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ORIGINAL ARTICLE

Rena Feinman · Kevin O. Clarke · Lawrence E. Harrison

Phenylbutyrate-induced apoptosis is associated with inactivation of NF-κB IN HT-29 colon cancer cells

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Abstract *Purpose*: Cytotoxic chemotherapy has been used to treat patients with metastatic colorectal cancer with limited success. Therefore novel chemotherapeutic approaches are needed. Based on encouraging preclinical data, there has been an interest in developing derivatives of butyrate as clinically applicable agents. The purpose of this study was to investigate the effects of phenylbutyrate (PB), a butyrate analogue, on the cell growth and apoptosis in a colon cancer cell model. *Methods*: Growth curves, flow cytometric studies, Western blotting, DNA binding assays and transient transfection experiments were performed in vitro using the colon cancer cell line HT-29 after exposure to PB. *Results*: Exposure of HT-29 colon cancer cells to PB resulted in growth inhibition and induction of apoptosis as measured by annexin V staining. This increase in apoptosis was associated with a decrease in mitochondrial membrane potential, an increase in caspase-3 activity and a decrease in intact PARP protein levels. Since NF-κB plays a pivotal role in the regulation of apoptosis, we explored the effects of PB on the DNA binding and transcriptional activity of this transcription factor. After PB treatment, NF-κB-DNA binding was markedly decreased and specifically, this decreased DNA binding was observed in the p50:p65 heterodimer. The decreased NF-κB DNA binding was observed as early as 3 h after PB treatment, while no apparent changes in annexin V binding were detected until 12 h after PB treatment. Untreated HT-29 cells transfected with a κB-luciferase reporter plasmid demonstrated significant constitutive activity of the κB binding site, which was markedly decreased after treating the cells with PB. *Conclusion*: These results suggest that PB-induced apoptosis may be partly regulated through the inactivation of NF-κB. PB, an oral butyrate analogue, may have therapeutic potential in colon cancer.

Keywords Phenylbutyrate · Apoptosis · Caspase-3 · NF-κB

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R. Feinman · K.O. Clarke
Division of Surgical Oncology,
UMDNJ-New Jersey Medical School,
185 South Orange Avenue, Newark,
New Jersey 07103, USA

L.E. Harrison (✉)
Department of Surgery, Division of Surgical Oncology,
UMDNJ-New Jersey Medical School,
185 South Orange Avenue, MSB G588,
Newark, New Jersey 07103, USA
E-mail: L.Harrison@UMDNJ.edu
Tel.: +1-973-972-5583
Fax: +1-973-972-6803

Introduction

Epidemiological and experimental studies suggest that dietary fiber is protective against the development of colon carcinoma and recently the salutary effects of dietary fiber have been attributed in part to the production of short-chain fatty acids (SCFA) [1]. SCFAs are natural constituents of the colonic lumen produced during anaerobic fermentation of dietary fiber by endogenous intestinal bacteria [2]. Butyrate has been identified as one such SCFA and has been reported to inhibit proliferation and stimulate differentiation in multiple cancer cell lines [3, 4, 5]. Specifically, exposure of colon carcinoma cells to butyrate results in growth arrest and cellular differentiation, and increased colonic butyrate levels have been shown to correlate with reduced colon cancer cell proliferation in a rodent model [6].

While it has been known for years that butyrate is an effective differentiation agent in vitro, clinical trials evaluating sodium butyrate as a therapeutic agent for malignancy have been disappointing. The lack of a clinical response is believed to be related to butyrate's rapid metabolism and very short plasma half-life (6 min), leading to the inability to achieve adequate serum concentrations (1–5 m*M*) [7]. Based on encouraging

preclinical data, there has been an interest in developing derivatives of butyrate as clinically applicable differentiating agents [8].

Phenylbutyrate (PB) is an analogue of butyrate, which is closely related to the aromatic fatty acid, phenylacetate. PB has been suggested as a more logical compound clinically, since it is an oral agent that can achieve millimolar concentrations in humans [9]. PB is an effective differentiation agent in multiple cell types, including malignant gliomas, prostate cancer, and melanoma [10, 11, 12, 13] and recent a report suggests that PB appears to be a promising therapeutic option for patients with leukemia [12]. Although the biochemical basis of PB's antitumor activity is not well established, there is evidence that changes in lipid metabolism and cell cycle regulation are important mechanisms [14, 15]. While PB has been shown to induce apoptosis in a variety of cancer cell lines [13, 16, 17], the mechanism of this observed programmed cell death has not been described.

Studies have implicated the transcription factor NF-κB as a critical regulator of apoptosis and NF-κB activation can either promote or inhibit apoptosis, depending on cell type and conditions [18]. NF-κB is a family of several structurally related proteins, including p50, p52, p65 (RelA), and RelB, that form homo- and heterodimers. As dimers, NF-κB proteins bind to a set of DNA target sites, collectively called κB sites, and directly regulate expression of genes involved in cell cycle regulation and apoptosis. Endogenous cytoplasmic inhibitors, known as IκBs, tightly regulate NF-κB activity by complexing with the transcription factor and trapping it in the cytoplasm. The most characterized IκB is IκBα, which avidly binds p65. Upon phosphorylation of IκB's serine residues, p65 is released allowing nuclear transmigration. The phosphorylated IκB subsequently undergoes ubiquitination and subsequent degradation [19].

