ELSEVIER

Journal of Nutritional Biochemistry 15 (2004) 220–228

THE JOURNAL OF Nutritional Biochemistry

Butyrate inhibits cytokine-induced VCAM-1 and ICAM-1 expression in cultured endothelial cells: the role of NF-κB and PPARα

Danuta Zapolska-Downar[a,c], Aldona Siennicka[a], Mariusz Kaczmarczyk[a], Blanka Kołodziej[b], Marek Naruszewicz[a,c,*]

[a]*Department of Clinical Biochemistry and Laboratory Diagnostic, Pomeranian Academy of Medicine, ul. Powstańców Wlkp. 72, Szczecin, Poland*
[b]*Department of Pathomorphology, Pomeranian Academy of Medicine, ul. Unii Lubelskiej 1 Szczecin, Poland*
[c]*National Institute of Food and Nutrition, ul. Powsińska 71/72 Warsaw, Poland*

Received 5 June 2003; received in revised form 2 October 2003; accepted 1 November 2003

Abstract

Adhesion and migration of leukocytes into the surrounding tissues is a crucial step in inflammation, immunity, and atherogenesis. Expression of cell adhesion molecules by endothelial cells plays a leading role in this process. Butyrate, a natural short-chain fatty acid produced by bacterial fermentation of dietary fiber, has been attributed with anti-inflammatory activity in inflammatory bowel disease. Butyrate in vitro is active in colonocytes and several other cell types. We have studied the effect of butyrate on expression of endothelial leukocyte adhesion molecules by cytokine-stimulated human umbilical vein endothelial cells (HUVEC). Pretreatment of HUVEC with butyrate–inhibited tumor necrosis factor–α (TNFα)–induced expression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecule-1 (ICAM-1) in a time and concentration-dependent manner. Butyrate at 10 mmol/L inhibited interleukin-1 (IL-1)–stimulated VCAM-1 and ICAM-1 expression. The effect of butyrate on cytokine-stimulated VCAM-1 expression was more pronounced than in the case of ICAM-1. Butyrate decreased TNFα-induced expression of mRNA for VCAM-1 and ICAM-1. Suppressed expression of VCAM-1 and ICAM-1 was associated with reduced adherence of monocytes and lymphocytes to cytokine-stimulated HUVEC. Butyrate inhibited TNFα-induced activation of nuclear factor–κB (NF-κB) in HUVEC. Finally, butyrate enhanced peroxisome proliferator-activated receptor–α (PPARα) expression in HUVEC. These results demonstrate that butyrate may have anti-inflammatory properties not only in colonocytes but also in endothelial cells. The anti-inflammatory and (perhaps) antiatherogenic properties of butyrate may partly be attributed to an effect on activation of NF-κB and PPARα and to the associated expression of VCAM-1 and ICAM-1. The present findings support further investigations on the therapeutic benefits of butyrate in several pathological events involving leukocyte recruitment.

Keywords: Butyrate; Inflammation; Endothelial cells; Cell adhesion molecules; Transcription factors

1. Introduction

Leukocyte recruitment from the blood is a critical step in inflammation, immunity, and atherogenesis [1–3]. Endothelial cells play an active role in the control of leukocyte recruitment by expressing membrane molecules for adherence of leukocytes. Vascular cell adhesion molecule–1 (VCAM-1) and intracellular cell adhesion molecule–1 (ICAM-1) of the immunoglobulin superfamily as well as selectins E and P of the selectin superfamily are among the most prominent adhesion molecules involved in this process [1,2,4].

Leukocyte recruitment is a multistep process that includes initial tethering of leukocytes to the endothelium followed by leukocyte rolling on the vascular wall, which activates the leukocytes to firm adherence [1,4]. Tethering and rolling depend on the interaction of P and E selectins present on endothelial cells with their carbohydrate ligands on leukocytes. Firm adhesion follows if leukocytes are activated while rolling on the endothelium, and is facilitated by the interaction of VCAM-1 with very late antigen–4 (VLA-4) or of ICAM-1 with lymphocyte function antigen–1 (LFA-1). Diapedesis and transendothelial migration are dependent on the chemotactic gradient, ICAM-1, and

* Corresponding author. Fax: +48 91 466 1490.
E-mail address: mnarusze@sci.pam.szczecin.pl (M. Naruszewicz).

0955-2863/04/$ – see front matter
doi:10.1016/j.jnutbio.2003.11.008

VCAM-1 activation, and homeotypic interactions between platelet/endothelial cell adhesion molecule–1 (PECAM-1) on endothelial cells and leukocytes [3]. It has recently been suggested that endothelial adhesion molecules may play a role in tumor progression and metastasis [5].

Most adhesion molecules described above are not present on resting endothelial cells. Their expression is the effect of stimulation by specific molecules such as tumor necrosis factor–α (TNFα), interleukin-1 (IL-1), interferon-γ (IFNγ), and oxidatively modified LDL (oxyLDL) [1,6]. Stimulation is mediated by NF-κB, a key transcription factor implicated in the regulation of genes during the immune and inflammatory responses. Among them are genes encoding VCAM-1, ICAM-1, and selectin E [7]. It has been suggested that peroxisome proliferator–activated receptor α, a nuclear receptor involved in the transcriptional response to fatty acids, exerts its anti-inflammatory action as a result of negative transcriptional regulation of NF-κB [8]. Recently it has been reported that fibric acid derivatives known to be PPARα activators inhibit cytokine–induced VCAM-1 expression in human endothelial cells [9].

Butyrate is a natural short-chain fatty acid present in dairy products and produced in the colon by anaerobic fermentation of undigested carbohydrates [10]. This four-carbon acid plays an important role in maintaining normal function and integrity of the colonic mucosa [11]. Clinical trials suggest that butyrate has anti-inflammatory properties in inflammatory bowel disease [12–15]. Butyrate administered to patients with ulcerative colitis suppressed mucosal inflammation and was associated with reduction of NF-κB activation in lamina propria macrophages [16]. Butyrate differentially modulates cytokine secretion in peripheral blood monocytes, abolishes lipopolisacharide (LPS)–induced cytokine expression [17], and inhibits production of proinflammatory IL-1 and TNFα cytokines by bacteria-stimulated monocytes [18].

Butyrate has also been reported to act as a potent antiproliferative agent and cell differentiation promoter [19] and to induce cancer cell apoptosis [20,21]. These effects may contribute to antitumor activity of butyrate and dietary fiber [22–24].

Recent studies suggest that butyrate may have antiatherogenic properties by inhibiting vascular smooth muscle cells [25]. It has also been shown that butyrate protects macrophages against free radicals and diminishes generation of proatherogenic oxyLDL [26].

It is evident from the above observations that butyrate participates in multiple activities in a number of cell types. So far, the action of this fatty acid on endothelial cell function and expression of endothelial adhesion molecules has not been investigated. We therefore examined the ability of butyrate to modulate the expression of ICAM-1 and VCAM-1 and transcription factors NF-κB and PPARα by human umbilical vein endothelial cells (HUVEC).

