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The dietary histone deacetylase inhibitor sulforaphane induces human β-defensin-2 in intestinal epithelial cells

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Summary

Antimicrobial peptides like human β-defensin-2 (HBD-2) play an important role in the innate immune system protecting the intestinal mucosa against bacterial invasion. The dietary histone deacetylase (HDAC) inhibitors sulforaphane (SFN) and butyrate have received a great deal of attention because of their ability to simultaneously modulate multiple cellular targets involved in cellular protection. In this study the influence of SFN and butyrate on HBD-2 expression as well as the molecular pathways involved in SFN-mediated induction of HBD-2 were scrutinized. Treatment of Caco-2, HT-29 and SW480 cells with SFN led to a time- and dose-dependent upregulation of HBD-2 mRNA expression as determined by semi-quantitative reverse transcription–polymerase chain reaction. Moreover, HBD-2 protein production increased in response to SFN, measured by enzyme-linked immunosorbent assay. Induction of HBD-2 was also observed in response to butyrate. Immunofluorescence analysis revealed that the protein was localized in the cytosol. Coincubation of SFN with a vitamin D receptor (VDR), or an extracellular-regulated kinase 1/2 or a nuclear factor-κB inhibitor all reduced HBD-2 mRNA upregulation. In contrast, transfection of cells with a dominant-negative peroxisome proliferator-activated receptor γ (PPARγ) mutant vector to inhibit PPARγ wild-type action and inhibition of p38 mitogen-activated protein kinase (MAPK) signalling did not affect SFN-mediated upregulation of HBD-2 mRNA. Moreover, SFN induced the expression of VDR, PPARγ and phosphorylated ERK1/2 but did not affect p38 MAPK activation. The data clearly demonstrate for the first time that the dietary HDAC inhibitor SFN is able to induce antimicrobial peptides in colonocytes. In this process HBD-2 expression is regulated via VDR, mitogen-activated protein kinase kinase/extracellular-regulated kinase and nuclear factor-κB signalling.

Keywords: β-defensin-2; innate immunity; MEK/ERK signalling pathway; sulforaphane; vitamin D receptor

Introduction

The gastrointestinal tract is constantly in contact with the many commensal microorganisms residing in the colon and distal small intestine. Although the presence of this native flora in general is mutually beneficial, the host also requires protection against these microorganisms. The role of antimicrobial peptides of the defensin family mediating

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindol; DMEM, Dulbecco's modified Eagle's medium; ECACC, European Collection of Cell Cultures; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBD-2, human β-defensin-2; HDAC, histone deacetylase; JNK, cJun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK/ERK, mitogen-activated protein kinase kinase/extracellular-regulated kinase; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PPARγ, peroxisome proliferator-activated receptor γ; SCFA, short-chain fatty acid; SFN, sulforaphane; VDR, vitamin D receptor.

protective responses has been established.[1–4] Disturbances in their expression in the intestinal tract have been linked to inflammatory bowel diseases.[5–8]

Defensins are small cationic peptides with a broad spectrum of antimicrobial activity having characteristic pairs of intramolecular disulphide bonds, a beta-sheet structure and a mass of 3000–5000.[9,10] They are classified as α- and β-defensins based on the position of three disulphide bridges.[11] The β-defensins are ubiquitously expressed throughout the gastrointestinal tract, including the colon.[2] Among the β-defensins, human β-defensin-1 (HBD-1) is constitutively expressed,[12,13] whereas HBD-2 is inducible in response to infection, proinflammatory mediators such as interleukin-1β, tumour necrosis factor-α, and probiotic bacteria.[14,15] Permeabilization of target membranes is the crucial step in defensin-mediated antimicrobial activity and cytotoxicity.[3] The positively charged defensin molecules are inserted into the bacterial membranes under the influence of cell-generated transmembrane potentials and local electrostatic fields, resulting in the cessation of RNA, DNA, and protein synthesis in bacteria.[3,16] Besides their direct antimicrobial effects, defensins exert further functions related to host defence such as the induction of histamine release by mast cells and chemoattraction of various cells of the immune system including neutrophils and T cells.[15,17] Moreover, they seem to be involved in carcinogenesis.[18–20]

The dietary histone deacetylase (HDAC) inhibitor sulforaphane (SFN) is one of the most biologically active phytochemicals in the human diet and is present at high concentrations in some cruciferous vegetables, especially broccoli (*Brassica oleracea*).[21] Sulforaphane has received a great deal of attention because of its ability to simultaneously modulate multiple cellular targets involved in cellular protection.[22,23] It is regarded as a highly promising dietary preventative agent because of its capacity to induce apoptosis and to inhibit cell proliferation in various tumour cells, including colorectal cancer.[21,22] In addition, several studies indicate that SFN exhibits immunomodulatory capacities by interfering with the actions of the proinflammatory transcription factor nuclear factor-κB (NF-κB) and by the modulation of the production of proinflammatory and anti-inflammatory cytokines.[24–26] Recent studies, including our own, report that HDAC inhibitors like butyrate and trichostatin A induce the expression of antimicrobial peptides in several colorectal cancer cells.[27–30] The aim of this work was to explore a possible role for SFN in the induction of HBD-2 expression as well as to scrutinize the molecular pathways involved in this regulatory process.

