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1,25-Dihydroxycholecalciferol Enhances Butyrate-Induced p21$^{Waf1/Cip1}$ Expression

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Butyrate, a short-chain fatty acid produced in the colon, as well as its prodrug tributyrin, reduce proliferation and increase differentiation of colon cancer cells. p21$^{Waf1/Cip1}$ and p27^{Kip1} are negative regulators of cell cycle and are thought to have a key function in the differentiation of various cell lines. We studied the effects of butyrate on differentiation, VDR expression, as well as on p21$^{Waf1/Cip1}$ and p27^{Kip1} expression in human colon cancer cells (Caco-2). Butyrate induced cell differentiation, which was further enhanced after addition of 1,25-dihydroxycholecalciferol. Synergistic effect of butyrate and dihydroxycholecalciferol in Caco-2 cells was due to butyrate-induced overexpression of VDR. While butyrate as well as dihydroxycholecalciferol increased p21$^{Waf1/Cip1}$ and p27^{Kip1} expression, in contrast combined exposure of butyrate and dihydroxycholecalciferol resulted in a synergistic amplification of p21$^{Waf1/Cip1}$, but not of p27^{Kip1} expression. These data imply that butyrate selectively increases p21$^{Waf1/Cip1}$ expression via upregulation of VDR in Caco-2 cells.

Key Words: **butyrate; Caco-2 cells; differentiation; 1,25-dihydroxycholecalciferol; p21$^{Waf1/Cip1}$; p27^{Kip1}; tributyrin; vitamin D receptor.**

Cell cycle is regulated by a family of cyclin-dependent kinases (cdk), whose activity is modified by negative regulators of the cycle, p21$^{Waf1/Cip1}$ and p27^{Kip1}. The cyclin-cdk inhibitor p21$^{Waf1/Cip1}$ seems to be a particularly important link between apoptosis, differentiation and cell cycle alterations: first, it is known to exert a G1 cell cycle arrest in response to a variety of stimuli, e.g., by DNA damage or during cellular differentiation [1]. Second, p21$^{Waf1/Cip1}$ is upregulated during the differentiation of enterocytes, and third, it may also be involved in the apoptotic pathway after terminal differentiation [2–5]. As for p27^{Kip1}, Tian and Quaroni [3] as well as Yamamoto *et al.* [5] excluded a significant role in growth inhibition and rather proposed a more important role in the induction of differentiation, suggesting the use of agents increasing the expression of p27^{Kip1} as useful in the therapy of human colon cancer. In turn, loss of p27^{Kip1} expression was associated with a poor prognosis in human carcinogenesis [4–6]. In contrary, we recently demonstrated that differentiation of Caco-2 cells occurs independently from p27^{Kip1}, rather proposing a role of p27^{Kip1} in the apoptotic process [7].

Butyrate, a normal constituent of the colonic luminal content, is formed by bacterial fermentation of unabsorbed complex carbohydrates in the mammalian digestive tract [8]. It acts as a potent antineoplastic agent in a wide variety of neoplastic cells [9, 10]. Whereas butyrate exerts its growth-inhibiting actions mainly by upregulating p21$^{Waf1/Cip1}$ [1], the process of butyrate-induced differentiation and apoptosis in Caco-2 cells is discussed to be associated with both induction of p21$^{Waf1/Cip1}$ and p27^{Kip1} [2, 11]. Tributyrin, a precursor of butyrate and a neutral short-chain fatty acid triglyceride containing three butyrate moieties, does exert antiproliferative, proapoptotic, and differentiation-inducing effects similar to natural butyrate in cell culture systems. *In vitro,* tributyrin is about two- to fourfold more active than butyrate, being dependent on the cell type [12–15]. This, as well as its favorable pharmacokinetics, contributed that tributyrin has been under clinical evaluation as antiproliferative agent in the treatment of cancer [16].

Growth-inhibiting and differentiation-inducing effects of 1,25-dihydroxycholecalciferol, the active form of vitamin D_3, have been demonstrated in a number of cell types [17–19] and is also associated with increased expression of p21$^{Waf1/Cip1}$ and p27^{Kip1} [20, 21]. Suppression of tumor growth was not observed in tumors lacking the VDR, suggesting that this effect is receptor

Abbreviations used: AP, alkaline phosphatase; cdk, cyclin-dependent kinase; VDR, vitamin D receptor.

