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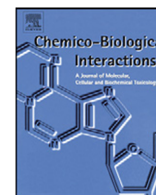

Butyrate induces cell apoptosis through activation of JNK MAP kinase pathway in human colon cancer RKO cells

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ABSTRACT

Butyrate has been shown to display anti-cancer activity through the induction of apoptosis in various cancer cells. However, the underlying mechanism involved in butyrate-induced apoptosis is still not fully understood. Here, we investigated the cytotoxicity mechanism of butyrate in human colon cancer RKO cells. The results showed that butyrate induced a strong growth inhibitory effect against RKO cells. Butyrate also effectively induced apoptosis in RKO cells, which was characterized by DNA fragmentation, nuclear staining of DAPI, and the activation of caspase-9 and caspase-3. The expression of anti-apoptotic protein Bcl-2 decreased, whereas the apoptotic protein Bax increased in a dose-dependent manner during butyrate-induced apoptosis. Moreover, treatment of RKO cells with butyrate induced a sustained activation of the phosphorylation of c-jun N-terminal kinase (JNK) in a dose- and time-dependent manner, and the pharmacological inhibition of JNK MAPK by SP600125 significantly abolished the butyrate-induced apoptosis in RKO cells. These results suggest that butyrate acts on RKO cells via the JNK but not the p38 pathway. Butyrate triggered the caspase apoptotic pathway, indicated by an enhanced Bax-to-Bcl-2 expression ratio and caspase cascade reaction, which was blocked by SP600125. Taken together, our data indicate that butyrate induces apoptosis through JNK MAPK activation in colon cancer RKO cells.
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1. Introduction

SCFAs (short-chain fatty acids) are end products of the physiological bacterial fermentation of alimentary fibers in the colonic lumen. It has long been established that SCFAs play a critical role in maintaining homeostatic cell turnover in the colonic epithelium [1]. Besides its role as a preferred energy source for normal colonic epithelial cells, butyrate was shown to play an important role in preventing the development of colon cancer [1–3]. Various mechanisms have been proposed to explain how butyrate inhibits tumorigenesis. The main mechanism responsible for this anti-tumoral effect of butyrate is induction of apoptosis [4–9]. Although butyrate is considered to be an anti-tumor agent, its particular effects on colon cancer cells and the mechanisms involved remain unknown.

Apoptosis is characterized by a number of well-defined features including cellular morphological change, chromatin condensation, DNA fragmentation, and activation of a family of cysteine proteases called caspases [10]. Caspase activation is generally considered to be a key hallmark of apoptosis. Activated caspase-9 activates downstream events by directly cleaving and activating pro-caspase-3, generating a fragment that activates the mitochondrial pathway [11]. Caspase activation is mediated by the Bcl-2 family of proteins, which includes both anti-apoptotic members, such as Bcl-2, Bcl-xL, and Bcl-w, and pro-apoptotic members, such as Bak, Bax, and Bad [12]. In the mitochondrial death pathway, the ratio of expression of the pro-apoptotic Bax protein and the anti-apoptotic Bcl-2 proteins ultimately determines cell death or survival [13,14].

Cellular behavior in response to extracellular stimuli is intervened by intracellular signaling pathways such as the mitogen-activated protein (MAP) kinase pathways. These pathways are critical targets in tumorigenesis that regulate cell proliferation, cell migration, including extracellular signal-regulated protein kinase 1/2 (ERK1/2), C-Jun N-terminal kinase (JNK) and p38 MAPK (p38) [15]. The ERK activation is associated with cell proliferation, differentiation, and cell survival. High level activation of ERK MAPK signaling exists in various colorectal cancer cells. In contrast, p38 and JNK are involved in growth arrest and, in some

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contexts, apoptosis [15–17]. In colon cancer and other cancer cells, chemical-induced apoptosis is thought to be fundamental to cancer treatment. Various well-known chemotherapeutic drugs are known to be capable of activating the JNK/p38 MAPK pathway and play critical roles in triggering apoptosis in different cancer cells [18]. Moreover, JNK MAPK signal pathways are responsible for apoptosis mediated by many anti-tumor agents [19–21]. For example, it was shown that the Smilax glabra Roxb. extract (SGRE)-mediated mitochondria-caspase dependent apoptotic pathway involves activation of p38, JNK, and ERK signaling [22], while Sulforaphane induced apoptosis via JNK-caspase-2 in advanced colon carcinoma [23]. Furthermore, cytotoxic lipid peroxides 4-hydroxy-2-nonenal (HNE) induces apoptosis of PC12 cells also through the activation of the JNK pathway without activation of ERK or p38 MAPK [24]. Thus, JNK MAPK was shown to play an essential role in chemical-induced apoptosis, and the MAPK signaling pathway may be also a potential target of butyrate action.