The purpose of this study was to investigate the pathway of PB-induced apoptosis and PB's effect on NF-κB activity in a colon cancer cell system. We report that exposure of HT-29 colon cancer cells to PB resulted in a time- and dose-dependent growth inhibition and apoptosis. This increase in apoptosis was associated with a decrease in mitochondrial membrane potential and an increase in caspase-3 activity. Decreased p65-p50 heterodimer DNA binding was observed as early as 3 h after PB treatment, while no apparent changes in annexin V binding were detected until 12 h after PB treatment. These results suggest that PB-induced apoptosis may be regulated in part through inactivation of NF-κB.

Materials and methods

Materials and cell culture

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.). PB was a kind gift from Dr. Hokan Cederberg (Triple Crown America, Philadelphia, Pa.). HT-29 cells were maintained in McCoy's supplemented with 10% complement-inactivated bovine calf serum.

MTT assay

Cell mass was determined by a standard MTT assay. Specifically, 3×10^3 cells were plated in 96-well plates and treatment with PB was initiated 24 h afterwards. At the completion of treatment, MTT (0.5 mg/ml) was added to each well and cells were incubated for 3 h at 37°C in an atmosphere containing 5% CO_2. Formazan crystals were dissolved with DMSO and the color intensity was measured using an ELISA reader at 570 nm (reference filter 690 nm).

Flow cytometry

For annexin V staining, FITC-conjugated annexin and propidium iodide were added to 3×10^6 cells and the fluorescence intensity was determined using a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.) and analyzed by CellQuest software (Becton Dickinson). Mitochondrial membrane potential ($\Delta\psi_{mt}$) was determined by flow cytometry using the dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimadazolcarbocyanine iodide; Molecular Probes, Eugene, Ore.). Briefly, cells were harvested, washed with PBS, and incubated with 10 μM JC-1 at 37°C for 15 min. Fluorescence intensity was determined using a FACScan flow cytometer (Becton Dickinson) and analyzed by CellQuest software (Becton Dickinson).

Whole-cell, nuclear and cytoplasmic extracts

Whole-cell extracts were prepared by lysing cell pellets with a Dounce microtip homogenizer in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% NP40, 50 mM NaF, 0.2 mM $NaVO_4$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 25 μg/ml pepstatin A). For nuclear extracts, cells were harvested, washed with PBS and resuspended in cytoplasmic extraction buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT) for 15 min. After Dounce homogenization, the cytoplasmic fraction was separated by centrifugation (14,000 g for 5 min at 4°C). The pellet was resuspended in nuclear extraction buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT) for 30 min on ice and centrifuged (14,000 g for 10 min at 4°C).

Western blot analysis

Samples for immunoblotting were prepared by mixing aliquots of the protein extracts with 3× SDS sample buffer (150 mM Tris, pH 6.8, 30% glycerol, 3% SDS, bromophenol blue dye 1.5 μg/100 ml, 100 mM DTT) and denatured by heating to 100°C for 4 min. Protein samples were then separated by SDS-PAGE, electrotransferred to a nitrocellulose membrane (Amersham, Arlington Heights, Ill.). The membrane was subjected to immunoblot analysis and proteins were visualized by the enhanced chemiluminescence method (Amersham).

Caspase-3 activity

Caspase-3 activity was assayed by cleavage of DEVD-pNA using a commercially available kit (Clontech, Palo Alto, Calif.). Briefly, cells were treated in the presence or absence of PB, and then 2×10^6 cells were incubated with DEVD-pNA in the presence of DTT for 60 min at 37°C. Color intensity was measured using an ELISA reader at 405 nm.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSA) were performed as follows. A reaction mixture of binding buffer (50 m*M* KCl, 20 m*M* HEPES-KOH, pH 7.5, 10 m*M* $MgCl_2$, 10% glycerol, 0.5 m*M* DTT, 1% NP40), 0.5 ng of ^{32}P-labeled oligonucleotide probe, 10 μg sonicated salmon sperm DNA, 2 μg poly(di-DC) (Pharmacia Biotech, Piscataway, N.J.) and 10 μg nuclear protein were incubated at 25°C for 10 min and the reaction products separated on a 4% polyacrylamide gel in 0.25× TBE (22.5 m*M* Tris-borate and 0.5 m*M* EDTA). For antibody perturbation experiments, 2.5 μg antibody was added 10 min prior to the addition of the oligonucleotide probe followed by incubation at 25°C. For oligonucleotide competition experiments, a 50-fold excess of unlabeled competitor oligonucleotide was added 10 min prior to the addition of the oligonucleotide probe followed by incubation at 25°C. The double-stranded DNA oligonucleotide for NF-κB is commercially available from Santa Cruz Biotechnology and contains the consensus binding site for NF-κB (5′-TCAAATGTGGGATTTTCCCATGAG-3′).