2. Methods and materials

2.1. Materials

Medium 199, fungizone, penicillin, streptomycin, gentamycin, heat-inactivated fetal bovine serum (FBS), collagenase (type Ia), gelatin, glutamine, trypsin/EDTA solution, and butyric acid were obtained from Sigma (St. Louis, MO). Recombinant TNFα (10 mg, 1×10^8 U/mg) and IL-1β (5×10^7 U/mg), random hexamers, reverse transcriptase–polymer chain reaction (PCR) buffer, and *Taq* polymerase were from Boehringer Mannheim (Mannheim, Germany). Tissue culture dishes from Costar were supplied by Sigma (St. Louis, MO, USA) and Ficoll-Paque by Pharmacia (Upsala, Sweden). Monoclonal antibodies against VCAM-1 (CD106:FITC), ICAM-1 (CD54:FITC), CD14 (CD14:PE), CD45 (CD45:FITC), platelet endothelial cell adhesion molecule-1 (CD31:PE), and IgG_1 (IgG_1:FITC) were from Becton Dickinson (San Diego, CA, USA). dATP, dTTP, dCTP and dGTP and RNAsin were from Promega (Madison, WI, USA). An ELISA-based kit for NF-κB activation measurements was obtained from Active Motif (Belgium).

2.2. Endothelial cell isolation and culture

HUVEC were obtained from umbilical cords by collagenase digestion as described by Jaffe [27]. In brief, veins of umbilical cords were perfused with PBS to remove blood cells, filled with 0.1% collagenase, and left for 8 minutes at 37°C. The resulting cellular suspension was supplemented with FBS and centrifuged ($400 \times g$) for 10 minutes. HUVEC were cultured in gelatin-coated 25-cm^2 flasks, 6-well or 24-well tissue culture plates, at 37°C under humidified 5% CO_2 in room air and in medium 199 supplemented with Earle's salts, 20 mmol/L HEPES, 100 mU/L penicillin, 100 mg/L streptomycin, 2.5 mg/L fungisone, 2 mmol/L glutamine, and 20% FBS. The medium was replaced every 2 days until confluence (3–5 days). HUVEC purity was ascertained by the cobblestone morphology typical for quiescent endothelial cells and by staining for PECAM-1 (CD31).

2.3. Isolation of peripheral blood mononuclear cells

Heparinized blood was diluted with PBS, and 25 mL was immediately layered over 15 mL Ficoll-Paque and centrifuged ($400 \times g$, 40 min, 22°C). The mixed mononuclear cell band was removed by aspiration, washed with PBS, centrifuged, and suspended in medium containing the same compounds as used for HUVEC culture, except that FBS concentration was reduced to 5%. The suspended cells were counted and used in the adhesion assay. Peripheral blood mononuclear cells (PBMC) were composed of approximately 10% monocytes and 90% lymphocytes.

2.4. Measurement of VCAM-1 and ICAM-1 expression in HUVEC

Second-passage HUVEC were cultured in gelatin-coated 24-well plates. Upon confluence, FBS concentration was reduced to 5% and culture was continued for 30 minutes, 12 hours, or 24 hours without or with butyrate added to a concentration of 0.1, 1.0, or 10.0 mmol/L (stock solution of 100 mmol/L butyric acid was neutralized with 1 mol/L NaOH). Next, HUVEC were incubated for 8 hours with TNFα (100 mU/L) or IL-1β (100 mU/L) in the absence or presence of butyrate. Under all conditions, cell viability was greater than 90% as judged by trypan blue exclusion.

After stimulation, cells were washed with PBS and treated for 30 minutes at 4°C with saturating amounts of PE-conjugated anti–PECAM-1 (CD31), FITC-conjugated anti–ICAM-1 (CD54), or FITC-conjugated anti–VCAM-1 (CD106) monoclonal antibodies (Mab). For isotype control, cells were treated with FITC-conjugated mouse anti-IgG_1 Mab. Cells were next washed with PBS, fixed in 1% paraformaldehyde, harvested by mild trypsinization, washed again, and centrifuged at 400 $\times$ g for 10 minutes before analysis (10,000 cells per sample) by flow cytometry (FACS Calibur, Becton Dickinson). After correction for nonspecific binding (isotype control), mean specific fluorescence intensity was measured in each channel.

2.5. Adhesion assay

HUVEC grown to confluence in 24-well plates were pretreated with butyrate (10 mmol/L) for 24 h and stimulated with TNFα (100 mU/L) or IL-1β (100 mU/L) for 8 hours before the adhesion assay. Briefly, HUVEC were washed with PBS and coincubated for 30 minutes with PBMC suspension (1×10^6/mL). The PBMC suspension was withdrawn and HUVEC were washed twice with PBS to remove nonadherent cells. Next, endothelial cells and the adhering monocytes and lymphocytes were treated for 30 minutes at 4°C with saturating amounts of FITC-conjugated mouse anti-CD45 and PE-conjugated anti-CD14 Mab. Cells were next washed with PBS, fixed in 1% paraformaldehyde, washed again, harvested by mild trypsinization, centrifuged, and counted before analysis (10,000 cells/sample) by flow cytometry. The proportion of monocytes and lymphocytes in the suspension was determined by measuring fluorescence I (FLI–CD45) and fluorescence II (FLII–CD14). The absolute number of monocytes and lymphocytes adhering to endothelial cells was calculated in relation to the total number of cells obtained after trypsinization. The results were expressed as percentage of monocytes and lymphocytes added.

2.6. Quantitation of VCAM-1, ICAM-1, and PPARα gene expression

For VCAM-1 and ICAM-1 gene quantitation, HUVEC were grown to confluence in 6-well plates, pretreated with butyrate (10 mmol/L) for 24 hours, and stimulated with TNFα (100 mU/L) for 4 hours. For PPARα gene quantitation, HUVEC were grown to confluence in 6-well plates and incubated with butyrate (0.1, 1.0, and 10.0 mmol/L) for 24 hours. Total RNA (from 10^6 cells) was isolated according to Chomczyński and Sacchi [28] using TRIZOL and its concentration was determined by spectrophotometry at 260 nm. RNA (500 ng) was dissolved in 20 μL of the reaction solution containing 2.5 mmol/L of dATP, dTTP, dCTP, and dGTP, 20 U of RNAsin, 100 pmol/L of random hexamers, and 20 U of MMLV reverse transcriptase. Incubation was carried out at 37°C for 60 minutes. The temperature of the reaction was then raised to 94°C for 5 minutes to inactivate the enzyme and finally reduced to 4°C. An aliquot of cDNA (5 μL of RT mixture) was dissolved in 25 μL of the reaction solution containing 10x PCR buffer (final Mg^{2+} concentration was 1.5 mmol/L), 2.5 mmol/L of dATP, dTTP, dCTP, and dGTP, 10 pmol/L of up- and downstream primers (PPARα, VCAM-1, ICAM-1, GAPDH, and β-actin), and 1 U *Taq*polymerase. For semiquantitative analysis, linearity of amplification of PPARα, VCAM-1, ICAM-1, GAPDH, and β-actin cDNAs depending on PCR cycle number was established in preliminary experiments. A total of 28 cycles for PPARα, 16 for VCAM-1, 18 for ICAM-1, 22 for GAPDH and for β-actin, there was 24 cycles, resulted in the best amplification profile to detect differences among the samples. The following sets of primers were used in PCR amplification: for VCAM-1 [29]: sense 5′-CCCTTGACCG-GCTGGAGATT-3′, antisense 5′-CTGGGGGCAACATT-GACATAAAGTG-3′, ICAM-1 [Primer Select 4.00 DNASTAR]; sense: 5′-TGAAGGCCACCCCAGAGGACAAC-3′, antisense 5′-CCCATTATGACTGCGG CTGCTGCTACC-3′, GAPDH [30]: sense 5′-GAGTCAACGGATTTGGTCGT-3′, antisense 5′-GTTGTCATGGATGACCTTGG-3′, PPARα [Primer Select 4.00 DNASTAR]: sense 5′-GCCCCTCCTCG-GTGACTTATC-3′, antisense 5′-ATGACCCGGGCTTT-GACCTT-3′, β-actin [31]: sense 5′-CCTCGCCTTTGC-CGATCC3′, antisense 5′-GGATCTTCATGAGGTAG TCAGTC-3′.