In this study, we investigated the effects of butyrate on colon cancer cell proliferation and apoptosis. The mechanisms involved in these processes were studied using the colon cancer RKO cell line. Our results clearly demonstrate that butyrate can induce apoptosis in a dose-dependent manner in RKO cells. Given the particular importance of apoptosis regulatory proteins of the Bcl-2 family and caspases during tumorigenesis, we focused on the role of these molecules in butyrate-induced apoptosis. Meanwhile, we also investigated the role of the MAPK signaling pathway in butyrate-induced growth inhibition and apoptosis in the RKO cell line.

2. Materials and methods

2.1. Cell lines

Human colorectal cancer cell line (RKO) were cultured in appropriate medium with 10% fetal bovine serum (TBD Science, Tianjin, China), 100 U/mL penicillin and 100 μg/mL streptomycin (Ameresco, USA), at 37 °C, 5% CO_2.

2.2. Antibodies and reagents

Antibodies against Bax, Bcl-2, p38, and JNK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies against cleaved caspase-9 and caspase-3, p-ERK, p-JNK, p-P38, and ERK were purchased from Cell Signaling Technology (Beverly, MA). Butyrate was purchased from Sigma. DAPI (4,6-diamidino-2-phenylindole) was purchased from Beyotime (Shanghai, China). ERK1/2 inhibitor PD98059 and U0126, p38 inhibitor SB203580, and JNK inhibitor SP600125 were purchased from Calbiochem (San Diego, CA, USA). DNA Marker λ-Hind III digest and DL2000 were purchased from TaKaRa Biotechnology (Dalian, China).

2.3. Cell proliferation analysis

The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to quantify the effect of butyrate on cell growth and viability. Cells were plated in 96-well plates (3×10^3 cells/well) in 100 μL of growth medium and allowed to grow for 24 h. The cells were then treated with 0, 1, 10, and 40 mM butyrate in the presence of 3% serum. At 24, 48, and 72 h after treatment, 20 μL of 5 mg/mL MTT (Sigma Chemical Co.) in phosphate-buffered saline (PBS) was added to each well for an additional 4 h of incubation. The blue MTT formazan precipitate was dissolved in 100 μL of dimethylsulfoxide (DMSO). The absorbance at 570 nm was measured on a microELISA reader (Bio-Rad, CA, USA). Cell viability was expressed as a percentage of the control, and data are shown as the mean ± standard deviation (SD) of three independent experiments.

2.4. Nuclear staining

Cells were treated with butyrate (0, 10, and 40 mM) for 24 h, and then the cells were harvested, washed with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were washed with PBS, and stained with DAPI (final concentration 0.5 μg/mL) for 10 min at room temperature. The cells were washed 2 more times with PBS and analyzed using a fluorescence microscope. The tests were performed in triplicate, and a minimum of 100 cells/field and least 4 fields in each well were counted.

2.5. DNA fragmentation analysis

Cells were treated with butyrate (0, 10, and 40 mM) for 24 h, after which they were harvested, washed with ice-cold PBS, and lysed in 50 μL lysis buffer (5 mM Tris–HCl pH 8.0, 10 mM EDTA, and 0.5% Triton X-100) on ice for 20 min. Then, the cell lysates were centrifuged at 12,000 × *g* for 20 min. The supernatant was treated with RNase A (100 mg/mL) at 37 °C for 60 min and proteinase K (200 mg/mL) at 50 °C for 120 min. Then, the DNA was extracted by phenol/chloroform before loading and analyzed by 1.4% agarose gel electrophoresis.

2.6. Immunofluorescence detection

Colon cancer RKO cells were seeded at a subconfluent density on sterile coverslips in six-well tissue culture plates. After incubating the attached cells in serum-free medium for 12 h, they were treated with 0, 10, and 40 mM butyrate for 24 h. Butyrate-treated RKO cells were fixed in 3.7% formalin and washed three times with PBS. Non-specific sites were then blocked with PBS containing 5% bovine serum albumin (BSA) for 30 min at room temperature with gentle rocking. Thereafter, a solution of specific antibody (anti-caspase-3) was flooded over the cells, and the cultures were incubated at 4 °C overnight. After washing with PBS, the cells were further incubated with Cy3-conjugated goat anti-rabbit IgM (Beyotime, Shanghai, China) for 1 h at room temperature, followed by washing with PBS, and then analyzed using an Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan).