Materials and methods

Cell culture

The human colorectal cancer cell lines Caco-2, HT-29 and SW480 were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were cultured in a humidified incubator at 37° in an atmosphere of 95% air and 5% CO_2. Caco-2 and SW480 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids, 1% sodium pyruvate and 1% penicillin/streptomycin. HT-29 cells were grown in McCoy's 5A medium, supplemented with 10% FCS and 1% penicillin/streptomycin. Medium from the dominant-negative peroxisome proliferator-activated receptor γ (PPARγ) mutant and empty-vector HT-29 cells was supplied with 400 μg/ml Geneticin 418 sulphate (G418; Gibco-BRL, Eggenstein, Germany). Cells were regularly screened for *Mycoplasma* contamination using the VenorGem *Mycoplasma* detection kit (Minerva Biolabs, Berlin, Germany).

For experiments, cells were seeded in plastic cell culture wells and were cultivated in either DMEM or McCoy's 5A medium, as indicated above, until 80% confluency was reached. Media were then removed and replaced with media containing either the solvent, SFN (1–20 μM), butyrate (1–5 mM) or one of the combinations of SFN (10 μM) with the vitamin D receptor (VDR) inhibitor ZK191732 (10 μM), the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 (20 μM), the extracellular signal-regulated kinase (ERK) 1/2 inhibitor PD98059 (40 μM), or the NF-κB inhibitor helenalin (20 μM). In these experiments, cells were pretreated with the inhibitors for 5 hr, followed by challenge with SFN for up to 48 hr. Sodium butyrate (Merck–Schuchardt, Hohenbrunn, Germany) was dissolved in phosphate buffered saline (PBS), SFN (Axxora, San Diego, CA) was rendered soluble in *aqua ad iniectabilia* (DeltaSelect, Pfufflingen, Germany). PD98059, SB203580 and helenalin (all from Calbiochem, Schwalbach/Taunus, Germany) were dissolved in dimethyl sulphoxide (Fluka; Sigma-Aldrich-Chemie, Steinheim, Germany), ZK191732 (supplied by the Department of Medicinal Chemistry at Schering AG, Berlin, Germany) was dissolved in ethanol. The maximum concentration of solvents in each medium was kept below 0·1% volume/volume and the media were changed every day. Cells were then harvested at the times indicated in the figure legends. Concentrations of SFN and butyrate used in our experiments correspond to appropriate physiological concentrations.[31–33]

Transfection assay

The following plasmids were used for transfection: pcDNA3 (Invitrogen, Karlsruhe, Germany), as an empty-vector for control transfection, and the plasmid pcDNA3-PPARγ$_{L468A/E471A}$, a dominant-negative PPARγ double-mutant, which was kindly provided by V.K. Chatterjee (Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Cambridge, UK).[34] These constructs were transfected into subconfluent HT-29 cells

with lipofectamine 2000 (Invitrogen) in serum-free conditions. After 6 hr, the cells were supplied with fresh medium containing 10% FCS. Twenty-four hours later, the cells were supplied with medium containing G418 (400 μg/ml) and this G418-supplemented culture medium was replaced twice a week. G418-resistant colonies were collected and used for further analysis. Successful transfection of the dominant-negative PPARγ cell system was recently demonstrated.[35,36]

Cytotoxicity

Cytotoxicity in all experiments was excluded by lactate dehydrogenase release assay using a commercial kit (LDH kit; Roche, Mannheim, Germany).

Messenger RNA isolation

Cells were cultivated in six-well plates and were treated at 80% confluency with SFN, butyrate, or the combination of SFN with one of the inhibitors for 24 and 48 hr, respectively. Total RNA was isolated from cells using an RNA isolation reagent (TRIR, Abgene, Epsom, UK), followed by phenol extraction and ethanol precipitation.

Semi-quantitative RT-PCR

Reverse transcription–polymerase chain reaction (RT-PCR) was conducted with the Gene Amp RNA PCR kit (Applied Biosystems, Branchburg, NJ) according to the manufacturer's protocol, starting from 1 μg total RNA. All RNA samples were subjected to DNase-treatment during the RT-step to remove traces of genomic DNA (Shrimp Nuclease, Abgene). Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control. From the results of preliminary experiments, 20 PCR cycles for GAPDH and 35 cycles for HBD-2 were selected as the optimal amplification conditions to produce a log-linear relationship between the amount of each messenger RNA (mRNA) and the intensity of the PCR product. The PCR contained 0·2 mM dNTPs (Invitrogen Corporation, Carlsbad, CA), 0·05 U/μl AmpliTaqGold DNA polymerase (Applied Biosystems), 1·5 mM Mg_2Cl (Applied Biosystems) and 0·2 μmol/l of either of the primers of HBD-2 and GAPDH (Biospring, Frankfurt, Germany). The PCR conditions for HBD-2 were: initial denaturation at 94° for 1 min followed by annealing at 58° for 1 min, extension at 72° for 2 min and a final extension at 72° for 7 min after the last cycle. The respective annealing temperature for GAPDH was 45°. Primers for amplification were as follows: HBD-2 sense 5′-ggtggta taggcgatcctgtt-3′, HBD-2 antisense 5′-agggcaaaagactggatg aca-3′; GAPDH sense 5′-gcaccgtcaaggctgagaac-3′, GAPDH antisense 5′-ccaccaccctgttgctgtag-3′. The expected sizes of HBD-2 and GAPDH were 66 and 803 base pairs, respectively. Aliquots of the PCR mixtures (10 μl) were analysed by electrophoresis using a 1·5% agarose gel containing ethidium bromide. Gels were placed on a UV transilluminator and digitalized using the DocuGel V-System (Scananalytics, Biozym, Landgraaf, the Netherlands). For semi-quantitative analysis of amplified PCR products, the fluorescent dye PicoGreen (Molecular Probes, Eugene, OR) was used according to the manufacturer's instructions. In brief, 2 μl of amplified DNA in 100 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7·5) was mixed with an equal volume of diluted PicoGreen reagent (1 : 200, volume/volume in TE buffer). Samples were incubated for 5 min at room temperature and protected from light in a microtitre plate. The fluorescence was measured (λ_{ex} = 485 nm; λ_{em} = 538 nm) in the fluorescence microplate reader Tecan SpectraFluor PLUS (MTX Lab Systems, Vienna, VA). A λ DNA linear standard curve was applied for each experiment.

