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G1 Arrest of U937 Human Monocytic Leukemia Cells by Sodium Butyrate, an HDAC Inhibitor, Via Induction of Cdk Inhibitors and Down-regulation of pRB Phosphorylation

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We investigated the effects of sodium butyrate, a histone deacetylase inhibitor, on the cell cycle progression in human monocytic leukemia U937 cells. Exposure of U937 cells to sodium butyrate resulted in growth inhibition, G1 arrest of the cell cycle and induction of apoptosis in a dose-dependent manner as measured by MTT assay and flow cytometry analysis. The increase in G1 arrest was associated with the down-regulation in cyclin D1, E, A, cyclin-dependent kinase (Cdk) 4 and 6 expression, and up-regulation of Cdk inhibitors such as p21 and p27. Sodium butyrate treatment also inhibited the phosphorylation of retinoblastoma protein (pRB) and p130, however, the levels of transcription factors E2F-1 and E2F-4 were not markedly modulated. Furthermore, the down-regulation of phosphorylation of pRB and p130 by this compound was associated with enhanced binding of pRB and E2F-1, as well as p130 and E2F-4, respectively. Overall, the present results demonstrate a combined mechanism involving the inhibition of pRB/p130 phosphorylation and induction of Cdk inhibitors as targets for sodium butyrate that may explain some of its anti-cancer effects in U937 cells.

Key words : Sodium butyrate, G1 arrest, Cdk inhibitors, pRB/p130

Introduction

The cell cycle progression in eukaryotic cells is orchestrated by the sequential activation-inactivation of a series of cyclin-dependent kinases (Cdks) and their respective substrates [11,17,20]. The activity of Cdks is also negatively regulated by binding to Cdk inhibitors in response to a variety of antiproliferative signals and thus modulates phosphorylation events of retinoblastoma protein (pRB) family members, which are essential for various cell cycle transitions [18,21,22]. These observations suggest new approaches that could alter uncontrolled cancer cell growth by modulating cell cycle regulators causing cell cycle arrest and could be useful in cancer preventing and/or intervention [10,11,20].

Sodium butyrate is a short chain fatty acid normally produced as a result of bacterial fermentation of fiber in mammalian intestines, represents one of the end products of carbohydrate breakdown [5]. Previous reports revealed that sodium butyrate is the most effective of the numerous fatty acids produced in the colon for arresting cell proliferation [12]. Although the molecular mechanisms by which sodium butyrate exerts these effects are not well understood, sodium butyrate is known as an inhibitor of histone deacetylase (HDAC) [3], which leads to chromatin remodeling and transcriptional modulation of genes expression implicated in diverse cellular processes such as cell cycle progression [1,2,4,8,19,24-26,28], cell differentiation and/or apoptosis [9,13,14,16,23]. Several studies have highlighted that sodium butyrate-treated cancer cells down-regulate the anti-apoptotic molecules [13,16] or upregulate pro-apoptotic molecules [9,14,23], however, the molecular mechanisms of its inhibitory action on cell cycle progression in human leukamia cells are not known completely.

The present study was carried out to characterize the probable mechanisms involved in sodium butyrate-mediated growth inhibitory effect in human monocytic leukamia U937 cells. We demonstrated that sodium butyrate induced cell cycle arrest at G1 phase through a combined mechanism involving the induction of Cdk inhibitors and the down-regulation of phosphorylation of pRB and p130.

Materials and Methods

Cell culture, sodium butyrate and cell growth inhibition study

U937 cells were purchased from the American Type

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Culture Collection (Rockville, MD), and maintained at 37°C in a humidified condition of 95% air and 5% CO_2 in DMEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Sodium butyrate was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in dimethyl sulfoxide. Measurement of cell growth inhibition was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzyme. For the morphological study, cells were treated with sodium butyrate for 48 hr and directly photographed with an inverted microscope.