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mediated. The VDR, present in "classical" vitamin D-responsive organs such as bone, kidney, and intestine, has also been localized in a variety of other normal tissues and several cancer cell lines [22] including the Caco-2 cell line [23]. In various cancer cell lines it has been shown that butyrate and dihydroxycholecalciferol act synergistically in reducing proliferation and enhancing differentiation of neoplastic cells [24–26].

Here, we present evidence that increased $p21^{Waf1/Cip1}$ expression induced by butyrate is at least in part due to upregulation of VDR expression in the human colon cancer cell line Caco-2.

MATERIALS AND METHODS

Cell culture. The human colorectal cancer cell line Caco-2 was obtained from German Cancer Research Center (Heidelberg). The stock was maintained in 175 cm^2 flasks in a humidified incubator at 37°C in an atmosphere of 95% air and 5% CO_2. Caco-2 cells of passages 45–54 were grown in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% sodium pyruvate, and 1% nonessential amino acids. Cells were passaged using Dulbecco's PBS containing 0.25% trypsin and 1% EDTA. The medium was changed three times per week. The cells were screened for possible contamination with mycoplasma in monthly intervals. For experiments, cells were seeded on plastic cell culture wells in serum-containing medium and allowed to attach for 24 h. Prior to treatment, the cells were synchronized in medium containing 1% FCS.

1,25-Dihydroxycholecalciferol (Sigma, Deisenhofen, Germany) was dissolved in ethanol (final maximal concentration of ethanol in medium was 0.1%) to yield a 10^{-3} mol/L stock solution which was stored at −20°C. Butyrate (Merck-Schuchardt, Munich, Germany) was dissolved in PBS (final maximal concentration of PBS in medium was 0.1%). Tributyrin was dissolved in DMSO to yield a 30% (v/v) stock solution. The solution was vigorously mixed and freshly prepared every day. To assess whether the solvents may influence the experimental conditions, control cells were treated with either 0.1% ethanol, 0.1% PBS or with DMSO in concentrations used in stock solution. No difference was observed. The medium of treated and control cells was changed every day.

Cell differentiation. Alkaline phosphatase (AP) activity was used to assess differentiation of Caco-2 cells. For the assay, the cells were washed with cold PBS, scraped, sonicated (2 × 5 s) and centrifuged at 10000 rpm for 10 min. AP activity in the supernatant was measured by hydrolysis of *p*-nitrophenyl phosphate at pH 9.8 and 25°C (Ecoline Alkaline Phosphatase Assay, Merck, Darmstadt, Germany). Cellular protein was determined by Coomassie blue assay using a commercial kit (Bio-Rad Laboratories GmbH, Munich, Germany). Enzyme activity was expressed as milliunits per milligram of protein, one unit representing the enzyme activity hydrolyzing 1 μmol of substrate per min.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Caco-2 cells were cultured in the presence of 5 or 500 μmol/L tributyrin for 12 h. Total RNA was isolated by Rneasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions, and quantified by measuring the absorbance at 260 nm. RT-PCR was performed as described [27]. RT and PCR primers were deduced from VDR sequence [28]. After DNase digestion, 2 μg of total RNA were reverse transcribed into cDNA using oligo d(T)-primer. After cDNA synthesis, PCR amplification was performed using the cDNA template with the specific primer pair (F1, 5′-GCCCACCACAAGACCTAT-3′ and R2, 5′-CCTTTTGGATGCTGTAACTG-3′). The amplification profile consisted of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s after a five-min denaturation step at 94°C in a Perkin Elmer Gene Amp PCR System 9600. The amplified products (50 cycles, 297 bp) were detected by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining and ultraviolet transillumination. As control, β-actin mRNA was used, resulting in a PCR product of 405 bp. The optical densities of the PCR products were analyzed by a commercially available computed program (Bio-1 D V.96., Vilber Lourmat, France), normalized for the density of β-actin, and verified by sequencing.

Nuclear extraction. Cells were washed twice with PBS containing 100 μM of sodium vanadate, scraped into tubes and centrifuged for 10 s. The pellet was rinsed with 400 μl of buffer A (10 mM Hepes-KOH pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF, 1 mM sodium vanadate) and then centrifuged for 20 s at +4°C (3500 rpm). The resulting pellet was again rinsed with 400 μl of buffer A containing 0.1% NP-40, incubated on ice for 15 min and then centrifuged for 5 min at +4°C (3500 rpm). After resuspending the pellet in 100 μl of a high salt buffer C (20 mM Hepes-KOH pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 1 mM sodium vanadate), incubating on ice for 20 min and centrifuging for 5 min at +4°C (12,000 rpm), protein of the resulting supernatant (nuclear extract) was determined by Coomassie blue assay.