Transfections and luciferase assay

The thymidine kinase-driven luciferase (tk-luc) reporter plasmids with or without three copies of the consensus κB site were kindly provided by J. Cleveland (St Judes Children's Hospital, Memphis, Tenn.). The CMV-p65 and CMV-p50 plasmids were kindly provided by A. Baldwin (University of North Carolina). The IκBα dominant negative vector was purchased from Clontech (Palo Alto, Calif.). For transient transfections, HT-29 cells were grown in six-well plates to 60–80% confluency. To each well was added 1 μg plasmid with 4 μl Lipofectamine 2000 (Life Technologies, Rockville, Md.) under serum-free conditions and after a 5-h incubation, complete medium was added. After transfection, cells were allowed to recover for 12 h and were then treated with PB (3 m*M*) for an additional 18 h. Cells were harvested, lysed and assayed for luciferase activity (Luciferase Assay System, Promega, Madison, Wis.). Luciferase activity was normalized according to protein content.

Results

PB induces apoptosis through activating the caspase-3 pathway

Initial experiments were designed to determine the growth-inhibitory activity of PB in a colon cancer cell model. HT-29 cells were not exposed to PB (control) or treated with 0.5, 1, 3 and 5 m*M* PB for 24, 48 and 72 h and viable cell mass was measured by the MTT assay. Exposure of PB to HT-29 colon cancer cells resulted in a significant decrease in viable cells in a time- and dose-dependent fashion (Fig. 1A). This decrease in cell mass was associated with cellular phenotypic changes, including a decrease in the cytoplasmic to nuclear ratio (Fig. 1B).

To determine whether an increase in apoptosis was associated with the observed decrease in cell number after PB treatment, HT-29 cells were not exposed to PB (control) or treated with 0.5, 1, 3 and 5 m*M* PB for 48 h or with 3 m*M* PB for 3, 6, 12, 24, 48 and 72 h, and annexin V staining was performed (Fig. 2A). Cells staining positive for annexin only (Fig. 2A, lower right panel) represent early apoptosis, while those cells staining positive for both annexin and propidium iodide represent cells in late apoptosis (upper right panel). Exposure to PB resulted in an increase in the number of apoptotic cells in a time-dependent fashion. While no significant increase in annexin V binding was identified by 3 h (8.3%) or 6 h (4.3%) of treatment as compared to control cells (9.8%), a significant increase in apoptosis was detected after 12 h exposure (21.7%), which further

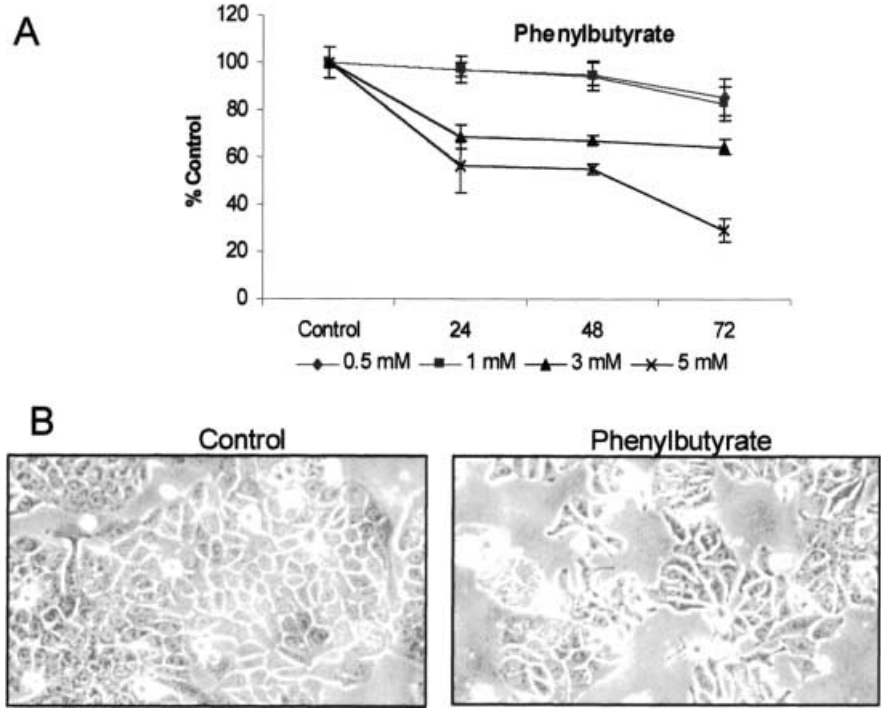


Fig. 1A, B PB induces growth inhibition in HT-29 colon cancer cells. **A** HT-29 cells were treated with PB (0, 0.5, 1, 3, 5 m*M*) for 24, 48 and 72 h and cell viability was measured by the MTT assay. **B** The phenotypic changes following PB treatment (3 m*M* for 48 h) include a decrease in the cytoplasmic to nuclear ratio as observed by light microscopy

