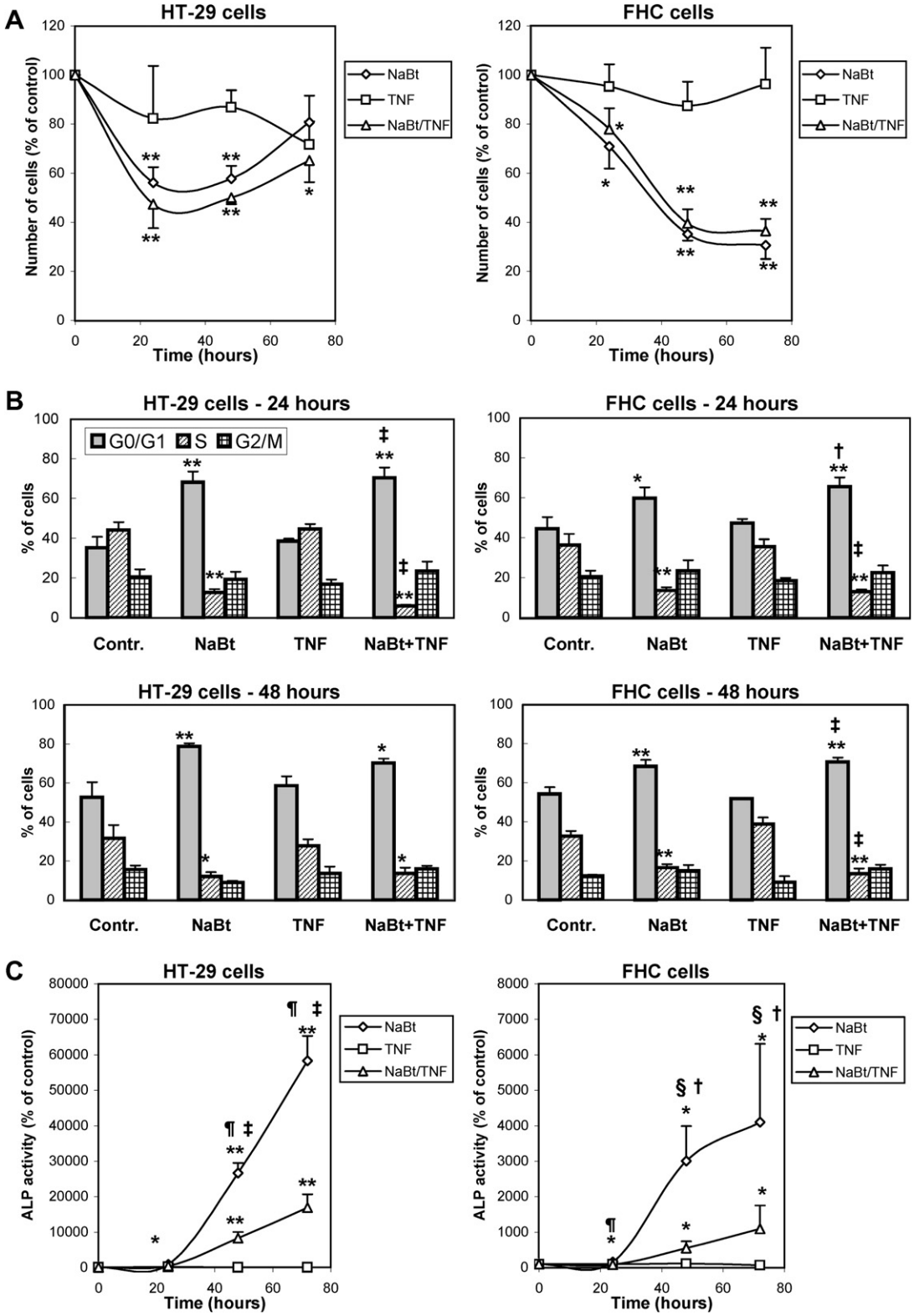

Fig. 1. Cell number (A), cell cycle analysis (B) and alkaline phosphatase (ALP) activity (C) in HT-29 and FHC cells treated for 24, 48, or 72 h with either NaBt (5 mM), TNF-α (15 ng/ml; TNF), or their combination. Data are presented as means ± SD from three independent experiments. $^{*}P < 0.05$ ($^{**}P < 0.01$) versus untreated controls considered as 100%; $^{\S}P < 0.05$ ($^{\P}P < 0.01$) versus NaBt alone; $^{\dagger}P < 0.05$ ($^{\ddagger}P < 0.01$) versus TNF-α alone.

29 and FHC cells compared to untreated control (Fig. 1C). However, the intensity of this effect varied depending on the cell type (at the maximum 583-fold after 72 h in HT-29 cells and 41-fold after 72 h in FHC cells). While TNF-α alone had no effect, it significantly suppressed the NaBt-induced ALP activity (approximately 3.2-fold in HT-29 cells and 5.5-fold in FHC cells after 48 h of TNF-α and NaBt co-treatment).

