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ARTICLES

The TGFβ/Smad 3-Signaling Pathway Is Involved in Butyrate-Mediated Vitamin D Receptor (VDR)-Expression

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Abstract Previously, we demonstrated the pivotal role of the vitamin D receptor (VDR) in mediating the butyrate-induced differentiation in colon cancer cells. Smad 3, a downstream component of transforming growth factor-β (TGFβ) signaling, has been shown to act as a coactivator of VDR and to possibly regulate the vitamin D signaling pathway. In this study, we demonstrate a distinct impact of the TGFβ/Smad 3-signaling pathway in the butyrate-mediated VDR expression and induction of differentiation. Butyrate treatment resulted in a significant induction of the phosphorylation level of Smad 3, while the combination of butyrate and a specific TGFβ1-antibody or a TGFβ-receptor inhibitor considerably diminished the butyrate-induced upregulation of VDR expression. Using a specific inhibitor, we were also able to demonstrate an involvement of the p38 MAPK in the increase of Smad 3 phosphorylation following butyrate treatment, thus opening the view to further elucidate possible mechanisms mediating the upregulation of VDR expression following butyrate treatment in colon cancer cells. J. Cell. Biochem. 102: 1420–1431, 2007.

Key words: butyrate; short chain fatty acids; vitamin D receptor; transforming growth factor-β; Smads; cellular differentiation; colon cancer cells

The short chain fatty acid butyrate is a normal constituent of the colonic luminal contents, formed by bacterial fermentation of unabsorbed complex carbohydrates in the digestive tract [Wachtershauser and Stein, 2000]. It serves as a primary source of energy for the colonic mucosa showing growth promoting effects on normal colonic epithelial cells in vivo and in vitro [Milovic et al., 2000]. In contrast, in a wide variety of neoplastic cells, butyrate reveals growth-arresting effects and induces apoptosis and differentiation of cultured cells at millimolar concentrations [Scheppach et al., 1995; Wachtershauser and Stein, 2001]. Regarding to its mechanism, butyrate has been revealed to mediate its effects by altering the expression of a variety of genes by hyperacetylation of histones via inhibition of histone deacetylases [Christman et al., 1980] and by causing hypomethylation [Parker et al., 1986] or hypermethylation [Tanaka et al., 1989]. Butyrate and 1,25-$(OH)_2D_3$ show synergistic actions concerning the induction of cellular differentiation and the reduction of cell growth [Yoneda et al., 1984; Yoshida et al., 1992; Gaschott et al., 2001a] in several neoplastic cell lines including the human colon cancer cell line Caco-2 [Yoneda et al., 1984; Yoshida et al., 1992; Gaschott et al., 2001b,c]. 1,25-$(OH)_2D_3$ was also shown to induce Smad 2 and 3

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phosphorylation in several cell lines including the human promyelocytic leukemia cell line, HL-60 [Cao et al., 2003]. It was further indicated that $1,25(OH)_2D_3$ induces phosphorylation of Smad proteins indirectly through a mechanism dependent on transforming growth factor-β (TGFβ)[Cao et al., 2003].

Previously, we demonstrated that the butyrate-induced cell differentiation in colon cancer cells is mediated by the vitamin D receptor (VDR) [Giuliano et al., 1991; Segaert and Bouillon, 1998; Daniel et al., 2004]. Besides its expression in classical vitamin D-responsive organs, such as bone, kidney, and intestine, the VDR has also been shown to be localized in nonclassical vitamin D target tissues like the skin and several cancer cell lines including Caco-2 [Schrader et al., 1994; Jones et al., 1998]. Following ligand binding, the VDR forms homodimers or dimerizes with other nuclear receptors such as retinoid receptor (RXR) to form heterodimers thus opening the possibility to initiate or inhibit gene expression via binding to vitamin D-responsive elements resulting in an interaction with the general transcription apparatus [Jones et al., 1998; Sunn et al., 2001]. However, the signaling interactions regulating VDR expression are far from being completely understood.

The TGFβ superfamily, comprising TGFβs, bone morphogenetic proteins (BMPs), activins and related proteins, regulate cell function and have key roles in development, cellular differentiation, apoptosis and carcinogenesis [Hoodless et al., 1996; Itoh et al., 2000; Massague, 2000; Derynck and Zhang, 2003]. They exert their effects through heteromeric receptor complexes consisting of type I and type II serine/threonine kinase receptors. Following ligand binding, the type II receptor phosphorylates the type I receptor to activate it. Intracellular signaling downstream of these receptor complexes is mediated by the recently identified Smad family [Hoodless et al., 1996; Heldin et al., 1997; Itoh et al., 2000; Massague, 2000; Derynck and Zhang, 2003]. Smads have been revealed to be directly phosphorylated and activated by TGFβ receptors type I. TGFβ and activin receptors phosphorylate Smad 2 and 3, whereas Smads 1,5, and 8 are phosphorylated by BMP receptors [Nakao et al., 1997; Itoh et al., 2000; Miyazono, 2000; Moustakas et al., 2001; Derynck and Zhang, 2003]. The signals for TGFβ are mediated by Smad 2 and 3, whereas Smad 1, 5, and 8 are specific for the signal transduction of BMP signals. Additionally to this pathway-restricted Smads, Smad 4 is necessary for the functional heterooligomerization with the pathway-restricted Smads. Upon heteroligomerization with Smad 4, these complexes move into the nucleus where they act as coactivators and/or DNA-binding transcription factors. In contrast to these positive transducers of TGFβ/BMB signaling, inhibitory Smads (Smads 6 and 7) have been identified, which bind directly to the TGFβ type I receptors, thus resulting in an interference with the phosphorylation of the pathway-restricted Smads and thereby inhibiting TGFβ/BMP-signalings. Smad 3 has been shown to act as a coactivator of the VDR and to possibly regulate the vitamin D signaling pathway [Yanagi et al., 1999; Yanagisawa et al., 1999; Subramaniam et al., 2001].