Flow cytometric analysis

After treatment with sodium butyrate, the cells were collected, washed with cold phosphate-buffered saline (PBS), and resuspended in PBS. DNA contents of cells were measured using a DNA staining kit (CycleTEST^{TM} PLUS Kit, Becton Dickinson, San Jose, CA). Propidium iodide (PI)-stained nuclear fractions were obtained by following the kit protocol. Fluorescence intensity was determined using a flow cytometer and analyzed by CellQuest software (Becton Dickinson).

Measurement of apoptosis by annexin-V FITC and PI double staining

The magnitude of the apoptosis elicited by sodium butyrate treatment was determined using an Annexin-V fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BD Pharmingen, San Diego, CA). In brief, the cells were washed with PBS and resuspended in annexin-V binding buffer containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$ according to the manufacturer's protocol. Aliquots of the cells were incubated with annexin-V FITC, mixed, and incubated for 15 min at room temperature in the dark. PI at a concentration of 5 µg/ml was added to identify the necrotic cells. The apoptotic cells were measured by a flow cytometer.

RNA extraction and reverse transcription-PCR

Total RNA was prepared using an RNeasy kit (Qiagen, La Jolla, CA) and primed with random hexamers to synthesize complementary DNA using AMV reverse transcriptase (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out in a Mastercycler (Eppendorf, Hamburg, Germany) with indicated primers (Table 1). Conditions for PCR reactions were 1×(94°C for 3 min); 35×(94°C for 45 sec; 58°C for 45 sec; and 72°C for 1 min) and 1×(72°C for 10 min).

Table 1. Gene-specific primers for RT-PCR

Name		Sequence of primers
Cyclin A	sense	5′-TCC-AAG-AGG-ACC-AGG-AGA-ATA-TCA-3′
	antisence	5′-TCC-TCA-TGG-TAG-TCT-GGT-ACT-TCA-3′
Cyclin B1	sense	5′-AAG-AGC-TTT-AAA-CTT-TGG-TCT-GGG-3′
	antisence	5′-CTT-TGT-AAG-TCC-TTG-ATT-TAC-CAT-G-3′
Cyclin D1	sense	5′-TGG-ATG-CTG-GAG-GTC-TGC-GAG-GAA-3′
	antisence	5′-GGC-TTC-GAT-CTG-CTC-CTG-GCA-GGC-3′
Cyclin E	sense	5′-AGT-TCT-CGG-CTC-GCT-CCA-GGA-AGA-3′
	antisence	5′-TCT-TGT-GTC-GCC-ATA-TAC-CGG-TCA-3′
Cdc2	sense	5′-GGG-GAT-TCA-GAA-ATT-GAT-CA-3′
	antisence	5′-TGT-CAG-AAA-GCT-ACA-TCT-TC-3′
Cdk2	sense	5′-GCT-TTC-TGC-CAT-TCT-CAT-CG-3′
	antisence	5′-GTC-CCC-AGA-GTC-CGA-AAG-AT-3′
Cdk4	sense	5′-ACG GGT GTA AGT GCC ATC TG-3′
	antisence	5′-TGG TGT CGG TGC CTA TGG GA-3
Cdk6	sense	5′-CGA ATG CGT GGC GGA GAT C-3′
	antisence	5′-CCA CTG AGG TTA GAG CCA TC-3′
p21	sense	5′-CTC-AGA-GGA-GGC-GCC-ATG-3′
	antisence	5′-GGG-CGG-ATT-AGG-GCT-TCC-3′
p27	sense	5′-AAG-CAC-TGC-CGG-GAT-ATG-GA-3′
	antisence	5′-AAC-CCA-GCC-TGA-TTG-TCT-GAC-3′
GAPDH	sence	5′-CGG-AGT-CAA-CGG-ATT-TGG-TCG-TAT-3′
	antisence	5′-AGC-CTT-CTC-CAT-GGT-GGT-GAA-GAC-3′

Amplification products obtained by PCR were electrophoretically separated on 1% agarose gel and visualized by ethidium bromide (EtBr, Sigma) staining.

