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PROPIONATE REDUCES THE CYTOKINE-INDUCED VCAM-1 AND ICAM-1 EXPRESSION BY INHIBITING NUCLEAR FACTOR- κ B (NF- κ B) ACTIVATION

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Adhesion and migration of leukocytes into the surrounding tissue are crucial steps in inflammation, immunity and atherogenesis. Expression of cell adhesion molecules by endothelial cells plays a role in these processes. Propionate is a naturally occurring short chain fatty acid produced by bacterial fermentation of dietary fibre. High intake of dietary fibre has been associated with an improved bowel function and with a reduced risk of cardiovascular disease. However, the molecular mechanisms responsible for these effects remain unknown. In this study, the effects of propionate on the expression of endothelial leukocyte adhesion molecules by cytokine-stimulated human umbilical vein endothelial cells (HUVEC) were investigated. Pretreatment of HUVEC with propionate significantly inhibited the tumor necrosis factor- α (TNF- α)-induced expression of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) in a time- and dose-dependent manner. At 10 mM, propionate also inhibited the interleukin-1 (IL-1)-mediated VCAM-1 and ICAM-1 expression, with the latter effect being more pronounced, as well as decreased the TNF- α -induced VCAM-1 and ICAM-1 mRNA expression in a similar manner. The decrease in VCAM-1 and ICAM-1 expression was associated with a reduction of adherence of monocytes and lymphocytes to the cytokine-stimulated HUVEC. In addition, propionate significantly inhibited the TNF- α -induced activation of nuclear factor- κ B (NF- κ B) and significantly increased the expression of peroxisome proliferator-activated receptor alpha (PPAR α) in HUVEC. These results demonstrate that propionate may have antiinflammatory and possibly antiatherogenic properties. Our findings warrant further investigation into the therapeutic effects of propionate on a number of pathological events involving leukocyte recruitment.

Key words: *propionate, inflammation, endothelial cells, cell adhesion molecules, transcription factors*

INTRODUCTION

The mechanisms of inflammatory response play a crucial role in the pathology of many diseases, including inflammatory bowel disease (IBD) (1) and diseases of atherosclerotic origin (2). Leukocyte infiltration into the intestinal or vascular wall is known to be central to their pathogenesis. Cellular influx into these sites is mediated by a chemical gradient of released chemotactic compounds and an expression of adhesion molecules on endothelial and inflammatory cells (3, 4). The most extensively studied endothelial adhesion molecules include intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), both belonging to immunoglobulins, as well as two selectins: E-selectin and P-selectin. Interactions between endothelial selectins and their ligands located on leukocytes mediate the temporary adhesion and rolling of leukocytes on endothelial cells, while those occurring between ICAM-1 and VCAM-1 and their leukocyte ligands play a key role both in the temporary and permanent adhesion of leukocytes, the latter being dependent on leukocyte activation. Upregulation of endothelial cell adhesion molecules, particularly ICAM-1, in the gut epithelial and vascular tissue is implicated in the pathogenesis of inflammatory bowel disease

(5, 6). Furthermore, an increased expression of endothelial adhesion molecules was also observed in atherosclerotic plaques (7), while the levels of circulating soluble form of VCAM-1 were shown to correlate with cardiovascular disease (8).

Most adhesion molecules described above are not present on resting endothelial cells. Their expression is a result of stimulation by specific molecules, such as tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), interferon- γ (INF- γ) and oxidatively modified LDL (oxLDL) (4, 9). Stimulation is mediated by NF- κ B, a key transcription factor implicated in the regulation of genes during the immune and inflammatory responses (10). It has been suggested that peroxisome proliferator-activated receptor alpha (PPAR α), a nuclear receptor involved in the transcriptional response to fatty acids, exerts its anti-inflammatory action through a negative transcriptional regulation of NF- κ B (11). It has been reported that fibric acid derivatives, known to be PPAR α activators, inhibit the cytokine-induced expression of VCAM-1 in human endothelial cells (12).

Fibre is an important nutritional component of the diet. Dietary fibre is a mixture of non-starch polysaccharides from undigested plant cell wall, which serve as substrates for anaerobic fermentation by bacteria in the colon. The majority of

cellulose fibres present in the diet are broken down in the large intestine to short chain fatty acids (SCFAs), mainly acetic, propionic and butyric acid, of which primarily the former two are being absorbed into the blood (13, 14). High intake of dietary fibre has been associated with an improved bowel function (15). Most of the effects of fibre on gastrointestinal cell function stem from the generation of SCFAs. Harig *et al.* (16), for instance, found that SCFAs decreased colonic inflammation in patients with colitis.

Many studies have showed a protective effect of dietary fibre on the outcomes of cardiovascular disease (CVD) (17-20). However, the mechanisms underlying the fibre's influence on the cardiovascular system have yet to be fully elucidated. Recent studies have suggested that the observed action on inflammatory response mechanisms may be of significance for the beneficial effects of high-fibre diet in CVD (20).

Butyrate is, by far, the most extensively studied SCFA, and its anti-inflammatory properties are well documented (21, 22). However, there is little information on acetate and propionate in this respect, although a report published recently has shown that propionic, but not acetic acid accelerated the healing of TNBS-induced colitis (23).

Since it has been previously shown that mononuclear cell recruitment plays a crucial role in the pathogenesis of inflammation and atherosclerosis, we decided to investigate 1) whether propionate inhibits the cytokine-induced expression of VCAM-1 and ICAM-1 and - in consequence - also the monocyte adhesion to endothelial cells, and 2) whether NF- κ B and PPAR α play a role in the interactions between cytokines and propionate.