SDS–polyacrylamide gel electrophoresis and immunoblot analysis. Cells were washed twice with ice-cold PBS and lysed in protein lysis buffer (Biolabs, Beverly, USA) containing protease inhibitors (Boehringer Mannheim, Germany). Protein extracts were obtained after sonication of cell lysates 2 × 5 s and centrifugation at 10000 rpm at +4°C. Protein content was again quantified with the Bio-Rad protein colorimetric assay. After addition of sample buffer to the whole cellular or to the nuclear extract and boiling samples at 95°C for 5 min 18 μg of total protein lysate ($p21^{Waf1/Cip1}$/$p27^{Kip1}$ blot) or 60 μg of nuclear protein (VDR blot) were separated on a 15% ($p21^{Waf1/Cip1}$/$p27^{Kip1}$) or on a 10% SDS–polyacrylamide gel (VDR). Proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) and the membrane was blocked overnight at +4°C with 3% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). Next, blots were washed and incubated 1 h in TBS-T with a 1:500 dilution of primary antibody for $p21^{Waf1/Cip1}$ (Oncogene, Cambridge, UK) or for VDR (Santa Cruz Biotechnologies, Santa Cruz, CA) or one h in 3% skim milk in TBS-T with a 1:1000 dilution of primary antibody for $p27^{Kip1}$ (Santa Cruz Biotechnologies, Santa Cruz, CA). The secondary, horseradish peroxidase-conjugated antibodies (Vector-Lab., Burlingame, USA) were diluted at 1:2000 and incubated with the membrane for another 30 min. After chemoluminescence reaction (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK), bands were detected after exposure to Hyperfilm-MP (Amersham International Plc, Buckinghamshire, UK). Blots were reprobed with actin antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). For quantitative analysis, bands were detected and evaluated densitometrically by ProViDoc system (Desaga, Wiesloch, Germany), normalized for the density of β-actin.

RESULTS

Cell differentiation. 1,25-Dihydroxycholecalciferol [10^{-6} mol/L], butyrate [2 mmol/L], tributyrin [1 mmol/L], and the combination of tributyrin with 1,25-dihydroxycholecalciferol all stimulated differentiation in Caco-2 cells 2–10-fold in comparison to spontaneous differentiation occurring in nontreated cells (Fig. 1). Whereas 1,25-dihydroxycholecalciferol increased AP activity only moderately, butyrate and tributyrin were more potent inducers of cell differentiation. Simultaneous treatment of Caco-2 cells with tributyrin and 1,25-dihydroxycholecalciferol resulted in a synergistic amplification of AP activity.