3.1.4. Cell death

In comparison to control, TNF-α did not significantly affect the mitochondrial enzymatic activity (measured by MTT assay), percentage of floating cells or the number of apoptotic cells (DAPI staining) in both cell lines used. The mitochondrial enzymatic activity of the both HT-29 and FHC cells was decreased by NaBt alone with the high effectiveness in cancer cells (approximately 40% decrease versus control). The effect was significantly elevated after 72 h of NaBt and TNF-α co-treatment in both cell lines (Fig. 2A). The percentage of floating cells was increased by NaBt alone in both cell lines, and the effects were most apparent after 48 and 72 h of treatment. The accumulation of floating cells was further significantly increased after co-treatment with NaBt and TNF-α in a longer cultivation time compared to both NaBt and TNF-α as single agents (Fig. 2B). While NaBt did not induce apoptosis in FHC cells, it increased the number of cells with morphologically detected apoptosis (up to 10%) of HT-29 cells (Fig. 2C). However, after 48 or 72 h of TNF-α and NaBt co-treatment, the level of apoptosis was significantly elevated in both cancer and normal cells in comparison with NaBt and TNF-α alone.

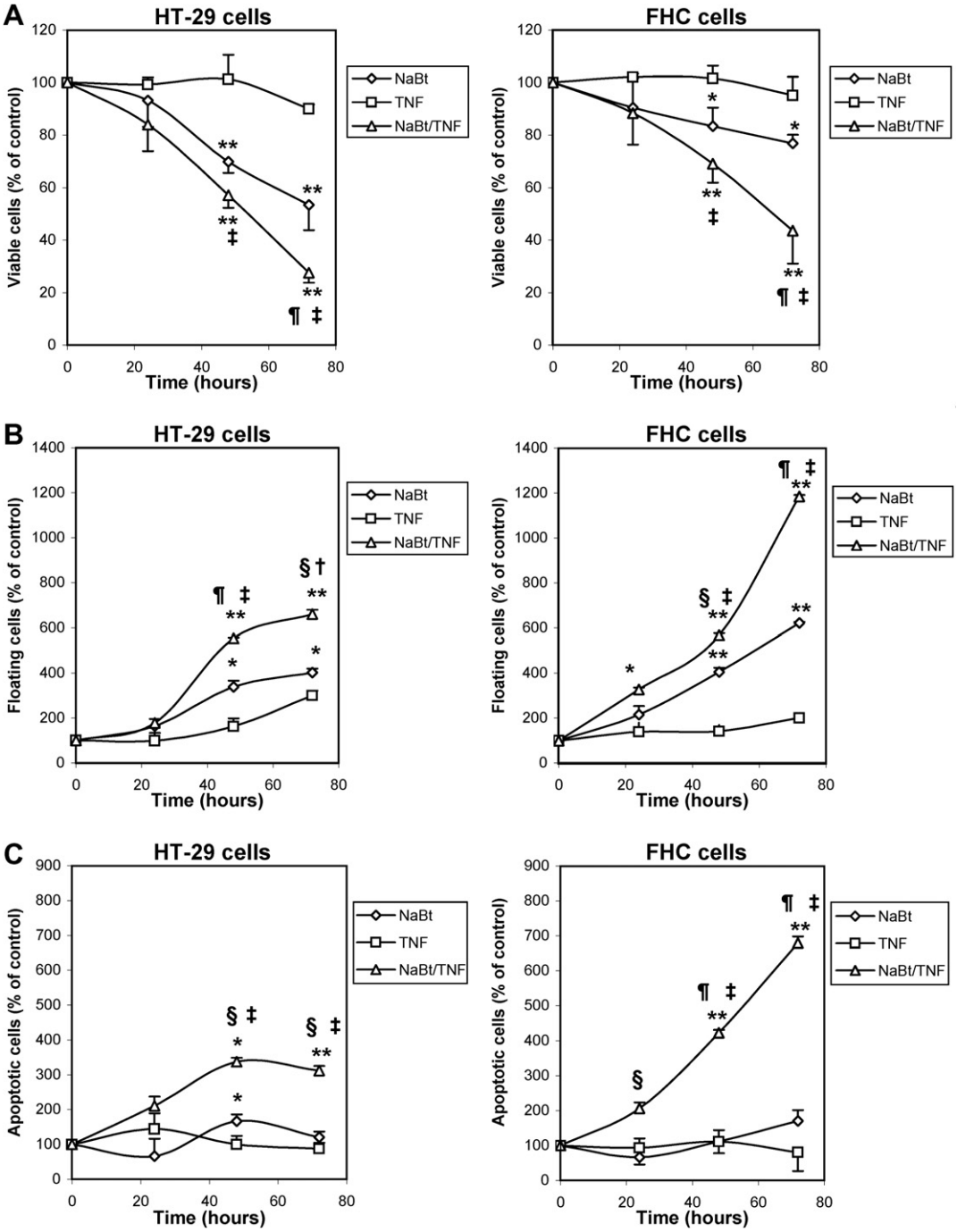

Fig. 2. Viable cell number measured as metabolic active cell percentage using MTT assay (A), floating cell number determined as percentage from whole cell population (B) and apoptotic cell number measured as percentage of cells with apoptotic morphology using DAPI staining and fluorescent microscopy (C) HT-29 and FHC cells treated for 24, 48, or 72 h with either NaBt (5 mM), TNF-α (15 ng/ml; TNF), or their combination. Data are presented as mean ± SD from three independent experiments. $^{*}P < 0.05$ ($^{**}P < 0.01$) versus untreated controls considered as 100%; $^{\S}P < 0.05$ ($^{\P}P < 0.01$) versus NaBt alone; $^{\dagger}P < 0.05$ ($^{\ddagger}P < 0.01$) versus TNF-α alone.