Immunoprecipitation, gel electrophoresis and Western Blot Analysis

The cells were harvested, lysed, and protein concentrations were quantified using the Bio Rad protein assay (BioRad Lab., Hercules, CA), following the procedure described by the manufacturer. For immunoprecipitation, cell extracts were incubated with an immunoprecipitating antibody in extraction buffer for 1 hr at 4°C. The immuno-complex was collected on protein G/A-Sepharose beads (Sigma). For the Western blot analysis, an equal amount of protein was subjected to electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) by electroblotting. Blots were probed with the desired antibodies for 1 hr, incubated with diluted enzyme-linked secondary antibodies and then visualized by the enhanced chemiluminescence (ECL) according to the recommended procedure (Amersham Corp.). The primary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Calbiochem (Cambridge, MA, USA). Peroxidase-labeled secondary antibodies were purchased from Amersham Corp.

Results

Inhibition of the cell viability and G1 arrest of the cell cycle by sodium butyrate

To test the effect of sodium butyrate on the growth of U937 cells, the cells were treated with different concentrations of sodium butyrate for 48 hr and viable cells were measured by MTT assay. As shown in Fig. 1, sodium butyrate had a strong inhibitory effect on cell proliferation in a dose-dependent manner, which was associated with a distinct morphological change including membrane ruffling. To determine whether sodium butyrate treatment of cells resulted in the alteration of cell cycle progression and induction of apoptosis, the cell cycle patterns and magnitude of the apoptosis were examined. Analysis of the cell cycle distribution of cells after exposure to sodium butyrate showed that these cells had marked accumulation in the G1 phase of the cell cycle (Fig. 2A), which was accompanied by a decrease in their S and G2/M phase. Additionally, the sub-G1 and annexin V-positive cells increased concentration-dependently in the sodium butyrate-treated U937 cells compared with the untreated control cells (Fig. 2B and C). Taken together, these results suggest that the growth inhibitory effect of sodium butyrate in U937 cells was the result of a block during this G1 phase and induction of apoptosis.

Effects of sodium butyrate on the levels of G1 phase cell cycle regulators

Since sodium butyrate arrested U937 cells in the G1 phase of the cell cycle, it was determined the expression levels of the cell cycle regulating factors such as cyclins and Cdks by RT-PCR and Western blotting. As shown in Fig. 3, the protein and mRNA levels of cyclin A, D1 and E were significantly decreased by sodium butyrate treatment however the levels of cyclin B1 were not affected. Sodium butyrate