2.7. Protein extraction and Western blotting assay

RKO cells were treated with butyrate (0, 10, and 40 mM) for 24 h, after which they were re-suspended in cell lysis buffer (1% Triton X-100, 0.015 M NaCl, 10 mM Tris–HCl, 1 mM EDTA, 1 mM PMSF, 10 μg/mL of each leupeptin and pepstain A) and then incubated on ice for 30 min. The cell lysates were centrifuged at 12,000 × *g* for 20 min at 4 °C, and the supernatants were mixed with one-quarter volume of 4× SDS sample buffer, boiled for 5 min, and then separated through a 12% SDS–PAGE gel. After electrophoresis, proteins were transferred to PVDF membranes, and blocked with 5% nonfat dry milk in TBST buffer (20 mM Tris–HCl pH 7.6, 150 mM NaCl and 0.05% Tween-20) for 1 h at room temperature. The membranes were then probed with diluted primary antibodies in 1% milk/TBST for 24 h at 4 °C, washed three times, incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 30 min at room temperature, and washed extensively before detection by chemiluminescence with the ECL-Plus kit (Beyotime, Shanghai, China). Proteins were visualized by exposing the blots to Kodak film. Western blotting data were quantified using Image J software.

Mitochondrial and cytosolic proteins were isolated using the Mitochondria/Cytosol Fractionation Kit according to the manufacturer's protocol (Beyotime Inst. Biotech, Peking, China).

2.8. Statistical analysis

All the presented data and results were confirmed in at least three independent experiments. Statistical comparisons were made by Student's *t*-test. The significance level was set as $^{*}P < 0.05$ and $^{**}P < 0.01$. Error bars denote SD.

3. Results

3.1. Effects of butyrate on RKO cell proliferation

We first examined the effect of butyrate on cell viability by treating the human colon cancer RKO cells with three concentrations of butyrate (1, 10 and 40 mM) in the presence of 3% serum medium. After 24, 48, and 72 h of treatment, the viability of the cells was determined by the MTT assay. Fig. 1 shows butyrate appeared to be an effective inhibitor of RKO cell viability, which was inhibited in a dose- and time-dependent manner.

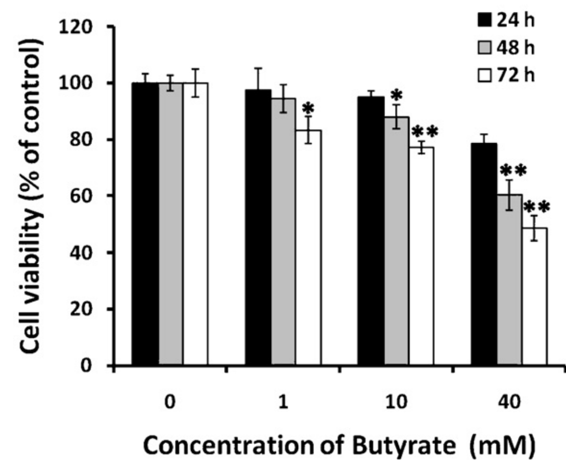

Fig. 1. Butyrate suppresses the viability of RKO cells. RKO cells were treated with 0, 1, 10, and 40 mM butyrate in the presence of 3% serum medium. At 24, 48, and 72 h after treatment, 20 μL MTT was added to each well for an additional 4 h of incubation. The data are presented as the relative proportion of viable cells (%) by comparing the butyrate-treated group with the untreated cells, the viability of which was assumed to be 100%. The results represent the mean ± SD of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ as determined by Student's *t*-test, difference of treated vs. the control group.

3.2. Effects of butyrate on RKO cell apoptosis

Physiological cell death is characterized by apoptotic morphology, including chromatin condensation, membrane blebbing, internucleosome degradation of DNA, and apoptotic body formation. To determine whether the inhibition of cell growth by butyrate resulted from the induction of apoptosis, DNA fragmentation, a hallmark of apoptosis, was demonstrated by incubating RKO cells with butyrate for 24 h; the genomic DNA was isolated and analyzed by agarose gel electrophoresis. The gel analyses showed a typical ladder pattern of DNA fragmentation in the treatment of two concentrations of butyrate (10 and 40 mM) (Fig. 2A).

The induction of apoptosis by butyrate in RKO cells was further confirmed in fluorescence photomicrographs of RKO cells stained with DAPI after treatment with 10 or 40 mM butyrate for 24 h. The morphological features of apoptosis, condensation of chromatin, and fragmentation of the nucleus were examined. Control cells showed round and homogeneous nuclei, whereas butyrate-treated cells showed condensed and fragmented nuclei (arrows) (Fig. 2B). As shown in Fig. 2C, just 10 mM butyrate can induce a significant increase of apoptosis in RKO cells.