3.2. NF-κB activation in HT-29 and FHC cells during TNF-α and NaBt co-treatment

We investigated the role of NF-κB in the above-described effects comparing fetal FHC and cancer HT-29 cells. To study NF-κB activation, four different approaches were used. Firstly, we established HT-29 cell clones with a pBIIX-LUC reporter construct for NF-κB activity detection. TNF-α induced an increase in NF-κB activity in HT-29 cells compared with the untreated control, and this effect was time-dependent (approximately 41- or 27-fold increase after 4 h, 47- or 36-fold increase after 8 h, and 75- or 48-fold increase after 24 h treatment in clone 3 or clone 93, respectively; Fig. 3). Secondly, the time-dependent increase of TNF-α-induced NF-κB binding activity in HT-29 and FHC cells was also observed by electrophoretic mobility shift assay (EMSA; Fig. 4). Thirdly, translocation of p65 protein into the nucleus was confirmed in nuclear extracts of HT-29 and FHC cells using Western blotting (Fig. 5). Finally, expression of NF-κB inhibitory IκB-α protein was detected in whole HT-29 and FHC cell extracts using Western blotting. TNF-α induced a decrease of IκB-α expression after 4, 8, and 24 h in both cell lines (Fig. 6).

NaBt alone had no effect on NF-κB activity during a short-time interval, but after 24 h the elevation of the activity compared to control was apparent in both HT-29 and FHC cell lines. In HT-29 cells, this increased activity was detected by clones with the reporter construct (Fig. 3), p65 protein expression in nuclear extracts (Fig. 5), and by a decrease of IκB expression (Fig. 6). However, it did not appear in EMSA analysis (Fig. 4). On the other hand, in FHC cells the elevation of NF-κB activity mediated by NaBt 24 h treatment was observed by all methods used except for Western blot of p65 in nuclear extracts (Fig. 5).

After co-treatment with TNF-α and NaBt, NF-κB activity was differently modulated in cancer and fetal cells compared to the effect of TNF-α alone. While the NF-κB binding activity (Figs. 3 and 4), its nuclear translocation (Fig. 5), and IκB degradation (Fig. 6) were initially increased after 4 h of the TNF-α and NaBt co-treatment in comparison with TNF-α alone in HT-29 cells, these effects did not appear in FHC cells (Figs. 4–6). After 8 h of TNF-α and NaBt co-treatment, the activity (Figs. 3 and 4) and nuclear translocation (Fig. 5) of NF-κB and IκB degradation (Fig. 6) were similar with that of TNF-α alone treatment in both cell lines. Finally, these parameters were decreased after 24 h (TNF-α and NaBt co-treatment compared to treatment with TNF-α alone; Figs. 3–6).

3.3. Effects of NF-κB inhibition on HT-29 and FHC cell differentiation and apoptosis during TNF-α and NaBt co-treatment

In order to inhibit NF-κB binding activity, we searched for optimal dose and time of parthenolide pre-treatment of HT-29 and FHC cells. As indicated in Fig. 7, we used both EMSA (Fig. 7A and C) and Western blot of nuclear extracts for p65 expression (Fig. 7B and D) to test the effects of 1 or 3 h pre-treatment with parthenolide (20 or 10 μM) on inhibition of TNF-α-induced NF-κB activation. A significant decrease of NF-κB translocation to the nucleus was detected after 3 h of parthenolide pre-treatment in both cell lines used, but in FHC cells, the decrease was apparent only after higher