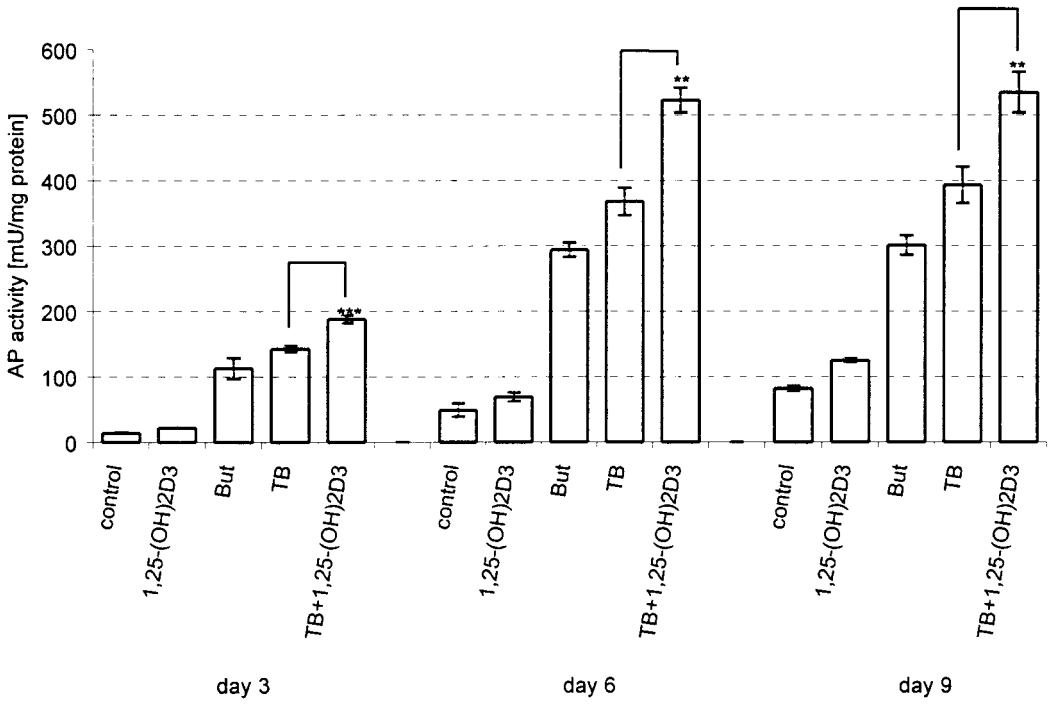

FIG. 1. Effect of butyrate, tributyrin, 1,25-dihydroxycholecalciferol, and tributyrin + dihydroxycholecalciferol on cell differentiation as assessed by AP activity. Caco-2 cells were treated daily with medium supplemented with 1 μmol/L dihydroxycholecalciferol, 2 mmol/L butyrate, 1 mmol/L tributyrin, or with medium supplemented with the combination of 1 mmol/L tributyrin and 1 μmol/L dihydroxycholecalciferol. Values are expressed in milliunits of AP activity per milligram cellular protein, and are means ± SD, n = 3. ** $P < 0.01$; *** $P < 0.001$.

VDR expression. As assessed by RT-PCR, butyrate enhanced VDR expression in a dose-dependent manner (Fig. 2). Incubation of Caco-2 cells for 12 h with a butyrate prodrug, tributyrin, in two different concentrations ([5 × 10^{-6} mol/L] and [5 × 10^{-4} mol/L]) increased VDR mRNA by 70% and 150%, respectively.

Western blot analysis confirmed this result after incubating Caco-2 cells with butyrate [2 mmol/L] for 48 h, increasing VDR protein content in the nucleus by 30% (Fig. 2).

$p21^{Waf1/Cip1}$ expression. To examine the effect on the cell cycle inhibitor $p21^{Waf1/Cip1}$, Caco-2 cells were incubated with 1,25-dihydroxycholecalciferol [10^{-6} mol/L], butyrate [2 mmol/L], or their combination. Western blot analysis (Fig. 3) revealed that 1,25-dihydroxycholecalciferol and butyrate alone caused a significant increase in the cdk inhibitor $p21^{Waf1/Cip1}$ after 24 h incubation. Combined treatment of Caco-2 cells with butyrate and 1,25-dihydroxycholecalciferol resulted in an amplification of the increase in $p21^{Waf1/Cip1}$, which was higher than that expected if the effects were additive.

$p27^{Kip1}$ expression. To investigate the effect on $p27^{Kip1}$, Caco-2 cells were grown under standard conditions or incubated with medium containing 1,25-dihydroxycholecalciferol [10^{-6} mol/L], butyrate [2 mmol/L], or both, and cells were harvested after 24 or 48 h. Western blots (Fig. 4) demonstrated that 1,25-

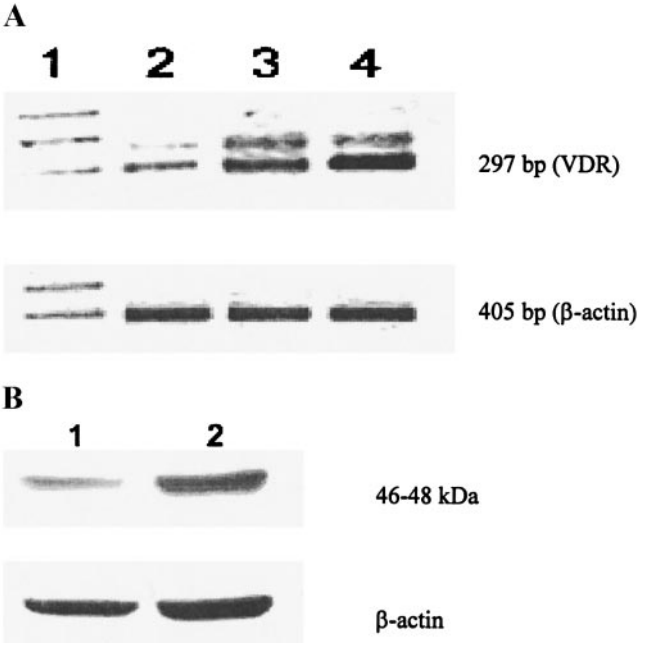

FIG. 2. Effect of tributyrin on VDR expression. (A) Caco-2 cells were grown for 12 h in serum-free medium in the absence (lane 2) or presence of tributyrin [5 μmol/L] (lane 3) or tributyrin [500 μmol/L] (lane 4). Total RNA from each cell culture was analyzed by RT-PCR for VDR-mRNA content. Lane 1, marker (100 bp). (B) Caco-2 cells were treated for 48 h with 2 mmol/L butyrate (lane 2) or with PBS only (lane 1) and then harvested for Western blot analysis. The band at 46–48 kDa corresponds to the VDR protein.