MATERIALS AND METHODS

Materials

Medium 199, fungizone, penicillin, streptomycin, gentamycin, heat-inactivated bovine serum (FBS), collagenase, gelatine, glutamine, trypsin/EDTA solution and propionic acid were obtained from Sigma (USA). Recombinant TNF- α (10 mg, 1×10^8 U/mg) and IL-1 β (5×10^7 U/mg), random hexamers, reverse transcriptase, PCR buffer, Tag polymerase were purchased from Boehringer Mannheim (Germany). Tissue culture dishes were supplied by Costar (USA) and Ficoll-Paque by Pharmacia (Sweden). Monoclonal antibodies against: VCAM-1 (CD 106; FITC), ICAM-1 (CD 54; FITC), CD14 (CD14:PE), CD 45 (CD45:FITC), platelet/endothelial cell adhesion molecule-1 (PECAM-1) (CD31:PE) and IgG1 (IgG1:FITC) were from Becton Dickinson (USA). Nucleotide dATP, dTTP, dCTP and dGTP as well as RNasin were from Promega (USA). ELISA-based kit for measuring the NF- κ B activation was obtained from Active Motif (Belgium).

Endothelial cell isolation and culture

The experimental protocols were approved by the local Ethic Committee.

Human umbilical vein endothelial cells (HUVEC) were obtained from umbilical cords by collagenase digestion as described by Jaffe (24). In brief, veins of umbilical cords were perfused with PBS to remove blood cells, filled with 0.1% collagenase (type Ia) and incubated for 8 min at 37 °C. The resulting cellular suspension was supplemented with FBS and centrifuged (300 x g for 10 min). HUVEC were cultured in gelatine-coated 25 cm² flask and on 6-well or 24-well tissue culture plates, under humidified 5% CO₂ in room air at 37 °C, in Medium 199 supplemented with Earle's salts, 20 mM HEPES,

100 μ U/ml penicillin, 100 μ g/ml streptomycin, 2.5 μ g/ml fungisone, 2 mM glutamine and 20% FBS. The medium was changed every 2 days up to obtaining confluence (3-5 days). HUVEC purity was confirmed by the cobblestone morphology, typical for quiescent endothelial cells, and by PECAM-1 (CD31) staining.

All cells used in this study were of first or second passage.

Peripheral blood mononuclear cells (PBMC) preparation

Heparinized blood was diluted with PBS and 25 ml was immediately layered over 15 ml Ficoll-Paque and centrifuged (400 x g, 40 min, 22 °C). The mixed mononuclear cell band was removed by aspiration, washed with PBS, centrifuged and suspended in HUVEC culture medium with the FBS concentration reduced to 5%. The suspended cells were counted and used in the adhesion assay. PBMC consisted of approximately of 10% monocytes and 90% lymphocytes.

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

Second passage HUVEC were cultured in gelatine-coated 24-well plates. Upon confluence, FBS concentration was reduced to 5% and cultured without or with propionate at different concentrations (0.1, 1.0 or 10.0 mM - prepared from a stock solution of 100 mM by neutralization with 1 M NaOH) for 30 min, 12 h or 24 h. Next, HUVEC were incubated for 8 h with TNF- α (100 U/ml) or IL-1 β (100 U/ml) in the absence or presence of propionate. Cell morphology and viability were examined by measuring lactate dehydrogenase activity and trypan blue dye exclusion, and were found to remain unchanged under experimental condition.

Following stimulation, cells were washed with PBS and treated for 30 min at 4 °C with a saturating amount of PE-conjugated anti-PECAM-1 (CD31) and FITC-conjugated either anti-ICAM-1 (CD54) or anti-VCAM-1 (CD106) monoclonal antibody. For isotype control, FITC-conjugated mouse anti-IgG₁ was used. Next, cells were washed with PBS, fixed in 1% paraformaldehyde, harvested by mild trypsinization, washed again and centrifuged at 300 x g for 10 min. After that, cells were analysed (10,000 cells per sample) by flow cytometry (FACS Calibur, Becton Dickinson). After correction for unspecific binding (isotype control), specific mean fluorescence intensity (MFI) was measured in each channel.

Adhesion assay

HUVEC were grown to confluence on 24-well plates, pretreated with propionate (10 mM) for 24 h and stimulated with TNF- α (100 U/ml) or IL-1 β (100 U/ml) for 8 h, then the adhesion assay was performed. Briefly, HUVEC were washed with PBS and co-incubated for 30 min with a PBMC suspension (1×10^6 /ml). The PBMC suspension was removed and the cells were washed twice with PBS to remove nonadherent cells. Next, the cells consisting of endothelial cells, monocytes and lymphocytes were incubated for 30 min at 4 °C with saturating amounts of FITC-conjugated mouse anti-CD45 and PE-conjugated anti-CD14. Following that, cells were washed with PBS, fixed in 1% paraformaldehyde, washed again, harvested by mild trypsinization, centrifuged and counted. Cells prepared in this manner were analysed (10 000 cells per sample) by flow cytometry. The proportion of monocytes and lymphocytes in the suspension was determined by measuring fluorescence I (FLI - CD45) and fluorescence II (FL II - 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 a percentage of monocyte and lymphocyte added.

