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Journal of Nutritional Biochemistry 18 (2007) 736–745
Journal of Nutritional Biochemistry

Physiological concentrations of butyrate favorably modulate genes of oxidative and metabolic stress in primary human colon cells☆

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Received 7 September 2006; received in revised form 15 November 2006; accepted 6 December 2006

Abstract

Butyrate, a metabolite of gut flora-mediated fermentation of dietary fibre, was analysed for effects on expression of genes related to oxidative stress in primary human colon cells. An induction of detoxifying, antioxidative genes is expected to contribute to dietary chemoprevention. Cells were treated with butyrate (3.125–50 mM; 0.5–8 h), and kinetics of uptake and survival were measured. Gene expression was determined with a pathway-specific cDNA array after treating colon epithelium stripes with nontoxic doses of butyrate (10 mM, 12 h). Changes of *hCOX-2*, *hSOD2* and *hCAT* expression were confirmed with real-time polymerase chain reaction (PCR) and by measuring catalase-enzyme activity. Primary colon cells consumed 1.5 and 0.5 mM butyrate after 4- and 12-h treatment, respectively. Cell viability was not changed by butyrate during 0.5–2-h treatment, whereas cell yields decreased after 1 h. Metabolic activity of remaining cells was either increased (4 h, 50 mM) or retained at 97% (8 h, 50 mM). Expression of *hCAT* was enhanced, whereas *hCOX-2* and *hSOD2* were lowered according to both array and real-time PCR analysis. An enhanced catalase-enzyme activity was detected after 2 h butyrate treatment. Healthy nontransformed colon cells well tolerated butyrate (50 mM, 2 h), and lower concentrations (10 mM, 12 h) modulated cyclooxygenase 2 (COX-2) and catalase genes. This points to a dual role of chemoprotection, since less COX-2 could reduce inflammatory processes, whereas more catalase improves detoxification of hydrogen peroxide (H_2O_2), a compound of oxidative stress. Changes of this type could reduce damaging effects by oxidants and protect cells from initiation.

Keywords: Butyrate; Human colon cells; Oxidative stress; Gene expression

1. Introduction

Colorectal cancer remains one of the major health problems and is the second most common cause of death due to cancer in countries with Western style diets [1]. Epidemiological and animal studies suggest that a diet high in fat, red meat and protein may increase the risk of colon cancer, whereas a high intake of fibre and complex carbohydrates may be protective [2,3].

There are some controversial findings in other epidemiological surveys either negating the protective roles of fibre [4,5] or the causative role of red meat [6]. By and large, however, most experimental studies directed at elucidating molecular, toxicological and chemoprotective associations support the roles of fibre in chemoprevention or of meat in posing a risk. In extension of these epidemiological findings, experimental studies suggest that a diet high in red meat might pose a risk on account of the iron contained in the heme [7,8]. The iron of heme or haemoglobin can, for example, catalyse the formation of reactive oxygen species that contribute to colon cancer development by inducing genotoxic damage [8]. Alternatively, heme from red meat can also increase endogenous *N*-nitroso compounds in the faeces, which has been positively correlated with the formation of alkylating DNA adducts like *O*(6)-carboxymethyl guanine in exfoliated colon cells [9]. In vivo, it seems feasible that such reactive compounds damage the colon crypt cells, resulting in initiation or in an enhanced progression of initiated cells [10]. Indeed, it has been shown that the faecal matrix itself is also capable of generating reactive oxygen species and acting genotoxic after a diet high in meat and low in fibre [11,12].

☆ This research was supported by the German Research Foundation Deutsche Forschungsgemeinschaft (DFG). The work of this study was carried out with financial support of the DFG, project PO 284/8–1 and 8–2.
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URL: http://www.uni-jena.de/biologie/ieu/et

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doi:10.1016/j.jnutbio.2006.12.012

One mechanism of chemoprevention could involve protection against genotoxic compounds at the preinitiation stage [13,14]. Thus, it has been shown that colon cells contain several compounds which can capture free radicals. Moreover, the cells express different types of detoxifying and stress-response enzyme systems [15], including glutathione *S*-transferases (GSTs), peroxidases and catalase. GSTs are known to be phase II enzymes that can detoxify a number of carcinogens, and some have clear peroxidase activity [16]. Catalase is one of the key defense systems against oxidative stress since it very rapidly detoxifies H_2O_2 to yield H_2O and O_2. Therefore, a high level of expression could be associated with less genetic damage due to the exposures that involve colorectal cancer risk [17].

A basic preliminary finding has shown that a short-term preincubation of primary colon cells of rats and humans with butyrate significantly reduced the genotoxic effects of H_2O_2 [18,19]. Nothing, however, is known on the potential mechanisms behind this effect. Since butyrate, a product formed in millimolar concentrations during gut fermentation of dietary fibres is able to modulate gene expression in human colon cells [20], e.g., via inhibition of histone deacetylases or via genomic response elements [21], it is now hypothesized that butyrate could enhance expression of genes that reduce oxidative and metabolic stress [20].

Another risk factor for the development of colon cancer is inflammation. It is known that, in colon tumors, cyclooxygenase 2 (COX-2) is overexpressed, which increases formation of inflammatory prostaglandins. Since inflammatory processes may also result in oxidative stress and generate free radicals, inhibition of COX-2 is possibly an effective mode of cancer chemoprevention [22].