The Smad pathway may not be viewed at a unique mean for TGFβs to regulate cellular functions, as other signaling pathways including the mitogen-activated protein kinase (MAPK) can either be induced by TGFβ, or can modulate the outcome of TGFβ-induced Smad signaling. Smad phosphorylation by MAPKs as p38 MAPK may serve to regulate either Smad transcriptional activity or capacity to translocate into the cell nucleus [Javelaud and Mauviel, 2005]. Previously, we demonstrated that the butyrate induced upregulation of the VDR occurs via an increase of p38 MAPK phosphorylation indicating a possible interplay of these signaling pathways [Daniel et al., 2004].

To address the complex interactions of the Smad/TGFβ-signaling being involved in the induction of VDR-expression following butyrate treatment, we used both, a selective TGFβ1-antibody and the specific inhibitor SB431542 which was identified as an inhibitor of activin receptor-like kinase (ALK)5 (the TGFβ type I receptor) resulting in a block of phosphorylation of Smad 2 and 3 in response to activin or TGFβ [Inman et al., 2002]. To further analyze the previously demonstrated role of the p38 MAPK pathway in this multistep signaling system, we also used the specific p38 MAPK inhibitor SB203580.

Herein, we assessed the role of the TGFβ/Smad 3-signaling pathway in the butyrate-mediated upregulation of VDR expression via p38 MAPK activation followed by phosphorylation of Smad 3 in the human colon cancer cell

line Caco-2. To further address the complex interplay of the vitamin D and TGFβ-signaling systems, we also assessed the impact of Smad 3 in the known calcitriol and butyrate synergy to induce Caco-2 cell differentiation.

MATERIALS AND METHODS

Chemicals and Supplies

Disposable cell culture ware was purchased from Nalge Nunc International (Wiesbaden, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), sodium pyruvate, nonessential amino acids, and PBS were obtained from GIBCO BRL (Eggenstein, Germany), penicillin/streptomycin was from Biochrom (Berlin, Germany).

Butyrate (Merck-Schuchardt, Munich, Germany) was dissolved in PBS (final maximum concentration of PBS in medium was 0.1% (v/v)). Control experiments with either 0.1% DMSO, 0.1% PBS, or 0.1% ethanol excluded effects of the solvents on the results of our investigations. Calcitriol ($1,25(OH)_2D_3$, Biomol, Hamburg, Germany) was dissolved in ethanol and used at a concentration of 1 μmol/L. The medium of treated and control cells was changed every day. The TGFβ1-antibody (Sigma, Deisenhofen, Germany) was dissolved in PBS and used at a concentration of 40 μg/ml. SB431542, a potent and selective inhibitor of transforming growth factor superfamily type 1 activin receptor like kinase, was used to block TGFβ1-receptor function, the final application was performed at a concentration of 10 μmol/L, dissolved in DMSO. The specific p38 MAPK inhibitor SB203580 was dissolved in DMSO and used at a concentration of 20 μmol/L.

Cell Culture

The human colon cancer cell line Caco-2 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in a humidified incubator at 37°C in an atmosphere of 95% air and 5% CO_2. The Caco-2 cells of passages 18–22 were cultured in DMEM supplemented with 10% FCS, 1% penicillin/streptomycin, 1% sodium pyruvate, and 1% nonessential amino acids. The medium was changed three times per week. The Caco-2 cells were passaged weekly using Dulbecco's PBS containing 0.25% trypsin and 1% EDTA. Screening of the cells for possible contamination with mycoplasma was performed at monthly intervals using the VenorGem Mycoplasma detection kit (Minerva Biolabs, Germany).

Cytotoxicity

Cytotoxicity was excluded by lactate dehydrogenase (LDH) release assay using a commercial kit (LDH kit, Roche, Germany).

Protein Extraction

Cellular protein extraction was performed using the Active Motif Nuclear cell extraction kit according to the manufacturer's instructions (Active Motif Nuclear extract kit, Rixensart, Belgium). Aliquots of the resulting extracts were analyzed for their protein content using the BioRad colorimetric assay according to the method of Bradford (BioRad Laboratories, Muenchen, Germany).

SDS–Polyacrylamide Gel Electrophoresis and Immunoblot Analysis

Caco-2 cells were seeded in 80 cm^2 flasks and were allowed to attach overnight and then exposed to the different substances for different time intervals ranging from 1 to 96 h. After the cells were washed three times with ice-cold PBS, they were incubated with cell lysis buffer (Cell signaling, Beverly MA) containing multiple protease inhibitors (Complete, Roche, Mannheim, Germany) for 20 min at 4°C. Protein extracts were obtained after sonication of cell lysates (2×5 s) and centrifugation at 10,000 rpm at +4°C (10 min). Protein was again quantified with the Bio-Rad protein colorimetric assay. After addition of sample buffer to the cellular or to the nuclear extract and boiling samples at 95°C for 5 min 40 μg of total protein lysate (phospho Smad 3), 30 μg of nuclear protein (VDR blot) or 20 μg of protein lysate (E-cadherin, phospho p38, p38) were separated on a 7.5% (E-cadherin), 10% (VDR, phospho Smad 3), or on a 12.5% (pp38/p38) SDS–polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell, Kassel, Germany) and the membrane was blocked overnight at +4°C with 3% skimmed milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T). The level of proteins was assayed using the primary antibodies for 1 h with agitation at room temperature. Immunoreactivity was demonstrated by enhanced chemiluminescence system (Amersham pharmacia biotech, Buckinghamshire, UK) using appropriate horseradish peroxidase

conjugated secondary antibodies (1:2,000). 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, the bands were detected with scanning densitometry, using a Desaga CabUVIS scanner and Desaga ProViDoc software (Desaga, Wiesloch, Germany).