Immunofluorescence assay

Caco-2 cells were grown on glass chamber slides (Lab-Tek; Nunc, Rochester, NY) and allowed to reach 80% confluency. Cells were fixed in ice-cold ethanol (95%) for 10 min and permeabilized by addition of 0·1% Triton X-100 in PBS for 10 min. After washing, unspecific binding of antibodies was blocked by incubating cells in 8% horse serum in PBS containing 0·1% Tween-20 for 1 hr at room temperature. HBD-2 antibody (Immundiagnostik, Bensheim, Germany) was diluted 1 : 25 in blocking buffer. After 1·5 hr of incubation, cells were washed three times with PBS before secondary antibody (cy3 conjugated rabbit–anti-goat; Sigma, St Louis, MO) was added for 1 hr. Following washing and air-drying, the cells were embedded in 4′,6-diamidino-2-phenylindol (DAPI) mounting medium (Vector Laboratories, Burlingame, CA) and evaluated by immunofluorescence microscopy (Eclipse E 600; Nikon, Tokyo, Japan) with a digital camera DX 20H (Kappa, Monrovia, CA).

β-defensin-2 enzyme-linked immunosorbent assay (ELISA)

Caco-2 cells were grown in six-well plates and were treated at 80% confluency with SFN (20 μM), butyrate (3 mM), or one of the combinations of SFN (20 μM) with the p38 MAPK inhibitor SB203580 (20 μM) or the ERK1/2 inhibitor PD98059 (40 μM). Cells were washed three times with ice-cold PBS and incubated with cell lysis buffer (Cell Signaling, Beverly, MA) containing multiple protease inhibitors (Complete, Roche, Mannheim, Germany) for 5 min at 4°. Protein extracts were obtained after sonication of cell lysates (twice for 5 seconds each time) and centrifugation at 7500 ***g*** at 4° (10 min). Protein content was determined using a colorimetric assay according to the method of

Bradford (Bio-Rad Laboratories, Munich, Germany). Cell lysates were diluted 1 : 3 with PBS containing 0·1% bovine serum albumin. 100 µl was used for each reaction. The HBD-2 ELISA Kit (Immundiagnostik) was then used according to the manufacturer's instructions. Protein concentration of HBD-2 was analysed and samples were normalized to equal protein concentrations.

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis and immunoblot analysis

Caco-2 and HT-29 cells were stimulated with SFN at 80% confluency. Cells were than harvested and protein content was determined using a Bio-Rad colorimetric assay (see β-defensin-2 ELISA section). Equal amounts of total protein lysates were separated on a 10% SDS–polyacrylamide gel for VDR, PPARγ and on a 12·5% SDS–polyacrylamide gel for ERK1/2, phospho-ERK1/2, p38 MAPK and phospho-p38 MAPK. Proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Subsequently, membranes were blocked for 1 hr with 5% (weight/volume; wt/v) non-fat dried milk in Tris-buffered saline containing 0·05% Tween-20 (TBS-T). Membranes were then incubated overnight with a 1 : 1000 dilution of VDR, ERK1/2 and phospho-ERK1/2 antibodies (all from Santa Cruz Biotechnologies, Santa Cruz, CA) or with p38 MAPK and phospho-p38 MAPK antibodies (both from Cell Signaling), or with a 1 : 2000 dilution of PPARγ antibody (Calbiochem, LaJolla, CA) in 0·05% TBS-T and 5% (wt/v) non-fat dried milk. After washing, the blots were incubated for 30 min with the corresponding horseradish peroxidase-conjugated antibody (Vector Laboratories, dilution 1 : 2000) in 0·05% TBS-T and 5% (wt/v) non-fat dried milk. The washing steps were repeated, and subsequently enhanced chemoluminescence detection was performed according to the manufacturer's instructions (Amersham Pharmacia Biotech, Buckinghamshire, UK) on Hyperfilm-MP (Amersham International plc, Buckinghamshire, UK). Blots were then reprobed with β-actin antibody (Sigma). For quantitative analysis, bands were detected by scanning densitometry, using a Desaga CabUVIS scanner and DESAGA PROVILDOC software (Desaga, Wiesloch, Germany).

Statistics

All statistical analyses were performed using GRAPHPAD PRISM 4·01 (San Diego, CA). Analysis of variance was performed when more than two groups were compared and, when significant ($P < 0·05$), multiple comparisons were performed using Tukey's test. Data are expressed as means ± SD from three independent experiments. A P value < 0·05 was considered to be significant.