The aim of this study was to find out whether expression of genes involved in these pathways of stress and toxicity (GSTs, peroxidases, catalase, COX-2) can be changed by physiologically relevant concentrations of butyrate in human primary colonocytes. In extension of this, it was a further aim to assess whether these changes result in functional consequences that could explain the previously observed reduction of H_2O_2 genotoxicity in butyrate-pretreated nontransformed colon cells. These mixed cell suspensions contain also the actual target cells of colon carcinogenesis (stem cells and dividing daughter cells [23]). It is, however, technically challenging to work with these cells in vitro since they have only a limited life span after isolation [24,25].

We present new findings on effects of butyrate in healthy nontransformed colon cells and report that genes associated with oxidative stress can be favorably modulated by this important short chain fatty acid.

2. Methods and materials

2.1. Primary colon tissue preparation and isolation of cells

The study was approved by the ethics committee of the Friedrich–Schiller University of Jena, and patients gave their informed consent. Primary human colon cells were isolated from tissue specimens obtained during surgery of colorectal tumors, diverticulitis and colon polyps (Table 1). The tissues were taken from the very edges of the resected colon segments. The surgeon and the pathologist confirmed that they did not show any microscopic or macroscopic signs of malignant or inflammatory pathology. Table 1 summarizes all diagnoses that were the basis for performing each surgery. Stenotic anastomosis was a reason for one of the surgical procedures. The anastomosis of this patient was constricted and, thus, made it necessary to resect the colon

Table 1
Summary of the available information about the donors whose cells were used for the different experiments

Donor	Gender (male/female)	Age (years)	BMI (kg/m^2)	Diagnosis	Used in experiment
1	F	40	20.8	Anastomosis stenosis	Array, real-time PCR
2	M	70	25.8	Adenoma	Array, real-time PCR
3	F	52	25.4	Sigma carcinoma	Array, real-time PCR
4	M	57	23.9	Sigma diverticulitis	Real-time PCR
5	M	64	24.2	Sigma diverticulitis	Real-time PCR
6	M	53	30.3	Sigma carcinoma	Real-time PCR
7	M	62	28.7	Sigma carcinoma	Viability, cell number, metabolic activity
8	F	77	25.0	Morbus Crohn	Viability, cell number, metabolic activity
9	M	63	31.6	Sigma diverticulitis	Viability, cell number, metabolic activity
10	F	66	48.8	Rectum carcinoma	Butyrate uptake
11	M	42	23.5	Sigma diverticulitis	Catalase activity
12	M	58	18.9	Colon carcinoma	Catalase activity, butyrate uptake
13	M	37	24.1	Rectum carcinoma	Catalase activity
14	F	54	27.3	Sigma carcinoma	Catalase activity
15	M	50	20.2	Rectum carcinoma	Butyrate uptake
16	F	52	23.1	Sigma diverticulitis	Butyrate uptake
17	M	68	23.7	Rectum carcinoma	Butyrate uptake
18	M	68	25.3	Rectum carcinoma	Catalase activity
19	F	59	23.0	Sigma carcinoma	Catalase activity
Mean±S.D.	(12/7)	57±11	26±6		

Mean age of the patients was 57 years, 12 were male and seven were female.

section, but the tissue was confirmed by histopathology to be nonmalignant, and the experimental slices were isolated from the edges of the resected bowel segment. Mean age (±S.D.) of the donors of colon cells for the experiments (metabolic activity, RNA isolation, cytosol preparation, butyrate consumption studies) was 57±11 years; 12 of the donors were male, seven were female. The tissue was stored in Hank's balanced salt solution (HBSS) (8.0 g/L NaCl; 0.4 g/L KCl; 0.06 g/L $Na_2HPO_4 \times 2\ H_2O$; 0.06 g/L K_2HPO_4; 1 g/L glucose; 0.35 g/L $NaHCO_3$; 4.8 g/L HEPES; pH 7.2), transported on ice to the laboratory within 1 h and worked up immediately. The human colon epithelium was separated from the tissue by perfusion-supported mechanical disaggregation [26].

Subsequently, single cells were isolated from the epithelial stripes by mincing and were incubated in 3 ml HBSS (60 min, 37°C) supplemented with 6 mg proteinase K (Sigma; Steinheim, Germany) and 3 mg collagenase P (Boehringer; Mannheim, Germany). The suspensions of primary human colon cells were diluted with HBSS, centrifuged and resuspended in phosphate-buffered saline (PBS) (8 g/L NaCl; 1.44 g/L Na_2HPO_4; 0.2 g/L KCl; 0.2 g/L KH_2PO_4; pH 7.3). Single cells were seeded into wells of 96-well microtiter plates and treated as indicated in tables and figures. Alternatively, intact tissue stripes were treated with butyrate up to 12 h for gene expression studies. Viability and cell yields were determined with trypan blue before performing the metabolic activity assay and before isolating RNA.