Quantitation of VCAM-, ICAM-1 and PPAR gene expression

For the quantitation of VCAM-1 and ICAM-1 gene expression, HUVEC were grown to confluence on 6-well plates, pretreated with propionate (10 mM) for 24 h and stimulated with TNF- α (100 U/ml) for 4 h. For PPAR α gene quantitation, HUVEC were grown to confluence in 6-well plates and then incubated with propionate (0.1, 1.0 or 10.0 mM) for 24h. Total RNA (10⁶ cells) was isolated by the method of Chomczynski (25) using TRIZOL, and its concentration was calculated from spectrophotometric measurements at 260 nm. RNA (500 ng) was dissolved in 20 μ l of the reaction mixture containing 2.5 mM of dATP, dTTP, dCTP, dGTP, 20 U of RNAsin, 100 pM 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 μ l of RT mixture) was dissolved in 25 μ l of the reaction mixture containing 10 x PCR buffer (final Mg²⁺ concentration: 1.5 mM), 2.5 mM of dATP, dTTP, dCTP, dGTP, 10 pM of up- and downstream primers (PPAR α , VCAM-1, ICAM-1, GAPDH and β -actin) and 1 U *Taq polymerase*. For semiquantitative analysis, linearity of amplification of PPAR α , VCAM-1, ICAM-1, GAPDH and β -actin cDNAs depending on the PCR cycle number was established in preliminary experiments. 28 cycles for PPAR α , 16 for VCAM-1, 18 for ICAM-1, 22 for GAPDH and for β -actin yielded the best amplification profile to allow for distinguishing the differences among the samples. The following sets of primers were used in PCR amplification:

VCAM-1: sense 5'-CCCTTGACCGGCTGGAGATT-3'
 antisense 5'-CTGGGGCAACATTGACATAAAGTG-3'
 ICAM-1: sense: 5'-TGAAGGCCACCCAGAGGACAAC-3'
 antisense 5'-CCATTATGACTGCGGCTGCTGCTACC-3'
 GAPDH : sense 5'-GAGTCAACGGATTTGGTCGT-3'
 antisense 5'-GTTGTCATGGATGACCTTGG-3'
 PPAR α : sense 5'-GCC CCT CCT CGG TGA CTT ATC-3'
 antisense 5'-ATG ACC CGG GCT TTG ACC TT-3'
 β -actin: sense 5'-CCTCGCCTTTGCCGATCC-3'
 antisense 5'GGATTCATGAGGTAGTCAGTC-3'

Amplification products obtained in PCR (241 bp for VCAM-1, 409 bp for ICAM-1 482 bp for GAPDH, 454 bp for PPAR α and β -actin) were separated by SDS-PAGE on a 3% agarose gel. Images of ethidium bromide-stained bands for PPAR α , VCAM-1, ICAM-1 and GAPDH were taken with a DS-34 Polaroid camera. The intensity of the bands was measured by densitometry using the gel analysis macro supplied with NIH

Image software. The signals were normalized (VCAM-1 and ICAM-1 to the cDNA levels of the GAPDH housekeeping gene, PPAR α to β -actin) and expressed as a ratio.

Measurement of the NF- κ B activation

To measure the NF- κ B activation, confluent HUVEC were preincubated with or without propionate (10 mM) for 24 h, followed by exposure to TNF- α (100 U/ml) for 2 h. NF- κ B activation was measured in a whole cell extract using the ELISA-based kit. Briefly, to obtain the whole cell extract, HUVEC were washed with ice-cold PBS, scraped into tubes and centrifuged. The pellet was lysed with the use of the Complete Lysis Buffer containing dithiothreitol and a protease inhibitor cocktail. After centrifugation at 14 000 rpm at 4 °C for 20 min, the protein concentration in the supernatant (whole cell extract) was determined by using the Bradford protein assay. To determine the NF- κ B activation with the use of the ELISA-based kit, 10 μ g of the whole cell extract was added to a 96-well plate on which an oligonucleotide containing the NF- κ B consensus binding site (5'-GGGACTTCC-3') has been immobilized. The active form of NF- κ B contained in the cell extract specifically bound to this oligonucleotide. After 1 h incubation, the plate was washed three times and the primary antibody against the active form of NF- κ B was added for the next 1 h. The antibody recognizes an epitope on p65 that is accessible only when NF- κ B is activated and bound to its target DNA. After washing, secondary antibody conjugated to horseradish peroxidase was added to obtain a sensitive readout by spectrophotometry at 450 nm. The experiment was performed in duplicates and results are expressed as OD_{450 nm}.

Statistical analysis

Results are expressed as mean \pm SD. Differences were analysed by 1-way ANOVA followed by Fisher's protected least significant difference test. A value of $p < 0.05$ was regarded as significant.

RESULTS

Effects of propionate on VCAM-a and ICAM-1 expression by TNF- α -stimulated HUVEC

VCAM-1 and ICAM-1 expression on the surface of endothelial cells was evaluated by flow cytometry. Expression was related to the mean fluorescence intensity from FCS (forward scatter) and PECAM-1 (CD 31) gated cells, representing 80-90% of the total pool of cells analysed. Expression of PECAM-1 was regarded as a marker of endothelial viability, in the light of the fact that it is always present on viable and disappears from dead cells (30). When compared with

Table 1. Propionate inhibits TNF- α - and IL-1-induced VCAM-1 and ICAM-1 expression in HUVEC.

Treatment	VCAM-1	ICAM-1
Control (medium only)	3,4 \pm 3,4	29,0 \pm 4,5
TNF α	100	100
Propionate + TNF α	41,3 \pm 5,1*	73,5 \pm 4,8*
IL-1	100	100
Propionate + IL-1	62,5 \pm 15,6*	70,3 \pm 9,4*

Cells were preincubated with or without propionate (10.0 mM) for 24 h prior to incubation without or with TNF- α (100 U/ml) or IL-1 (100 U/ml) for 8 h. Next, cells were stained for VCAM-1 and ICAM-1 and analyzed by flow cytometry. Results are expressed as percent of TNF- α - or IL-1-stimulated cells (% control) and presented as mean \pm SD from 7 experiments. * $p < 0.001$ - as compared to cells stimulated with TNF- α or IL-1 in the absence of propionate.