Cell Differentiation

Alkaline phosphatase (AP) activity was used to analyze differentiation of Caco-2 cells. AP activity is one of a number of accepted surrogate markers of colon cell differentiation [Gum et al., 1987; Witt et al., 2001]. For the assay, attached growing cells were washed with ice-cold PBS, scraped, sonicated (2×5 s), and centrifuged at 10,000 rpm for 10 min at 4°C. AP activity in the supernatant was measured by hydrolysis of *p*-nitrophenyl phosphate as substrate according to the manufacturer's instructions (Merck, Darmstadt, Germany). Cellular protein content was assayed according to the method described by Bradford [1976]. Enzyme activity was expressed as milliunits per milligram of protein, one unit representing the enzyme activity hydrolyzing 1 μmol of substrate per minute.

Statistics

All data presented in this paper are mean values from three different experiments ±SD. Differences between two values were tested for statistical significance using the Student's t unpaired *t*-test (SigmaStat, SPSS, Chicago, IL). A *P*-value <0.05 was considered to be significant.

RESULTS

Influence of the Specific Transforming Growth Factor Superfamily Type 1 Activin Receptor Like Kinase Inhibitor (ALK) SB431542 and the TGFβ1-Antibody on Butyrate-Induced Cell Differentiation

Incubation with butyrate (3 mmol/L) caused time-dependently (time-intervals ranging from 48 to 96 h) a significant induction of cellular differentiation in Caco-2 cells (fivefold increase vs. control) as assessed by analysis of AP activity. Simultaneous treatment of cells with butyrate and the specific transforming growth factor superfamily type 1 activin receptor like kinase inhibitor SB431542 (10 μmol/L) diminished the butyrate-mediated induction of cell differentiation at all investigated time points with the effect being most prominent after 96 h of treatment ($P < 0.001$ vs. butyrate). In addition, parallel incubation of butyrate with a specific TGFβ1-antibody (40 μg/ml) did also significantly inhibit the induction of cell differentiation following butyrate treatment, while the effect became most clear after 96 h of treatment ($P < 0.01$ vs. butyrate). The specific inhibitor SB431542 as well as the TGFβ1-antibody alone had no impact on the induction of cell differentiation (Fig. 1A–C). To further address the role of TGFβ in inducing Smad 3 phosphorylation with regard on the known synergy of $1,25(OH)_2D_3$ and butyrate in inducing Caco-2 cell differentiation, we also used the combination of butyrate and $1,25(OH)_2D_3$ as well as SB431542. $1,25(OH)_2D_3$ prominently induced AP activity in a time-dependent fashion. The specific transforming growth factor superfamily type 1 activin receptor like kinase inhibitor SB431542 (10 μmol/L) was able to clearly reduce the seen induction of AP activity at all investigated time points (Fig. 2). In addition, the p38MAPK inhibitor SB203580 was also demonstrated to be involved in the

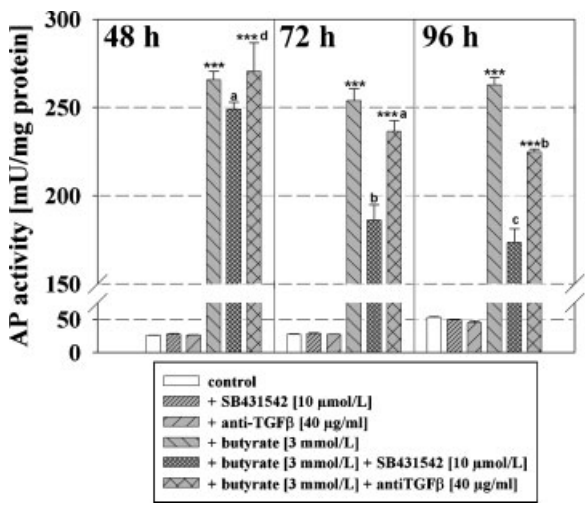

Fig. 1. Effect of butyrate, the activin receptor like kinase inhibitor SB431542, the specific TGFβ1-antibody as well as the combined application of butyrate and SB431542 or the TGFβ1-antibody, respectively, on differentiation of Caco-2 cells as assessed by analysis of alkaline phosphatase (AP) activity after 48, 72, or 96 h of treatment. Values are expressed in milliunits of AP activity per milligram cellular protein. Data are means ± SEM, n = 3. ***$P < 0.001$ versus control; a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$; d = nonsignificant versus butyrate.

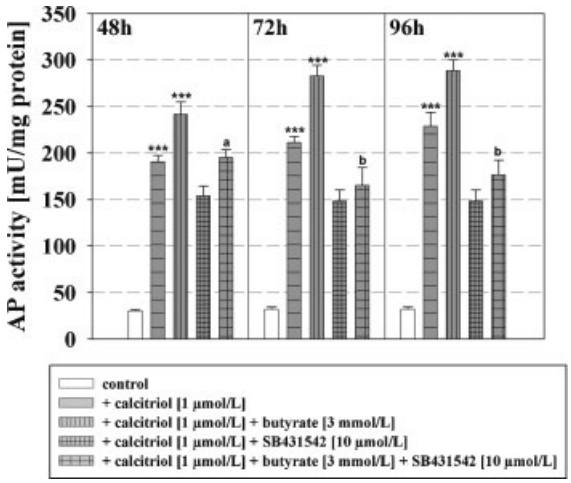

Fig. 2. Impact of the TGFβ/Smad 3-signaling pathway on differentiation of Caco-2 cells as assessed by analysis of alkaline phosphatase (AP) activity after 48, 72, or 96 h of treatment. Cells were treated with calcitriol, the combination of calcitriol and butyrate as well as each with the specific inhibitor SB431542. Values are expressed in milliunits of AP activity per milligram cellular protein. Data are means ± SEM, n = 3. ***$P < 0.001$ versus control; a = $P < 0.05$ versus calcitriol and butyrate.

observed increase in Caco-2 cell differentiation following treatment with the combined application of $1,25(OH)_2D_3$ and butyrate (Fig. 3).