3.3. Effects of butyrate on the expression of Bcl-2 family of proteins and caspases in RKO cells

Several gene products are known to be important in controlling the apoptotic process. The imbalance of expression of anti- and pro-apoptotic proteins after the stimulus is one of the major

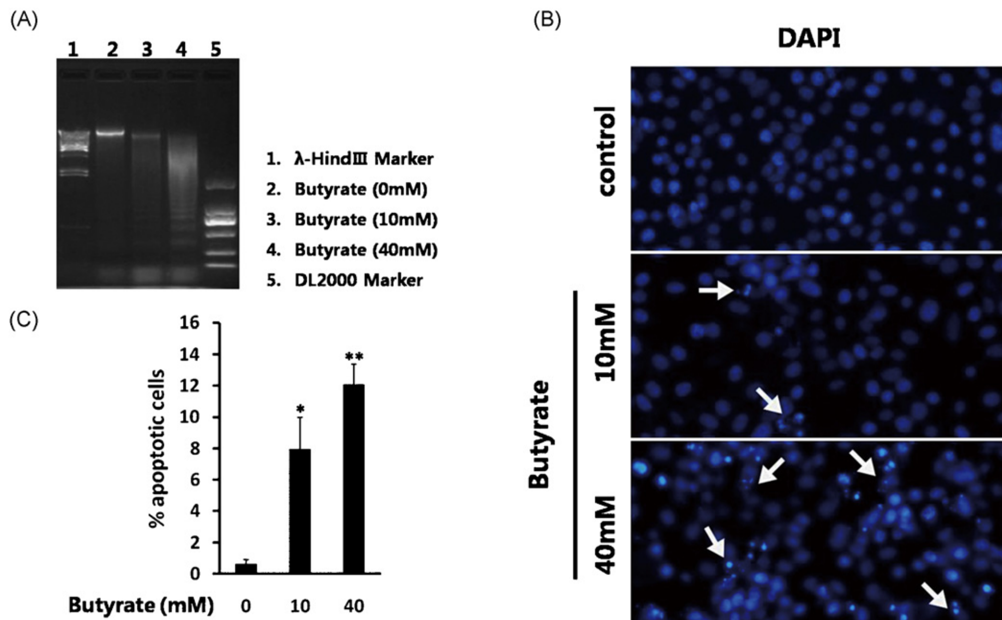

Fig. 2. Butyrate induces apoptosis of RKO cells. (A) RKO cells were treated with 0, 10, and 40 mM butyrate in serum-free medium for 24 h. DNA fragmentation was isolated and subjected to 1.4% agarose gel electrophoresis, followed by visualization of bands and photography. (B) The effect of butyrate on the morphology of the nuclear chromatin in RKO cells. RKO cells were treated with 0, 10, and 40 mM butyrate in serum-free medium for 24 h and fixed and stained with DAPI. The morphological changes in nuclear chromatin were then viewed under a fluorescence microscope. The arrow points to the apoptotic body in apoptotic RKO cells. The results were from one experiment representative of three experiments. (magnification, 200×). (C) Apoptotic cells were quantified by counting a minimum of 4 fields of 100 cells/field (magnification, 200×). The results are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ as determined by Student's *t*-test, difference of butyrate 10 and 40 mM treatment vs. the control group.