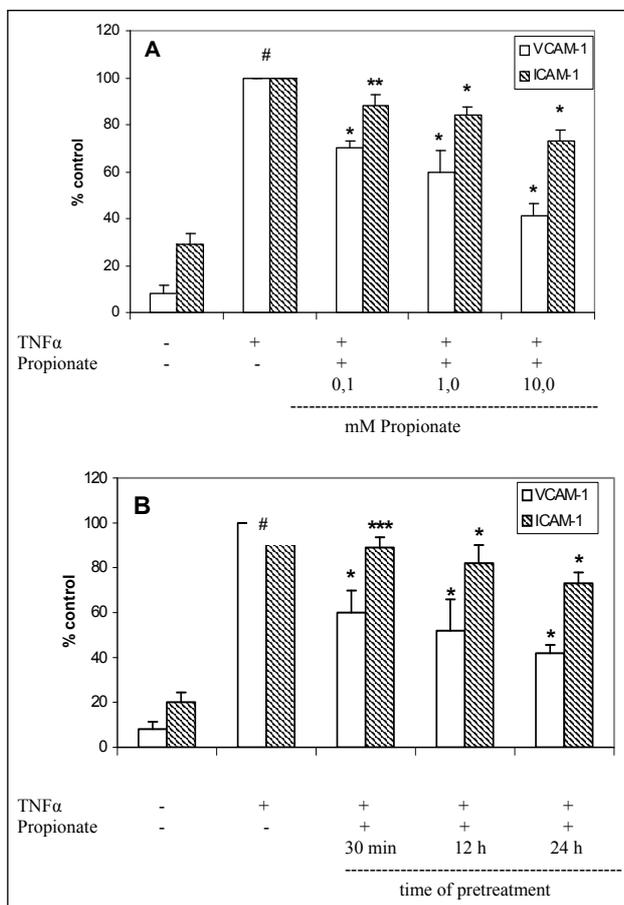


Fig. 1. Propionate inhibits TNF- α -induced cell surface expression of VCAM-1 and ICAM-1 in HUVEC. **A** - Cells were preincubated with or without propionate (0.1, 1.0, 10.0 mM) for 24 h prior to incubation without or with TNF- α (100 U/ml) for 8 h. **B** - Cells were preincubated with or without propionate (10.0 mM) for 30 min, 12 h or 24 h and then stimulated with TNF- α (100 U/ml) for 8 h. Cells were next stained for VCAM-1 and ICAM-1 and analyzed by flow cytometry. Results are expressed as percent of TNF- α -stimulated cells (% control), and are given as mean \pm SD from 7 experiments. # $p < 0.001$ as compared to control; *** $p < 0.05$, ** $p < 0.01$, * $p < 0.001$ - as compared to cells stimulated with TNF- α in the absence of propionate.

control cells, no significant changes were found in the PECAM-1 expression on cells exposed to propionate and TNF- α or IL-1.

As expected, HUVEC cultured for 8 h with TNF- α or IL-1 β demonstrated a marked increase in VCAM-1 and ICAM-1 expression as compared to resting cells (Table 1). Pretreatment of HUVEC with propionate (10 mM) for 24 h significantly reduced both VCAM-1 and ICAM-1 expression levels in TNF- α -stimulated cells: by 55-65% ($p < 0.001$, $n = 7$) and 22-32% ($p < 0.001$, $n = 7$), respectively. Expression of VCAM-1 and ICAM-1 on HUVEC stimulated with IL-1 β was reduced by 37-63% ($p < 0.001$, $n = 7$) for VCAM-1 and by 21-40% ($p < 0.001$, $n = 7$) for ICAM-1. It appeared that the effect of propionate on TNF- α -induced VCAM-1 expression was more pronounced as compared to ICAM-1 expression. Treatment of unstimulated HUVEC with propionate did not alter VCAM-1 and ICAM-1 expression (data not shown).

To investigate the time and concentration dependency of propionate treatment on the VCAM-1 and ICAM-1 expression, HUVEC were pretreated with propionate at different

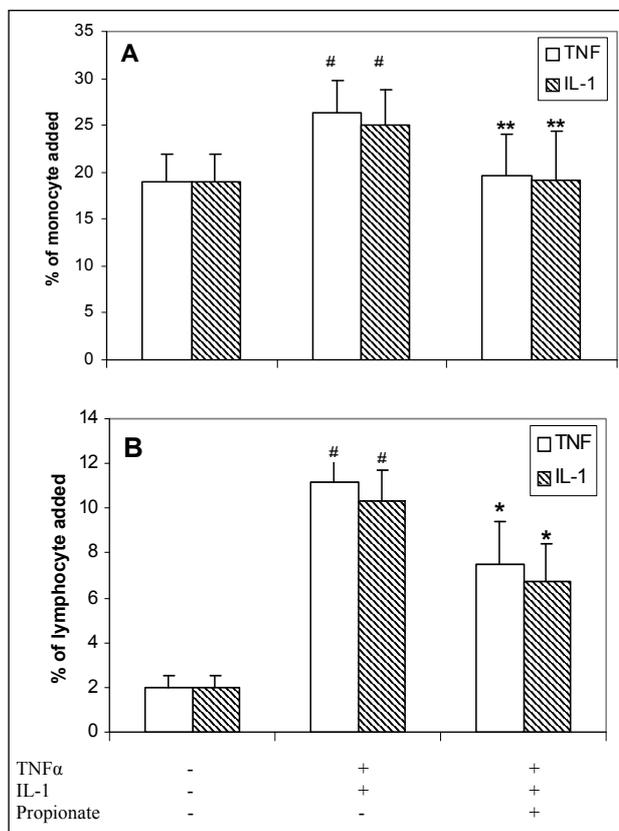


Fig. 2. Propionate inhibits adhesion of monocytes (A) and lymphocytes (B) to TNF- α -stimulated HUVEC.