Results

Sulforaphane is a direct inducer of HBD-2 mRNA expression in colonocytes

Challenge of HT-29 cells with increasing concentrations of SFN (1–20 µM) provoked a dose-dependent upregulation of HBD-2 mRNA after 24 hr (Fig. 1) and 48 hr of treatment (data not shown). Significant effects were seen

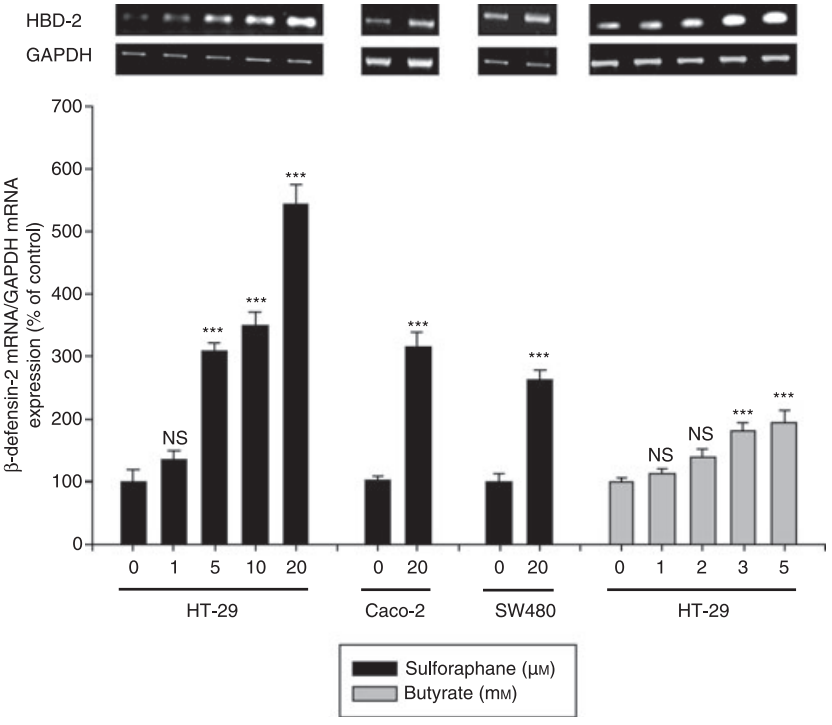

Figure 1. Dose-dependent effect of sulforaphane (1–20 µM) and butyrate (1–5 mM) on β-defensin-2 messenger RNA (mRNA) expression in HT-29 cells after 24 hr of treatment. Moreover the induction of β-defensin-2 mRNA expression in Caco-2 and SW480 cells in response to sulforaphane (20 µM) was demonstrated. The β-defensin-2 mRNA expression was measured by semi-quantitative reverse transcription–polymerase chain reaction using the fluorescent dye Pico Green. All values for mRNA levels are normalized to corresponding mRNA amounts for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). One representative gel of three independent experiments is shown. Induction of β-defensin-2 mRNA is displayed as relative percentage to solvent treated control cells. ***$P < 0·001$, NS, not significant.

at concentrations ≥ 5 μM. Increased expression of the HBD-2 gene in response to SFN (20 μM) was also obtained in the colon cancer cell lines Caco-2 and SW480 after 24 hr (Fig. 1), reflecting a common mechanism in colorectal cancer cells. Analogously, stimulation of HT-29 cells with increasing concentrations of the dietary HDAC inhibitor butyrate (1–5 mM) also resulted in a dose-dependent upregulation of HBD-2 mRNA compared to control cells (Fig. 1) after 24 hr. A similar upregulation was seen in the colorectal cancer cell line Caco-2 (data not shown).

Sulforaphane elevates HBD-2 protein expression in colonocytes

Upregulation of HBD-2 mRNA was accompanied by a similar increase in protein level as measured by ELISA. In Caco-2 cells, a time-dependent increase of HBD-2 protein level was observed after exposure to SFN (20 μM) or butyrate (3 mM) (Fig. 2a: SFN: 24 hr: + 62%, $P < 0{\cdot}05$; 48 hr: + 104%, $P < 0{\cdot}001$; butyrate: 24 hr: + 47%, $P < 0{\cdot}05$; 48 hr: + 161%, $P < 0{\cdot}001$). In addition, the subcellular localization of HBD-2 protein in Caco-2 cells was determined via immunofluorescence analysis (Fig. 2b). Caco-2 cells were treated with SFN (20 μM) up to 48 hr. Both control cells and SFN-treated cells showed strong staining for HBD-2 that was predominantly in the cytosol. Moreover, SFN challenge for 48 hr led to an increased expression of the HBD-2 protein, which is in line with the results obtained by ELISA. Similar results were obtained after challenging the cells with butyrate used at a concentration of 3 mM (data not shown).

The VDR but not the PPARγ is involved in SFN-induced HBD-2 mRNA expression

The nuclear receptors VDR and PPARγ are potent regulators of immune responses. To evaluate the involvement of PPARγ in SFN-induced HBD-2 mRNA expression, HT-29 cells transfected with a dominant-negative PPARγ mutant to inhibit wild-type receptor action were used (Fig. 3a). In these mutant cells, an equal time-dependent increase of HBD-2 mRNA compared to wild-type and empty-vector cells was observed in response to SFN, indicating that PPARγ activity is not required for HBD-2 regulation. However, stimulation of HT-29 wild-type cells with SFN (10 μM) for 48 hr caused significant upregulation of the expression of PPARγ (Fig. 3b), indicating that the receptor is involved in SFN-mediated signalling. Moreover, wild-type Caco-2 cells were treated with SFN (10 μM) in the presence of the VDR antagonist ZK191732. Inhibition of VDR significantly antagonized SFN-induced HBD-2 mRNA after 24 hr of incubation, demonstrating the