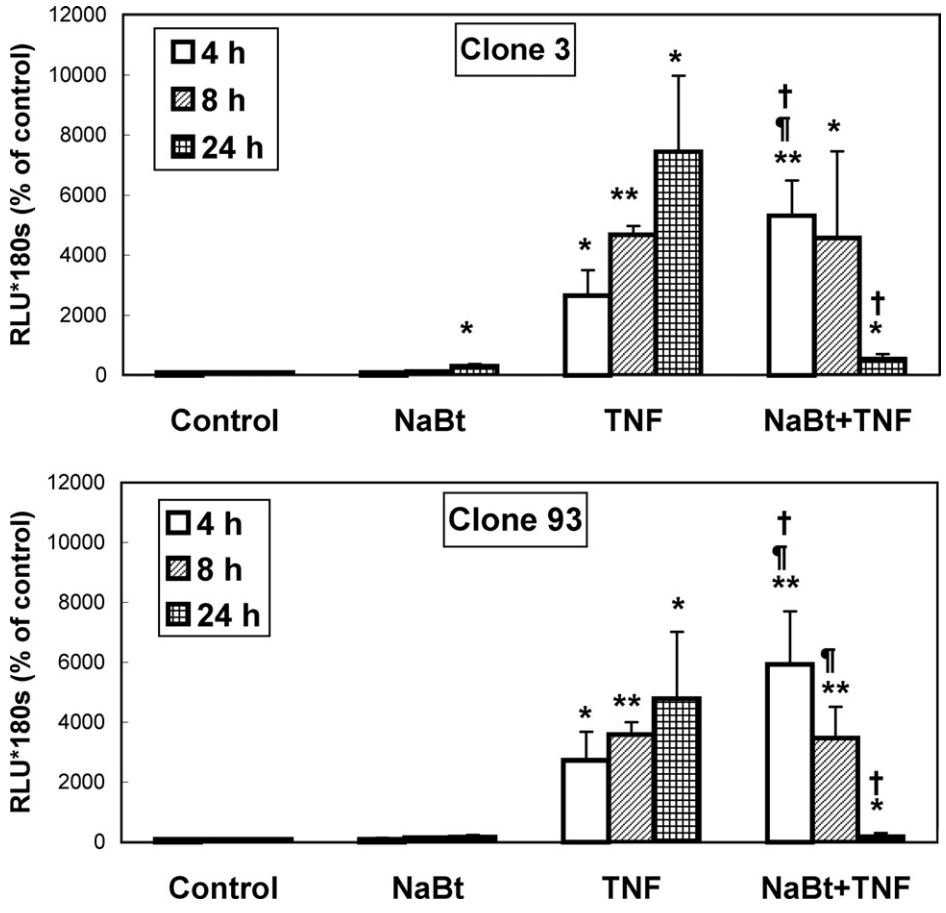

Fig. 3. NF-κB binding activity (expressed as relative luciferase activity) of two HT-29 clones (#3 and #93) with pBIIX-LUC construct after 4, 8, or 24 h treatment with either NaBt (5 mM), TNF-α (15 ng/ml; TNF), or their combination. Data are presented as means ± SD from three independent experiments. $^{*}P < 0.05$ ($^{**}P < 0.01$) versus untreated controls considered as 100%; $^{\S}P < 0.05$ ($^{\P}P < 0.01$) versus NaBt alone; $^{\dagger}P < 0.05$ versus TNF-α alone.

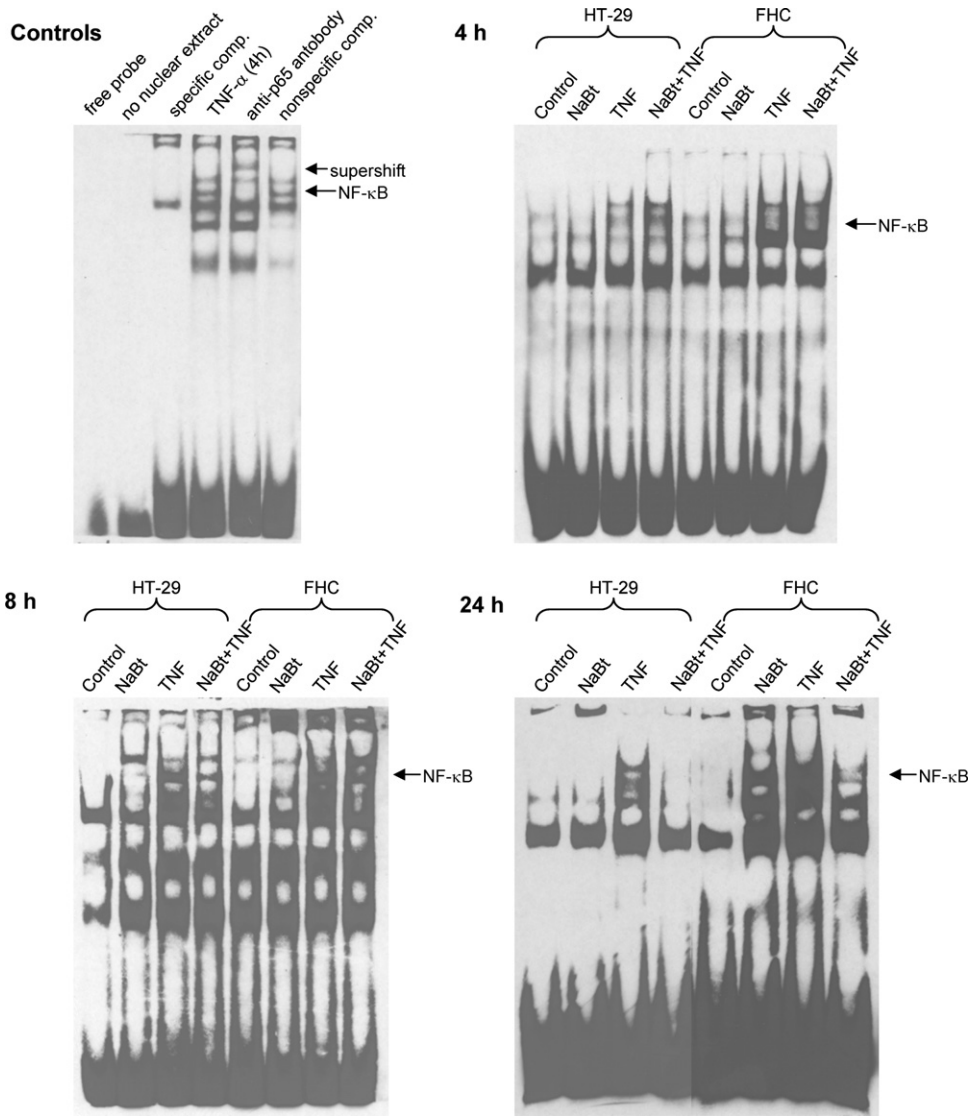

Fig. 4. NF-κB binding activity (using EMSA) of HT-29 and FHC cells after 4, 8, or 24 h of treatment with either NaBt (5 mM), TNF-α (15 ng/ml; TNF), or their combination; controls of shift specificity (free NF-κB probe labeled with biotin (lane 1), reaction mixture without nuclear extract (lane 2), specific competition with 100-molar excess of unlabeled NF-κB probe (lane 3), TNF-α (15 ng/ml) for 4 h (lane 4), TNF-α (15 ng/ml) for 4 h with anti-p65 antibody added to the reaction mixture (lane 5), and competition with 100-molar excess of unlabeled NF-κB non-specific oligonucleotides). Similar results were obtained in two other independent experiments.