In the next step, analysis of E-cadherin protein expression after 72 h (Fig. 4A,B) and 96 h (Fig. 4C,D) of treatment with butyrate and/

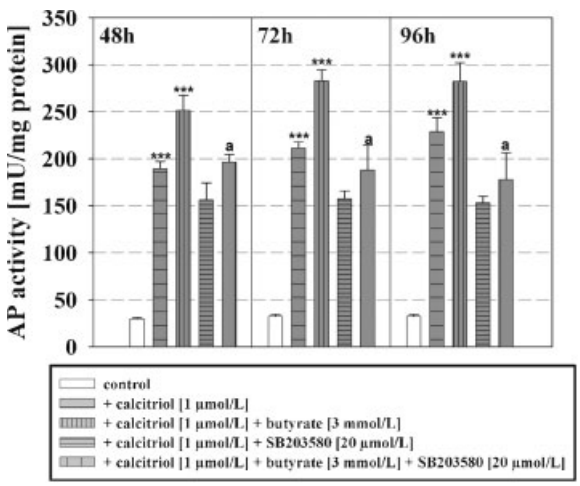

Fig. 3. Impact of the p38 MAPK on differentiation of Caco-2 cells as assessed by analysis of alkaline phosphatase (AP) activity after 48, 72, or 96 h of treatment. Cells were treated with calcitriol, the combination of calcitriol and butyrate as well as each with the specific p38 MAPK inhibitor SB203580. Values are expressed in milliunits of AP activity per milligram cellular protein. Data are means ± SEM, n = 3. ***$P < 0.001$ versus control; a = $P < 0.05$ versus calcitriol and butyrate.

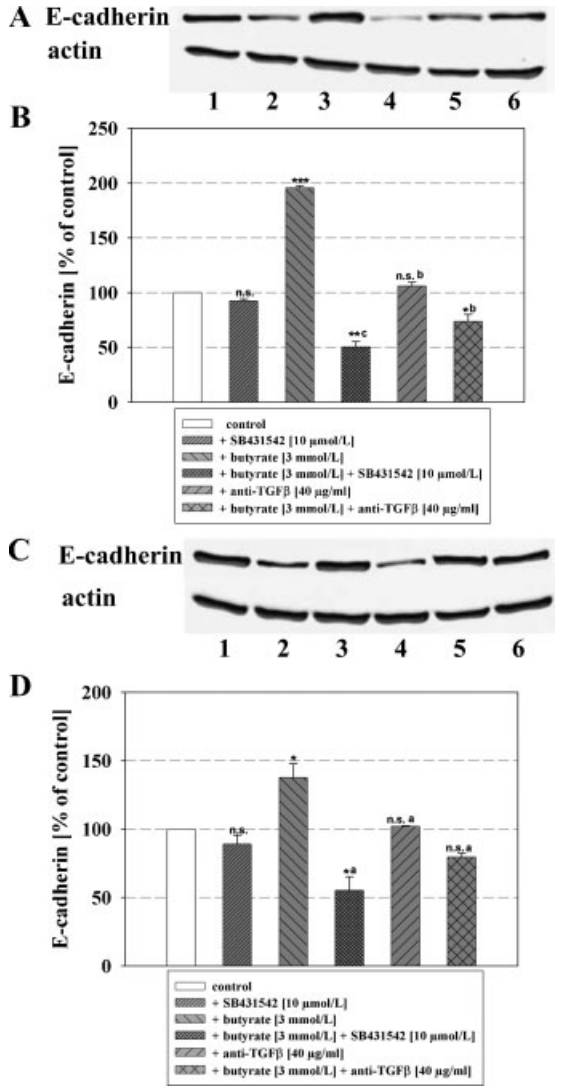

Fig. 4. E-cadherin protein expression by Western blot analysis after 72 h (**A**) or 96 h (**C**) of treatment following treatment with either control (**lane 1**), SB431542 (**lane 2**), butyrate (**lane 3**), butyrate and SB431542 (**lane 4**), the specific TGFβ1-antibody (**lane 5**), butyrate, and TGFβ1-antibody (**lane 6**). The graphs represent the densitometric evaluation of E-cadherin protein expression after 72 h (**B**) or 96 h (**D**) of treatment. Quantitative data are corrected for β-actin levels and are means ± SEM from three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.s. = nonsignificant versus control; a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$ versus butyrate.

or the specific inhibitor SB431542 or the TGFβ1-antibody confirmed the results seen from measuring AP activity. Butyrate significantly enhanced E-cadherin protein expression ($P < 0.001$ vs. control). In contrast, the combined application of butyrate and the specific ALK5-inhibitor SB431542 completely abolished the butyrate-induced upregulation of E-cad-

herin protein expression ($P < 0.001$ vs. butyrate). Similar effects could also be observed using the specific TGFβ1-antibody in combination with butyrate ($P < 0.01$ vs. butyrate), while treatment with SB431542 or the TGFβ1-antibody alone did not result in a significant change of E-cadherin protein expression (Fig. 4).

Effect of Butyrate, SB431542, and the TGFβ1-Antibody on the Phosphorylation Status of Smad 3

To assess the role of TGFβ/Smad 3-signaling in butyrate-mediated induction of differentiation in Caco-2 cells, we analyzed the phosphorylation status of Smad 3 by Western blot. In addition, we also used the combination of $1,25(OH)_2D_3$ to further address the impact of the TGFβ and Smad 3-signaling pathway mediating cellular differentiation.

As shown in Figure 5A,B, incubation of Caco-2 cells with butyrate resulted in a significant increase of phosphorylation of Smad 3 protein after 2 h of treatment (195% of control, $P < 0.01$). The combination of butyrate and the specific TGFβ1-antibody totally abolished the butyrate-mediated upregulation of the phosphorylation of Smad 3 (74% of control, $P < 0.01$ vs. butyrate). Incubation of Caco-2 cells with the TGFβ1-antibody alone also caused a moderate decrease of the phosphoryation status of Smad 3 to 68% of control level ($P < 0.05$ vs. control).