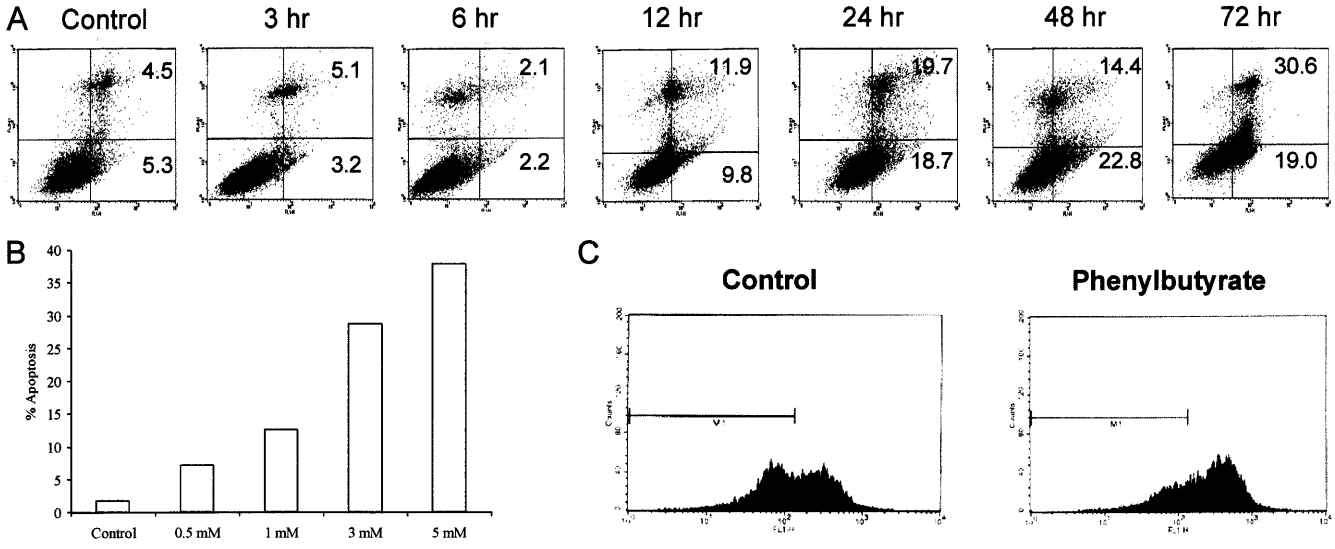


Fig. 2A–C Exposure of HT-29 cells to PB induces apoptosis. **A** HT-29 cells were treated with PB (3 m*M*) for 3, 6, 12, 24, 48 and 72 h and apoptosis was determined by annexin V binding. Cells staining positive for annexin only (lower right panel) represent early apoptosis, while those cells staining positive for both annexin and propidium iodide (PI) represent cells in late apoptosis. **B** HT-29 cells treated for 48 h with increasing concentrations of PB showed a dose-dependent increase in the number of apoptotic cells as measured by annexin V staining. **C** Mitochondrial membrane potential ($\Delta\psi_{mt}$) was measured in HT-29 cells treated with PB (3 m*M* for 48 h). At high $\Delta\psi_{mt}$, the JC-1 dye forms J-aggregates, which emit at 590 nm (orange range of visible light). However, at low $\Delta\psi_{mt}$, JC-1 exists as a monomer, emitting at 527 nm (green range). A marked increase in the cell population emitting at 527 nm (low $\Delta\psi_{mt}$) occurs after exposure to PB

increased to 49.6% after 72 h of PB treatment (Fig. 2A). Similarly, a dose-dependent increase in the number of apoptotic cells was observed in cells treated for 48 h (Fig. 2B). Similar growth-inhibitory and proapoptotic effects of PB were also been observed in the colon cancer cell line Caco-2 (data not shown).

Since the dissipation of $\Delta\psi_{mt}$ has been linked to the initiation of some apoptotic cascades, $\Delta\psi_{mt}$ was measured in HT-29 cells treated with PB (3 m*M* for 48 h). At high $\Delta\psi_{mt}$, the JC-1 dye forms J-aggregates, which emit at 590 nm (orange range of visible light). However, at low $\Delta\psi_{mt}$, JC-1 exists as a monomer, emitting at 527 nm (green range). Figure 2C demonstrates an increase in the cell populations emitting at 527 nm (low $\Delta\psi_{mt}$) after exposure to PB, suggesting that PB decreases mitochondrial membrane potential.

To investigate the apoptotic cascade initiated by PB, HT-29 cells were exposed to PB (3 m*M* for 24 and 48 h) and caspase-3 protein and activity levels were measured. PB exposure resulted in a decrease in the 32 kDa inactive precursor of caspase-3, with a concomitant increase in the 21 kDa active form. The increase in the active form of caspase-3 protein levels was associated with a significant increase in caspase-3 activity. Lysates equalized for protein from cells treated with PB (3 m*M*) for 24 and 48 h were assayed for in vitro caspase-3 activity using DEVD-pNA as a substrate. PB exposure significantly increased caspase-3 activity, which could be partially reversed by the addition of the caspase-3 inhibitor, Ac-DEVD-CHO. Associated with the increase in caspase-3 activity after PB exposure, protein analysis of PARP, a target of caspase-3, demonstrated a loss of intensity of its 112 kDa band after 24 and 48 h of exposure, suggesting cleavage by caspase-3 (Fig. 3A, B).