The amplification profile consisted of an initial denaturation at 94°C for 3 minutes (for GAPDH, β-actin) and 5 minutes (for PPARα, VCAM-1, and ICAM-1) followed by denaturation at 94°C for 25 seconds for all genes, annealing at 57°C (GAPDH), 60°C (β-actin, PPARα), and 62°C (VCAM-1, ICAM-1) for 50 seconds and extension at 72°C for 50 seconds for all genes.

Amplification products obtained with PCR (VCAM-1 241 bp, ICAM-1 409 bp, GAPDH 482 bp, PPARα 454 bp, and β-actin 626, cDNA) were electrophoretically separated on 3% agarose gel. Ethidium bromide–stained bands of PPARα, VCAM-1, ICAM-1, and GAPDH were photographed with the DS-34 Polaroid camera (Sigma, St. Louis, MO, USA). Band intensity was densitometrically measured with the gel analysis macro supplied with the National Institutes of Health (Bethdesda, MD) image (available free at: http://rsb.info.nih.gov/nih-image/). VCAM-1 and

ICAM-1 signals were normalized to cDNA levels of the housekeeping gene GAPDH, PPARα to β-actin and expressed as a ratio.

2.7. Measurement of NF-κB activation

To measure NF-κB activation, confluent HUVEC were pretreated without or with butyrate (10 mmol/L) for 24 hours and exposed to TNFα (100 mU/L) for 2 hours. NF-κB activation was measured in whole cell extract with an enzyme-linked immunoassay (ELISA)–based kit. Briefly, HUVEC were washed with ice-cold PBS, scraped into tubes, and centrifuged. The pellet was lysed with complete lysis buffer containing dithiothreitol and a protease inhibitor cocktail. After centrifugation at 5000 × g and 4°C for 20 minutes, the protein concentration in the supernatant (whole cell extract) was determined with a Bradford-based assay. To determine NF-κB activation with the ELISA-based kit, an oligonucleotide containing the NF-κB consensus binding site (5′-GGGACTTTCC-3′) specific for the active form of NF-κB was immobilized to a 96-well plate and the well was filled with 10 μg of the whole cell extract. After 1 hour incubation and three washings, the primary antibody against the active form of NF-κB recognizing an epitope on p65 that is accessible only when NF-κB is activated and bound to its target DNA was added for 1 hour. After washing, the secondary antibody conjugated to horseradish peroxidase was added to achieve a sensitive readout by spectrophotometry at 450 nm. The experiments were performed in duplicate and the results were expressed as OD_{450nm}.

2.8. Statistical analysis

The results were expressed as mean ± SEM. Differences were analyzed by one-way analysis of variance followed by Fisher's protected least significant difference test. The level of significance was taken as $P < 0.05$.

3. Results

3.1. Effect of butyrate on VCAM-1 and ICAM-1 expression by cytokine-stimulated HUVEC

VCAM-1 and ICAM-1 expression on the surface of endothelial cells was analyzed by flow cytometry. Expression was related to mean fluorescence intensity from FCS (forward scatter) and PECAM-1 (CD31) gated cells, representing 80–90% of all cells analyzed. Expression of PECAM-1 was taken as a marker of endothelial viability, considering the fact that the protein is always present on viable cells and disappears from dead cells [32]. When compared with control cells, no significant changes in percentage of PECAM-1–positive cells in experiments with either butyrate or butyrate and cytokines were found.

As expected, HUVEC cultured for 8 hours with TNFα or IL-1β demonstrated markedly ($P < 0.001$) enhanced VCAM-1 and ICAM-1 expression compared with resting cells (Table 1). Pretreatment with butyrate (10 mmol/L) for 24 hours significantly ($P < 0.001$; $n = 7$) reduced VCAM-1 and ICAM-1 expression by 67–80% and 29–37%, respectively, by TNFα-stimulated HUVEC. Expression on HUVEC stimulated with IL-1β was reduced by 47–73% ($P < 0.001$; $n = 7$) for VCAM-1 and by 23–36% ($P < 0.001$; $n = 7$) for ICAM-1.

Table 1
Butyrate inhibits TNFα- and IL-1–induced VCAM-1 and ICAM-1 expression in HUVEC

Treatment	VCAM-1	ICAM-1
Control (medium only)	3.4 ± 3.4	29.0 ± 4.5
TNFα	100*	100*
Butyrate + TNFα	26.2 ± 6.9†	67.4 ± 4.0†
IL-1	100*	100*
Butyrate + IL-1	40.1 ± 13.1†	70.3 ± 6.5†

Cells were pretreated with or without butyrate (10.0 mmol/L) for 24 h before incubation without or with TNFα (100 mU/L) or IL-1 (100 mU/L) for 8 h. Cells were next stained for VCAM-1 and ICAM-1 and analyzed by flow cytometry. Results are expressed as percentage of TNFα- or IL-1–stimulated cells (% control) and are given as mean ± SD from seven experiments.

* $P < 0.001$ compared to untreated cells.

† $P < 0.001$ compared to cells stimulated with TNFα or IL-1 in the absence of butyrate.

To investigate the time- and concentration-dependent effect of butyrate on VCAM-1 and ICAM-1 expression, HUVEC were pretreated for different times with butyrate at several concentrations before stimulation with TNFα. Inhibition of TNFα-induced VCAM-1 and ICAM-1 expression depended on the concentration used, with maximal reduction observed with 10 mmol/L butyrate (Fig. 1A). In addition, butyrate inhibited VCAM-1 and ICAM-1 expression induced by TNFα in a time-dependent manner, with maximal reduction of VCAM-1 expression noted after 12 hours and ICAM-1 expression after 24 hours of butyrate pretreatment (Fig. 1B).