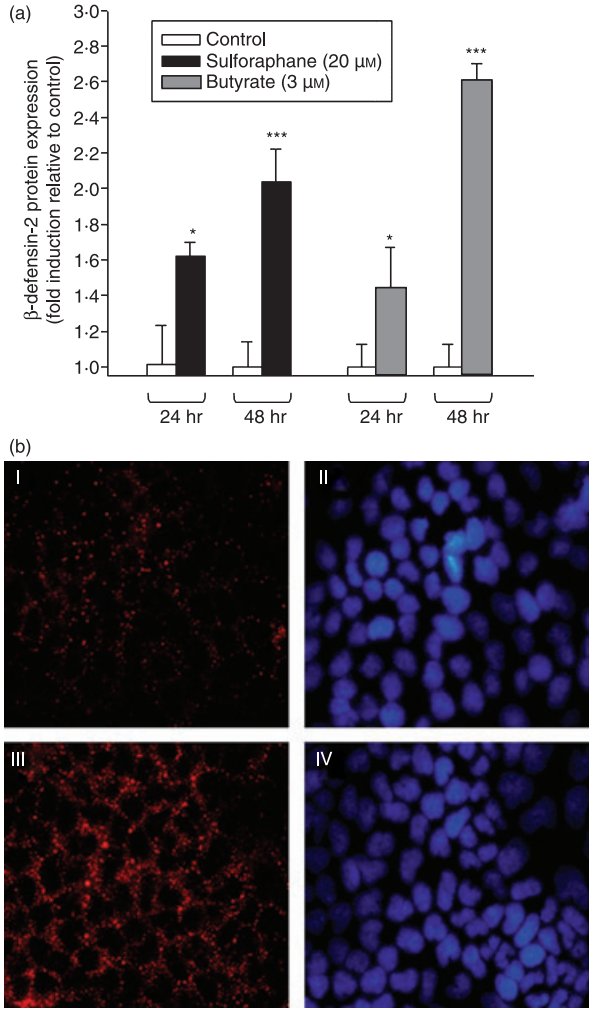

Figure 2. (a) β-defensin-2 protein expression in Caco-2 cells measured by enzyme-linked immunosorbent assay (ELISA). Cells were starved for 72 hr and then stimulated with sulforaphane (10–20 μM) or butyrate (3 mM) for 24 and 48 hr. Proteins were harvested and β-defensin-2 was measured by ELISA. The concentration range of all experiments was in ng/ml. Induction of β-defensin-2 protein is displayed as fold induction relative to solvent-treated control cells. $^{***}P < 0{\cdot}001$, $^{*}P < 0{\cdot}05$. (b) Representative immunofluorescence assays for β-defensin-2 in Caco-2 cells. Confluent cells were challenged with sulforaphane (20 μM) for 48 hr (III) or grown in culture medium as controls (I). (II, IV) Nuclear counterstaining with 4′,6-diamidino-2-phenylindol (DAPI) mounting medium for each of the adjacent panels. Magnification, × 400.

pivotal role of the receptor in SFN-induced HBD-2 upregulation (− 71% versus control, $P < 0{\cdot}001$, Fig. 4a). To reveal whether VDR is a target of the SFN signalling pathway, colonocytes were stimulated with SFN (10–20 μM) for 24 hr. As depicted in Fig. 4(b), SFN increased the production of VDR protein in HT-29 cells. A similar pattern was observed for the colorectal cancer cell line Caco-2 (Fig. 4b).

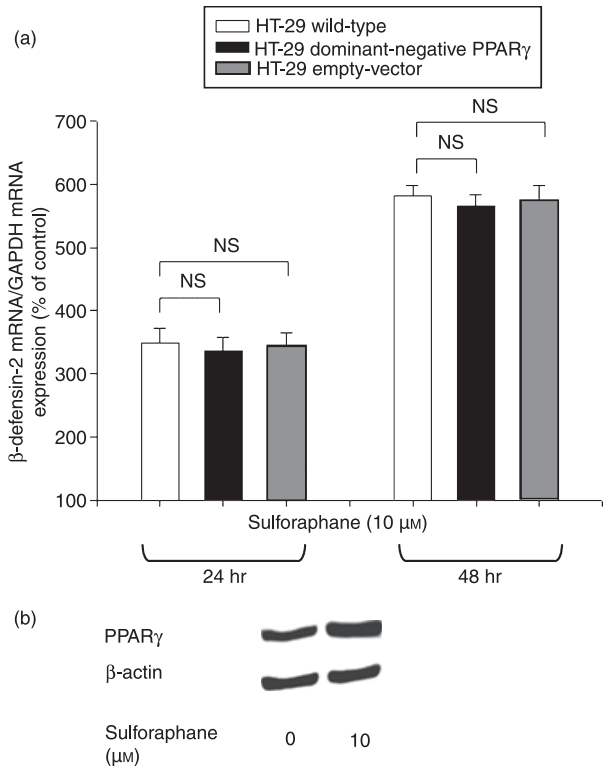

Figure 3. (a) Time-dependent effect of sulforaphane (10 μM) on β-defensin-2 messenger RNA (mRNA) expression in wild-type, empty-vector and peroxisome proliferator-activated receptor γ (PPARγ) mutant HT-29 cells after 24 and 48 hr of treatment. β-Defensin-2 mRNA was measured by semi-quantitative reverse transcription polymerase chain reaction with the fluorescent dye Pico Green. All values for mRNA levels are normalized to corresponding mRNA amounts for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Induction of β-defensin-2 mRNA is displayed as a relative percentage to solvent-treated control cells; NS, not significant. (b) Western blot for PPARγ expression after treatment of HT-29 wild-type cells with sulforaphane (10 μM) for 48 hr. One representative blot of three independent experiments is shown.