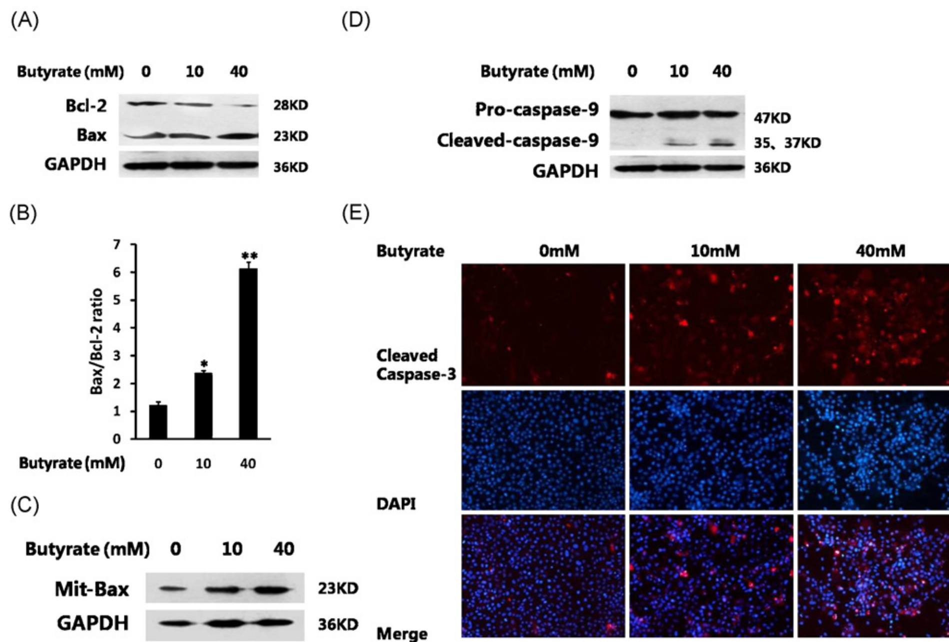

Fig. 3. Expression of Bcl-2 family proteins, caspase-9 and caspase-3 in butyrate-treated RKO cells. (A) RKO cells were incubated with 0, 10, and 40 mM butyrate for 24 h. Cell lysates were prepared, and subjected to SDS–PAGE. Bcl-2 and Bax were determined by Western blotting using specific antibodies. (B) A densitometric analysis of the Western blotting data was used to quantify the levels of Bax and Bcl-2 to evaluate the effect of butyrate on the Bax/Bcl-2 ratio. $^{*}P<0.05$ and $^{**}P<0.01$ as determined by Student's *t*-test, difference of butyrate (10 and 40 mM) treatment vs. the control group. (C) RKO cells were incubated with 0, 10, and 40 mM butyrate for 24 h. Mitochondrial proteins were isolated using the Mitochondria/Cytosol Fractionation Kit, and subjected to SDS–PAGE. Bax and GAPDH were determined by Western blotting using specific antibodies. (D) RKO cells were incubated with 0, 10, and 40 mM butyrate for 24 h. Cell lysates were prepared, and subjected to SDS–PAGE. Pro-caspase-9 and cleaved-caspase-9 were determined by Western blotting using specific antibodies. (E) Immunofluorescence detection of cleaved caspase-3 in butyrate-induced apoptosis. RKO cells were incubated with 0, 10, and 40 mM butyrate for 24 h. Cells were stained by an antibody recognizing activated cleaved caspase-3 (red fluorescence), and the nuclei were visualized with the nuclear dye DAPI (blue fluorescence). (magnification, 100×).

mechanisms underlying the ultimate fate of cells in the apoptotic process. It has been recognized that the Bcl-2 family plays crucial roles in regulating apoptosis by functioning as promoters (e.g., Bax) or inhibitors (e.g., Bcl-2) of cell death [25–27].

To assess the molecular mechanism of apoptosis induced by butyrate, we examined the expression of anti-apoptotic protein Bcl-2, at 24 h after 10 and 40 mM of butyrate treatment. There was a decrease of Bcl-2 expression in a dose-dependent manner following the butyrate treatment in RKO cells (Fig. 3A). We next examined the expression of a pro-apoptotic protein Bax, which is inserted into the outer membrane of the mitochondria and forms a large channel, allowing the release of cytochrome *c* [14]. Fig. 3A shows a dose-dependent increase of Bax protein induced by butyrate. Furthermore, the expression ratio of Bax and Bcl-2 was markedly increased in RKO cells treated with butyrate (Fig. 3B), which indicated that the apoptotic process is activated. To further analyze the role of Bax in butyrate-induced apoptosis, we analyze the Bax redistribution to the mitochondria. As shown in Fig. 3C, Bax is increased in mitochondrial fractions in a dose-dependent manner. Thus, the result showed that Bax redistribution from the cytosol to the mitochondria was dose-dependently involved in butyrate-induced apoptosis in RKO cells.

Because activation of caspases is affected by Bax and Bcl-2, we next investigated the involvement of caspase-9 and caspase-3 in butyrate-induced apoptosis. In Fig. 3D, the caspase-9 activation was determined by measurement of the active forms of caspase-9, and the result indicated that butyrate may cause apoptosis in RKO cells in part by increasing caspase-9 activity, as well as by the reduction of pro-caspase-9. In addition, the activation of caspase-3 in response to butyrate, which was considered to play a central role in many types of stimuli-induced apoptosis, was also examined by an immunofluorescence assay [6,10,16]. As shown in Fig. 3E, treatment of butyrate induced a dramatic increase of caspase-3 activity in RKO cells at 24 h. Just 10 mM butyrate can effectively induce caspase3-dependent apoptosis of RKO cells.

3.4. Butyrate effectively activates JNK MAPK pathways but slightly suppresses the ERK1/2 MAPK pathway

Next, we explored the underlying signal transduction pathways of butyrate-induced apoptosis. ERK1/2 are preferentially activated by growth factors, whereas JNK and the p38 MAPKs are preferentially activated by cell stress-inducing signals, such as oxidative stress, environmental stress, and toxic chemical insults [28,29]. Thus, we investigated whether butyrate-mediated apoptosis was regulated by stress-activated protein kinases, JNK, and p38 MAP kinase. We determined the effect of butyrate on the actions of JNK, p38, and ERK1/2 MAPKs, and the results are shown in Fig. 4A and B. RKO cells were first treated with various concentration of butyrate (2, 4, 6, 8, and 10 mM) for 24 h, and the actions of p38, JNK, and ERK1/2 MAPKs were measured by Western blot analysis using anti-phospho-p38, anti-phospho-JNK, and anti-phospho-ERK1/2 antibodies. JNK phosphorylation, indicating JNK activation, was apparent in butyrate-treated cells in a dose-dependent manner, whereas ERK1/2, which promoted cell survival, phosphorylation was inhibited upon being treated with butyrate. We found that just 8 mM butyrate can largely induce the activation of JNK. Therefore, we treated RKO cells with 8 mM butyrate for various times (1, 4, 8, 12, and 24 h), which showed the same trends (Fig. 4C and D). Taken together, our findings indicate that butyrate induces the activation of JNK in dose-and time-dependent manner, suggesting that JNK MAPK may be specifically activated in the butyrate-induced apoptotic pathway.