3.2. Effect of butyrate on adhesiveness of TNFα- or IL-1β–stimulated HUVEC to monocytes and lymphocytes

In an attempt to determine the functional importance of inhibitory properties of butyrate on TNFα- or IL-1β–stimulated expression of VCAM-1 and ICAM-1, we have studied the adhesion of freshly isolated monocytes and lymphocytes to HUVEC. Stimulation of HUVEC with TNFα significantly ($P < 0.001$) increased the number of adherent monocytes from 19.0% ± 2.9% to 26.3% ± 3.5% (Fig. 2A) and the number of lymphocytes from 2.0% ± 0.5% to 11.2% ± 1.5% (Fig. 2B). The same pattern was found when TNFα was replaced by IL-1β: the percentage of adherent monocytes increased from 19.0% ± 2.0% to 25.1% ± 3.7% (Fig. 2A) and lymphocytes from 2.0% ± 0.5% to 10.3% ±

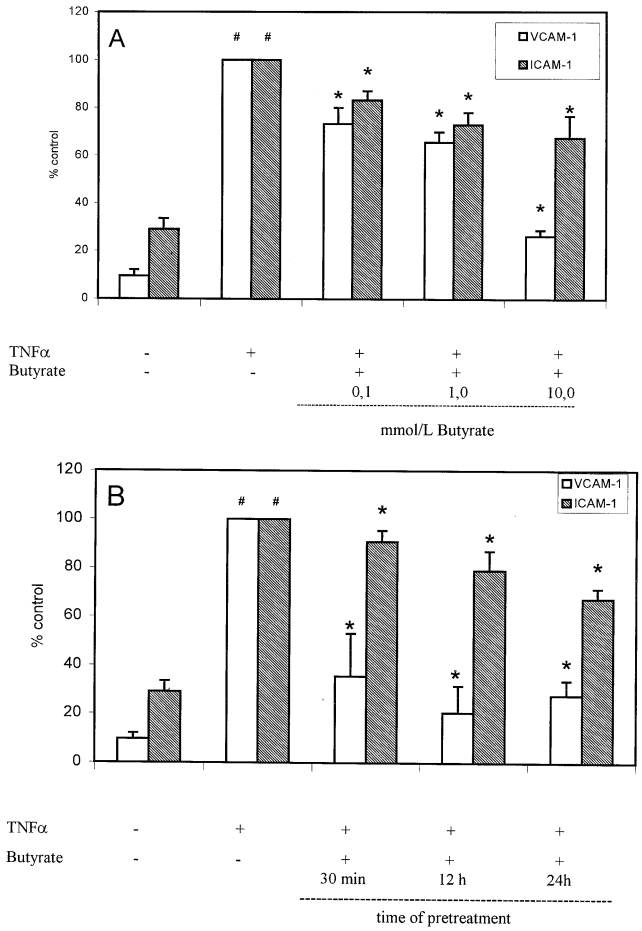

Fig. 1. Butyrate inhibits TNFα-induced cell surface expression of VCAM-1 and ICAM-1 in HUVEC. (*A*) Cells were pretreated without or with butyrate (0.1, 1.0, 10.0 mmol/L) for 24 hours before incubation without or with TNFα (100 mU/L) for 8 hours. (*B*) Cells were pretreated without or with butyrate (10.0 mmol/L) for 30 minutes, 12 hours, and 24 hours, and then stimulated with TNFα (100 mU/L) for 8 hours. Next, cells were stained for VCAM-1 and ICAM-1 and analyzed by flow cytometry. Results are expressed as percentage of TNFα-stimulated cells (100%) and are given as mean ± SD from seven experiments. $^{*}P < 0.001$ compared with cells stimulated with TNFα in the absence of butyrate; $^{\#}P < 0.001$ compared with untreated cells.

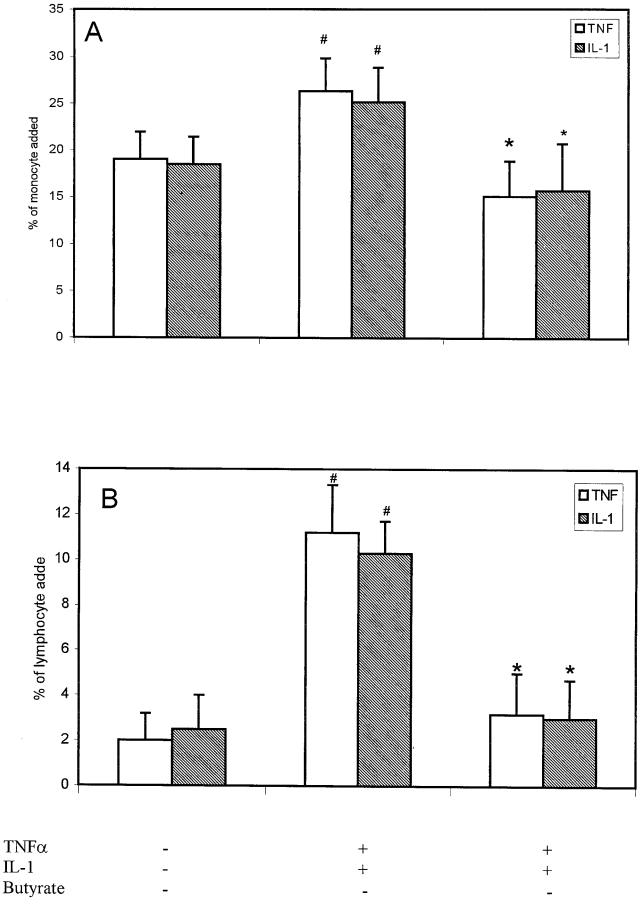

Fig. 2. Butyrate inhibits adhesion of monocytes (*A*) and lymphocytes (*B*) to TNFα or IL-1β–stimulated HUVEC. Endothelial cells were pretreated without or with butyrate (10.0 mmol/L) for 24 hours before incubation without or with TNFα (100 mU/L) or IL-1 (100 mU/L) for 8 hours. Freshly isolated PBMC were coincubated with these cells for 30 minutes. Subsequently, monocyte and lymphocyte adhesion was measured as described in the "Methods and material " section. Results are expressed as percentage of monocytes and lymphocytes added and are given as mean ± SD from nine experiments.$^{*}P < 0.001$ compared with cells stimulated with TNFα in the absence of butyrate; $^{\#}P < 0.001$ compared with untreated cells.

1.4% (Fig. 2B). Pretreatment with 10 mmol/L butyrate for 24 hours significantly ($P < 0.001$; $n = 9$) reduced the adhesion of monocytes to TNFα-stimulated HUVEC (Fig. 2A) from 26.3 ± 3.5% to 15.1 ± 3.7% and to IL-1β-stimulated HUVEC from 25.1% ± 3.7% to 15.7% ± 5.0%. Reduction varied with each population, but the mean percentage of adherent monocytes was lower than in the case of unstimulated cells, suggesting that butyrate not only completely abolished TNFα or IL-1β-induced increase in HUVEC adhesiveness for monocytes but also decreased the adhesion of monocytes to unstimulated cells. Adhesion of lymphocytes to TNFα-stimulated HUVEC was reduced from 11.2% ± 1.5% to 3.2% ± 1.2% ($P < 0.001$; $n = 9$) and to IL-1β–stimulated HUVEC from 10.3% ± 1.4% to 3.0% ± 1.7% ($P < 0.001$; $n = 9$) (Fig. 2B).