2.2. Quantification of butyrate uptake by gas chromatography

The concentration of butyrate in culture supernatants of butyrate-treated primary human cells was measured by gas chromatography using a GC17-A gas chromatograph (Shimadzu, Duisburg, Germany) [27,28]. For this, 5×10^6 cells were treated with 10 mM butyrate, which was dissolved in 3 ml cell culture medium. The culture supernatants, collected after 2, 4, 8 and 12 h of incubation, were stored at −20°C and were analysed for the remaining butyrate concentration, as described previously [29]. The difference between the concentration before and after the treatment is estimated to represent the amount of butyrate which is intracellular available. The samples were thawed and centrifuged to remove cellular components. Fifty microliters of isocaproic acid (0.89 μg/ml in concentrated formic acid; internal standard for calibration) were added to 0.5 ml of each sample of which 1 μl was injected and evaporated at 250°C. The internal standard isocaproic acid was the basis for calculating the butyrate concentrations.

2.3. Determination of viability, cell number and measurement of metabolic activity

Cell suspensions containing 2×10^6 cells/ml were incubated for 0.5–4 h, with 0–50 mM butyrate in a shaking thermomixer at 37°C. The trypan blue exclusion test was routinely used to determine cell viability and cell number after incubating the cells in suspension with butyrate.

For the metabolic activity assay, isolated cells were seeded into 96-well microtiter plates (50 000 cells per well) and incubated in minimal essential medium with Earle's salts enriched with 20% fetal calf serum, 2 mM glutamine, 1% penicillin/streptomycin, 100 μg/ml gentamycin, 2.5 μg/ml fungizone, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite [30].

To determine biological effects of physiological concentrations of butyrate (0–50 mM), the primary colon cells were incubated for 2, 4, 6 and 8 h. Metabolic activity as a surrogate parameter of cytotoxicity was assessed using the CellTiter-Blue assay (Promega, Mannheim, Germany) in 96-well microtiter plates with measurements after different time points. The CellTiter-Blue assay is a viability assay, which can be used to estimate the number of viable cells in nonadherent cell suspensions, such as primary colon cells. To measure the metabolic capacity of the cells, this assay uses the dye resazurin, which is reduced into resorufin only by viable cells. This product is highly fluorescent and was detected with Ex/Em 520/595 nm after 2 h incubation with the reagent. Mean values were calculated from means of three parallel determinations of three independent experiments.

2.4. Treatment with butyrate for gene expression analysis

Effects of butyrate on gene expression were studied after incubating the epithelial tissue stripes with 10 mM butyrate instead of using single cells, since this results in an improved survival of the target cells for up to 12 h [20]. Primary human colon tissue pieces were plated in Petri dishes (35 mm) and, after allowing the tissue pieces to settle for 15 min, they were treated with 10 mM butyrate-dissolved cell culture medium, as described in Section 2.3 [30]. After 12 h, single cells were isolated, as described above, quantified and further processed for RNA isolation.

2.5. RNA isolation

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany), dissolved in 50 μl RNase-free water and stored at −20°C. The ratio $A^{260}/_{280}$ and the concentration of total RNA was determined spectrophotometrically (Eppendorf BioPhotometer, Hamburg, Germany) for protein or phenol contamination. This was followed by formaldehyde denaturing RNA gel electrophoresis to check the integrity of the ribosomal RNA and DNA contamination. Contaminating DNA was eliminated by DNase-I treatment using the RNase-free DNase I Amplification Grade kit (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions. Subsequently macro array analysis was carried out.

2.6. Macroarray analysis

Hybridization was performed on 112 genes (three blanks, 13 reference spots and 96 human genes related to stress and toxicity) on cDNA gene macroarrays (GEArray Q Series Human Stress&Toxicity Gene Array HS12; SuperArray

Bioscience, Frederick, MD, USA). Genes were classified into functional categories, representing genes belonging to Proliferation/Carcinogenesis, Growth Arrest/Senescence, Inflammation and Necrosis/Apoptosis (Oxidative & Metabolic Stress, Heating Stress, DNA Damage & Repair and Apoptosis Signaling). A detailed gene list is available at the company's Web site (www.superarray.com). Workup of the array was performed according to the manufacturer's protocol and as has been previously reported for another array type from the same company [20]. Briefly, single-stranded cDNA was synthesized from total RNA (1 μg) in vitro. By applying a single-step ampo linear polymerase reaction technique, the cDNA was labelled with dUTP-biotin. The cDNA macroarray was hybridized overnight at 60°C with the biotin-labelled cDNA. The hybridized membrane was subjected to chemiluminesence analysis for quantification of the conjugation signals with streptavidin-linked alkaline phosphatase and CDPstar. The resulting signals were captured with a CCD camera (Fujifilm LAS-1000, Diana, Greenwoods, SC, USA) and analyzed with AIDA array analysis (Raytest GmbH, Straubenhardt, Germany) software to evaluate the differential gene expression of the various samples. Raw data were normalized between 0% and 100% expression, where the signals of the means of the negative controls (areas without spotted gene sequences or with genes not expressed in human cells) were set to equal 0% and the means of the signals of the positive controls (household genes) were fixed to equal 100%. Thus, the data shown here represent mean expression levels relative to negative and positive reference genes.