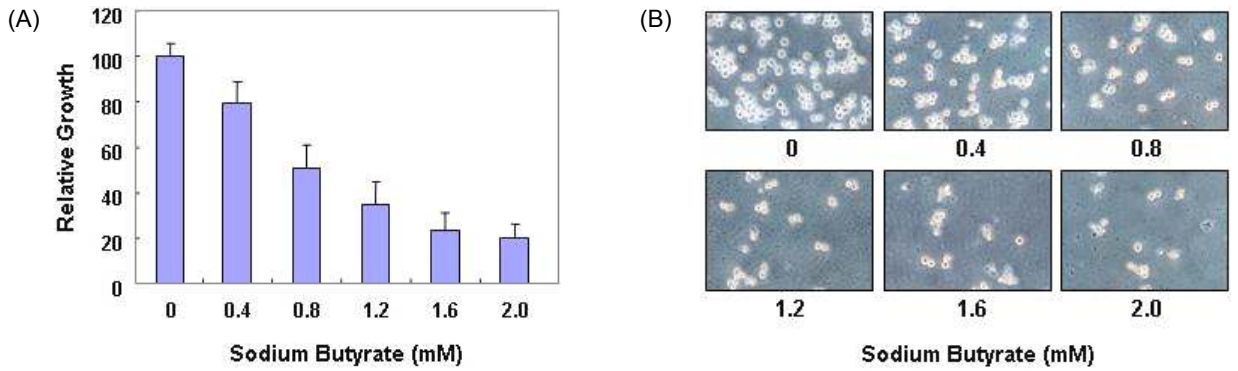

Fig. 1. Growth inhibition and morphological changes by sodium butyrate treatment in U937 cells. (A) U937 cells were plated at 4×10^4 cells per 60-mm plate, and incubated for 24 hr. The cells were treated with variable concentrations of sodium butyrate for 48 hr and growth inhibition was measured by the metabolic-dye-based MTT assay. Results are expressed as the means±S.E. of three independent experiments. (B) Cells were incubated with variable concentrations of sodium butyrate. After incubation with sodium butyrate for 48 hr, the cells were examined under an inverted microscope. Magnification, X200.

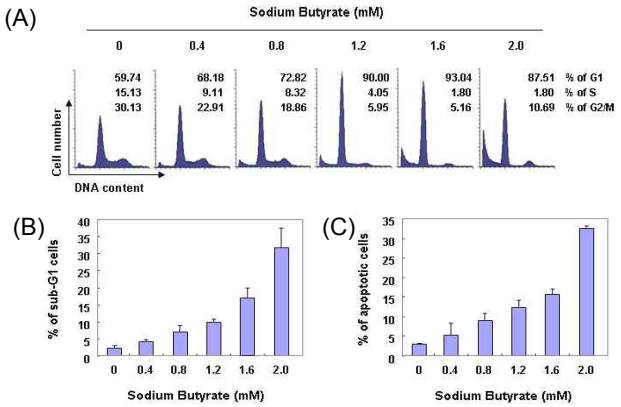

Fig. 2. Induction of G1 arrest of the cell cycle and apoptosis by sodium butyrate in U937 cells. (A) The cells were treated with sodium butyrate for 48 hr, collected, fixed, and stained with PI for flow cytometry analysis. Data are presented as the mean values obtained from three independent experiments. (B) The percentages of cells in the sub-G1 phase are presented. The results are expressed as the mean±SD of three independent experiments. (C) The cells grown under the same conditions were collected and stained with FITC-conjugated annexin-V and PI for flow cytometry analysis. The apoptotic cells were determined by counting the % of annexin V(+)/PI(-) cells and the % of annexin V(+)/PI(+) cells. The results are expressed as the mean±SD of three independent experiments.

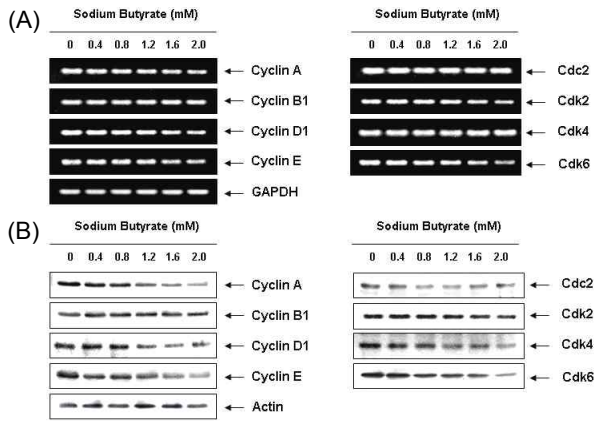

Fig. 3. Effects of sodium butyrate on the mRNA and protein levels of cyclins and Cdks in U937 cells. (A) After 48 hr incubation with sodium butyrate, total RNAs were isolated and reverse-transcribed. The resulting cDNAs were subjected to PCR with indicated primers and the reaction products were subjected to electrophoresis in a 1% agarose gel and visualized by EtBr staining. GAPDH was used as an internal control. (B) The cells were lysed and then cellular proteins were separated by SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were probed with the indicated antibodies. Proteins were visualized using an ECL detection system. Actin was used as an internal control.

also inhibited the levels of Cdk4 and Cdk6 in a concentration-dependent manner. However, the levels of Cdc2 and Cdk2 relatively remained unchanged in sodium butyrate-treated cells. These results suggest that the suppressive effects of the sodium butyrate at G1 phase of U937 cells are partly caused by down-regulating the levels of the cell cycle regulating factors at the G1 boundary.

