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Butyrate Stimulates Cyclin D and p21 and Inhibits Cyclin-Dependent Kinase 2 Expression in HT-29 Colonic Epithelial Cells

Samila Siavoshian, Herve M. Blottiere,*[,1] Christine Cherbut,* and Jean-Paul Galmiche
*Centre de Recherche en Nutrition Humaine de Nantes, CRI INSERM 95-08, CHU Hôtel-Dieu, *INRA-LTAN, Nantes, France*

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Sodium butyrate, a product of colonic fermentation of dietary fiber, has been shown to inhibit cell proliferation by blocking the cells in the G1 phase of the cell cycle. However, its mechanism of action is still unknown. We found that butyrate strongly stimulated cyclin D and p21/WAF1/CIP1 expression in HT-29 human colonic adenocarcinoma cells, in a dose dependent manner. These stimulations were associated with a decrease in cyclin-dependent kinase (cdk) 2 level, whereas cdk4 and cdk6 remained unchanged. Our results suggest that the inhibition of cell cycle progression by sodium butyrate may be explained by a modulation of cell cycle regulatory proteins such as cyclin D and p21.

n-Butyrate, a four-carbon fatty acid produced by colonic fermentation, can inhibit cell proliferation and stimulate cell differentiation in several cell lines including colonic adenocarcinoma cells (1, 2). The mechanisms by which butyrate can regulate cell proliferation is still unknown, however it has been shown that its effect results mainly in a blockade in the G1 phase of the cell cycle (3), prior to the onset of cyclin E expression (4).

The eukaryotic cell division cycle has been found to be governed by two families of proteins which are associated to form heterodimers : the cyclins and the cyclin dependent kinases (cdk) (5). D type cyclins appear early in the G1 phase and complex with cdk4 or cdk6 and then can directly bind to the unphosphorylated or underphosphorylated form of pRb, thereby targeting active cdks to phosphorylate pRb, and to allow E2F transcription factor to activate gene transcription (6, 7). Cdk2 kinase activity is first evident during the middle of G1. It assembles with cyclin E in the middle of G1 and then with cyclin A at the start of the S phase. Cyclin E-cdk2 may function in controlling progression through G1, and cyclin A-cdk2 may function in controlling the start of DNA synthesis (8). Recently, a new type of cell cycle regulatory proteins have been identified. These proteins which bind to and inhibit the activity of cyclin/cdk complexes are classified into the p21 and the p16/INK4 families (9). The p21/WAF1/Cip1 protein selectively inhibits the G1/S cdk-cyclin complexes (10). A further key cdk inhibitor family named p16/INK4 was also found. It seems to form a binary complex with cdk4 or cdk6, thus destroying or preventing the formation of the cdk-cyclin dimers (11).

In the present paper, we investigated the mechanisms of inhibition of the cell cycle progression in the G1 phase which was induced by the treatment with butyrate from the viewpoint of cell cycle regulatory proteins.

MATERIALS AND METHODS

Cell culture. HT-29 cells, were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin at 37°C in a 5% CO_2 incubator. HBL-100 human mammary epithelial cell line were cultured in RPMI supplemented with the same compound, and used as positive control for p16 expression (12). All tissue culture reagents were from Gibco (Cergy Pontoise, France).

Cells were plated onto 75 cm^2 flasks at a density of 2×10^6 cells per flask. HT-29 cells were synchronized by exposing the culture to fetal calf serum-deprived DMEM medium for 24 hours. Cells were then exposed to complete medium in the presence or absence of increasing concentration of sodium butyrate (from 1 to 8 mM) for 1 to 4 days.

Immunoblotting. Cell lysates were prepared by washing the cells three times with PBS. The cells were incubated for 1 h at 4°C in 500 ml lysis buffer consisting of 10 mM Tris-HCl pH 7.4; 20 mM NaCl, 5 mM $MgCl_2$, 0.5% Nonidet P40 and 0.1 mM phenylmethylsulfonyl-

[1] Address request for reprints to Hervé M. Blottière, INRA, BP 71627, rue de la Géraudière, 44316 Nantes Cedex 03, France. Fax: (33) 02-40-67-50-12; e-mail: Blottier@nantes.inra.fr.