Loss of NF-κB DNA binding is an early event in PB-induced apoptosis

Since PB treatment induced apoptosis in HT-29 colon cancer cells and NF-κB is an important regulator of programmed cell death, we next investigated the effects of PB on NF-κB DNA binding. HT-29 cells were treated in the presence or absence of PB (3 m*M*) for 48 h. Cells were harvested, nuclear extracts prepared and EMSA performed using an oligonucleotide containing the consensus binding site for κB. In untreated cells, two major complexes were identified and based on supershift experiments. The faster migrating band was determined to represent the p50:p50 homodimer and the slower migrating band, the p65:p50 heterodimer. Treatment with PB resulted in a decrease in the p65:p50 heterodimer DNA binding with a modest increase of the p50:p50 homodimer (Fig. 4A). To determine whether the inhibition of p65:p50 binding after PB exposure was a primary event leading to apoptosis, or a late event secondary to apoptotic cleavage, a time course investigation was performed. As compared to untreated controls, treatment with PB inhibited NF-κB DNA binding as early as 3 h after the start of exposure, which persisted throughout the time course (Fig. 4B).

To establish whether the decrease in NF-κB DNA binding correlated with in vivo κB transcriptional activity, HT-29 cells were transiently transfected with a κB-luciferase reporter plasmid. After 18 h, cells were treated or not treated with PB (3 m*M*) for and

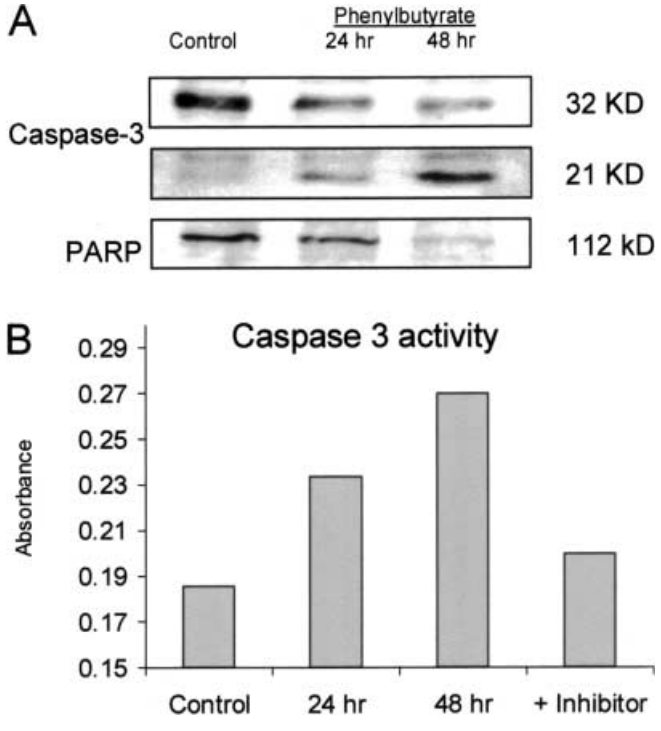


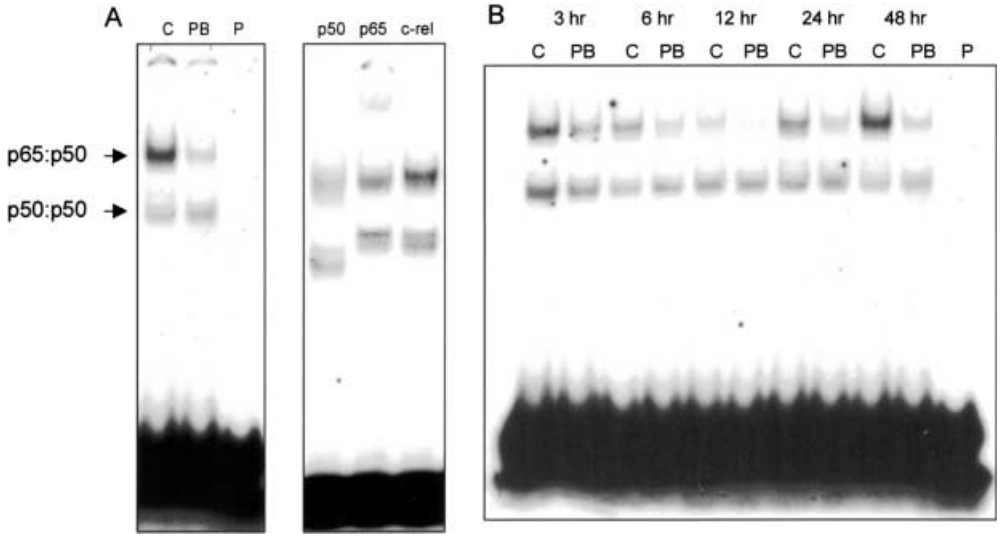
Fig. 3A, B PB exposure increases caspase-3 activity. **A** PB exposure (3 m*M*) resulted in a decrease in the 32 kDa inactive precursor of caspase-3, with a concomitant increase in the 21 kDa active form. Associated with the increase in caspase-3 activity after PB exposure, protein analysis of PARP, a target of caspase-3, demonstrated a loss of intensity of its 112 kDa band after 24 and 48 h exposure, suggesting cleavage by caspase-3. **B** Whole-cell lysates from cells treated with PB (3 m*M*) for 24 and 48 h were assayed for in vitro caspase-3 activity using DEVD-pNA as a substrate. PB exposure significantly increased caspase-3 activity, which could be partially reversed by the addition of the caspase-3 inhibitor, Ac-DEVD-CHO