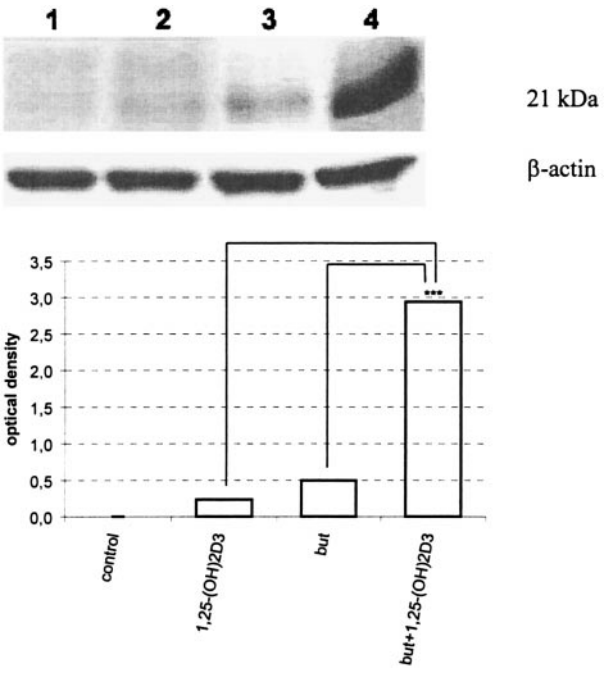

FIG. 3. Effect of butyrate on $p21^{Waf1/Cip1}$ expression in Caco-2 cells treated with 1,25-dihydroxycholecalciferol [1 μmol/L] (lane 2), butyrate [2 mmol/L] (lane 3), or dihydroxycholecalciferol + butyrate (lane 4) for 24 h. Control cells (lane 1) were treated with solvents only. The band at 21 kDa corresponds to the $p21^{Waf1/Cip1}$ protein. *** $P < 0.001$.

dihydroxycholecalciferol caused a time-dependent increase in $p27^{Kip1}$ (up to 120 and 180% after 24 and 48 h, respectively vs 100% (control)), whereas butyrate treatment resulted in a more prominent increase after 24 h (up to 200% after 24 and 48 h vs control). After incubation of Caco-2 cells with butyrate + 1,25-dihydroxycholecalciferol, $p27^{Kip1}$ expression increased in an approximately additive manner only (up to 230% [24 h] and 300% [48 h] vs 100%).

DISCUSSION

Our study shows that butyrate (in the form of its prodrug, tributyrin) combined with 1,25-dihydroxycholecalciferol acts synergistically in inducing differentiation of Caco-2 cells. The most likely mechanism of this effect is the dose-dependent upregulation of VDR by butyrate and subsequent enhanced binding of 1,25-dihydroxycholecalciferol to Caco-2 cells. We also demonstrated that combination of butyrate and dihydroxycholecalciferol acts synergistically in upregulating the expression of $p21^{Waf1/Cip1}$, but not $p27^{Kip1}$, in Caco-2 cells.

Although in various cancer cell lines it has been previously shown that butyrate and dihydroxycholecalciferol, if given simultaneously, have synergistic antiproliferative and differentiation inducing actions [24–26], the exact mechanisms of this effect have still not been elucidated in full. Recent data suggest that expression of calretinin, a calcium-binding protein possibly involved in maintaining the undifferentiated phenotype of WiDr cells, is potently downregulated by butyrate. Furthermore, butyrate as well as agents elevating $[Ca^{2+}]_i$ diminish the interaction of calretinin with the cytoskeleton [29]. The study by Nathan *et al.* [30] demonstrates that both butyrate and dihydroxycholecalciferol alter lipid dynamics of the cell membrane and membrane fluidity, occurring concomitantly with cell differentiation. Our own study may reveal another possible mechanism of potent and synergistic action of these two well-known differentiation inducing agents in colon cancer cells—that butyrate enhances the action of dihydroxycholecalciferol by increasing the expression of its receptor.