Endothelial cells were preincubated with or without propionate (10.0 mM) for 24 h prior to incubation without or with TNF- α (100 U/ml) or IL-1 (100 U/ml) for 8 h. Freshly isolated PBMC were coincubated with these cells for 30 min. Next, monocyte and lymphocyte adhesion was measured as described in Materials and Methods. Results are expressed as a percentage of monocyte and lymphocyte added, and are given as mean \pm SD from 9 experiments. # $p < 0.001$ as compared to control; ** $p < 0.01$, * $p < 0.001$ - as compared to cells stimulated with TNF- α in the absence of propionate.

concentrations or for different periods of time prior to stimulation with TNF- α . Inhibition of the TNF- α -induced VCAM-1 and ICAM-1 expression depended on propionate concentration, with a maximum reduction observed at 10 mM (Fig. 1A). In addition, propionate inhibited VCAM-1 and ICAM-1 expression induced by TNF- α in a time-dependent manner, with the maximum reduction occurring after 24h of preincubation with propionate (Fig. 1B).

Effects of propionate on adhesive properties of TNF- α - or IL-1-stimulated HUVEC towards monocytes and lymphocytes

In an attempt to determine the functional importance of inhibitory properties of propionate on the TNF- α - or IL-1 β -induced expression of VCAM-1 and ICAM-1, we have studied the adhesion of freshly isolated monocytes and lymphocytes to HUVEC. Stimulation of HUVEC by TNF- α increased the number of adherent monocytes from 19.0% \pm 2.9% to 26.3% \pm 3.5% of added monocytes (Fig. 2A) and the number of lymphocytes from 2.0% \pm 0.7% to 11.2% \pm 1.5% of delivered lymphocytes (Fig. 2B). The same pattern was observed when TNF- α was replaced by IL-1 β . The number of adherent monocytes increased from 19.0% \pm 2.0% to 25.11% \pm 3.7%

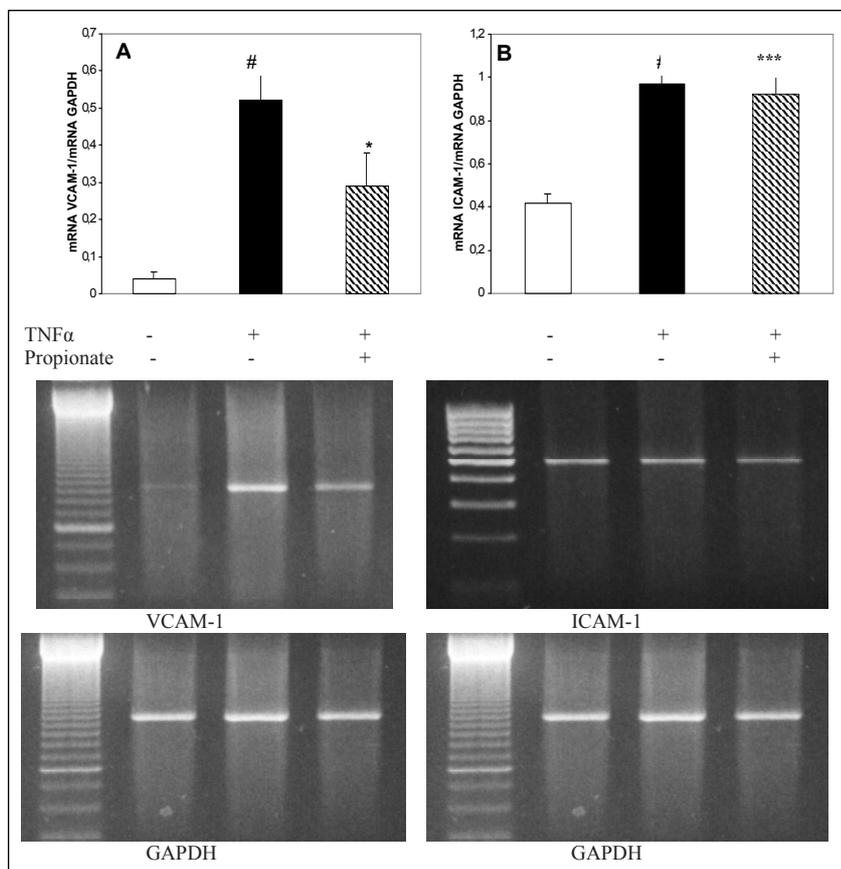


Fig. 3. Propionate inhibits VCAM-1 (A) and ICAM-1 (B) mRNA expression in HUVEC. Cells were preincubated with or without propionate (10.0 mM) for 24 h prior to incubation without or with TNF- α (100 U/ml) for 4 h. mRNA was extracted, reverse transcribed and amplified by PCR using specific primers for GAPDH, VCAM-1 and ICAM-1. A and B - Optical density from 5 separate experiments after normalization to GAPDH as a internal standard. Data are expressed as a ratio VCAM-1/GAPDH, and are given as mean \pm SD from 5 experiments. # $p < 0.001$ as compared to control; *** $p < 0.05$, * $p < 0.001$ - as compared to cells stimulated with TNF- α in the absence of propionate.

Bottom figures show photographs of a UV-illuminated electrophoretic gel from a representative experiment.