3.3. Effect of butyrate on VCAM-1 and ICAM-1 mRNA levels in HUVEC stimulated with TNFα

To explore the mechanism responsible for inhibition of VCAM-1 and ICAM-1 surface expression, we studied mRNA levels for these molecules using RT PCR. Stimulation of HUVEC with TNFα for 4 hours induced an approximately 13-fold increase ($P < 0.001$) in specific VCAM-1 PCR products (Fig. 3A). Pretreatment with butyrate (10 mmol/L) for 24 hours completely abolished induction of VCAM-1 transcripts by TNFα (from 0.52 ± 0.09 to 0.04 ± 0.02; $P < 0.001$; $n = 5$). Stimulation of HUVEC with TNFα for 4 hours induced an approximately 2-fold increase ($P < 0.001$) in specific ICAM-1 PCR products (Fig. 3B). As in the case of surface expression, the inhibitory action of butyrate on TNFα-induced levels of ICAM-1 mRNA was less pronounced than for VCAM-1 mRNA. Preincubation

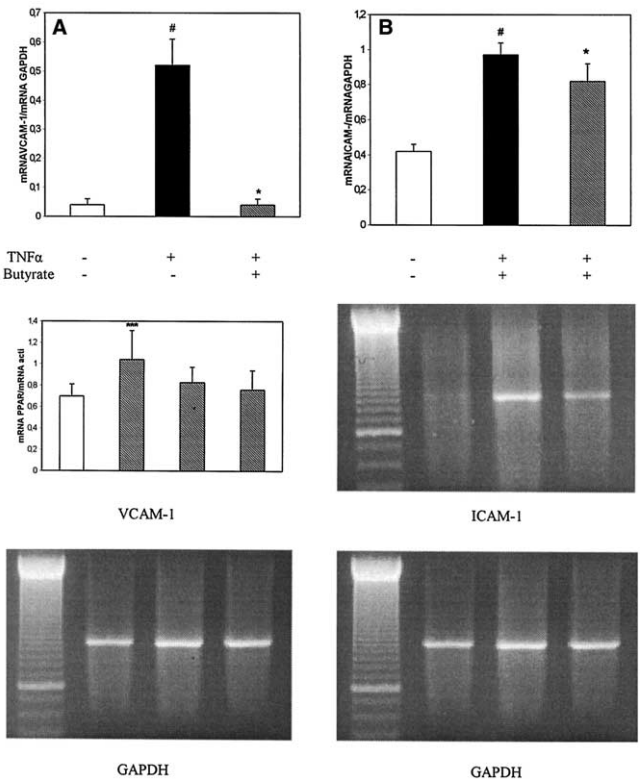

Fig. 3. Butyrate inhibits VCAM-1 (*A*) and ICAM-1 (*B*) mRNA expression in HUVEC. Cells were pretreated without or with butyrate (10.0 mmol/L) for 24 hours before incubation without or with TNFα (100 mU/L) for 4 hours. mRNA was extracted, reverse transcribed, and amplified by PCR using specific primers for GAPDH, VCAM-1, and ICAM-1. (*A and B*) Optical densities from five separate experiments after normalization to GAPDH (internal standard). Data are expressed as VCAM-1/GAPDH and are given as mean ± SD from five experiments. $^{\#}P < 0.001$ compared with untreated cells; $^{**}P < 0.01$, $^{*}P < 0.001$ compared with cells stimulated with TNFα in the absence of butyrate. Photographs below graphs show ultraviolet-illuminated gel electrophoregrams from a representative experiment.

with butyrate reduced the induction of ICAM-1 mRNA by 13–22% (from 0.97 ± 0.07 to 0.81 ± 0.1; $P < 0.01$; $n = 5$). Neither TNFα, nor butyrate altered GAPDH transcription.

3.4. Effect of butyrate on TNFα-induced NF-κB p65 activation

We decided to check whether NF-κB activation was involved in the effects of butyrate on TNFα-stimulated expression of adhesion molecules by measuring the amount of p65 with DNA-binding activity in whole cell extracts of treated HUVEC with a new ELISA-based assay. TNFα-stimulated HUVEC showed a marked ($P < 0.001$) increase (approximately 7-fold) in the levels of active form of NF-κB (Fig. 4). Pretreatment of HUVEC with butyrate (10 mmol/L) for 24 hours significantly reduced (from 0.44 ± 0.06 to 0.11 ± 0.05; $P < 0.001$; $n = 9$) NF-κB p65 activation compared with TNFα-treated cells. Reduction varied in individual populations, ranging from 69% to 84%.

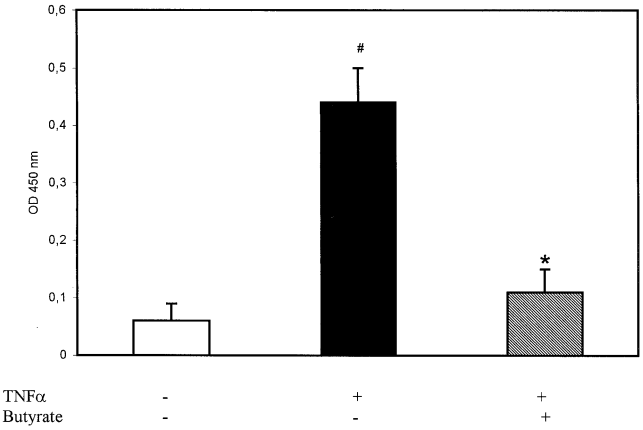

Fig. 4. Butyrate inhibits TNFα-induced activation of NF-κB. Cells were pretreated without or with butyrate (10.0 mmol/L) for 24 hours before incubation without or with TNFα (100 mU/L) for 2 hours. A whole cell extract was obtained and NF-κB activation was measured with an ELISA-based kit. Results are expressed as OD_{450nm} and are given as mean ± SD from nine experiments. $^{*}P < 0.001$ compared with cells stimulated with TNFα in the absence of butyrate; $^{\#}P < 0.001$ compared with untreated cells.

3.5. Effect of butyrate on PPARα expression

Because it has been suggested that PPARα negatively interacts with the activated NF-κB signaling pathway, we searched for an activatory effect of butyrate on PPARα. RT-PCR analysis revealed that incubation of HUVEC with butyrate (10 mmol/L) for 24 hours significantly induced the synthesis of specific PPARα PCR products from 0.7 ± 0.11 to 1.04 ± 0.27 ($P < 0.05$; $n = 6$) (Fig. 5). Induction varied in individual populations, ranging from 30% to 61%. Butyrate did not alter constitutive β-actin transcription.

4. Discussion

Adhesion and transendothelial migration of leukocytes into the surrounding tissues are crucial steps in inflammation, immunity, and atherogenesis [1–3]. Vascular endothelial cells play an active role in this process by expressing cell adhesion molecules which enhance their adhesiveness for leukocytes. Adhesion molecules have been implicated in tumor progression [5]. It now appears from our findings that butyrate is capable of interfering with these pathologically important processes.

We have demonstrated for the first time that butyrate inhibits TNFα or IL-1 stimulated expression of VCAM-1 and ICAM-1 by reducing both protein and specific mRNA production through inhibition of NF-κB activation. Inhibition was associated with decreased adhesiveness of monocytes and lymphocytes to stimulated endothelial cells.