Figure 5C,D reveals the influence of the specific TGFβ I receptor inhibitor SB431542 on butyrate-induced phospho Smad 3 up regulation. The combined incubation with butyrate (3 mmol/L) and SB431542 (10 μmol/L) caused a complete inhibition of the butyrate-mediated increase of phosphorylation of Smad 3 ($P < 0.01$

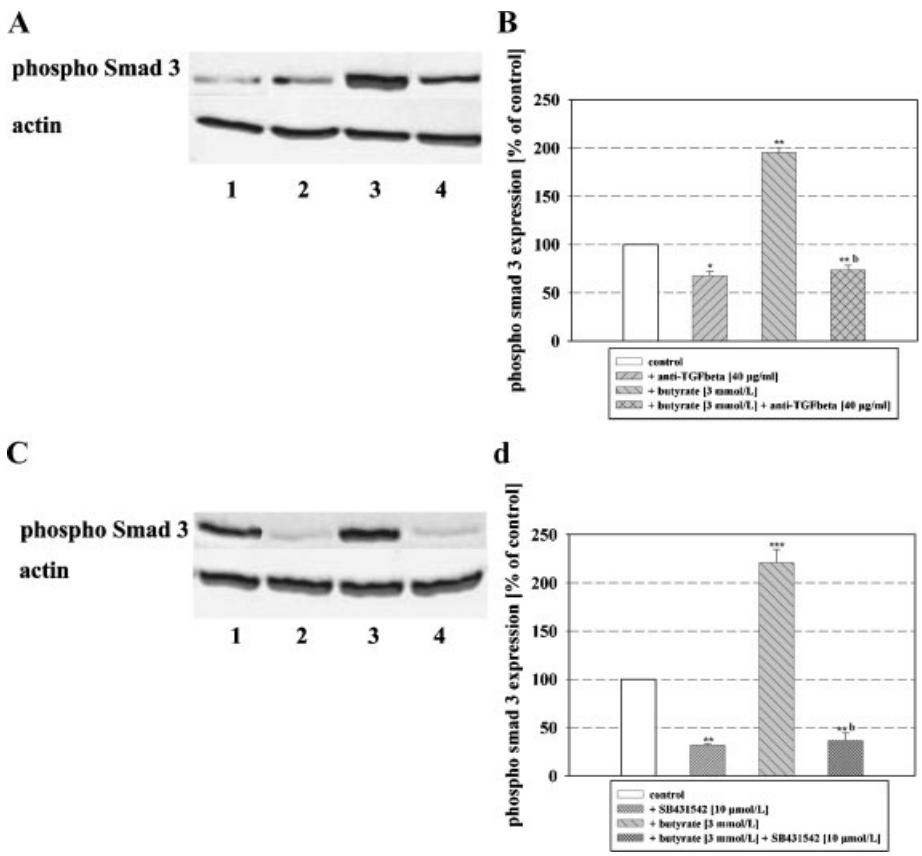

Fig. 5. Western blot analysis of phosphorylation status of Smad 3 after 2 h of treatment (**A**) with either the TGFβ1-antibody (**lane 2**), butyrate (**lane 3**), butyrate, and TGFβ1-antibody (**lane 4**). The graph shows the densitometric evaluation of phosphorylated Smad 3 (**B**). **C**: Phosphorylation of Smad 3 after 2 h of treatment with either SB431542 (**lane 2**), butyrate (**lane 3**), butyrate and SB431542 (**lane 4**), and the densitometric analysis, respectively (**D**). Quantitative data are corrected for β-actin levels and are means ± SEM from three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; n.s. = nonsignificant versus control; a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$ versus butyrate.

vs. butyrate) to 36% of control value after 2 h of treatment. SB431542 alone also significantly reduced the phosphorylation of Smad 3 in Caco-2 cells after 2 h of treatment to 32% of control value ($P < 0.05$ vs. control). The combined application of $1,25(OH)_2D_3$ (1 μmol/L) and butyrate prominently induced the phosphorylation of Smad 3 (up to 294% of control, $P < 0.001$), while the additional use of the inhibitor SB431542 distinctly reduced the mediated-induction of Smad 3 phosphorylation to 77% of control value ($P < 0.001$ vs. control, Fig. 6).

Influence of the Inhibitor SB431542 and the TGFβ1-Antibody on VDR Protein Expression

As we have demonstrated previously [Daniel et al., 2004], incubation with butyrate resulted in a time- and dose-dependent induction of VDR-expression (fourfold increase vs. control, $P < 0.001$) in Caco-2 cells with the effect being most evident after 6 h of treatment. In the following set of experiments, we assessed the impact of SB431542 and the TGFβ1-antibody on the butyrate-mediated upregulation of VDR expression. Treatment of Caco-2 cells with the combination of butyrate and the TGFβ1-antibody in parallel abolished the butyrate-mediated induction of VDR-expression (butyrate: 296% vs. butyrate + anti-TGFβ1: 150% of control level, $P < 0.01$). In contrast, incubation with the TGFβ1-antibody alone had no impact on the VDR-expression level ($P > 0.05$ vs. control value, Fig. 7A,B).

Additionally, as shown in Figure 7C,D, the TGFβRI-inhibitor SB431542 also significantly reduced the upregulation of VDR protein expression caused by butyrate (butyrate: 415% vs. butyrate + SB431542: 236% of control value, $P < 0.01$), while SB431542 treatment alone did not result in any significant change of VDR