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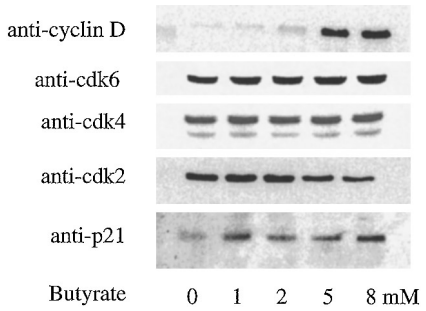

FIG. 1. Western blot analysis of cell cycle regulatory proteins expression in HT-29 cells treated during 4 days with increasing amount of butyrate. Equal volumes of whole cell extracts containing 15 mg of proteins were separated and electrophoretically blotted. Proteins were probed with indicated antibodies.

fluoride. After centrifugation at 10,000 × g during 10 min at 20°C, the protein content of these extracts was determined spectrophotometrically using the D_C Protein Assay (BioRad, Ivry sur seine, France). 15 mg of total protein from control and butyrate-treated samples were resolved on 12% SDS-PAGE gels along with prestained protein molecular weight standards (BioRad). Gels were then blotted onto PVDF membranes (Sigma, L'isle Dabeau Chesne, France). Upon completion of the transfer, the blots were blocked with PBS containing 6% non-fat milk overnight at 4°C. Membranes were incubated with the primary antibodies a 1:200 dilution in 3% milk/PBS for 1 h at room temperature. Rabbit polyclonal antibodies anti-cyclin D, anti-cdk2, anti-cdk4, anti-cdk6, anti-p16 and anti-p21 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The blots were washed four times, and then incubated with biotin-conjugated anti-rabbit antibody (Sigma) diluted 1:500 in 3% milk/PBS for 1 h at room temperature. After additional washes, the blots were incubated with the streptavidin-peroxydase complex (Sigma) diluted 1:500 in 3% milk/PBS for 1h. After additional washes, the cdk, cyclin D and p21 proteins were detected with a chemiluminescence assay system (Amersham, Les Ulis, France).

RESULTS

Butyrate Enhances Cyclin D, but Not cdk4 and cdk6 Protein Levels

We examined the protein level of cyclin D, 1 and 4 days after treatment with various concentrations of butyrate. The cyclin D protein was present in low amount in control cells. Its expression was greatly enhanced after incubation with sodium butyrate (Fig 1 and Fig 2). A 30 fold increase was obtained for a 8 mM butyrate concentration as measured after densitometric analysis of the blots. The cdk4 and cdk6 proteins were constitutively present in HT-29 cells. After incubation with sodium butyrate, the protein level of cdk4 and cdk6 remained unchanged (Fig 1).

Butyrate Decreases cdk2 Protein Level

We then studied the effect of butyrate on cdk2 protein level after 1 and 4 days treatment in HT-29 cells. Fig 1 shows that control cells expressed the cdk2 protein. After an incubation with sodium butyrate, the protein level of cdk2 decreased. This decrease was first observed at a 5 mM concentration, and it was maximal at 8 mM.

Butyrate Enhances p21, but Not p16 Proteins Level

We examined the effect of butyrate on the expression of p21 protein. On non-confluent cells, p21 protein was not detected (Fig 2), however it was detected on pre-confluent cells *i.e.* cells maintained during 4 days in complete medium (Fig 1). After 1 day incubation with sodium butyrate, the protein level of p21 was strongly induced (Fig 2). After 4 days, this stimulation was still detectable, but was less pronounced (Fig 1).

The expression of p16 was also investigated. p16 was not present in normally growing HT-29 cells, whereas it was detectable in HBL-100 breast epithelial cells. After treatment with sodium butyrate, p16 protein remained absent (Fig 2).

DISCUSSION

In the present report, we studied the effect of butyrate on cell cycle regulatory proteins, mainly those which participate in G1 to S phase transition. Indeed, butyrate was shown to block the cells in the G1 phase of the cell cycle as observed on L1210 and Molt-4 leukemia cell lines (3, 4). By flow cytometry and after staining with propidium iodide, we observed similar effect on HT-29 cells (data not shown). The treatment with butyrate strongly stimulated cyclin D and p21 expression, while unaffecting cdk4, cdk6 and p16 level. Moreover, a decrease in the amount of cdk2 protein was observed.