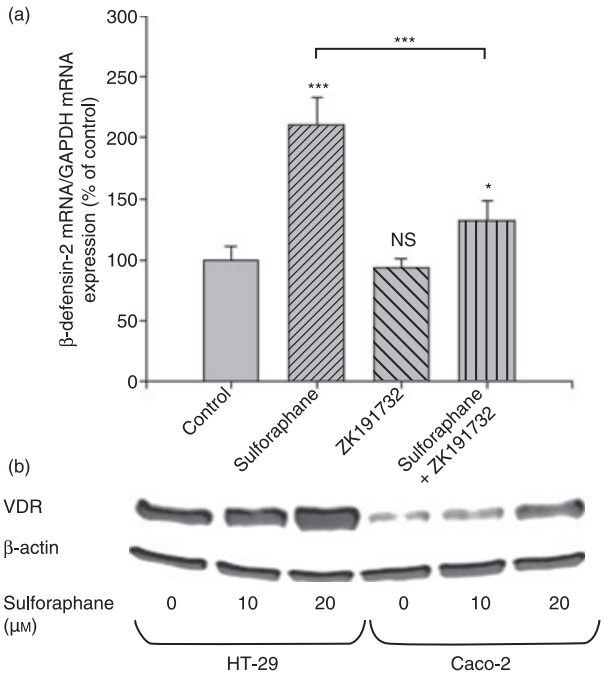

Figure 4. (a) β-defensin-2 messenger RNA (mRNA) expression in Caco-2 cells. Medium was supplemented with the solvent, sulforaphane (10 μM), the vitamin D receptor (VDR) inhibitor ZK191732 (10 μM) or a combination of sulforaphane and ZK191732 for 24 hr. Semi-quantitative analysis of polymerase chain reaction products was performed using Pico Green. All values for mRNA levels are normalized to corresponding mRNA amounts of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Induction of β-defensin-2 mRNA is displayed as relative percentage to solvent-treated control cells. ***$P < 0{\cdot}001$, *$P < 0{\cdot}05$, NS, not significant. (b) Western blot for VDR expression after treatment of HT-29 and Caco-2 cells with sulforaphane (10–20 μM) for 24 hr. One representative blot of three independent experiments is shown.

The mitogen-activated protein kinase kinase (MEK)/ERK signalling pathway and NF-κB play a role in SFN-induced HBD-2 expression

To determine the involvement of intracellular signal transduction pathways such as p38 MAPK and ERK1/2 in SFN-mediated HBD-2 expression, Caco-2 cells were preincubated for 5 hr with specific inhibitors before stimulation with SFN (10 μM). Combined treatment of SFN with the ERK1/2 inhibitor PD98059 reduced the induction of HBD-2 mRNA expression after 24 hr (Fig. 5a). In contrast, coincubation with the p38 MAPK inhibitor SB203580 did not affect the elevated HBD-2 mRNA expression caused by SFN at 24 hr (Fig. 5a). Similar to the observations on mRNA level, inhibition of the ERK1/2 pathway almost blocked SFN-induced HBD-2 protein expression (−87%, $P < 0{\cdot}001$) in Caco-2 cells after 24 hr, as detected by ELISA, while inhibition of the p38 MAPK trail did not influence the induction of the peptide by the drug (−16%, not significant). Moreover, exposure of Caco-2 cells with SFN (10–20 μM) resulted in a rapid phosphorylation of ERK1/2 after 8 hr, while the amount of total ERK1/2 protein was not affected. The expressions of phospho-p38 MAPK and p38 MAPK proteins were unchanged by SFN in Caco-2 cells (Fig. 5b). To gain more insights into the signalling events of HBD-2 mRNA expression in response to SFN, the involvement of the NF-κB pathway in this regulation was scrutinized. Treatment with the NF-κB inhibitor helenalin partially reversed the induction of HBD-2 mRNA (−23%, $P < 0{\cdot}05$, 24 hr) caused by SFN (10 μM) in Caco-2 cells.

Discussion

The discovery of antimicrobial peptides such as defensins has extended our knowledge of non-specific defence