2.7. Determination of hCOX-2, hSOD2 and hCAT expression with real-time polymerase chain reaction in primary colon cells

The expression of *hCOX-2*, *hSOD2* and *hCAT* was verified by quantitative real-time polymerase chain reaction (PCR) using the system of iCycler iQ (Biorad GmbH, München, Germany). One microgram of total RNA was subjected to reverse transcription (SuperScript II, First-Strand cDNA Synthesis System; Invitrogen, Karlsruhe, Germany) in 20 μl buffer with oligo $(dT)_{15}$ primers, according to manufacturers instructions. Fifty nanograms of cDNA, calculated as RNA equivalents, were used in a 25 μl PCR amplification reaction containing 2x iQ SYBR Green supermix (100 mM KCl, 40 mM Tris-HCl (pH 8.4), 0.4 mM each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, stabilizers) and 10 pmol gene-specific primers for the target genes and reference (*hGAPDH*) gene. The following primer sequences were used to amplify a region of *hCOX-2*, *hSOD2* and *hCAT* and *hGAPDH* mRNA:

COX-2-F (5′ -tcc tcc tgt gcc tga tga ttg c-3′)
COX-2-R (5′ -act gat gcg tga agt gct ggg-3′)
CAT-F (5′ -tgg aca agt aca atg ctg ag-3′)
CAT-R (5′ -tta cac gga tga acg cta ag-3′)
SOD2-F (5′ -gcc ctg gaa cct cac atc aac-3′)
SOD2-R (5′ -caa cgc ctc ctg gta ctt ctc-3′)
GAPDH-F (5′ -cca ccc atg gca aat tcc atg gc-3′)
GAPDH-R (5′ -agt gga ctc cac gac gta ctc ag-3′).

PCR cycles included 1 cycle of 95°C for 2 min, followed by 40 cycles each of 94°C for 30 s, annealing temperature of 57°C for 30 s and 72°C for 40 s and a final extension step of 72°C for 10 min. Product-specific amplification was confirmed by melting curve analysis and agarose gel electrophoresis. All experiments were performed in duplicates. The fluorescence threshold value (C_T) was calculated using the iCycler iQ optical v3.0a system software. The relative quantification of the target-*mRNA* expression was calculated with the comparative $\Delta\Delta C_T$ ($\Delta C_T = \Delta C_{T\,\text{control}} - \Delta C_{T\,\text{reference}}$) method. For normalization, ΔC_T values were calculated by subtracting the average of the C_T value in the control for the reference gene from the average of the C_T value for the target gene and subtracting the average of the C_T value in the treated sample of the reference gene from the target gene. Then, the difference between the ΔC_T values of control and treatment ($\Delta\Delta C_T$) was calculated. The fold change was calculated according to the efficiency method ($E=2$; fold change $= E^{\text{difference}}$) [31,32].

2.8. Preparation of cytosol, measurement of cytosolic protein and catalase activity

After incubation with butyrate, the cells were washed with PBS and then resuspended and lysed in cold phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.1% Triton-X. After centrifugation (10000*g*, 10 min, 4°C), the supernatant was aliquoted and frozen at −80°C until use. Total protein content was measured using the method by Bradford with bovine serum albumin as standard protein [33]. Catalase activity was assayed spectrophotometrically at 25°C by following the extinction of H_2O_2 at 240 nm by the method of Aebi [34]. Assay mixtures contained 10 mM H_2O_2 and 100 μl of cell lysates in 50 mM potassium phosphate buffer (pH 7.0). Enzyme activities were calculated using 0.0394 $mM^{-1} \times cm^{-1}$ as absorption coefficient at 240 nm.

2.9. Statistical analyses

GraphPad Prism software version 4 (GraphPad Software, San Diego, CA, USA) was used to calculate one- or two-way analysis of variance (ANOVA) with Bonferroni's posttest or Dunnett's multiple comparison tests, where appropriate. Microsoft Excel was used for *t* test and fold change analysis. Data of at least three ($n \geq 3$) experiments were evaluated to establish two-sided significance levels of independently reproduced determinations.

3. Results

3.1. Butyrate consumption by colon cells

Table 2 shows how primary nontransformed colon cells utilized butyrate, which was dissolved and diluted according

Table 2
Consumption of butyrate by human primary colon cells, determined as residual butyrate in the cell culture medium after 2, 4, 8 ($n=3$–5) and 12 h ($n=2$) incubation with butyrate

	mmol/L in the medium		Estimated cellular concentration (mmol/L)		Estimated uptake (μmol/l×10^6 cells)
	Mean	S.D.	Mean	S.D.	
2 h	8.9	1.2	1.2	1.2	0.72
4 h	9.0	1.1	1.5	0.5	0.9
8 h	9.0	0.2	1.1	0.3	0.66
12 h	10.7	0.4	0.5	0.1	0.3

10.5±0.7 mM butyrate were detected as the starting concentration using gas chromatography.

to its molar mass (110.09 g/mol, sodium butyrate) to yield 10 mM in the culture medium. The estimated concentrations consumed by the cells were determined by analyzing the residual concentration of butyrate in the supernatants of treated cells.

As the starting concentration, 10.5 mM butyrate were detected with gas chromatography. This aberration from the calculated concentration can be due to technical conditions.

After 2, 4 and 8 h, by calculating the difference between the measured starting and the residual butyrate concentration, on average, 1.2, 1.5 and 1.1 mM were consumed by the cells, whereas after 12 h, the concentration in the cell culture medium was lowered by approximately 0.5 mM. This was estimated to be the available amount of butyrate in the intracellular compartment. Thus, most of the added butyrate (86% after 4 h and 95% after 12 h) stayed detectable in the culture medium, pointing to a limited absorption process within 2–12 h ex vivo.