concentration of parthenolide. Thus, we used a 3 h pre-treatment with 10 μM of parthenolide for HT-29 cells and 20 μM for FHC cells for further experiments. To exclude the effects of this NF-κB inhibitor on MAPK pathways, Western blot analysis for phosphorylated and total protein expression of ERK1/2 and p38 after 3 h of parthenolide pre-treatment (10 μM for HT-29 and 20 μM for FHC cells) and/or 10 min treatment with EGF (25 ng/ml), which was used as a positive control for MAPK pathway activation, was performed (Fig. 7E). No changes in ERK1/2 and p38 phosphorylation and no inhibition of EGF-mediated MAPK activation was detected after parthenolide pre-treatment.

Both HT-29 and FHC cells presented decreased sensitivity to NaBt-mediated inhibition of cell growth after parthenolide pre-treatment (Fig. 8A). However, cell growth was not significantly affected by TNF-α and NaBt co-treatments in both parthenolide pre-treated and non-pre-treated cells compared to NaBt alone.

Parthenolide increased apoptosis induced by NaBt (DAPI staining) in both HT-29 and FHC cells (Fig. 8B). However, apoptosis induced by TNF-α and NaBt co-treatment was modulated by parthenolide differently in HT-29 and FHC cells. While in HT-29 cells, parthenolide significantly potentiated apoptosis induced by this type of treatment (3.6-fold increase versus NaBt alone), no significant effect of was apparent in FHC cells (1.6-fold increase versus NaBt alone).

Unlike FHC cells, the differentiation ability of HT-29 cells induced by NaBt was strongly decreased after parthenolide pre-treatment (Fig. 8C). In FHC cells, the suppressive effect of TNF-α on NaBt-mediated cell differentiation (5.8-fold decrease of ALP activity versus NaBt alone) was attenuated after parthenolide pre-treatment (2.7-fold decrease of ALP activity versus NaBt alone).

4. Discussion

Colon epithelial cell kinetics may be affected by endogenous growth regulators or by dietary factors like TNF-α and butyrate, respectively. We demonstrated that TNF-α markedly reduced NaBt-induced differentiation in HT-29 colon adenocarcinoma cells contrary to its potentiation of apoptosis [26]. The ability of TNF-α to synergize with NaBt in promoting apoptosis in colon cancer cells

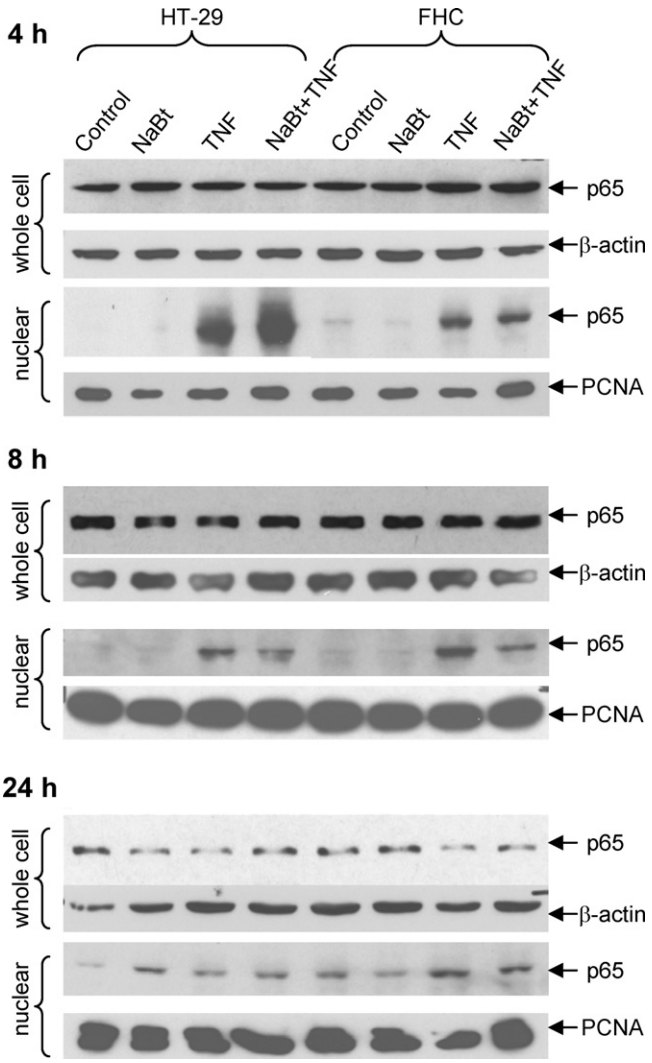

Fig. 5. NF-κB p65 protein expression in whole cell lysates or nuclear extracts of HT-29 and FHC cells treated for 4, 8, or 24 h with NaBt (5 mM), TNF-α (15 ng/ml; TNF), or their combination. Expression of β-actin or PCNA was used as equal loading control. Similar results were obtained in two other independent experiments.