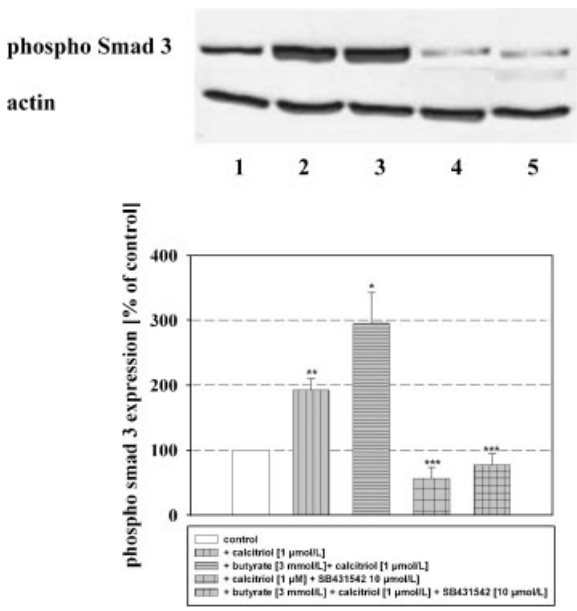

Fig. 6. Western blot analysis of phosphorylation status of Smad 3 after 2 h of treatment (**A**) with either calcitriol (**lane 2**), calcitriol and butyrate (**lane 3**), calcitriol, and SB431542 (**lane 4**), as well as the combination of calcitriol, butyrate and SB431542 (**lane 5**). The graph shows the densitometric evaluation of phosphorylated Smad 3 (**B**). Quantitative data are corrected for β-actin levels and are means ± SEM from three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s. = nonsignificant versus control.

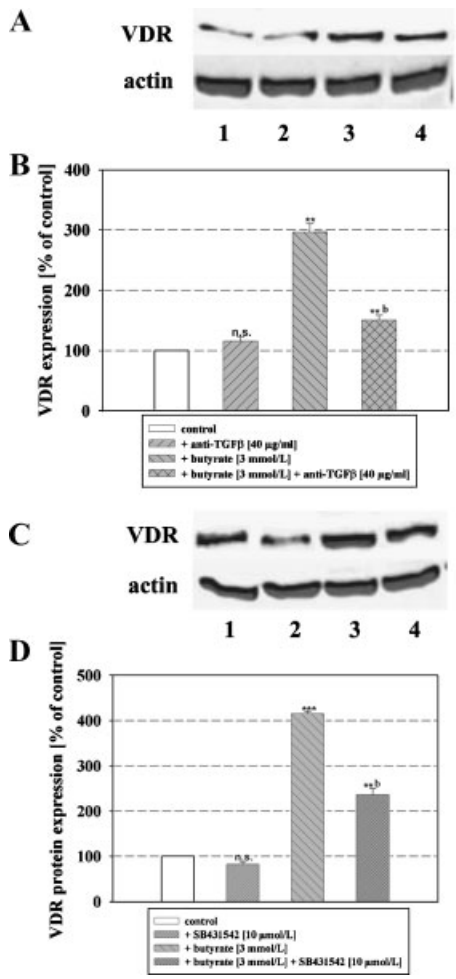

Fig. 7. Effect of the specific TGFβ1-antibody (**A**) and SB431542 (**C**), respectively, on butyrate-mediated VDR-protein expression after 6 h of treatment. One representative gel of three independent experiments is shown, densitometric analysis of the respective blots is shown in (**B**,**D**). Quantitative data are corrected for β-actin levels. $^{**}P < 0.01$; $^{***}P < 0.001$ versus control; n.s. = nonsignificant, b = $P < 0.01$ versus butyrate.

protein expression (82% of control level, $P > 0.05$).

Influence of the p38 MAPK Pathway on the Phosphorylation Level of Smad 3

To test in a further set of experiments, a possible involvement of the p38 MAPK pathway in the induction of Smad 3 phosphorylation following butyrate treatment the specific p38 MAPK inhibitor SB203580 was used. The combined application of butyrate and the specific p38 MAPK inhibitor SB203580 (20 μmol/L) completely abolished the butyrate-mediated induction to 38% of control value ($P < 0.001$ vs. butyrate) indicating that p38 MAPK might directly be involved in the regulation of phosphorylation of Smad 3 in the response to butyrate, while on the other hand the application of SB203580 alone did not cause any significant change in phosphorylation of Smad 3 (Fig. 8A,B). In this set of experiments, we also used the synergistic combination of butyrate and $1,25(OH)_2D_3$, which as revealed in Figure 5 significantly induced Smad 3 phosphorylation. The use of the specific p38 MAPK inhibitor clearly abrogated the seen increase of the phosphorylation status of Smad 3 to 69% of control value (Fig. 9).

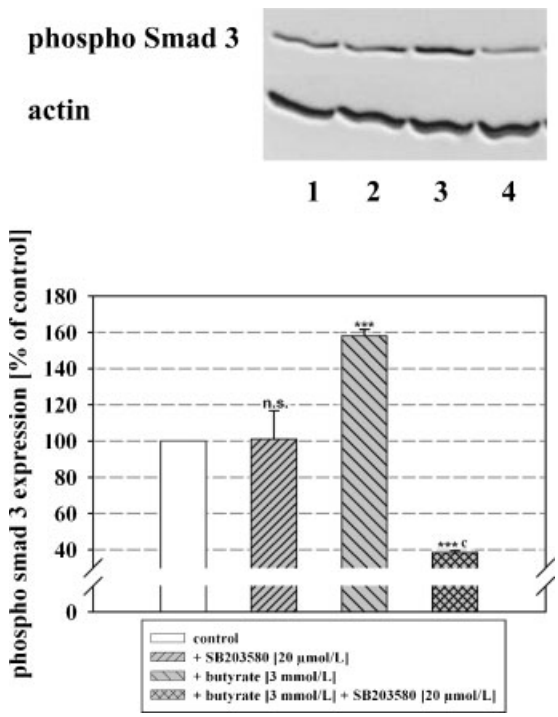

Fig. 8. Effect of the specific p38 MAPK inhibitor SB203580 on phosphorylation of Smad 3 as assessed by Western blot analysis (**A**) after 2 h of treatment with either SB203580 (**lane 2**), butyrate (**lane 3**), butyrate, and SB203580 (**lane 4**). Densitometric analysis is shown in (**B**). Quantitative data are corrected for β-actin levels and are means ± SEM from three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; n.s. = nonsignificant versus control; a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$ versus butyrate.