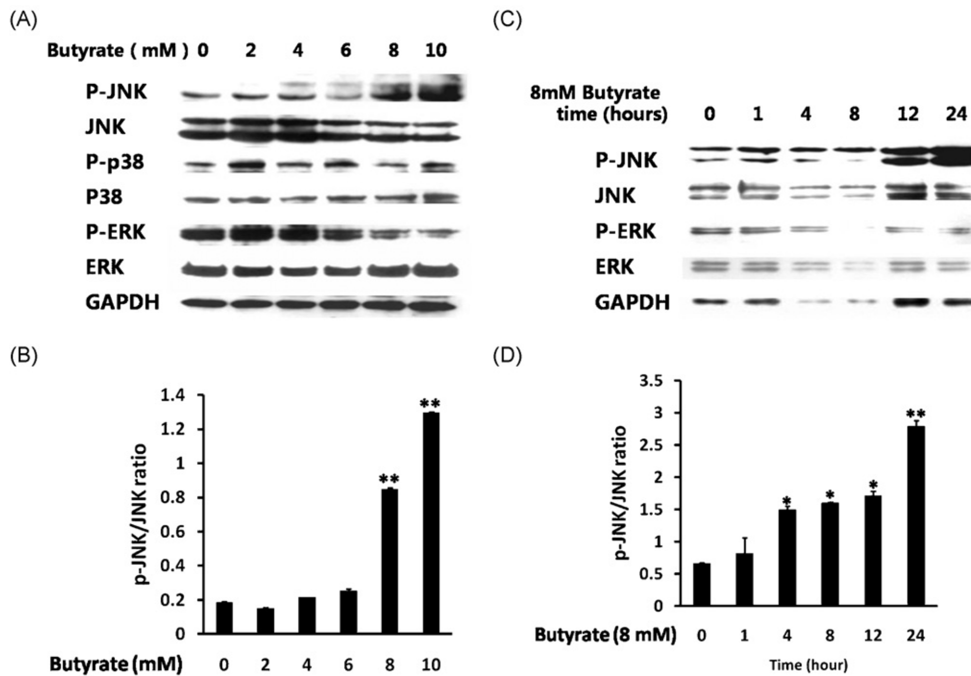

Fig. 4. Butyrate triggers JNK MAPK activation by inducing phosphorylation of proteins. (A) Dose-dependent changes in the activities of ERK1/2, JNK, and p38 in RKO cells after exposure to various concentration of butyrate for 24 h. Cell lysates were prepared and subjected to SDS–PAGE. ERK1/2, JNK, p38, and their phosphorylated forms were determined by immunoblotting using specific antibodies. Antibodies against total ERK1/2, total JNK, and total p38 were used to normalize protein loading. (B) Quantitative analysis was performed by Image J software and normalized by the anti-GAPDH signal. The Western blotting data presented are representative of that obtained in at least three separated experiments. $^{**}P < 0.01$ as determined by Student's *t*-test compared with the untreated group. (C) Time-dependent changes in the activities of ERK1/2 and JNK in RKO cells after exposure to butyrate (8 mM) for various times (1, 4, 8, 12, and 24 h). Cell lysates were prepared and subjected to SDS–PAGE. JNK and ERK1/2, and their phosphorylated forms were determined by immunoblotting using specific antibodies. Antibodies against total JNK and total ERK1/2 were used to normalize protein loading. (D) Quantitative analysis was performed by Image J software and normalized by the anti-GAPDH signal. The Western blotting data presented are representative of that obtained in at least three separated experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ as determined by Student's *t*-test compared with the untreated group.

3.5. The role of JNK MAPK on butyrate-induced apoptosis in RKO cells

We then used pharmacological inhibitors to determine the role of JNK MAPK in butyrate-induced apoptosis and growth inhibition. Cells were pre-incubated for 30 min with SP600125 (JNK inhibitor) in serum-free medium. Thereafter, the cells were exposed to 8 mM butyrate for 24 h. As shown in Fig. 5A and B, butyrate-induced cell death was reversed by SP600125, and SP600125 indeed suppressed the butyrate-induced JNK MAPK signaling pathway, which suggested that butyrate acts on RKO cells via JNK. Similar results were shown in the DNA fragmentation analysis (Fig. 5C), but the inhibition of p38 with 10 μM of SB203580 did not attenuate butyrate-induced growth inhibition (Fig. 5D). Taken together, these data indicate that activation of JNK MAPK is involved in butyrate-induced apoptotic pathway in colon cancer RKO cells.