3.2. Viability, cell number and metabolic activity

Cell viability and cell number were determined by trypan blue exclusion after short-term treatments of single cells with butyrate. The treatment of primary colon cells for 0.5 to 4 h with the highest concentration of butyrate (50 mM) resulted in a number of biological effects. After 30 min, viability remained unchanged by the treatments, but cell recovery was below 100% for all data points, including the untreated control. After 1 or 2 h, the different concentrations again caused no impairment of the cells' viabilities, but the recovered numbers of cells decreased, mostly independent of increasing butyrate concentrations by almost 50%. After 4 h, cell viability was reduced in the three highest test concentrations (78.1%, 73.9% and 73.1%) in comparison to the baseline viability (90.4%). There was also a more pronounced reduction of 60% of cell number which, however, again was only marginally related to the butyrate concentrations (data not shown).

There were also different effects on metabolic activity by the butyrate treatment. After 2 and 4 h, the increase of the metabolic activity by butyrate (18.6% after 2 h and 31.5% after 4 h, 50 mM) may reflect trophic effects, which were lost and not any longer apparent for the extended treatment durations of 6 and 8 h (data not shown). In parallel studies (Sauer et al., in preparation), butyrate did not increase but rather reduced the metabolic activity more after 12 and 24 h at the same concentrations of ≥25–50 mM in cells from other donors.

When comparing the absolute levels of metabolic activity, the level increased from 2 to 4 h (3229±1455–3991±966 fluorescence units) and then was again reduced after 6 and 8 h (3609±1118 and 3113±1136 fluorescence units; data not shown).

3.3. Gene expression analysis

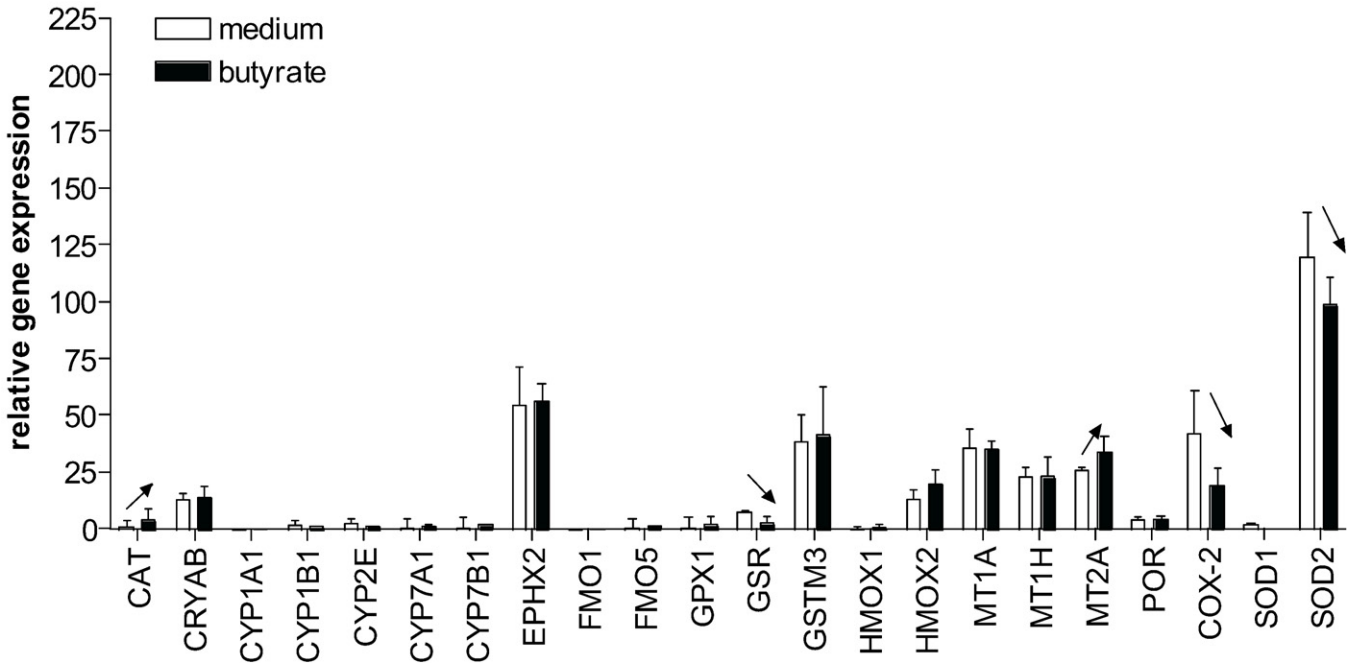
In analogy to previous studies [20], we incubated the intact epithelial stripes for 12 h (which was the maximal possible exposure time) with 10 mM butyrate (amount still within the nontoxic concentration range) to determine effects on patterns of gene expression using the HS12 cDNA macroarray (Superarray). Here, only results are reported on expression of genes related to oxidative and metabolic stress (Table 3). Further results of other gene groups will be described and discussed in another context elsewhere (Scharlau et al., in preparation), but are already posted on our Web site (www.uni-jena.de/biologie/ieu/et).

In the gene cluster of 22 oxidative and metabolic stress-associated genes, *hCAT* (2.9-fold) and *hMT2A* (1.3-fold; $P<.05$) were enhanced; *hGSR* (0.4-fold), *hPTGS2* (COX-2; 0.5-fold; $P<.001$) and *hSOD2* ($P<.05$) were lowered. As we have reported previously using the drug metabolism array (Superarray), a number of genes coding for cytochrome P450 enzymes were expressed only at very low levels and hardly altered by the butyrate treatment [20]. Similar results were obtained for the CYP450, spotted on the membrane used in this study (data not shown).