Cyclin D/cdk4 and cdk6 complexes are thought to play a major role in cell cycle progression in early G1

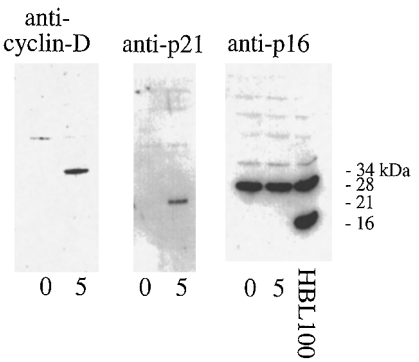

FIG. 2. Effect of butyrate on the expression of cyclin D, p21 and p16 proteins in HT-29 cells after one day of incubation without or with 5 mM butyrate. Cells were harvested and analyzed by immunoblot analysis. Equal volumes of whole cell extracts containing 15 mg of proteins were separated and electrophoretically blotted. For p16, HBL-100 cell extracts was used as positive control.

phase. The stimulation of cyclin D expression after butyrate treatment may be a "marker" of the blockade of cells in G1. Indeed Pagano *et al.* showed a transient overexpression of cyclin D1 in fibroblasts arrested in G1 phase (13). Alternatively, cyclin D may directly participate to the inhibition of proliferation. It was shown that cyclin D1 overexpression using retrovirus-mediated transduction markedly inhibited the growth of HC11 mouse mammary epithelial cells through a blockade in G1 phase (14). Moreover, the expression of transcription factors involved in cell growth, namely *c-myc* and *c-jun* were also reduced by cyclin D1 overexpression. Butyrate which blocks the cells in G1 phase was also shown to reduce the level of expression of *c-myc* in a colonic carcinoma cell line (15). Cycloheximide was able to inhibit the reduction of *c-myc*, indicating that the butyrate effect needed the synthesis of a protein. Our observation that cyclin D was strongly stimulated after butyrate treatment may explain these results on *c-myc* expression. However, the exact role played by the overexpression of cyclin D by butyrate on cell cycle blockade deserve more studies.

Cyclins A and E can associate with the cdk2 and have been suggested to control initiation or continuation of DNA replication itself. It has become apparent that cyclin E and A sequentially activate cdk2 around the start of S phase, and that cyclin A activates cdk2 shortly after cyclin E does, concomitant with the onset of measurable DNA synthesis (21). The diminution of cdk2 level by butyrate may lead to inhibition of the event that is essential for DNA replication. Our results provide evidence for a specific cellular mechanism of butyrate induced differentiation and cell cycle arrest, because after treatment with sodium butyrate, cdk4 and cdk6 expressions were not modified.

p21 level can be increased in response to physiologic and chemical inducers of differentiation (16), including sodium butyrate (17). p21 seems a particularly important link between apoptosis, differentiation and cell cycle alterations in response to exogenous stimuli. Its mRNA expression was shown to be slightly increased in HT-29 cells cultured after confluence (17). In our experiments, p21 was not detected in non-confluent cells, but it was detected in pre-confluent cells. The increased expression of this inhibitory protein observed in HT-29 cells may induce growth inhibition in the G1 phase. Indeed, p21 inhibits cyclin D1-cdk4, cyclin D1-cdk6, cyclin E-cdk2 and cyclin A-cdk2 kinase activity to varying degrees (18, 19). Moreover, the strong stimulation of p21 expression may explain cyclin D overexpression. Actually, Chen *et al.* showed that the increased expression of cyclin D1 either in human or murine cells through the accumulation of the wild-type p53 protein was mediated at least in part by the p21 gene product (20).

Cyclin-cdk complexes have also been shown to be regulated by another group of inhibitors *i.e.* p16/INK4 family. We demonstrated that p16 was not involved in butyrate mediated arrest of proliferation in HT-29 cells.

In conclusion, our results indicate that inhibition of cell cycle progression after treatment with butyrate can be explained by modifications of the level of cyclin D, cdk2 and p21, which regulate cell cycle progression. We hypothesize that butyrate induces the expression of p21 which led to the inhibition of cyclins/cdks activity, resulting in underphosphorylation of pRb and a blockade in G1 phase. In fact, this underphosphorylation of pRb in the presence of butyrate was shown previously in HT-29 cells (22). Finally, this underphosphorylation of pRb will stimulates cyclin D expression, since p21 does not induce cyclin D1 in pRb deficient cells (20).

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