4. Discussion

Butyrate is a well-known inhibitor of histone deacetylase [30], which can induce apoptosis in various cancer cells [31,32], especially in colon cancer cells. Butyrate acts as a critical factor in the physiological turnover of the colonic epithelium [33], and plays an essential role in cell differentiation, morphology, motility, as well as induction of cell cycle arrest and apoptosis [8,34,35]. Butyrate induces both mitochondria-mediated apoptosis and caspase-independent autophagic cell death in HeLa cells [36]. In addition, butyrate blocked the TNF-α activation of Cox-2 protein, and dramatically suppressed Cox-2 activity in HT-29 cells [37]. Butyrate can induce Smad3 and potentiates TGF-β signaling and its tumor suppressor activity in gut epithelial cells [38]. Moreover, butyrate exerts immunomodulatory effects and anti-inflammatory properties in the gastrointestinal tract and influences cytokine-activated gene expression in colonic epithelial cells via inhibition of NFκB signaling [39–41]. Accumulating evidence suggests that activation of caspases and the consequent cleavage of substrate proteins contribute to the effects of butyrate in colon carcinoma cells [42–44]. Therefore, butyrate induces apoptosis in various cancer cells via different mechanisms, yet its detailed anticancer mechanism in colon cancer is still elusive. Therefore, in this study, we clarified the molecular mechanism underlying butyrate-induced apoptosis in human colon cancer RKO cells. The results showed for the first time that butyrate induces apoptosis in a dose- and time-dependent manner in RKO cells (Figs. 1 and 2).

Mitochondria are involved in a variety of key events leading to apoptosis, such as releasing of caspase activators, changes in electron transport, the production of ROS, and participation in regulation of both pro- and anti-apoptotic Bcl-2 family proteins [45]. It has recently been reported that butyrate-induced terminal differentiation of HT29 colon carcinoma cells involved caspase activation [9]. In this study, butyrate had anti-tumor effects in RKO cells including a typical ladder pattern of internucleosomal DNA fragmentation (Fig. 2A), and activation of caspase indicated by decreased pro-caspase-9 and increased cleavage of caspase-3 (Fig. 3D and E).

The Bcl-2 family proteins, whose members may be anti-apoptotic or pro-apoptotic, regulate cell death by controlling the mitochondrial membrane permeability during apoptosis [46–48]. It is, therefore, inferred that Bcl-2 family proteins may participate in the event during apoptosis induced by butyrate. Bcl-2 is a potent anti-apoptotic factor, whereas Bax is an antagonist of Bcl-2 and acts to promote cell death. Bax is localized mostly in the cytoplasm, but redistributes to mitochondria in response to stress stimuli [49,50]. The ratio between Bax and Bcl-2 determines the cells' survival or