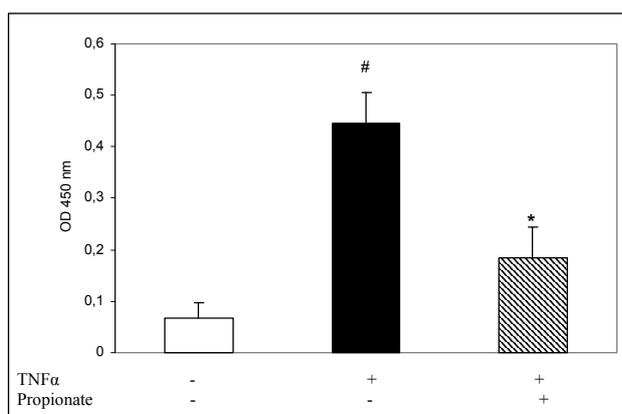


Fig. 4. Propionate inhibits TNF- α -induced activation of NF- κ B. Cells were preincubated with or without propionate (10.0 mM) for 24 h prior to incubation without or with TNF- α (100 U/ml) for 2 h. Next, a whole cell extract was obtained and NF- κ B activation was measured with the ELISA-based kit. Results are expressed as OD_{450nm}, and are given as mean \pm SD from 9 experiments. # $p < 0.001$ as compared to control; * $p < 0.001$ - as compared to cells stimulated with TNF- α in the absence of propionate.

(Fig. 2A) and lymphocytes from 2.0% \pm 0.5% to 10.3% \pm 1.4% (Fig. 2B) of added cells. Pretreatment with 10 mM propionate for 24 h significantly reduced ($p < 0.01$, $n = 9$) the adhesion of monocytes (Fig. 2A) to HUVEC from 26.3% \pm 3.5% to 19.6% \pm 6.0% of cells added to TNF- α -stimulated HUVEC, and from 25.11% \pm 3.7% to 19.11% \pm 5.2% of cells added to IL-1 β -stimulated HUVEC. The extent of reduction varied in individual populations, but the resultant mean value of adherent monocytes was similar to unstimulated cells. The results show that

propionate completely abolished the TNF- α - or IL-1 β -induced increase of HUVEC adhesive properties towards monocytes. The adhesion of lymphocytes to TNF- α -stimulated HUVEC was reduced from 11.2% \pm 1.5% to 7.5% \pm 1.9% of added cells ($p < 0.001$, $n = 9$) and to IL-1 β -stimulated HUVEC from 10.3% \pm 1.4% to 6.6% \pm 1.7% of added cells ($p < 0.001$, $n = 9$) (Fig. 2B).

Effects of propionate on mRNA levels of VCAM-1 and ICAM-1 in HUVEC stimulated by TNF- α

To explore the mechanism responsible for inhibition of VCAM-1 and ICAM-1 surface expression, we studied these molecules' mRNA levels using RT PCR. Stimulation of HUVEC with TNF- α for 4 h resulted in an approximately 13-fold increase in the amount of specific VCAM-1 PCR products (Fig. 3A). Pretreatment with propionate (10 mM) for 24 h significantly reduced the induction of VCAM-1 transcripts by TNF- α . The reduction varied in individual populations, ranging from 68% to 80% (from 0.52 \pm 0.09 to 0.29 \pm 0.09; $p < 0.001$, $n = 5$). Stimulation of HUVEC with TNF- α for 4 h induced only an approximately 2-fold increase in the amount of specific ICAM-1 PCR products (Fig. 3A). Similarly to the observed effect on the protein expression, the inhibitory action of propionate on the TNF- α -mediated ICAM-1 mRNA level was less pronounced than that on VCAM-1 mRNA. Pretreatment with propionate reduced the induction of ICAM-1 mRNA by 9-13% (from 0.97 \pm 0.07 to 0.91 \pm 0.08; $p < 0.05$; $n = 5$). Neither TNF- α nor propionate altered GAPDH transcription.

Effects of propionate on the TNF- α -induced NF- κ B p65 activation.

To determine the effect of propionate on the transcriptional regulation of adhesion molecule expression, we studied NF- κ B p65 protein levels in the whole cells extract of TNF- α -treated