additional 24 h. Transfections with the control plasmid (tk-luc) demonstrated negligible luciferase activity in both control and PB-treated cells. Untreated HT-29 cells transfected with the complete κB-luc reporter demonstrated constitutive activity of the κB binding site, which was significantly decreased after treating the cells with PB (Fig. 5A), suggesting that PB decreases both p65:p50 DNA binding and transcriptional activity.

Based on the observation that PB exposure resulted in a significant decrease in p65:p50 DNA binding with an increase in the p50:p50 homodimer and since it has been suggested that the p65:p50 activates κB transcriptional activity while the p50:p50 homodimer is inhibitory, cotransfections with the κB-luc reporter plasmid and p50 and p65 expression vectors were performed. While HT-29 cells overexpressing p50 demonstrated a significant decrease in luciferase activity as compared to an empty vector control, cells overexpressing p65 demonstrated a significant increase in κB binding. This increase in NF-κB transcriptional activity after p65 overexpression was partially abrogated by cotransfection with the p50 vector, suggesting competition for the κB DNA binding site (Fig. 5B).

To provide additional evidence that NF-κB participates in PB-induced apoptosis, we transiently transfected HT-29 cells with an NF-κB dominant-negative plasmid. The NF-κB dominant-negative model utilizes a super-repressor IκBα constitutive repressor expression plasmid. The IκBα mutant contains mutations at residues 32 and 36 (the two inducible phosphorylation sites) which disrupt phosphorylation and subsequent protea-

Fig. 4A, B p65:p50 heterodimer DNA binding is decreased after PB exposure. **A** EMSA was performed using an oligonucleotide containing the consensus binding site for κB. Two major bands are identified as the p65:50 heterodimer (slower migrating band) and the p50:p50 homodimer (faster migrating band). The p65:p50 heterodimer DNA binding was decreased after PB exposure (3 m*M* for 48 h), with a modest increase in the p50:p50 homodimer-DNA binding. B Cells were treated or not treated with PB (3 m*M*) and harvested at 3, 6, 12, 24 and 48 h after treatment. Treatment with PB inhibited p50:p65 DNA binding as early as 3 h after the start of exposure, which persisted throughout the time course. A modest increase in the p50:p50 homodimer DNA binding was also detected after 6 h of PB exposure (*C* control, *PB* phenylbutyrate, *P* probe alone)



some-mediated degradation [20]. The super-repressor IκBα mutant is still capable of binding NF-κB and inhibiting DNA binding and κB transcriptional activity [21]. PB treatment (3 m*M* for 24 h) increased the number of apoptotic cells in non-transfected HT-29 cells (6.6% vs 15.3%) and empty vector transfectants (19.8% vs 26.0%). In comparison, annexin V staining of NF-κB dominant-negative cells treated with PB showed a dramatic increase in the number of apoptotic cells (22.9% vs 53.3%; Fig. 6).

Discussion

Each year approximately 150,000 new cases of colorectal cancer are diagnosed in the United States, making it the third leading cause of cancer death [22]. Approximately 30% of patients present with advanced tumors and an additional 45% of patients with regional lymph node disease will eventual develop distant metastases. Cytotoxic chemotherapy has been used to treat patients with metastatic colorectal cancer with limited success, providing only 15–25% response rates with few 5-year survivors. Therefore novel chemotherapeutic approaches are needed. One novel approach is the use of differentiation agents, which use existing cellular systems to induce the cell to regain a normal phenotype and/or to cause growth arrest with subsequent apoptosis. The aromatic fatty acid PB has been shown to be an effective differentiation agent and inducer of apoptosis in a variety of cancer cell types [13, 17, 23]. The mechanism of PB's proapoptotic affect, however, has not been well studied. This is the first study examining the mechanism of PB-induced apoptosis. We demonstrated in a colon cancer model that PB induces apoptosis through activation of caspsase-3 which appears to be regulated by inhibiting the NF-κB pathway.