3.4. Confirmatory experiments with real time PCR

The reduction for *hCOX-2* and *hSOD2* mRNA and the induction of *hCAT* in primary colon cells after butyrate treatment (10 mM) was reassessed with real-time PCR. When investigating aliquots of the same RNA batches as those used in the array analysis ($n=3$), the directional changes of the gene expression levels were confirmed. Thus, expression levels for *hCAT* were increased with a fold change of 1.6±0.6, whilst expression levels of *hCOX-2* were reduced (fold change 0.7±0.2), in comparison to the medium control. Expression levels for *hSOD2* were also reduced (fold change 0.7±0.4). The available RNA was not sufficient to perform confirmatory analysis for glutathione reductase (*GSR*) and *MT2A*.

Table 3
Modulation of gene expression in primary colon cells after treatment with 10 mM butyrate for 12 h. Mean±S.D. of the medium controls of three independently reproduced gene arrays are shown using RNA of three different donors (No. 1–3)



Oxidative and metabolic stress	Expression level in the medium control		Fold change by treatment with butyrate		Analysis of significance	
	Mean	S.D.	Mean	S.D.	*t* test	2-way ANOVA
CAT	4	9	2.9			
GSR	24	4		0.4		
MT2A	84	4	1.3		$P<.05$	
COX-2	137	61		0.5		***
SOD2	384	62		0.9		*

For the butyrate treatment, fold change values are shown whereas a regulation is considered to be significant when ≤0.5 or ≥2.0, respectively. Statistical significance was checked using both an unpaired *t* test as well as a two-way ANOVA with Bonferoni's posttest, respectively.

RNA from additional donors of primary colon cells treated with butyrate under the same conditions was also used to assess expression of *hCOX-2*, *hSOD2* and *hCAT*. Fig. 1 shows that there is a high variability between the donors. With these samples (No. 4–6), the expression of *hCOX-2* was down-regulated in one of the additional

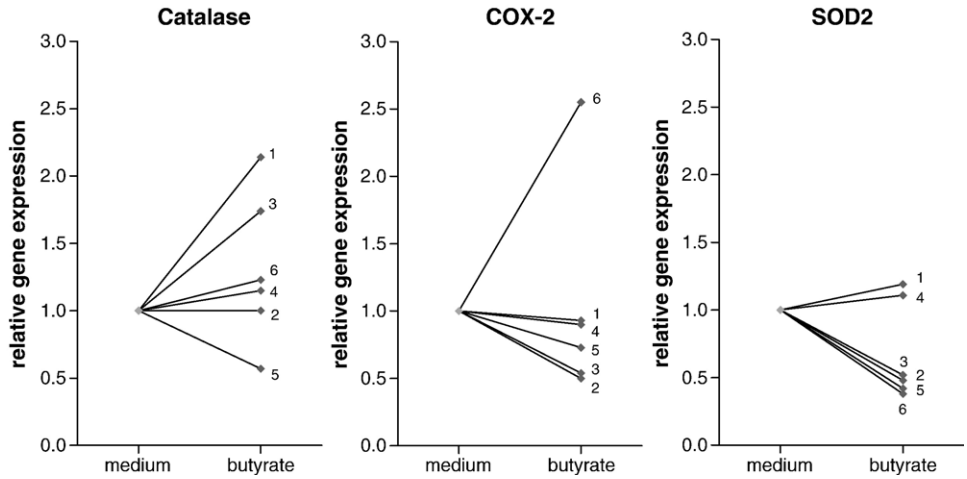


Fig. 1. Expression of *CAT*, *COX-2* and *SOD2* genes and variability of response with samples from different donors of primary colon cells after incubation with butyrate (12 h) analysed with real time PCR ($n=6$). Donors 1–3 also provided the RNA used in the gene expression analysis. When regarding results from all six donors, the changes mediated by butyrate treatment were nonsignificant using paired *t* test.

Table 4
GAPDH normalized expression levels (arbitrary units) calculated from the Ct values obtained in the real-time PCR experiments

Donor	Reason for surgery	CAT			COX-2			SOD2		
		Expression level		fold change	Expression level		fold change	Expression level		Fold change
		Medium	Butyrate		Medium	Butyrate		Medium	Butyrate	
1	Anastomosenstenosis	0.014	0.029	2.14	0.330	0.308	0.93	0.250	0.297	1.19
2	Adenoma	0.025	0.025	1.00	0.129	0.065	0.50	0.366	0.177	0.48
3	Sigma carcinoma	0.012	0.021	1.74	0.297	0.159	0.54	0.467	0.241	0.52
4	Sigma diverticulitis	0.074	0.085	1.15	0.225	0.203	0.90	0.203	0.225	1.11
5	Sigma diverticulitis	0.088	0.051	0.57	0.125	0.092	0.73	0.210	0.088	0.42
6	Sigma carcinoma	0.046	0.056	1.23	0.069	0.177	2.55	0.189	0.072	0.38

Donors 1–3 were also used for the gene array analysis. The expression levels of catalase were lower than those for *COX-2* and *SOD2*. Expression of all three genes was found to be highly variable between the different donors.

donors, remained almost unchanged in the second and was distinctly up-regulated in the third of the new donors. For the *hCAT*, we also saw a variation. In two of the further donors, the expression was only slightly enhanced, whereas in the third new donor, it decreased. We found a similar pattern of variation for *hSOD2*. For this gene, the expression of one additional donor (as well as of one array-RNA donor) was slightly increased, whereas when using RNA of the two more donors, expression levels were reduced. Regarding the absolute values of the expression levels, there was also a strong variability between the different donors (see Table 4), but we were not able to attribute the variability to the type of disease. Moreover, the findings were not due to possible experimental artifacts either since both melting curve analysis and gel electrophoresis ascertained the specificity of the PCR products.