Induction of Cdk inhibitors by sodium butyrate

To further understand the anti-proliferative mechanism of sodium butyrate, it was investigated whether Cdk inhibitors, such as p21 and p27, are involved in the sodium butyrate-induced growth arrest in U937 cells (Fig. 4). In the untreated control cells, the protein and mRNA levels of p21 and p27 were very low. However, the incubation of cells with sodium butyrate caused a striking concentration-dependent increase in the induction of p21 and p27 protein and mRNA. Because the p53 gene is deleted in U937 cells

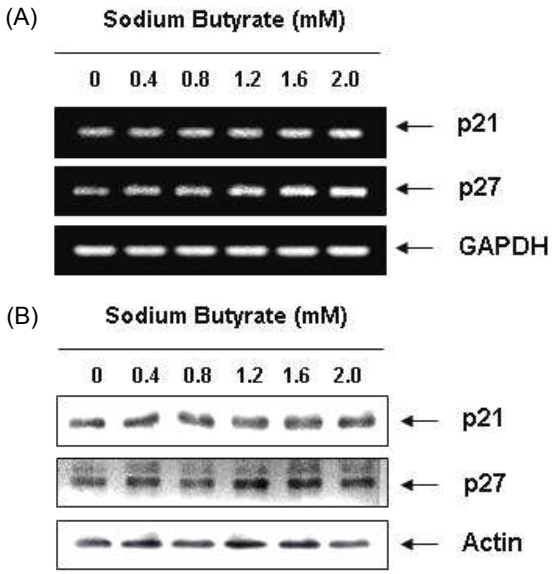

Fig. 4. Induction of Cdk inhibitors by sodium butyrate in U937 cells. (A) After 48 hr incubation with sodium butyrate, total RNAs were isolated and reverse-transcribed. The resulting cDNAs were then subjected to PCR with p21 and p27 primers and the reaction products were subjected to electrophoresis in a 1% agarose gel and visualized by EtBr staining. GAPDH was used as an internal control. (B) The cells were lysed and the cellular proteins were then separated by electrophoresis in SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Next, the membranes were probed with the anti-p21 and anti-p27 antibodies and then visualized using an ECL detection system. Actin was used as an internal control.

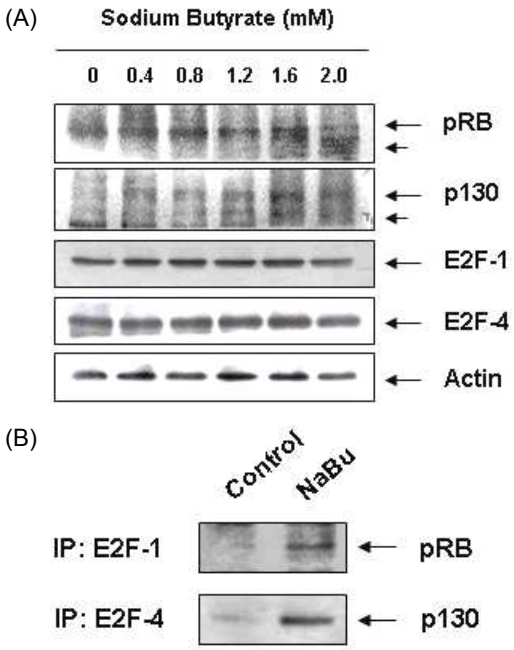

Fig. 5. Hypophosphorylation of pRB and p130, and enhanced association with E2Fs in U937 cells after exposure to sodium butyrate. (A) The cells were treated with the indicated concentrations of sodium butyrate for 48 hr and total cell lysates were prepared and separated by 8% or 10% SDS-polyacrylamide gel. Western blotting was performed using the indicated antibodies and an ECL detection system. Actin was used as an internal control. (B) Whole cell lysates (0.5 mg of protein) from control cells and cells treated with sodium butyrate were immunoprecipitated with anti-E2F-1 and E2F-4 antibodies. Immuno-complexes were separated by 8% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with anti-pRB and ant-p130 antibodies. Proteins were detected by ECL detection.