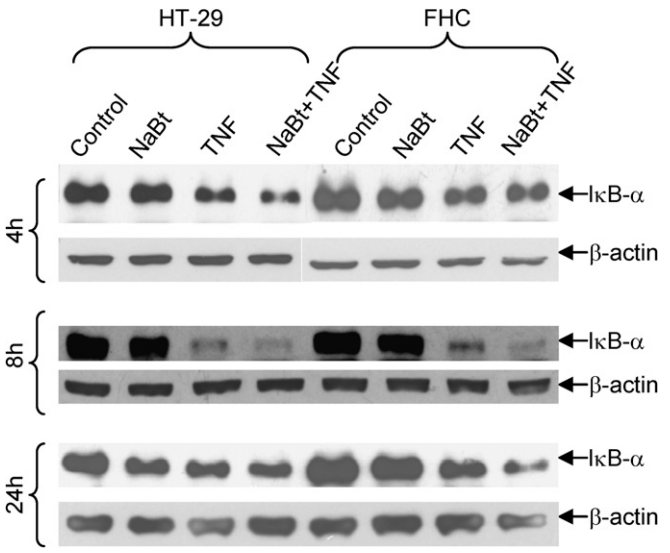

Fig. 6. IκB-α protein expression of HT-29 and FHC cells treated for 4, 8, or 24 h with NaBt (5 mM), TNF-α (15 ng/ml; TNF), or their combination. β-Actin expression was used as equal loading control. Similar results were obtained in two other independent experiments.

was shown also by other authors [27–29]. However, we newly demonstrate a TNF-α-mediated increase of apoptosis induced by NaBt in non-cancer cell line (FHC) derived from normal human fetal colon. We found a much more dramatic increase of NaBt-induced differentiation in cancer HT-29 than in fetal FHC cells. This different dynamic was already demonstrated by Fajkus et al. [30]. Interestingly, NaBt-mediated differentiation was significantly suppressed by TNF-α in both cell lines, with the higher effectiveness in FHC cells. Furthermore, our results suggest that induction of the apoptotic process by NaBt is reduced or delayed in fetal cells compared to cancer cells. On the other hand, the potentiation of apoptosis by TNF-α and NaBt co-treatment was observed in both cancer and fetal cells.

We suggested NF-κB to be involved in the mechanism of TNF-α-induced modulation of cell differentiation and apoptosis mediated by NaBt. The transcription factor NF-κB can play a pivotal role in regulating programmed cell death and can possess the ability to activate both pro-apoptotic and anti-apoptotic genes [31]. TNF-α belongs to the typical activators of the canonical pathway of NF-κB activation, which is completed by p50/p65 heterodimer binding to DNA. To elucidate the role of NF-κB activation during the investigated TNF-α and NaBt co-treatment, were compared cancer HT-29 and fetal FHC colon cell lines with regard to this parameter. We detected time-dependent TNF-α-induced NF-κB activation in HT-29 cells using a reporter constructs sensitive to NF-κB activity (pBIIX-LUC). Moreover, we indicated a strong activation of NF-κB by TNF-α not only in HT-29, but also in normal FHC cells (results of EMSA, p65 expression in nuclear extracts, and decreased level of IκB in whole cell extracts). From the report of Zwacka et al. it could be supposed that TNF-α-induced prolonged NF-κB activation is associated with TNF-α resistance [32]. Considering that both HT-29 and FHC cells are relatively resistant to TNF-α cytotoxicity this observation is in agreement with our results.

In accord with the results of Rouet-Benzineb et al. we demonstrated no changes in NF-κB binding activity after short-time treatment with NaBt (4 or 8 h) in both cell lines [33]. However, after 24 h of NaBt treatment, the NF-κB activity was enhanced in both cells of adenocarcinoma and fetal origin despite the previously published results that butyrate decreased the activity of this transcription factor [34]. It has been previously found that butyrate could alter NF-κB subunit composition induced by proinflammatory cytokines. Inan et al. demonstrated that NaBt pre-treatment of HT-29 cells inhibits the TNF-α-mediated p65 and p50 translocation to the nucleus [35]. These authors also suggested that NaBt prevented the NF-κB subunit translocation by suppressing cellular proteasome activity (and subsequently IκB degradation) through NaBt ability to inhibit histone deacetylases [24,36]. However, our results showed that NaBt increased TNF-α-mediated NF-κB binding activity, p65 nuclear translocation and IκB-α degradation in cancer HT-29 cells, but not in fetal FHC cells, after 4 h of NaBt and TNF-α co-treatment. The difference in NaBt ability to modulate TNF-α-mediated NF-κB activation between cancer and fetal colon epithelial cells represents the most important finding of the present study. The NaBt-mediated increase of TNF-α-induced NF-κB activity in HT-29 cells disappeared during the subsequent 4 h of treatment, and after 24 h NaBt significantly inhibited the TNF-α-induced NF-κB binding activity in both cell lines. The inhibition of TNF-α-induced p65 nuclear translocation by NaBt pre-treatment was also indicated in SW480 and SW620 human colon carcinoma cells [37,29]. While the NaBt concentration around 5 mM, which corresponds to the physiological concentration in the colon, was the same in the above-mentioned and our experiments, based on our previous works [38,39], we used significantly lower doses of