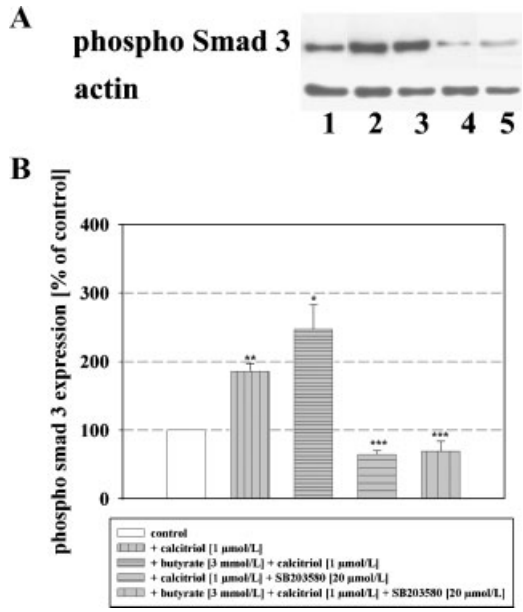

Fig. 9. Effect of the specific p38 MAPK inhibitor SB203580 on phosphorylation of Smad 3 as assessed by Western blot analysis (**A**) after 2 h of treatment with either calcitriol (**lane 2**), calcitriol and butyrate (**lane 3**), calcitriol and SB203580 (**lane 4**), as well as with the combination of calcitriol, butyrate, and SB203580 (**lane 5**). Densitometric analysis is shown in (**B**). Quantitative data are corrected for β-actin levels and are means ± SEM from three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; n.s. = nonsignificant versus control.

Effect of the ALK Inhibitor SB431542 on Phosphorylation of p38 MAPK

To further analyze the interrelationships in the signal transduction pathways mediating butyrate-induced VDR upregulation, we also investigated whether the TGFβI-receptor inhibitor SB431542 has any impact on the phosphorylation status of p38 MAPK. As demonstrated earlier, butyrate treatment resulted in a significant induction of p38 MAPK phosphorylation starting after 1 h of incubation (125% of control, $P < 0.001$). Here, we show the combined application the TGFβRI-inhibitor SB431542 did not cause any significant change in the induction of p38 MAPK phosphorylation in response to butyrate (125% of control, n.s. vs. butyrate, Fig. 10A,B).

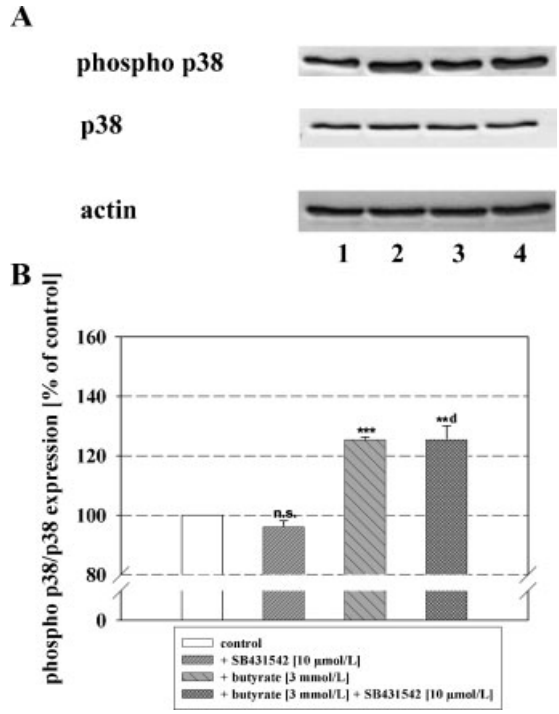

Fig. 10. Western blot of the phosphorylation level of p38 and p38 MAPK following treatment with either SB431542 (**lane 2**), butyrate (**lane 3**), or butyrate + SB431542 (**lane 4**) for 1 h. For both proteins one representative gel of three independent experiments is shown (**A**). Densitometric analysis of the phosphor p38/p38 protein ratio is represented in (**B**). Quantitative data are corrected for β-actin levels and are means ± SEM from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s. = nonsignificant versus control; a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$ versus butyrate.

DISCUSSION

In this study, we demonstrate a functional interplay between vitamin D-, p38 MAPK-, and TGFβ-signaling following Smad 3 phosphorylation to finally mediate butyrate-induced upregulation of VDR expression in Caco-2 cells.

Cell differentiation in response to butyrate is mediated via an upregulation of the VDR following p38 MAPK phosphorylation [Gaschott et al., 2001c; Daniel et al., 2004]. The lipophilic hormone vitamin D as well as the TGFβ have been shown to be key players in the regulation of cellular differentiation. Vitamin D was found to control transcription of target genes through the VDR [Schrader et al., 1994; Jones et al., 1998; Segaert and Bouillon, 1998]. Smad 3 was demonstrated to act as coactivator specific for ligand-induced transactivation of the VDR by forming a complex with a member of the steroid receptor coactivator-1 protein family in the nucleus, thus, Smad 3 may mediate the cross-talk between vitamin D and TGFβ signaling pathways [Yanagisawa et al., 1999]. In view of the complex interplay between vitamin D and TGFβ signalings, it is likely that other downstream components of the TGFβ-signaling pathway also modulate the transactivation function of VDR [Yanagi et al., 1999].

TGFβ is a multifunctional cytokine playing a pivotal role in the regulation of cellular differentiation. To examine the role of the TGFβ-signaling pathway in the butyrate-induced upregulation of VDR expression, we used SB431542 as well as a specific TGFβ1-antibody. Treatment with SB431542 or the TGFβ1-antibody abolished time-dependently the induction of cell differentiation by butyrate as documented by analysis of AP activity and E-cadherin protein expression. Regarding the intricate regulation of the relevant signaling pathways, in addition, the effect of SB431542 or the TGFβ1-antibody might be at least in part also butyrate or VDR independent, which might itself explain their effect on Caco-2 cell differentiation.