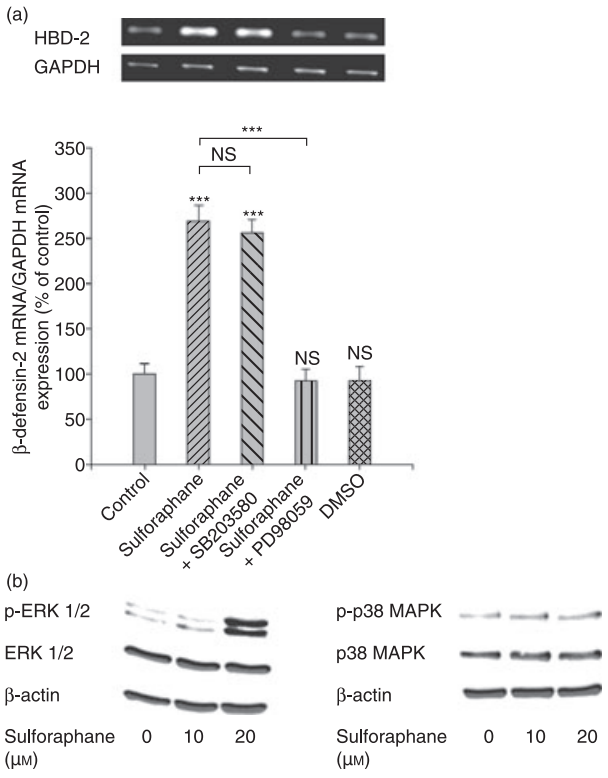

Figure 5. (a) Influence of the p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 (20 μM) and the extracellular signal-regulated kinase (ERK) 1/2 inhibitor PD98059 (40 μM) on sulforaphane-induced (10 μM) β-defensin-2 messenger RNA (mRNA) expression after 24 hr of treatment in Caco-2 cells. β-defensin-2 expression was analysed by semi-quantitative reverse transcription–polymerase chain reaction with the fluorescent dye Pico Green. All values for mRNA levels are normalized to corresponding mRNA amounts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). One representative gel of three independent experiments is shown. Induction of β-defensin-2 mRNA is displayed as relative percentage to solvent-treated control cells. ***$P < 0{\cdot}001$, NS, not significant. (b) Western blot for ERK1/2, phospho-ERK1/2, p38 MAPK and phospho-p38 MAPK expression after treatment of Caco-2 cells with sulforaphane (10–20 μM) for 8 hr. One representative blot of three independent experiments is shown.

mechanisms. A deficiency in the antimicrobial defence system of defensins may be a reasonable and plausible explanation for the breakdown of barrier functions with increased invasion of infectious pathogens, leading to inflammation or deleterious immune responses. Abnormalities in defensin expression have not only been observed in inflammatory bowel diseases[8,16] but also in cystic fibrosis, where the inactivation of the defensins as the result of high salt concentrations has been linked to recurrent bronchopulmonary infections.[37] In contrast to ulcerative colitis and ileal Crohn's disease, colonic Crohn's disease is characterized by an impaired induction of the epithelial HBD-2.[6,38,39] This defective regulation could be explained by the lower HBD-2 gene copy number in the HBD-2 locus in colonic Crohn's disease.[39] A normal HBD-2 copy number distribution was seen in ileal Crohn's disease and also in ulcerative colitis.[39] Eventually, the lack of HBD-2 induction may contribute to a defective antimicrobial barrier leading to chronic inflammation in Crohn's disease affecting the large bowel.[39] Detailed knowledge of HBD-2 expression and regulation is still lacking. The aim of this study was to determine a possible role for the dietary HDAC inhibitor SFN in the regulation of HBD-2. Similar experiments were accomplished with the dietary HDAC inhibitor butyrate. In this context, the involvement of the nuclear receptors VDR and PPARγ as well as the MEK/ERK and NF-κB pathways in SFN-mediated induction of HBD-2 were examined.

Accumulating evidence suggests that SFN is a highly promising dietary preventive agent as the result of its ability to exert immunomodulatory effects via multiple mechanisms of action including anti-inflammatory actions.[24,40] The anti-inflammatory effects of SFN and butyrate may be achieved via different pathways: The agents can affect the expression of a diverse array of genes potentially involved in immune modulation through their ability to inhibit HDAC activity via histone hyperacetylation.[40] This results in chromatin relaxation and increased transcription of target genes.[40] SFN and butyrate are also able to block tumour necrosis factor-α-induced and lipopolysaccharide-induced NF-κB translocation resulting in reduced expression of proinflammatory cytokines.[24,25,41] Moreover, the involvement of HDAC inhibitors such as butyrate and trichostatin A in the modulation of the intestinal antimicrobial peptides make these peptides possible targets for the actions of SFN.[27,30,42] Indeed, in a variety of human colon cancer cells, including HT-29 cells, and in a mouse model, SFN, when used under the same experimental conditions as in our study, inhibited HDAC activity accompanied by a global increase in histone H3 and H4 acetylation.[40,43,44] Similarly, inhibition of HDAC activity and histone hyperacetylation have also been demonstrated for the dietary HDAC inhibitors diallyl disulphide and butyrate in the colon cancer cell lines Caco-2 and HT-29.[29,45,46] However, the exact mechanism by which HDAC inhibitors induce the release of antimicrobial peptides is not completely understood. Recently, a novel approach was provided by the study of Schauber *et al.*, which demonstrated that VDR coactivators are important for HDAC-inhibitor-induced acetylation of histone proteins.[47] Increased histone acetylation caused by butyrate intensified the induction of the antimicrobial peptide cathelicidin in response to 1,25-dihydroxyvitamin D_3. For this regulation, correct functioning of both the VDR and of the steroid receptor coactivator 3 (SRC3)/p300, which possesses inherent histone acetyltransferase activity, were required.[47]

The present study demonstrated for the first time that the dietary HDAC inhibitor SFN is a direct inducer of

HBD-2 mRNA and protein in colonocytes. Similar results were obtained after butyrate treatment. Immunofluorescence experiments showed that the peptide exhibits a primarily cytoplasmic localization, as demonstrated by previous groups in colon cancer cells.[12,48] In our *in vitro* setting, butyrate has only a minor effect on HBD-2 transcription compared to SFN, but has a much stronger effect on HBD-2 peptide expression. The different amount of HBD-2 protein can be explained by variations in the modulation of intracellular signalling initiated by each HDAC inhibitor, concerning stability of mRNA transcripts as well as protein translation, e.g. via interaction with initiation factors. In the present study, SFN activates ERK1/2, whereas butyrate mainly induces p38 MAPK activation.[36] Recently it was demonstrated that ERK can regulate translation initiation via dephosphorylation of the initiation factor elF2α.[49] Moreover, downstream substrates of p38 MAPK were shown to regulate mRNA stability and also translation.[50] Activation of different mechanisms may therefore contribute to the observed diversity in HBD-2 protein expression compared to mRNA transcription.