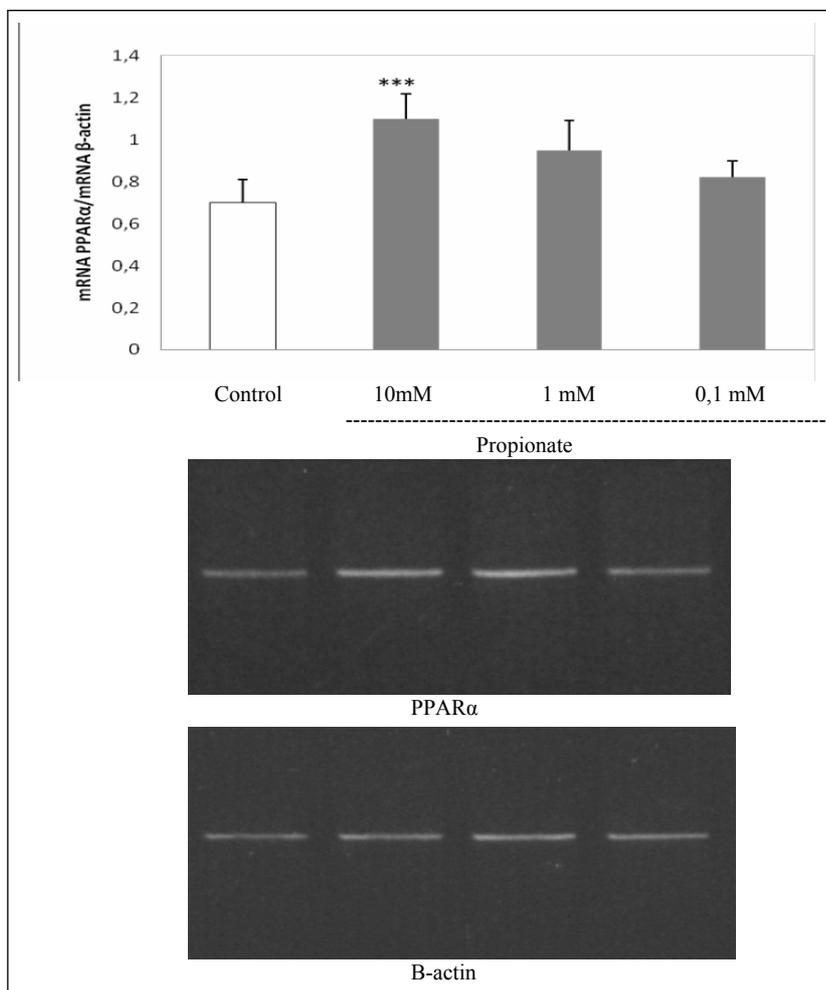


Fig. 5. Propionate induces PPAR α mRNA expression in HUVEC. Cells were incubated with propionate (10.0, 1.0 or 0.1 mM) for 24 h. mRNA was extracted, reverse transcribed and amplified by PCR using specific primers for β -actin and PPAR α . Top, optical density from 6 separate experiments after normalization to β -actin as a internal standard. Data are expressed as a ratio PPAR α / β -actin, and are given as mean \pm SD from 6 experiments. *** $p < 0.05$ - as compared to control cells.

Bottom figures show photographs of a UV-illuminated electrophoretic gel from a representative experiment.

HUVEC by new ELISA-based assay. TNF- α -stimulated HUVEC showed a marked increase (about 7-fold) in the NF- κ B p65 levels (*Fig. 4*). Pretreatment of HUVEC with 10 mM propionate for 24 h significantly reduced (from 0.44 ± 0.061 to 0.184 ± 0.069 ; $p < 0.001$; $n = 9$) the NF- κ B p65 activation as compared to TNF- α treated cells. The extent of reduction varied in individual populations, ranging from 46% to 76%.

Effects of propionate on the PPAR α expression

Since it has been previously suggested that PPAR α may negatively interact with the activated NF- κ B signalling pathway, we decided to examine whether propionate is a PPAR α activator. RT PCR analysis revealed that incubation of HUVEC with propionate (10 mM) for 24 h resulted in a significant increase in the amount of specific PPAR α PCR products from 0.7 ± 0.11 to 1.1 ± 0.12 ($p < 0.05$; $n = 6$) (*Fig. 5*). The observed effect varied in individual populations, ranging from 44% to 66%. Propionate had no effect on the constitutive β -actin transcription.

DISCUSSION

Adhesion and transendothelial migration of leukocytes into surrounding tissue are crucial steps in inflammation, immunity and atherogenesis (2, 4). Vascular endothelial cells play an active role in these processes by expressing cell adhesion molecules. It

now appears from our findings that propionate is capable of interfering with these pathologically important processes.

In the present study, we have demonstrated for the first time that propionate inhibits TNF- α or IL-1-induced expression of VCAM-1 and ICAM-1, by reducing both protein and specific mRNA levels through inhibition of NF- κ B. This effect was significantly more pronounced for VCAM-1 than for ICAM-1, and was time- and dose-dependent. The observed inhibition was associated with a decreased adhesion of monocytes and lymphocytes to stimulated endothelial cells.

Propionate is a naturally occurring SCFA and its levels may be increased by a more frequent introduction of fibre-rich products into the diet or consumption of the so-called functional food containing acidophilic bacteria (13, 14). Along with two other SCFAs, acetate and butyrate, propionate is produced in the large intestine in the process of bacterial fermentation of cellulose fibres. To date, butyrate has been the most extensively studied SCFA of the three. Its anti-inflammatory properties, stemming from its inhibitory effect on NF- κ B activation, among other things, as well as its clinical benefits in the treatment of inflammatory diseases of the alimentary tract are well documented (21). Recently, we have shown that butyrate inhibits the cytokine-induced expression of VCAM-1 and ICAM-1 by acting on NF- κ B (22). The observed effect was associated with a decreased adhesion of monocytes and lymphocytes to stimulated endothelial cells. The attention paid mainly to butyrate resulted from the fact that of the three SCFAs produced in the large intestine, acetic and propionic acid pass into the blood, whereas butyric acid is almost entirely absorbed by

colonic epithelium with only a small portion of butyrate passing into the blood. It is therefore generally assumed that it is butyrate that is the main contributor to the benefits of a diet rich in cellulose fibres for the prophylaxis and treatment of alimentary tract diseases. However, we have demonstrated, for the first time, that also propionate may attenuate an ongoing inflammatory response at the cellular level and that it may contribute to the anti-inflammatory properties of SCFAs mixture observed *in vivo*. Notably, it has been shown recently that sodium propionate administered orally significantly accelerated the healing of colitis, while administration of sodium acetate did not produce a similar effect (23).

Our results also show that the propionate-mediated inhibition of cytokine-induced ICAM-1 and VCAM-1 protein expression was concomitant with a decrease in the levels of specific mRNA. Thus, it may be suggested that the observed effects of propionate may have their origin in its influence on the mechanisms regulating the transcription of genes encoding these molecules. In order to examine further this thesis, we decided to investigate the influence of propionate on NF- κ B, the key transcription factor implicated in the regulation of variety of genes during immune and inflammatory response, including those for VCAM-1 and ICAM-1 (10). Most of NF- κ B present in unstimulated cells is regulated by association with one of the many inhibitory I κ B molecules. It can be activated by diverse signals for example TNF- α , IL-1 or oxyLDL, which induce the release of NF- κ B from its complex with I κ B. The release of NF- κ B is followed by a translocation of active p50/p65 dimers to the cell nucleus and their binding to gene promoter regions. We have shown that the levels of active form of p65 in endothelial cells are increased by the presence of TNF- α and decreased by a pretreatment with propionate. The amount of data on the influence of propionate on NF- κ B is limited. Recently, a report has been published demonstrating that propionate, similarly to butyrate, may inhibit the TNF- α -mediated activation of NF- κ B in colon adenocarcinoma cell line (26).