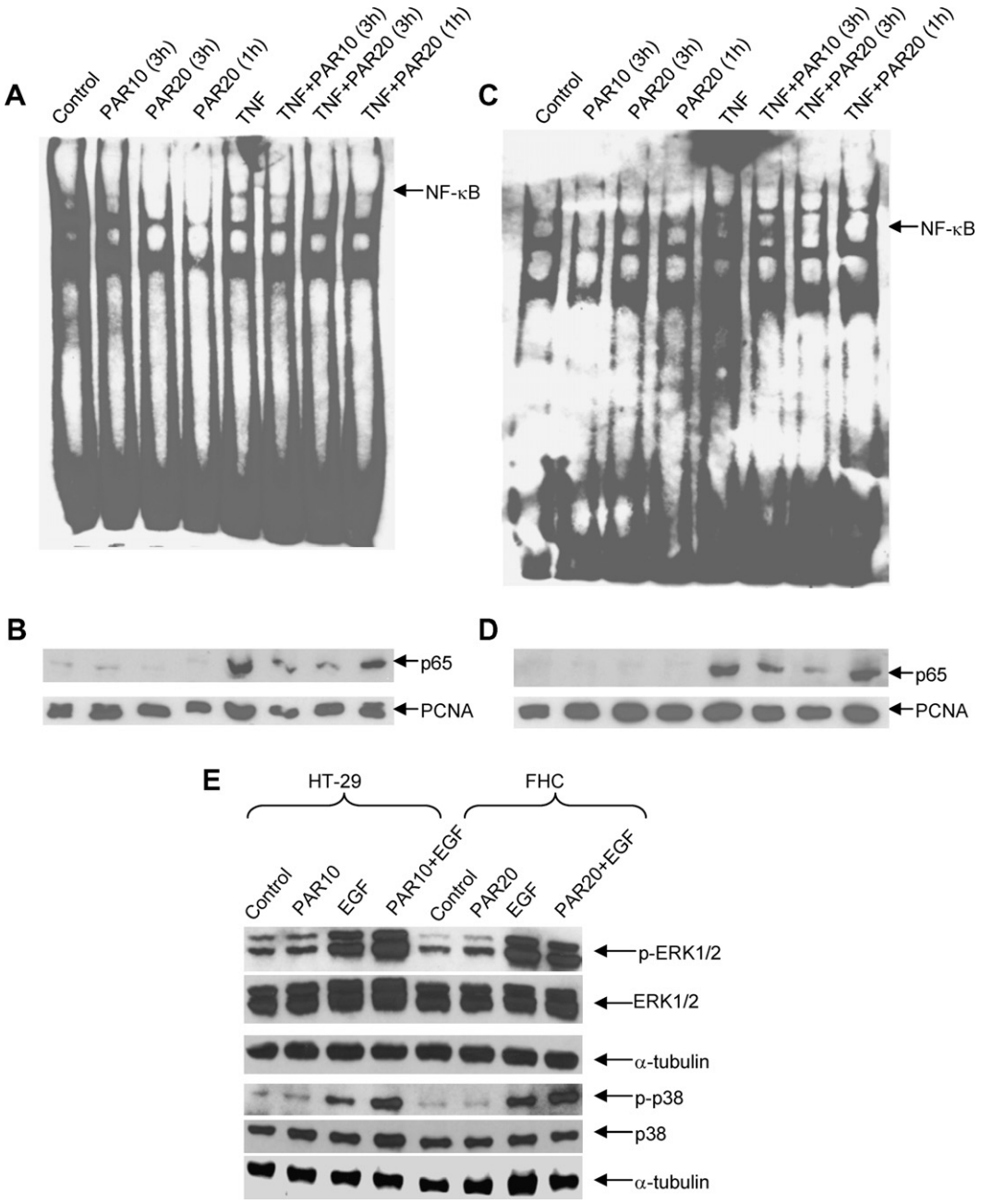

Fig. 7. NF-κB binding activity (using EMSA) (A and C) and NF-κB p65 protein expression in nuclear extracts (B and D) of HT-29 (A and B) and FHC (C and D) cells after 1 or 3 h pre-treatment with parthenolide (10 or 20 μM; PAR), 4 h treatment with TNF-α (15 ng/ml; TNF), or both (TNF + PAR). Phospho and total ERK1/2 and p38 protein expression (E) in whole cell extracts of HT-29 and FHC cells after 3 h pre-treatment with parthenolide (10 or 20 μM; PAR), 10 min treatment with EGF (25 ng/ml) or both (PAR + EGF). PCNA and α-tubulin expressions were used as equal loading control. Similar results were obtained in another independent experiment.

TNF-α (15 ng/ml versus 100 ng/ml) to simulate a bit more the real level of TNF-α in the colon during chronic inflammatory diseases (e.g. Crohn's disease) [6,11]. The difference in the concentrations of TNF-α used and also application of this cytokine simultaneously with NaBt (in contrast to NaBt pre-treatment used by the above-mentioned authors) may play a role in the dissimilarity of our results.

To study the involvement of NF-κB in the modulation of NaBt effects by TNF-α, we used an NF-κB inhibitor, sesquiterpene lactone parthenolide. It prevents the NIK- and MEKK1-induced activation of IKKs, but does not interfere with the induced activation of MAPKs [40]. We found a significant difference in parthenolide sensitivity between fetal and cancer cells, which may be corresponded to the distinct way of NF-κB activation in these two cell lines. Moreover, in cancer cell line, pre-treatment with parthenolide caused a significant increase of apoptosis induction by NaBt and TNF-α co-treatment, while, in fetal cells, it inhibited apoptosis induced by this type of treatment. This distinct response could be associated with lower differentiation status in NaBt- or both NaBt and TNF-α-treated HT-29 cells after parthenolide pre-treatment compared with FHC cells.