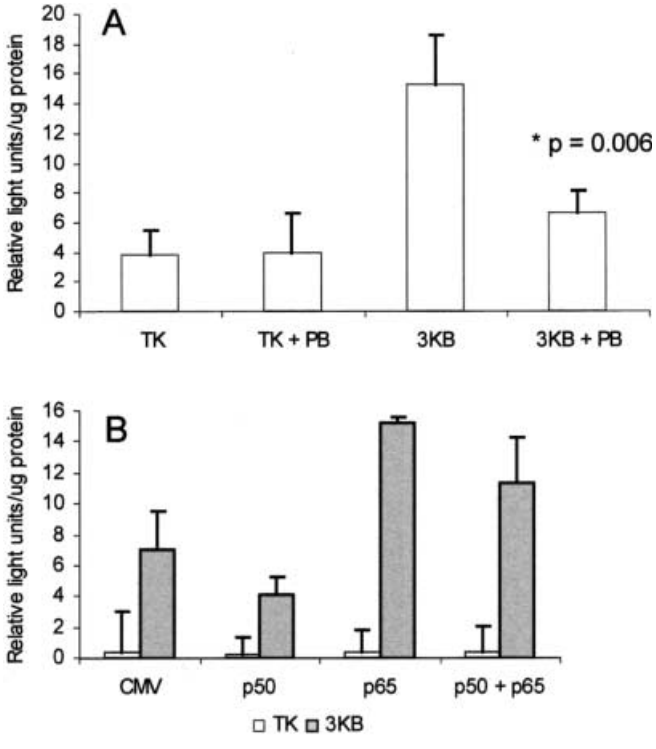


Fig. 5A, B PB inhibits κB-dependent transcriptional activity. **A** Transient transfections with a κB reporter gene were performed in the absence or presence of PB (3 m*M*). Transfections with a plasmid containing only the tk promoter without the κB sites demonstrated minimal luciferase activity, which was unaffected by exposure to PB (18 h). In contrast, untreated HT-29 cells transfected with the complete κB-luc reporter demonstrated significant constitutive activity of the κB binding site, which was significantly decreased after treating the cells with PB. **B** HT-29 cells were transiently transfected with the κB reporter gene and cotransfected with either p-CMV-50 or p-CMV-65. While overexpression of p65 increased κB-dependent transcriptional activity, p50 decreased constitutive activity

An early event in the apoptotic pathway is the dissipation of mitochondrial membrane potential ($\Delta\psi_{mt}$) with subsequent release of cytochrome *c* into the cytosol. Heerdt et al. have suggested that dissipation of $\Delta\psi_{mt}$ is an early event in the initiation of apoptosis in both colon and breast cancer cells treated with the fatty acids butyrate and tributyrin [24, 25]. Similar to the effects of these fatty acids, we demonstrated that PB-induced apoptosis was associated with a decrease in $\Delta\psi_{mt}$ (Fig. 2C). Once mitochondrial membrane potential is decreased and cytochrome *c* is released, the onset of apoptosis is associated with the proteolytic activation of caspases. Caspase-3, the main executioner of apoptosis, cleaves several important intracellular molecules, leading to the morphological and biochemical changes associated with apoptosis. We observed that the decrease in $\Delta\psi_{mt}$ after PB exposure was associated with the proteolytic activation of caspase-3 and cleavage of PARP, its downstream target (Fig. 3A). Activation of caspase-3 has also been observed with the fatty acid, butyrate [26]. These findings suggest that fatty acids, including PB, are proapoptotic agents and the pathway for programmed cell death is through activation of caspase-3.

While the downstream effector pathway of fatty acid-induced programmed cell death is mediated in part through caspase-3 activation, the proximal signals leading to the apoptotic cascade are undefined. The transcription factor NF-κB plays a pivotal role in the regulation of apoptosis by direct regulation of genes that inhibit or promote apoptosis, through regulation of the cell cycle, which sensitizes or desensitizes a cell to apoptotic signals and lastly, through interactions with other proteins involved in cell survival [27]. NF-κB is a family of several structurally related proteins (p50, p52, p65 and Rel B) that form dimers and subsequently bind to the κB DNA site. Transcriptional activity is dependent on both DNA binding as well as the constituents of the NF-κB dimer. In general, the p65 subunit activates κB transcriptional activity, while p50, which lacks the acidic transcriptional activation domain, is a weak activator and oftentimes acts as a repressor [28]. This is relevant, since constitutive expression of p65 has been

shown to be important in promoting survival and oncogenesis in a variety of cancers [29, 30, 31]. Supporting the role of p65 as an antiapoptotic factor includes the fact that NF-κB regulates the expression of multiple pro-survival Bcl-2 homologues [32] and that mouse p65 knockouts will die by day 10 of embryonic development and histological evaluation of these mice demonstrates massive hepatic apoptosis [33]. In addition, cells overexpressing p65 have been shown to be resistant to proapoptotic therapy [34]. Since p65 provides a survival advantage for these malignancies, it may provide a potential target for antitumor therapy.