In our study, we also show that propionate is an activator of PPAR α in endothelial cells. PPAR α exerts anti-inflammatory effects both in the vascular wall and the liver (11). It has been suggested that the anti-inflammatory properties of PPAR α stem from its inhibitory activity on NF- κ B activation. PPAR α may limit the endothelial responses that promote leukocyte adhesion and entry into the vessel wall. It has been previously shown that the inhibition of transcriptional expression of VCAM-1, but not ICAM-1, by a PPAR α agonist results, at least partially, from NF- κ B inhibition (12). Thus, the activation of PPAR α by propionate observed in this study could potentially explain the fact that this compound exerted a significantly more pronounced inhibitory effect on VCAM-1 than on ICAM-1. Notably, these findings appear to be of particular relevance for the antiatherogenic properties of propionate. It has been suggested that VCAM-1 plays a significant role in the selective accumulation of monocytes and lymphocytes, but not granulocytes, in the walls of blood vessels in atherosclerosis (27).

Several clinical trials have established an inverse relation between fibre intake and coronary heart disease (CHD) (17-19). A higher intake of dietary fibre was associated with a lower risk of CVD and MI (18), and was implicated in protection against intima-media thickness (19). Functional food containing acidophilic bacteria (ProViva) has been shown to lower the LDL cholesterol and fibrinogen levels and decrease the triglyceride levels and systolic blood pressure in patients with moderately elevated cholesterol levels (28) and in smokers (29). Monocytes freshly isolated from the blood of smokers consuming ProViva exhibited a statistically significantly decreased adhesion both to unstimulated and stimulated endothelial cells. Recently, it has been shown that high-fibre diet was associated with a reduction in oxidative stress

(30). Moreover, a reduction in sICAM-1, sE-selectin and CRP levels, as well as in MCP-1 production, was observed.

In view of the above findings, it transpires that consumption of high-fibre diet may significantly influence the course of diseases having their origin in atherosclerosis. Still, the underlying molecular mechanisms of effects of such a diet require further investigation. The anti-inflammatory mechanisms of propionate action observed in this study are most likely implicated in the beneficial action of high-fibre diet in diseases of the alimentary tract. They also seem to indicate that propionate may be at least partially responsible for the antiatherosclerotic effects of high-fibre diet. A recent study has demonstrated an association between dietary fibre and the levels of CRP, a clinical indicator of inflammation, suggesting that inflammation may be an important mediator in the association between dietary fibre and CVD (31). From our study it appears that the beneficial antiatherosclerotic properties of propionate may also stem from the fact that this molecule is an activator of PPAR α . Activation of PPAR α results in a reduction of plasma triglycerides (TG), through an induction of genes that decrease the ability of TG for hepatic very low-density lipoprotein (VLDL) secretion and which promote the lipoprotein lipase-mediated lipolysis of TG-rich plasma lipoproteins (32). The stimulatory effect of PPAR α on the plasma level of HDL appears to be mediated by an increased production of ApoA1 and ApoA2. Kawase *et al.* (33) have recently shown that supplementation of the diet with fermented milk containing *Lactobacillus casei* and *Streptococcus thermophilus* results in significant increase in HDL cholesterol and decrease in triglyceride levels in healthy volunteers. Trials in hyperlipidemic and diabetic patients showed that high dietary fibre can reduce the plasma levels of TG and cholesterol. The effect on TG was more marked than on cholesterol. It has been suggested that the observed hypotriglyceridemic effect may be due to a lower hepatic secretion rate of TG, through a selective exposure of the liver to an increased amount of propionic acid produced in the large intestine during fermentation of non-digestible carbohydrates. The hypocholesterolemic effect is likely due to a decreased secretion of VLDL. Activation of PPAR α has been also suggested to improve insulin resistance (32). It has been postulated that propionic acid may improve glucose tolerance and inhibit cholesterol synthesis in the liver by improving insulin resistance (35-37). On the other hand, you should not forget that in the case of chronic use of high-fat diet, overexpression of PPAR α is possible, which in turn leads to accumulation of triacylglycerols and ceramides in many organs, including the heart (38). Since ceramides are lipotoxicity mediators, this may even result in cardiomyopathy.

The particular utilitarian significance of our studies is associated with the fact that propionate could potentially have a strong inhibitory effect on the inflammatory process stimulating the progression of atherosclerosis. Unfortunately, previous attempts of using synthetic non steroidal anti-inflammatory drugs in clinical practice have failed due to their adverse impact on hypertension and coagulation (38). At the same time, an adequate amount of fiber in diet could constitute a safe source of natural anti-inflammatory factors, including propionate. According to the AHA recommendations, this amount should be 25 g of soluble fiber daily, while in countries of high atherosclerosis risk it usually reaches 50% of the demand at the most (39). Thus, although the best known chemical derivative of propionate the widely used ibuprofen - cannot be recommended for lowering the risk of atherosclerosis, the use of fiber as its alternative seems fully justified. It should be added that not only short-chain fatty acids originating from fibre are able to reduce inflammation but similar molecular mechanisms exist also in the case of plant polyphenols originating from the traditional

Mediterranean diet, which is currently commonly recommended in atherosclerosis prophylaxis (41).

In conclusion, it should be noted that propionate inhibits the cytokine-induced expression of VCAM-1 and ICAM-1 and, in consequence, the adhesion of mononuclear leukocytes to the endothelial cells, through inhibition of NF- κ B activation. The anti-inflammatory and antiatherosclerotic activity of propionate may be associated with its ability to activate PPAR α . Our findings warrant further investigations into the potential therapeutic usefulness of propionate in several pathological events involving leukocyte recruitment, including vascular disease. With this in mind, it could be of particular interest to consider different means of stimulating the production of propionate such as, for example, by ingestion of high amounts of dietary fibre.

Conflict of interests: None declared.

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