Our results indicated that NaBt-mediated differentiation and apoptosis of colon epithelial cells can be modulated by TNF-α. Furthermore, we found significant differences in the mechanism of the response to NaBt and TNF-α co-treatment between cells of non-cancer and adenocarcinoma origin, suggesting that the NF-κB pathway is more effectively involved in these processes in cancer cells. Our findings imply the possible role of TNF-α in the develop-

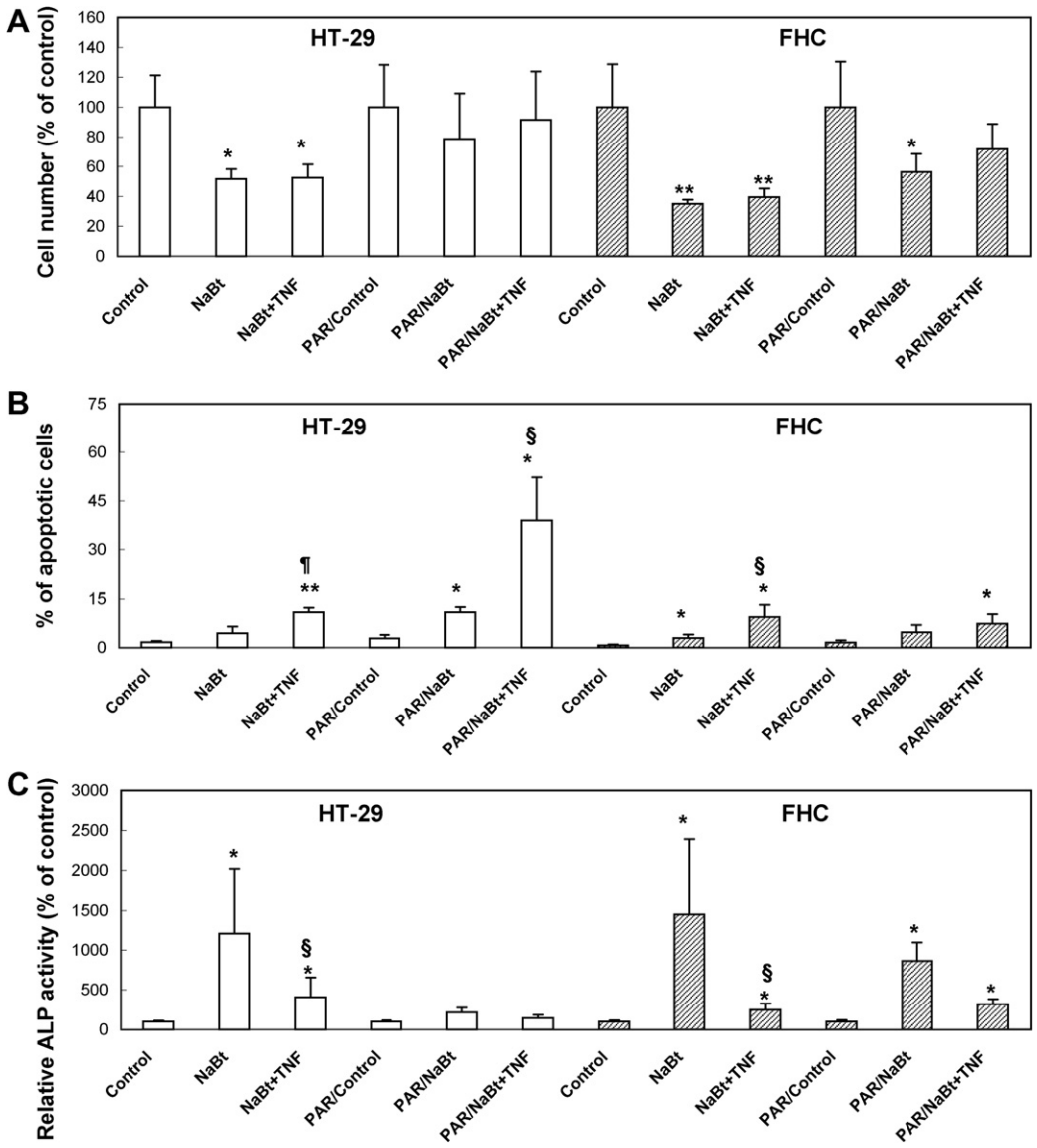

Fig. 8. Total cell number (A), number of apoptotic cells (B), and relative ALP activity (C) of HT-29 and FHC cells treated for 48 h with NaBt (5 mM) or its combination with TNF-α (15 ng/ml; TNF), with or without parthenolide pre-treatment (3 h, 10 (HT-29) or 20 (FHC) μM; PAR). Data are presented as mean ± SD from three independent experiments. $^{*}P < 0.05$ ($^{**}P < 0.01$) versus relevant untreated controls with or without PAR pre-treatment considered as 100% (A and C); $^{\S}P < 0.05$ versus NaBt alone or NaBt after PAR pre-treatment, respectively.

ment of adenoma or carcinoma during prolonged chronic inflammation of the colon via suppression of the differentiation and potentiation of apoptosis induced by butyrate in colon epithelial cells.

Acknowledgments

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