Both butyrate and dihydroxycholecalciferol arrest cell cycle in G_1, the effect of the latter being related to the expression level of its receptor [9]. In this study, administration of the dihydroxycholecalciferol analogue EB1089 increased the protein level of the cdk inhibitor $p27^{Kip1}$ and markedly enhanced the binding of $p27^{Kip1}$ with cdk2 in comparison to nontreated cells. Whereas this study suggests strong mutual relationship between dihydroxycholecalciferol-induced cell differentiation and $p27^{Kip1}$ expression, our data rather suggest that $p21^{Waf1/Cip1}$, and not $p27^{Kip1}$, is the most

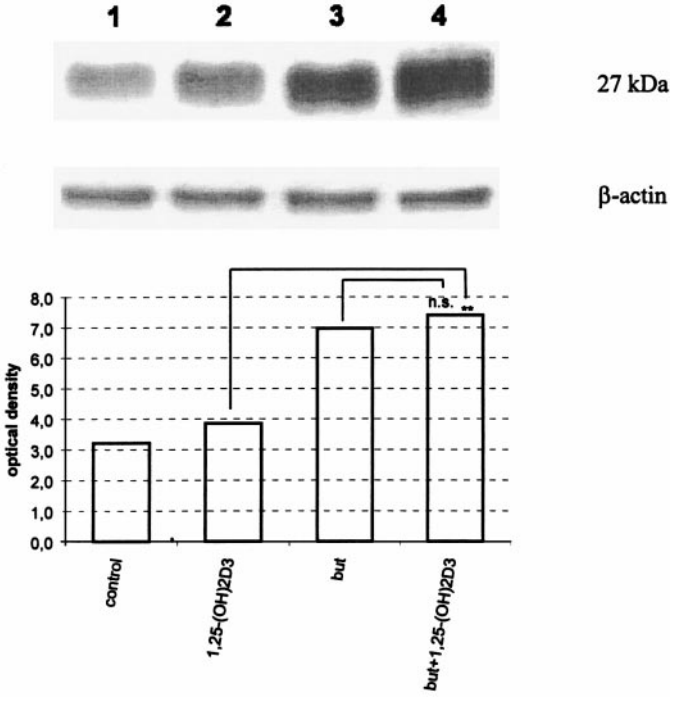

FIG. 4. Effect of butyrate on $p27^{Kip1}$ expression in Caco-2 cells treated with 1,25-dihydroxycholecalciferol [1 μmol/L] (lane 2), butyrate [2 mmol/L] (lane 3), or dihydroxycholecalciferol + butyrate (lane 4) for 24 h. Control cells (lane 1) were treated with solvents only. The band at 27 kDa corresponds to the $p27^{Kip1}$ protein. ** $P < 0.01$; ns, not significant.

important negative regulator of cell cycle when the cells are forced to differentiate by butyrate and dihydroxycholecalciferol in combination. Simultaneous increase in $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ could be observed during the normal differentiation process of enterocytes [3, 4]. Moreover, $p27^{Kip1}$ expression was limited to quiescent cells of the mid- to upper villus, implying that $p27^{Kip1}$ is indeed involved in the differentiation of normal enterocytes [31]. In contrast, our recent data showed that upregulation of $p27^{Kip1}$ may rather be related to apoptosis than to differentiation in colon cancer cells, as the latter was shown to be clearly suppressed by mevastatin [7]. The expression of $p27^{Kip1}$ is regulated mainly at the post-translational level via ubiquitin-proteasome mediated proteolysis [6]; the mechanisms of this regulation may differ in normal and neoplastic cells, as well as in different neoplastic cell populations in culture. While $p21^{Waf1/Cip1}$ is clearly involved in spontaneous and induced differentiation of Caco-2 cells, $p27^{Kip1}$ may have other functions more related to the induction of apoptosis in this cell line [7].

It may be concluded from this study that butyrate exerts, at least in part, its differentiation-inducing effect in Caco-2 cells by an upregulation of VDR. This is followed by a selective stimulation of $p21^{Waf1/Cip1}$ expression, which may provide an useful therapeutic approach in chemoprevention and treatment of colorectal cancer by the two nutrients occurring naturally in human diet. The improved bioavailability of butyrate in the form of tributyrin may create an opportunity for its possible therapeutic and chemopreventive applications, especially if synergy with dihydroxycholecalciferol and its analogs can be demonstrated *in vivo*, confirming *in vitro* studies.

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