The present study was undertaken in pursuit of the possibility that butyric acid, a natural short-chain fatty acid produced inter atia from ingested polysaccharides, may be

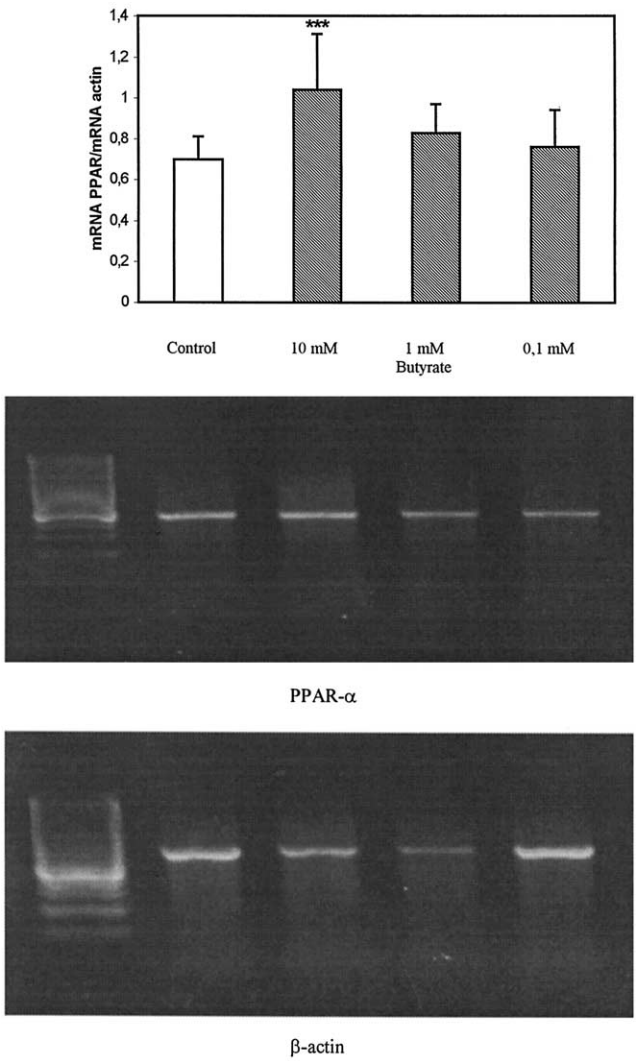

Fig. 5. Butyrate induces PPARα mRNA synthesis in HUVEC. Cells were incubated with butyrate (10.0, 1.0, and 0.1 mmol/L) for 24 hours. mRNA was extracted, reverse transcribed, and amplified by PCR using specific primers for β-actin and PPARα. (*Top*) Optical densities from six separate experiments after normalization to β-actin (internal standard). Data are expressed as PPARα/β-actin and are given as mean ± SD from six experiments. $^{***}P < 0.05$ compared with cells stimulated with TNFα in the absence of butyrate. Photographs below graph show ultraviolet-illuminated gel electrophoregrams from a representative experiment.

behind the reported benefits of dietary fiber and some food products containing bacterial strains [33]. Most dietary fibers are fermented in the colon to short-chain fatty acid (mainly acetic, propionic, and butyric acids) [34]. Acetic and propionic acids pass into the blood and are taken up by the liver, whereas butyric acid is almost entirely absorbed by the colonic epithelium with only a small portion of butyrate passing into the blood. This fact has prompted research on the benefits of butyrate for the prevention and treatment of gastrointestinal diseases. Butyrate has been shown to increase wound healing and to reduce inflammation in the intestine [35]. As an agent inducing growth arrest and apoptosis, butyrate seems capable of preventing colorectal cancer [20,21,36,37].

Evidence indicates that butyrate is a pleiotropic agent inducing numerous biochemical changes in different cell types. This fatty acid has been shown to inhibit T cell activation in response to mitogens or allo-antigens and to downregulate the capacity of monocytes to stimulate T cells [38–40]. Butyrate was able to inhibit bacteria-stimulated production of proinflammatory IL-12 and TNFα cytokines by purified peripheral blood monocytes [18]. Recent studies suggest that butyrate exhibits antiatherogenic properties by inhibiting vascular smooth muscle cell proliferation [25].

We have shown that butyrate at millimolar (physiological) concentrations inhibits the cytokine-stimulated expression of VCAM-1 and ICAM-1, with the effect being more potent in the case of the former adhesion molecule. These findings appear to be of relevance for the antiatherogenic and antitumor properties of butyrate. The recruitment of monocytes and lymphocytes into the vascular intima is a characteristic feature of atherosclerosis from its early stages [2]. It has been suggested that the selectivity of this process for mononuclear leukocytes depends on the presence of VCAM-1 [41]. Interactions between VCAM-1 and VLA-4 have recently been implicated in tumor intravasation [42].

Our results offer additional evidence for the selective inhibition of VCAM-1 expression previously observed with several substances. Cominacini et al. [43] found that probucol and vitamin E suppress the oxyLDL-stimulated expression of VCAM-1 and ICAM-1, the effect being greater in the case of the former adhesion molecule. Drugs with antioxidant properties, such as N-acetylcysteine, verapamil, or flavonoids, are selective inhibitors of cytokine-stimulated synthesis of VCAM-1 [44–46]. We have recently reported that probucol and vitamin E selectively inhibit VCAM-1 but not ICAM-1 expression [47,48]. One possible explanation for the different effects on VCAM-1 and ICAM-1 is that both adhesion molecules share common regulatory signaling pathways after receptor activation, but their expression is modulated by gene-specific signal transduction.

The decrease in VCAM-1 and ICAM-1 expression prompted us to examine the effect of butyrate on NF-κB, a key transcription factor implicated in the regulation of a variety of genes participating in immune and inflammatory responses, including those encoding VCAM-1 and ICAM-1 [7]. We have demonstrated that butyrate inhibits cytokine-simulated NF-κB activation in endothelial cells, as already observed with other cell types. For example, butyrate was found to inhibit IL-1–mediated NF-κB activation in human epithelial cells [49]. In the human HT-29 colonic cell line, butyrate reduced both constitutive and cytokine-stimulated NF-κB activation [50]. LPS-induced expression of cytokines and activation of NF-κB was abolished by butyrate in peripheral mononuclear cells [17]. In unstimulated cells, NF-κB is mostly present in association with its inhibitory molecule (IκB) and appears to be activated by TNFα or IL-1 through phosphorylation of the IκB subunit [7]. It has

been suggested that release of NF-κB from its complex with IκB is mediated by free radicals and hydrogen peroxide. Release of NF-κB is followed by translocation of active p50/p65 dimers to the cell nucleus and their binding to gene promoter regions. We have shown that levels of the active form p65 in endothelial cells are increased by TNFα and decreased by pretreatment with butyrate. In stimulated endothelial cells expressing VCAM-1 and ICAM-1, antioxidants such as N-acetylcysteine, pyrrolidine, dicarbamate, or probucol suppress NF-κB activation and VCAM-1 expression without any effect on ICAM-1 [32,51].