In a following step, we also used the combination of butyrate and $1,25(OH)_2D_3$, which results in an even more pronounced induction of cellular differentiation at all analyzed time points. As demonstrated for butyrate, also in the combined application form with calcitriol, the specific transforming growth factor superfamily type 1 activin receptor like kinase inhibitor SB431542 was able to significantly inhibit Caco-2 cell differentiation at all investigated time points. $1,25(OH)_2D_3$ has been indicated to induce an autocrine TGFβ-pathway in several different cell types [De et al., 1990; Cao et al., 2003].

In addition, we observed that coincubation of butyrate with the inhibitor SB431542 or the TGFβ1-antibody led to a significant reduction of the butyrate-induced increase of VDR expression. The involvement of the TGFβ-signaling pathway in the induction of VDR expression in response to butyrate was further demonstrated by a significant increase of Smad 3 phosphorylation following butyrate treatment. As already revealed in other investigations [Cao et al., 2003], $1,25(OH)_2D_3$ was able to distinctly enhance the phosphorylation status of Smad 3. The combination of $1,25(OH)_2D_3$ and butyrate was shown to most prominently induce phosphorylation of Smad 3, this induction was also clearly reduced using SB431542. In HL-60 cells $1,25(OH)_2D_3$ was revealed to

phosphorylate Smad 3 and to stimulate cell differentiation, which was distinctly abrogated using a neutralizing antibody to TGFβ, suggesting that $1,25(OH)_2D_3$ acts indirectly by activating signaling from either autocrine or paracrine TGFβ in these cells [Cao et al., 2003]. In this study, it was also clearified that reduction in the levels of Smad 3 phosphorylation was sufficient to reduce the commitment of these cells to differentiation. The authors concluded that these effects might also be independent of TGFβ, or possibly dependent on synergistic interaction of the VDR with Smad 3 to regulate and coordinate the expression of certain target genes containing both $1,25(OH)_2D_3$-response elements and Smad-binding elements, which has been demonstrated previously in other studies [Yanagisawa et al., 1999; Subramaniam et al., 2001].

In a previous study, we demonstrated a possible link between p38 MAPK activation and butyrate-induced cell differentiation via an upregulation of VDR expression [Daniel et al., 2004]. To assess the implication of the p38 MAPK pathway in the observed increase of Smad 3 phosphorylation caused by butyrate, we co-incubated the Caco-2 cells with the specific p38 MAPK inhibitor SB203580. SB203580 totally abolished the induction of Smad 3 phosphorylation in response to butyrate. This reduction in the phosphorylation level of Smad 3 using SB203580 could also be observed using the combined treatment regimen of $1,25(OH)_2D_3$ and butyrate. In contrast, combined treatment with the TGFβ I receptor SB4315342 and butyrate had no impact on the induction of p38 MAPK phosphorylation following butyrate treatment.

Also warranting the intricacies of the relevant signaling pathways, the above discussed data let us hypothesize a model of the possible mechanisms whereby Caco-2 cells might integrate a multiplicity of differentiation signals impinging on the cells simultaneously. This might open the view to better understand the butyrate-mediated induction of cell differentiation via upregulation of VDR expression through p38 MAPK activation resulting in Smad 3 phosphorylation in Caco-2 cells based on the following observations:

(1) Butyrate causes an increase of p38 MAPK phosphorylation thereby leading to Smad 3 phosphorylation.

(2) The VDR upregulation in response to butyrate depends on Smad 3 phosphorylation as inhibition of the TGFβ-signaling pathway completely abolished the induction of VDR expression and the observed increase of cell differentiation.

The biological response to TGFβ and probably in this manner also to butyrate seems to depend on the coordinated interplay of different signaling pathways. The ability to selectively manipulate a biological response to butyrate will therefore rely on our understanding of this interplay and the relative contribution of a specific pathway to complex cellular outcomes such as differentiation.

The complex interplay between the TGFβ- and p38 MAPK signaling pathway could recently also be demonstrated in vascular smooth muscle cells. Pharmacological inhibition of the kinase activity of TGFβRI using SB431542 completely blocked downstream phosphorylation events, including Smad and p38 phosphorylation, which led to an attenuation of TGFβ-dependent growth inhibition [Seay et al., 2005]. More specifically, pharmacological inhibition of the p38 pathway using SB203580 resulted in complete attenuation of the TGFβ-dependent growth inhibition.

However, several studies clearly indicated, that activation of the TGFβ-signaling pathway by MAPK activation is not only cell-type specific, but the activation of a given MAPK combination is also cell-type dependent [Javelaud and Mauviel, 2005]. Finally, it has also been pointed out that MAPKs, and in particular the p38 pathway, is involved in the control of post-translational modification of Smads [Ohshima and Shimotohno, 2003]. The activation of MAPK pathways may have positive or negative regulatory effects on Smads, depending on the nature of MAPK activation, which, in turn, may affect both the specificity and multiplicity of MAP kinase-dependent phosphorylation events [Javelaud and Mauviel, 2005].

The induction of cellular differentiation in response to butyrate have been revealed in a wide variety of tumor cells as well as in several in vivo models [Parker et al., 1986; Yoshida et al., 1992; Rocchi et al., 1998; Witt et al., 2001]. Effective cellular differentiation following butyrate treatment has been clearly shown for malignant cells from distinct tumor types

including hepatoma [Nakagawa et al., 1985], colon carcinoma [Augeron and Laboisse, 1984; Gum et al., 1987], neuroblastoma [Rocchi et al., 1998], and breast cancer [Graham and Buick, 1988], opening the view for a common mechanism mediating the differentiation inducing potential of butyrate. Thus, our described hypothesis regarding the induction of cell differentiation via upregulation of VDR expression through p38 MAPK activation followed by Smad 3 phosphorylation might hold true for several tumor cell lines.

To conclude, the data provided in this study might be supportive for our contention that the TGFβ/Smad3-signaling might play a distinct role in the regulation of butyrate's induction of cell differentiation, which might expand our understanding of the complex signaling interplay mediating butyrate-induced upregulation of VDR expression followed by p38 MAPK activation leading to Smad 3 phosphorylation.

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