3.5. Measurement of catalase activity

After treatment of single cells for 30 min and 2 h with butyrate, we determined catalase activity in six different donors. Since the level of enzyme activity varied between the different individuals, we analysed changes of enzyme activity due to the treatment with butyrate as relative values after setting the corresponding medium controls to equal 100%.

After 30 min, we did not detect any change of catalase activity after butyrate treatment, whereas after 2 h, catalase activity was enhanced by about 65% subsequent to the butyrate treatment (Fig 2; $P=.0646$, unpaired t test).

We also treated cells isolated from three other donors for 4 h and 8 h. After 4 h, catalase activity also tended to be induced (18.4%), whilst after 8 h, the induction was less apparent (10.1%) (results not shown).

4. Discussion

Butyrate has been suggested to reduce cancer risks by acting as a cancer-suppressing type of agent [35,36]. If these effects also would also occur in vivo, it may be of exceptional protective activity under the "real-life situation," since in normal colon cells, it serves as a survival factor, and in transformed cell lines, it has been shown to inhibit the motility and to induce cell cycle arrest, differentiation and apoptosis [37,38]. Since humans steadily develop preneoplastic and neoplastic lesions with increasing age [39], a continuous exposure to butyrate could thus be meaningful in this context. In normal cells, which reflect more the types of cells available in a healthy, nondiseased colon mucosa, butyrate has trophic and growth-promoting effects and acts as a nutrient [40], enhances survival [38] and helps maintaining the integrity of the healthy colon mucosa [41,42]. Using normal primary human colon cells in culture is a unique in vitro possibility to study direct effects of butyrate in the actual target cells of colon carcinogenesis and to evaluate how the detected activities may be associated with potential mechanisms of chemoprevention. The approach, however, is relying on only a model system, and studies in humans will be necessary to determine more precisely the relevance of the findings for the in vivo situation.

Here, it was shown that 1.5 mM of butyrate was taken up by primary cells treated with 10 mM butyrate after 4 h, whereas 0.5 mM were taken up after 12 h. These amounts

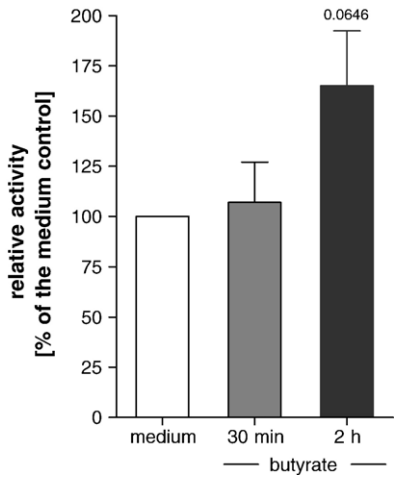


Fig. 2. Quantification of catalase activity after treating primary colon cells with butyrate (10 mM) for 30 min and 2 h. Cells treated with plain medium served as a control, which was set to equal 100% ($n=6$). Statistical evaluation with an unpaired t test (Welch's correction) revealed that the changes did not reach significance ($P=.0646$). The measured activity was based on the total protein concentration, as determined with the method of Bradford [32].

represented only 14 and 5% of the exposure. To our knowledge, this is the first time butyrate consumption was directly measured in human colon cells in vitro. In rats, butyrate absorption increased linearly with luminal concentrations. It must be kept in mind, however, that higher butyrate concentrations may be needed in vivo than in vitro to achieve the same intracellular concentrations due to the presence of mucus or to differences in the absorptive surface of the cells in vivo [43]. Another study has shown that there is a regional variation of nutrient utilization in the colon using biopsy specimens [44]. In the colon, the absorbed butyrate is partly secreted out of the cell and reaches the blood stream [43], which may be an explanation for the increase of the detected butyrate amounts after 12 h in this study. Recently, we had shown that HT29 cells consumed 0.17 mM after 24-h treatment with 1 mM butyrate or 0.27 mM after treatment with 2 mM, equalling approximately 20% of the exposing dose. LT97 adenoma cells consumed 0.83- and 0.76-mM treatment with 1 or 2 mM butyrate for 24 h (80% and 75% of the original dose), respectively [29]. So far, we have not performed uptake studies with higher doses or shorter incubation times in the cell lines, but a comparison is currently being performed. Thus, in the half of the incubation time (12 vs. 24 h) primary colon cells consumed the double amount of butyrate (0.5 vs. 0.2–0.3 mM), which would speak for the trophic effects in primary cells, in comparison to HT29 cells, albeit culture media and exposure conditions were different. Different butyrate uptakes may be explained by the availability of the monocarboxylate transporter 1 (MCT1) which is necessary for butyrate uptake and is down-regulated from normal cells to malignant cells. Especially, normal cells require both the MCT1 transporter and butyrate for their homeostasis [45,46]. It also has to be considered that there is a time-dependant loss of cells, which means that the uptake per 1×10^6 cells must be regarded as an approximation.