Oxidative stress is another factor that appears to be involved in VCAM-1 gene regulation [52]. Interestingly, butyrate was attributed with antioxidative properties when it was revealed that it suppresses LDL oxidation and reduces oxidative stress in macrophages stimulated with azobisamidinopropane dihidrochloride [26]. We are of the opinion that preincubation with butyrate triggers an antioxidant-sensitive mechanism suppressing NF-κB activation and VCAM-1 expression in cytokine-stimulated endothelial cells. The markedly weaker inhibition of ICAM-1 expression by butyrate exposes other mechanisms implicated in the suppression of NF-κB activation and ICAM-1 expression. It has been shown that butyrate prevents IκBα degradation in a human colon cell line stimulated with TNFα by reducing cellular proteasome activity [53]. Butyrate was found to inhibit NF-κB activation by stabilizing IκBα and IκBβ [54]. The ability of butyrate to inhibit NF-κB activation seems to be derived in part from its inhibitory effect on histone deacetylase [50].

In the present study, exposure of endothelial cells to butyrate was associated with activation of PPARα. The inhibitory action of PPARα on NF-κB activation has already been suggested [8]. Moreover, inhibition of VCAM-1 transcription by fenofibrate, an activator of PPARα, was partly attributed to suppression of NF-κB activation [9]. Activation of PPARα by butyrate appears to be relevant to the antiatherogenic properties of this fatty acid, considering the fact that fenofibrate reduces triglyceride-rich lipoprotein release and increases HDL-cholesterol levels [8]. Recently has been shown that butyrate in the presence of ^{14}C-oleic acid led to a significant reduction in triglyceride synthesis and diminished the secretion of very–low density lipoproteins by Cac0-2 cells [55].

In conclusion, our findings indicate that anti-inflammatory, and perhaps antiatherogenic, action of butyrate is partly attributable to an inhibitory effect on NF-κB activation and to the associated expression of VCAM-1 and ICAM-1. This possibility merits further investigations with the hope of demonstrating the therapeutic usefulness of butyrate in several pathological events involving leukocyte recruitment. A butyrate preparation needs to be developed to ensure effective levels of this fatty acid in blood. Recently it has been shown that clobetasone butyrate, an analogue of butyrate, exhibits greater anti-inflammatory activity than hydrocortisone [56].

References

[1] Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 1994;76:301–14.

[2] Li H, Cybulsky MI, Gimbrone MA, Libby P. An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. Arterioscler Thromb 1993;13:197–204.

[3] Jang Y, Lincoff AM, Plow EF, Topol EJ. Cell adhesion molecules in coronary artery disease. J. Am. Coll. Cardiol 1994;24:1591–601.

[4] Price DT, Loscalzo J. Cellular adhesion molecules and atherogenesis. Am J Med 1999;107:85–97.

[5] Alexiou D, Karayiannakis AJ, Syrigos KN, Zbar A, Kremmyda A, Bramis I, Tsigris C. Serum levels of E-selectin, ICAM-1 and VCAM-1 in colorectal cancer patients: correlations with clinicopathological features, patient survival and tumour surgery. Eur J Cancer 2001;37:2392–7.

[6] Kume N, Cybulsky MI, Gimbrone MA Jr. Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. J Clin Invest 1992;90:1138–44.

[7] Collins T. Endothelial nuclear factor-kappa B and the initiation of the atherosclerotic lesion. Lab. Invest 1993;68:499–508.

[8] Fruchart JC, Duriez P, Staels B. Peroxisome proliferator-activated receptor-alpha activators regulate genes governing lipoprotein metabolism, vascular inflammation and atherosclerosis. Curr Opin Lipidol 1999;10:245–57.

[9] Marx N, Sukhova GK, Collins T, Libby P, Plutzky J. PPARalpha activators inhibit cytokine-induced vascular cell adhesion molecule-1 expression in human endothelial cells. Circulation 1999;99:3125–31.

[10] Bugaut M, Bentejac M. Biological effects of short-chain fatty acids in nonruminant mammals. Annu Rev Nutr 1993;13:217–41.

[11] Scheppach W. Effects of short chain fatty acids on gut morphology and function. Gut 1994;35(1 Suppl):S35–8.

[12] Vernia P, Marcheggiano A, Caprilli R, Frieri G, Corrao G, Valpiani D, Di Paolo MC, Paoluzi P, Torsoli A. Short-chain fatty acid topical treatment in distal ulcerative colitis. Aliment Pharmacol Ther 1995; 9:309–13.

[13] Steinhart AH, Brzezinski A, Baker JP. Treatment of refractory ulcerative proctosigmoiditis with butyrate enemas. Am J Gastroenterol 1994;89:179–83.

[14] Patz J, Jacobsohn WZ, Gottschalk-Sabag S, Zeides S, Braverman DZ. Treatment of refractory distal ulcerative colitis with short chain fatty acid enemas. Am J Gastroenterol 1996;91:731–4.

[15] Pinto A, Fidalgo P, Cravo M, Midoes J, Chaves P, Rosa J, dos Anjos Brito M, Leitao CN. Short chain fatty acids are effective in short-term treatment of chronic radiation proctitis: randomized, double-blind, controlled trial. Dis Colon Rectum 1999;42:788–96.

[16] Luhrs H, Gerke T, Muller JG, Melcher R, Schauber J, Boxberge F, Scheppach W, Menzel T. Butyrate inhibits NF-kappaB activation in lamina propria macrophages of patients with ulcerative colitis. Scand J Gastroenterol 2002;37:458–66.

[17] Segain JP, Raingeard de la Bletiere D, Bourreille A, Leray V, Gervois N, Rosales C, Ferrier L, Bonnet C, Blottiere HM, Galmiche JP. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. Gut 2000;47:397–403.

[18] Saemann M. D, Bohmig GA, Osterreicher C.H, Burtscher H, Parolini O, Diakos C, Stockl J, Horl WH, Zlabinger G.J. Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. FASEB J 2000;14: 2380–2.

[19] Feng P, Ge L, Akyhani N, Liau G. Sodium butyrate is a potent modulator of smooth muscle cell proliferation and gene expression. Cell Prolif 1996;29:231–41.

[20] Luhrs H, Kudlich T, Neumann M, Schauber J, Melcher R, Gostner A, Scheppach W, Menzel TP. Butyrate-enhanced TNFalpha-induced ap-