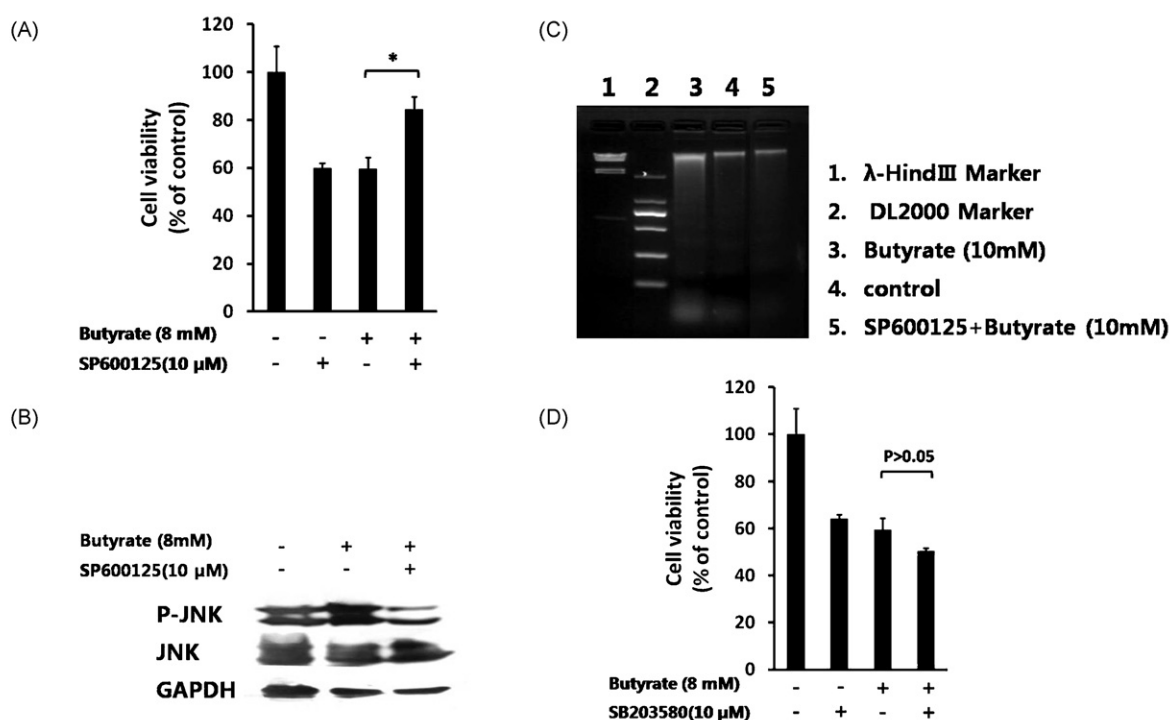

Fig. 5. Pharmacological inhibition of butyrate-induced JNK and p38 activation attenuates butyrate-induced apoptosis. (A) Effects of SP600125 on butyrate-induced growth inhibition of RKO cells, determined by MTT assay. RKO cells were pre-treated with or without 10 μM JNK inhibitor SP600125 for 30 min. Cells were then exposed or not exposed to 8 mM butyrate in serum-free medium for 24 h. The experiment was repeated three times, and similar results were obtained each time. (B) Effects of SP600125 on butyrate-induced activation of the JNK MAPK signaling pathway. RKO cells were pre-treated with or without 10 μM JNK inhibitor SP600125 for 30 min. Cells were then exposed or not exposed to 8 mM butyrate in serum-free medium for 24 h. Cell lysates were prepared and subjected to SDS–PAGE. JNK and their phosphorylated forms were determined by immunoblotting using specific antibodies. (C) Effects of SP600125 on butyrate-induced apoptosis of RKO cells. DNA fragmentation was analyzed by 1.4% agarose gel electrophoresis as described in Section 2. (D) Effects of SB203580 on butyrate-induced growth inhibition of RKO cells, determined by MTT assay. RKO cells were pre-treated with or without 10 μM p38 inhibitor SB203580 for 30 min. Cells were then exposed or not exposed to 8 mM butyrate in serum-free medium for 24 h. The experiment was repeated three times and similar results were obtained each time.

death, and this study finds that the expression of the Bcl-2 showed a decrease in butyrate-induced apoptosis, whereas the amount of Bax increased under the same conditions (Fig. 3A and B). In addition, butyrate promotes Bax translocation to mitochondria (Fig. 3C). A decrease in the levels of Bcl-2 and an increase in Bax lead to the loss of mitochondrial transmembrane potential, a key event in the induction of apoptosis, and opens mitochondrial permeability transition pores. These results indicate that the treatment of butyrate leads to a shift from an anti-apoptotic to a pro-apoptotic state, which results in the activation of capsase-3.

However, the mechanism of the butyrate-induced apoptosis in RKO cells was not fully elucidated. There may be other factors involved in butyrate-induced apoptosis in RKO cells. The family of MAPKs including ERK1/2, JNK and p38 MAPKs play central roles in the signaling pathways of cell proliferation, survival, and apoptosis [45]. The ERK activation has been implicated in cell proliferation and cell cycle progression, while JNK and p38 are more commonly activated in response to stress and cellular damage [16–19,51–53]. There is a large body of evidence suggesting that persistent JNK activation induces apoptosis. The stimuli reported to induce JNK-dependent apoptosis are stress inducers such as UV- and gamma-irradiation as well as cytotoxic drugs [54–60] and anti-carcinogenic compounds [61]. Thus, it has been suggested that JNK is required for stress-induced mitochondrial death signaling pathways [16]. Additionally, activation of JNK promotes Bax translocation to mitochondria in stress-induced apoptosis [62]. As shown in this study, JNK MAPK was phosphorylated, whereas ERK1/2 phosphorylation was slightly inhibited in human colon cancer RKO cells treated with butyrate (Fig. 4). In addition, the pharmacological inhibition of JNK MAPK activation attenuated butyrate-induced apoptosis, while activation of p38 was not involved in butyrate-induced apoptosis (Fig. 5A and B). Thus, activation of the JNK MAPK pathway is required for butyrate-induced apoptosis.

In conclusion, we examined the molecular mechanisms involved in butyrate-induced apoptosis in human colon cancer RKO cells. Butyrate induced apoptosis in RKO cells via activation of caspase-9 and caspase-3, and activation of the JNK MAPK pathway plays an important role in butyrate-induced apoptosis. In addition, inactivation of ERK MAPK was involved in butyrate-induced growth inhibition of RKO cells. These findings establish a mechanistic link between the MAPK pathway and butyrate-induced growth inhibition and apoptosis.

Conflict of interest statement

None declared.

Acknowledgments

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