[6], it is most likely that the induction of p21 is mediated in a p53-independent fashion.

Down-regulation of pRB and p130 phosphorylation and increased binding with E2Fs by sodium butyrate

Since the pRB family members are important checkpoint proteins in G1 phase of the cell cycle, it was next determined the kinetics between phosphorylation of pRB and p130 and the transcription factors, E2F-1 and E2F-4 in U937 cells treated with sodium butyrate. The levels of pRB and p130 expression were remarkably changed from hyperphosphorylated form to hypophosphorylated form by sodium butyrate treatment without marked altering E2Fs expression (Fig. 5A). Co-immunoprecipitation analysis indicated that association of pRB/p130 and E2Fs was almost undetectable in the untreated log phase cells, however, there was a strong increase in the association of pRB and E2F-1, and p130 and E2F-4, respectively, in sodium butyrate-treated cells (Fig. 5B) suggesting that sodium butyrate inhibits the releasing of E2F-1 and E2F-4 protein from pRB and p130.

Discussion

In this study, we tested sodium butyrate, a HDAC inhibitor, for its activity in inhibiting the growth of human monocytic leukamia U937 cells. We found that treatment of cells with sodium butyrate resulted in a concentration-dependent inhibition of cell viability, which was associated with gross morphological changes (Fig. 1). Subsequent experiments addressed the issue of whether this compound perturbs the cell cycle using DNA flow cytometric analysis. The data revealed a cell-cycle block at G1 to S phase transition and an accumulation of cells at sub-G1 apoptotic region (Fig. 2A and B), which contained less DNA than G1 cells [15]. The apoptotic activity of sodium butyrate was also confirmed using annexin-V FITC and PI double staining (Fig. 2C). These results suggested that sodium butyrate interferes with the proliferation of U937 cells and induces apoptosis in close association with the G1 arrest by modulation of cell cycle-regulators expression as a possible molecular mechanism of the effect of sodium butyrate. Thus, we investigated the effects of sodium butyrate on the expression of G1/S transition regulatory proteins to analyze the mechanism of G1 arrest.

In terms of regulation of the cell cycle, Cdks play a most critical role. Two major mechanisms for Cdk regulation are binding with its catalytic subunit cyclin, followed by activation of Cdk/cyclin complexes, and binding with Cdk inhibitors followed by inactivation of Cdk/cyclin complexes [18,20,27]. An alteration in the formation of these complexes could lead to increased cell growth and proliferation, and decreased cell growth and proliferation followed by differentiation and/or cell death by apoptosis [10,11,20]. In general, D-type cyclins are synthesized in a pre-DNA-synthetic gap (early G1 phase) and are key regulators of the signal transduction in the G1 phase cell proliferation. And, the progression through the G1/S transition is regulated by cyclin E, which is expressed in late G1 preceding cyclin A expression. Thus, as cells enter G1, the cyclin D/Cdk4 (and/or Cdk6) complex appears to be necessary for tran-

sition through early G1, whereas cyclin E /Cdk2 complex is required in transition from late G1 into S phase. Furthermore, cyclin A accumulates during S phase and associates with and activates primarily Cdk2 [17,20,27]. In the present study, the results from RT-PCR and immunoblotting analyses clearly demonstrated that levels of cyclin D1, E and A were markedly decreased by sodium butyrate treatment in both transcriptional and translational levels, but the levels of cyclin B1 were not affected. The levels of Cdc2, Cdk4 and Cdk6 also downregulated by sodium butyrate treatment in a concentration-dependent manner without changes of the expression of Cdk2 (Fig. 3). The data indicated that sodium butyrate-induced G1 arrest of the cell cycle in U937 cells was associated with modulation of several G1 phase-related cyclin/Cdk complexes.