Both PPARγ and VDR are highly expressed in the colonic epithelium, indicating that both receptors are important agents in the physiology of the human colon.[36,51,52] The receptors partly mediate their anti-inflammatory actions through negative interference with proinflammatory transcription factors such as NF-κB.[35,53,54] Moreover, there is a great influx of information that PPARγ ligands may influence the inflammatory response in inflammatory bowel diseases and colon cancer.[55,56] Similarly, the active form of vitamin D, 1,25-dihydroxyvitamin D_3, was shown to inhibit the development of various autoimmune diseases, including inflammatory bowel diseases.[57] In addition, a VDR gene polymorphism has been associated with susceptibility to Crohn's disease.[58] All these features imply the possible involvement of both receptors in SFN-mediated HBD-2 regulation. Using the VDR inhibitor ZK191732 and a Caco-2 dominant-negative PPARγ mutant cell line in our experiments, we demonstrated that VDR activity, but not PPARγ activity, is required for HBD-2 expression induced by SFN. The properties of the VDR inhibitor ZK191732 and the functional successful transfection of the dominant-negative PPARγ mutant cell system have been described recently.[35] Our findings are supported by the presence of a consensus vitamin D response element in the promoter of the human HBD-2 gene.[59] Accordingly, stimulation of several human cell types with 1,25-dihydroxyvitamin D_3 resulted in increased expression of HBD-2 mRNA.[59] Moreover, our *in vitro* model demonstrates for the first time the upregulation of VDR and PPARγ after SFN stimulation in colonocytes, not only corroborating the involvement of both receptors in SFN-mediated signalling but also making them attractive targets involved in the HBD-2 pathway. A similar increase of the receptors was observed after butyrate treatment.[60–62]

Stimulation with SFN may activate different MAPKs in colorectal cancer cells.[31,63,64] Kinase pathways act as signal sorters and conduct a variety of upstream signals to the nucleus of the eukaryotic cell, where transcription of specific target genes will be affected.[65] To gain insight into the regulatory pathways by which SFN augments HBD-2 expression in Caco-2 cells, our *in vitro* model focused on two major MAPKs: p38 MAPK and ERK1/2. These MAPKs are involved in a large variety of cellular activities, including cell survival, proliferation and inflammatory responses.[65–68] Increased phosphorylation of ERK1/2 by SFN in various cell lines, including human colon adenocarcinoma Caco-2 cells, has been demonstrated by several groups, although the amount of ERK1/2 protein was not affected. In contrast, SFN treatment had no impact on p38 MAPK activation.[31,63] These observations are confirmed by our experiments. Moreover, inhibition of the ERK1/2 pathway in this study prevented SFN-mediated induction of HBD-2 mRNA expression in Caco-2 cells, while blocking of the p38 MAPK trail did not affect HBD-2 levels. Our data are in accordance with observations in middle-ear epithelial cells, demonstrating transcriptional activation of the HBD-2 gene caused by interleukin-1α which is mediated through a Raf-MEK1/2-ERK1/2 signalling pathway.[69] Conflicting results were obtained in the lung epithelial cell line A549, in which interleukin-1β-induced upregulation of HBD-2 was partly attenuated by inhibiting the p38 MAPK but not by the ERK1/2 signalling pathway.[70] These different observations indicate a cell-specific and stimulus-dependent kinase pathway leading to induction of HBD-2. However, all these studies underscore the importance of the MAPK signalling pathways in innate immunity through the regulation of HBD-2 levels.

Nuclear factor-κB plays a key role in regulating the transcription of several members of a proinflammatory gene programme in intestinal epithelial cells that is induced in response to inflammation, especially in inflammatory bowel diseases, or to infection with enteroinvasive bacteria.[12] The NF-κB pathway has been described as an important regulator of HBD-2 induction in colon epithelial cells.[12,14,71] Moreover, this pathway has been shown to participate in SFN signalling.[24,25] Our data demonstrate that pretreatment of Caco-2 cells with the NF-κB inhibitor helenalin slightly diminished HBD-2 upregulation caused by SFN. This finding is not only supported by former studies demonstrating the necessity of NF-κB in the induction of HBD-2 by inhibitor experiments, but also by the identification of a NF-κB consensus sequence in the proximal promoter of the hBD-2 gene, which appears to be necessary for optimal HBD-2 gene expression.[12,14,71,72] Nevertheless, we suggest that the induction of HBD-2 in response to SFN is more complex

than if it were based solely on the actions of VDR, ERK1/2 and NF-κB. Since several mechanisms of action have been identified as modulating HBD-2 signalling, for instance the pathways of Ap1, protein kinase C, phosphatidylinositol-3-kinase and cJun N-terminal kinase, and because these pathways are also targets of SFN in colonocytes, multiple signalling pathways may be involved in the upregulation of the peptide.[14,31,70,71,73,74]

In summary, we have demonstrated for the first time that SFN induces the expression of the antimicrobial peptide HBD-2 at both mRNA and protein levels in colorectal cancer cells. Similar effects were obtained after butyrate treatment. Furthermore, we revealed that SFN-mediated induction of HBD-2 is modulated via VDR, MEK/ERK and the NF-κB signalling pathway. These data support the potential usefulness of dietary HDAC inhibitors in the therapy of colonic Crohn's disease. Further *in vivo* studies are required to establish the relevance of these findings.

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