optosis is associated with inhibition of NF-kappaB. Anticancer Res 2002;22:1561–8.
[21] Gaschott T, Maassen CU, Stein J. Tributyrin, a butyrate precursor, impairs growth and induces apoptosis and differentiation in pancreatic cancer cells. Anticancer Res 2001;21:2815–9.
[22] Velazquez OC, Rombeau JL. . Butyrate. Potential role in colon cancer prevention and treatment Adv Exp Med Biol 1997;427:169–81.
[23] Hassig CA, Tong JK, Schreiber SL. Fiber-derived butyrate and the prevention of colon cancer. Chem Biol 1997;4:783–9.
[24] Whiteley LO, Klurfeld DM. Are dietary fiber-induced alterations in colonic epithelial cell proliferation predictive of fiber's effect on colon cancer? Nutr Cancer 2000;36:131–49.
[25] Ranganna K, Yatsu F. M, Hayes BE, Milton SG, Jayakumar A. Butyrate inhibits proliferation-induced proliferating cell nuclear antigen expression (PCNA) in rat vascular smooth muscle cells. Mol Cell Biochem 2000;205:149–61.
[26] Blache D, Gesquiere L, Loreau N, Durand P. Oxidant stress: the role of nutrients in cell-lipoprotein interactions. Proc Nutr Soc 1999;58: 559–63.
[27] Jaffe EA. Culture and identification of large vessel endothelial cells In: Jaffe EA, editor. Biology of Endothelial Cells. Boston: Martinus Niijhoff, 1984. p 1–13.
[28] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
[29] Polte T, Newman W, Gopal TV. Full length vascular cell adhesion molecule 1 (VCAM-1). Nucleic Acids Res 1990;18:5901.
[30] Ercolani L, Florence B, Denaro M, Alexander M. Isolation and complete sequence of a functional human glyceraldehyde-3-phosphate dehydrogenase gene. J Biol Chem 1988;263:15335–41.
[31] Raff T, van der Giet M, Endemann D, Wiederholt T, Paul M. Design and testing of beta-actin primers for RT-PCR that do not co-amplify processed pseudogenes. Biotechniques 1997;23:456–60.
[32] Weber C, Erl W, Pietsch A, Strobel M, Ziegler-Heitbrock HW, Weber PC. Antioxidants inhibit monocyte adhesion by suppressing nuclear factor-kappa B mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulated to generate radicals. Arterioscler Thromb 1994;14:1665–73.
[33] Salminen S, Bouley C, Boutron-Ruault MC, Cummings JH, Franck A, Gibson GR, Isolauri E, Moreau MC, Roberfroid M, Rowland I. Functional food science and gastrointestinal physiology and function. Br J Nutr 1998;80(Suppl 1):S147–71.
[34] Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut 1987;28:1221–7.
[35] Wachtershauser A, Stein J. Rationale for the luminal provision of butyrate in intestinal diseases. Eur J Nutr 2000;39:164–71.
[36] Barnard JA, Warwick G. Butyrate rapidly induces growth inhibition and differentiation in HT-29 cells. Cell Growth Differ 1993;4:495–501.
[37] McIntyre A, Gibson PR, Young GP. Butyrate production from dietary fibre and protection against large bowel cancer in a rat model. Gut 1993;34:386–91.
[38] Kyner D, Zabos P, Christman J, Acs G. Effect of sodium butyrate on lymphocyte activation. J Exp Med 1976;1976:144 :1674–8.
[39] Suthanthiran M, Rubin AL, Novogrodsky A, Stenzel KH. Immunosuppressive properties of polar organic compounds that induce cellular differentiation in Friend erythroleukemia cells. Transplantation 1982;33:534–40.
[40] Bohmig GA, Krieger PM, Saemann MD, Wenhardt C, Pohanka E, Zlabinger GJ. n-Butyrate downregulates the stimulatory function of peripheral blood-derived antigen-presenting cells: a potential mechanism for modulating T-cell responses by short-chain fatty acids. Immunology 1997;92:234–43.
[41] Huo Y, Ley K. Adhesion molecules and atherogenesis. Acta Physiol Scand 2001;173:35–43.
[42] Bogetto L, Gabriele E, Cariati R, Dolcetti R, Spessotto P, Doglioni C, Boiocchi M, Perris R, Colombatti A. Bidirectional induction of the cognate receptor-ligand alpha4/VCAM-1 pair defines a novel mechanism of tumor intravasation. Blood 2000;95:2397–2406.
[43] Cominacini L, Garbin U, Pasini AF, Davoli A, Campagnola M, Contessi GB, Pastorino AM, Lo Cascio V. Antioxidants inhibit the expression of intercellular cell adhesion molecule-1 and vascular cell adhesion molecule-1 induced by oxidized LDL on human umbilical vein endothelial cells. Free Radic Biol Med 1997;22:117–27.
[44] Marui N, Offermann MK, Swerlick R, Kunsch C, Rosen CA, Ahmad M, Alexander RW, Medford RM. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. J Clin Invest 1993;92:1866–74.
[45] Yamaguchi M, Suwa H, Miyasaka M, Kumada K. Selective inhibition of vascular cell adhesion molecule-1 expression by verapamil in human vascular endothelial cells. Transplantation 1997;63:759–64.
[46] Wolle J, Hill RR, Ferguson E, Devall LJ, Trivedi BK, Newton RS, Saxena U. Selective inhibition of tumor necrosis factor-induced vascular cell adhesion molecule-1 gene expression by a novel flavonoid. Lack of effect on transcription factor NF-kappa B. Arterioscler Thromb Vasc Biol 1996;16:1501–8.
[47] Zapolska-Downar D, Zapolski-Downar A, Markiewski M, Ciechanowicz A, Kaczmarczyk M, Naruszewicz M. Selective inhibition by alpha-tocopherol of vascular cell adhesion molecule-1 expression in human vascular endothelial cells. Biochem Biophys Res Commun 2000;274:609–15.
[48] Zapolska-Downar D, Zapolski-Downar A, Markiewski M, Ciechanowicz A, Kaczmarczyk M, Naruszewicz M. Selective inhibition by probucol of vascular cell adhesion molecule-1 (VCAM-1) expression in human vascular endothelial cells. Atherosclerosis 2001;155:123–30.
[49] Luhrs H, Gerke T, Boxberger F, Backhaus K, Melcher R, Scheppach W, Menzel T. Butyrate inhibits interleukin-1-mediated nuclear factor-kappa B activation in human epithelial cells. Dig Dis Sci 2001;46: 1968–73.
[50] Inan MS, Rasoulpour RJ, Yin L, Hubbard AK, Rosenberg DW, Giardina C. The luminal short-chain fatty acid butyrate modulates NF-kappaB activity in a human colonic epithelial cell line. Gastroenterology 2000;118:724–34.
[51] Chen YH, Lin SJ, Ku HH, Shiao MS, Lin FY, Chen JW, Chen YL. Salvianolic acid B attenuates VCAM-1 and ICAM-1 expression in TNF-alpha-treated human aortic endothelial cells. J Cell Biochem 2001;82:512–21.
[52] Offermann MK, Medford RM. Antioxidants and atherosclerosis: a molecular perspective. Heart Dis Stroke 1994;3:52–7.
[53] Yin L, Laevsky G, Giardina C. Butyrate suppression of colonocyte NF-kappa B activation and cellular proteasome activity. J Biol Chem 2001;276:44641–6.
[54] Chakravortty D, Koide N, Kato Y, Sugiyama T, Mu MM, Yoshida T, Yokochi T. The inhibitory action of butyrate on lipopolysaccharide-induced nitric oxide production in RAW 264.7 murine macrophage cells. J Endotoxin Res 2000;6:243–7.
[55] Marcil V, Delvin E, Seidman E, Poitras L, Zoltowska M, Garofalo C, Levy E. Modulation of lipid synthesis, apolipoprotein biogenesis, and lipoprotein assembly by butyrate. Am J Physiol Gastrointest Liver Physiol 2002;283:G340–6.
[56] Parneix-Spake A, Goustas P, Green R. Eumovate (clobetasone butyrate) 0.05% cream with its moisturizing emollient base has better healing properties than hydrocortisone 1% cream: a study in nickel-induced contact dermatitis. J Dermatol Treat 2001;12:191–7.