Here, additional studies on the cytotoxic potential of a butyrate treatment in primary colon cells using metabolic activity, viability and cell number as parameters, were performed. The observed time-dependent effects indicated that individual primary cells in suspension culture remained viable for only relatively short periods of time (<<8 h). The loss of cells was time-related but was not enhanced with increasing concentrations of butyrate. After treating intact epithelial stripes, however, the viability of the subsequently isolated primary cells was retained for 12 h. Using these in vitro conditions, it was, moreover, possible to isolate sufficient intact RNA for further gene expression analysis. In the donors used for these experiments, butyrate did not impair the cells' metabolic activity but enhanced this parameter after short treatment durations (2 and 4 h) which again pointed to trophic effects.

The studies on modulation of gene expression by butyrate identified new target genes related to stress response in primary, nontransformed colon cells. The gene products are known to protect against factors of oxidative and metabolic stress. A confounding result was, however, that there was a high variability of gene induction by butyrate in cells from different donors. Thus, butyrate was strongly effective in cells of some donors, whereas cells of other donors did not respond. This finding necessitates further in-depth studies to elucidate the reasons for individuals being responders or nonresponders. In particular, for catalase, it is known that there is a common polymorphism in the promoter region of the *CAT* gene, which results in lower enzyme activity. It was also shown that the catalase activity is strongly affected by diet, especially by the consumption of fruits and vegetables, which deliver exogenous antioxidants [47]. Therefore, lifestyle factors of the donors of colon cells may be the reason for the variability of induction. The interindividual variability of gene expression levels is possibly due to unavoidable experimental differences encountered during the surgical isolation. However, the isolation procedure and the duration were always performed according to one standard protocol. In summary, it is clear from the present studies that butyrate does modulate gene expression in nontransformed primary human colon cells in vitro, as was shown by array analysis and real-time PCR.

Major findings were that *hCAT* and *hMT2A* were inducible in the primary colon cells. This suggests a better protection of butyrate-treated cells during situations of metabolic and oxidative stress. For example, the induction of catalase can protect the cells against H_2O_2, which is also produced endogenously [48]. A lower exposure of the cells to H_2O_2 (and, thus, to resulting reactive oxygen species) can protect cells from DNA damage, risk of mutations and possibly, initiation [49]. The effects by butyrate can be regarded to be chemoprotective for the untransformed cells, since exposure would be reduced. The induction of *MT2A,* as observed here, and of *GSTT2*, as observed previously [20], can both also protect from oxidative stress due to antioxidative capacities, particularly if the induction is present prior to oxidative stress [50]. For these genes, however, further confirmatory data is needed before coming to final conclusions.

This study also reports a reduced expression of *COX-2* in primary cells treated with butyrate, possibly resulting in anti-inflammatory mechanisms. The effect can be regarded to be protective [51], since the inhibition of chronic inflammatory processes might prevent enhanced proliferation in inflamed tissue [22]. An overexpression of *COX-2* has been reported in various types of tumors and some precancerous tissues. For instance, the inhibition of COX-2 activity was able to reduce growth of polyps in adenomatous polyposis coli knockout mice [52]. Cyclooxygenases are responsible for the metabolism of arachidonic acid into prostaglandins. There are two isoforms, the constitutively expressed COX-1 and the inducible COX-2, of which the latter is implicated in tumorigenesis and cancer progression [53]. Altogether, the reduction of inflammatory processes may represent a feasible approach of chemoprevention in

healthy cells. Of cause, it still remains to be demonstrated that similar effects can be measured in vivo.

In contrast, *SOD2* and *GSR* were reduced in primary cells. These genes code for two enzymes that are also important for the detoxification of products derived from oxidative stress, as are catalase and MT2A. Superoxide dismutase scavenges superoxide anions, which are reduced to H_2O_2 that is damaging. In following reactions, a glutathione peroxidase can cleave H_2O_2 to yield H_2O. For this reaction, glutathione is oxidised and can be reduced by the GSR. Since *SOD2* is expressed at a high level in the studied donors, the reduction might be not of such potent biological relevance. Moreover, our previous studies have shown that a preincubation of colon cells with butyrate could reduce the genotoxicity of H_2O_2 [18], possibly pointing to a more clear-cut functional consequence resulting from the induction of catalase and *MT2A* than by the reduction of *SOD2* and *GSR*.

In conclusion, even though only relative small quantities of butyrate were consumed by the primary colon epithelium, the intracellular concentrations were apparently sufficient to modulate gene expression. Our studies showed that physiological butyrate concentrations were not toxic to primary human colon cells. In particular, here, we present new insights into feasible approaches of chemoprevention in a nontransformed primary colon cell model since the enhancement of catalase, and potentially of other genes involved in the defense against reactive oxygen species, could protect cells from oxidative stress, whereas the repression of the COX-2 expression level could decrease inflammatory reactions posing a risk for the development of colon cancer.

Acknowledgments

We acknowledge Prof. Jahreis and D.C. Möckel, Department of Nutritional Physiology, Friedrich–Schiller University of Jena for analytical assistance. We thank all donors of colon tissue samples for giving their informed consent and supporting our studies.

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