We detected both p50:p50 and p65:p50 nuclear complexes in HT-29 colon cancer cells and demonstrate that PB treatment resulted in a decrease in the p65:p50 heterodimer DNA binding, with a concomitant increase in the p50:50 homodimer binding (Fig. 4). This would suggest that in our model, p65:p50 provides transcription of pro-survival genes and that PB, by inhibiting p65 binding and transcriptional activity, allows the cell to undergo apoptosis. This observed inhibition of NF-κB by PB may represent an upstream event leading to apoptosis or conversely, the result of apoptosis and caspase activation, since p50 and p65 are known proteolytic targets of activated caspase-3 [26]. Our findings suggest that the decreased NF-κB binding and transcriptional activity is an upstream event to caspase-3 activation since the decreased NF-κB DNA binding was observed as early as 3 h after PB treatment, while no apparent changes in annexin V binding were detected until 12 h after PB treatment. A similar early inhibition of NF-κB which precedes apoptosis in glucocorticoid-treated multiple myeloma cells has also been reported [35].

Decreases in NF-κB binding and transcriptional activity have also been reported with butyrate-treated HT-29 cells [36]. However, in contrast to the findings in this study, Inan et al. have reported that the decrease in NF-κB binding is manifested by a decrease binding of the p50:50 homodimer after butyrate exposure. They suggest that since butyrate is a histone deacetylase (HDAC) inhibitor, p50 binding may be regulated by acetylation. While, like butyrate, PB is a fatty acid and also a HDAC inhibitor (data not shown), they are different agents, which may provide different effects on the same cell line. This rationale may explain the disparate results observed regarding their effects on p50 and p65.

In summary, we demonstrated that PB-induced apoptosis of HT-29 colon cancer cells is preceded by the inhibition of NF-κB-DNA binding and transcriptional

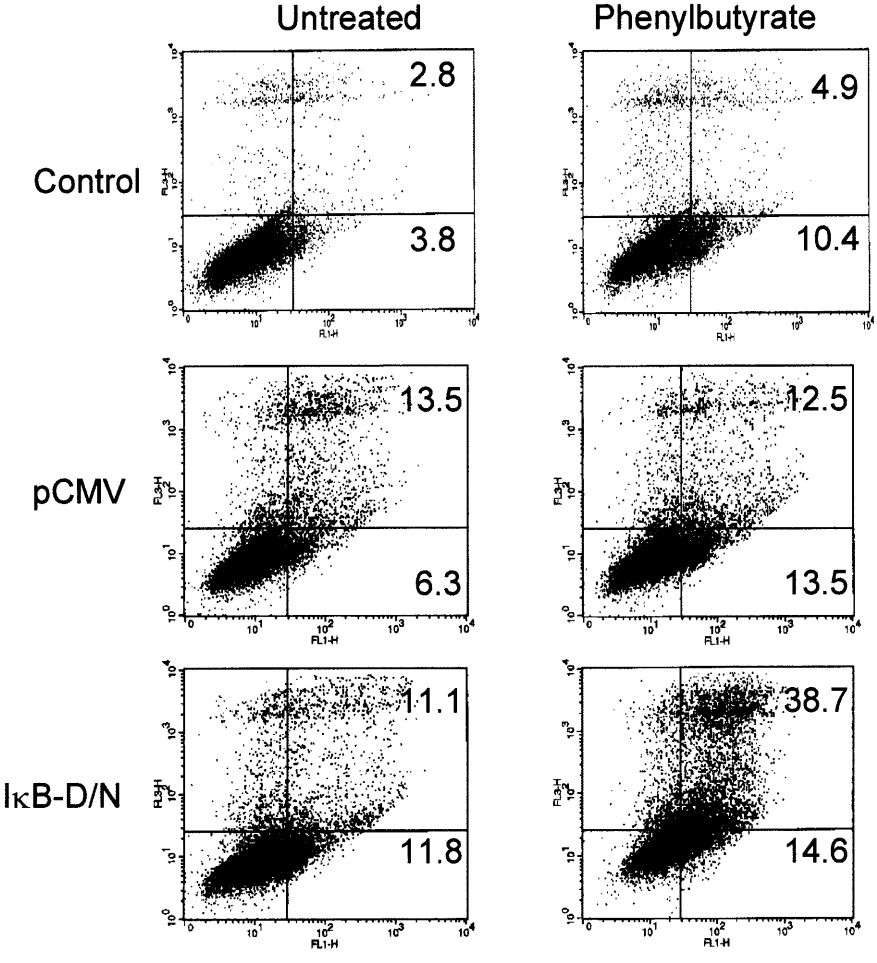


Fig. 6 PB-induced apoptosis is enhanced in a NF-κB dominant-negative model. Transient transfections with IκBα constitutive repressor expression plasmid were performed in the absence or presence of PB (3 m*M* for 24 h). After treatment, annexin V staining was performed. As compared to PB-treated empty vector control (pCMV) and untreated dominant-negative transfectants, NF-κB dominant-negative cells (IκB-D/N) treated with PB showed an increase in the number of apoptotic cells

activity, suggesting that this represents an early event in the apoptotic cascade. Despite some concerns about the ability to achieve and maintain millimolar serum concentrations, PB may have therapeutic potential in colon cancer.

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