The activity of Cdks is also negatively regulated by binding to Cdk inhibitors in response to a variety of antiproliferative signals and thus modulates phosphorylation events of pRB family members (pRB, p130 and p107), which are essential for various cell cycle transitions. Cdk inhibitors are divided into two families according to substrate specificity. In mammalian cells, these are the CIP/KIP family (p21, p27 and p57), and the INK4a family (p15, p16 and p18). Cdk inhibitors mediate cell cycle arrest in response to several antiproliferative signals. Among them, p21 and p27 bind to cyclins/Cdk complexes and prevent kinase activation, subsequently blocking the progression of the cell cycle at the G1 or G2/M phases [7,18,21]. Under normal conditions, pRB family proteins bind to the members of the E2F family of transcription factors. However, growth factors induce phosphorylation and dissociation of the pRB family proteins from E2Fs, which triggers G1 cell cycle progression [20-22]. Therefore, the cyclin E/Cdk2 complex is an obvious candidate for control of pRB phosphorylation. If decreased levels of either protein or the association between respective binding partners were observed, a concomitant decrease in the degree of pRB phosphorylation would be expected. Sodium butyrate has previously been shown to increase the protein levels of the Cdk inhibitor p21 and p27 in human oral squamous carcinoma and neuroendocrine tumor cells [2,26], and to inhibit pRB phosphorylation in several cancer cells [8,19,24,28]. The data generated in this study demonstrate that in the U937 cells, sodium butyrate concentration-dependently enhanced expression of the Cdk inhibitors, p21 and p27 (Fig. 4). Furthermore, it was found that sodium butyrate blocked pRB and p130 phosphorylation and increased the binding of pRB to E2F-1 and p130 to E2F-4 in U937 cells (Fig. 5). Additionally, our data also suggest that sodium butyrate-caused p21 upregulation involves a p53-independent pathway because U937 cells lack functional p53 [6].

In summary, the present study demonstrates that sodium butyrate, a HDAC inhibitor, inhibited the U937 human monocytic leukamia cell proliferation by inducing G1 cell cycle arrest. Although further studies are needed, the present work suggests that CIP/KIP family (p21 and p27) and pRB family (pRB and p130) play important roles in G1 cell cycle arrest induced by sodium butyrate in human leukamia cells.

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초록 : Cdk inhibitors의 발현 증가 및 pRB 인산화 저해에 의한 HDAC inhibitor인 sodium butyrate에 의한 인체백혈병세포의 G1 arrest 유발

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대표적인 histone deacetylase inhibitor 저해제의 일종인 sodium butyrate에 의한 인체백혈병 U937 세포의 증식억제에 관한 기전 연구를 세포주기 조절 측면에서 조사하였다. MTT assay 및 flow cytometry 분석을 통하여 sodium butyrate의 처리 농도 증가에 따른 U937 세포의 증식억제는 세포주기 G1 arrest 및 apoptosis 유발에 의한 것임을 확인하였다. RT-PCR 및 Western blotting 결과에서 sodium butyrate에 의한 G1 arrest는 세포주기 G1기에서 S기로의 진입에 중요한 역할을 하는 cyclin D1, E, A, cyclin-dependent kinase (Cdk) 4 및 Cdk6 발현의 저해와 p21 및 p27과 같은 Cdk inhibitor의 발현 증가와 연관성이 있었다. Sodium butyrate는 또한 retinoblastoma protein (pRB) 및 p130 단백질의 인산화를 저해시켰으나, S기 진행에 중요한 전사조절인자인 E2F-1 및 E2F-4의 발현에는 큰 영향이 없었다. 그러나 sodium butyrate에 의한 pRB 및 p130 단백질의 인산화 저해는 pRB와 E2F-1 및 p130과 E2F-4와의 결합력을 증가시켰다. 본 연구의 결과는 U937 세포의 증식억제에 pRB/p130 인산화 억제 및 Cdk inhibitors의 발현 증가가 중요한 역할을 하고 있음을 보여 주는 것으로, sodium butyrate의 항암기전 이해에 중요한 자료가 될 것이다.