Short-chain fatty acids stimulate the migration of neutrophils to inflammatory sites

Marco A. R. VINOLO*, Hosana G. RODRIGUES*, Elaine HATANAKA*, Cristina B. HEBEDA†, Sandra H. P. FARSKY† and Rui CURI*

*Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of Sao Paulo, 05508-900, Butantã, Sao Paulo, Brazil, and †Department of Clinical and Toxicological Analyses, Faculty of Pharmaceutical Sciences, University of Sao Paulo, 05508-900, Butantã, Sao Paulo, Brazil

ABSTRACT

SCFAs (short-chain fatty acids) are produced by anaerobic bacterial fermentation. Increased concentrations of these fatty acids are observed in inflammatory conditions, such as periodontal disease, and at sites of anaerobic infection. In the present study, the effect of the SCFAs acetate, propionate and butyrate on neutrophil chemotaxis and migration was investigated. Experiments were carried out in rats and *in vitro*. The following parameters were measured: rolling, adherence, expression of adhesion molecules in neutrophils (L-selectin and β2 integrin), transmigration, air pouch influx of neutrophils and production of cytokines [CINC-2$\alpha\beta$ (cytokine-induced neutrophil chemoattractant-2$\alpha\beta$), IL-1β (interleukin-1β), MIP-1α (macrophage inflammatory protein-1α) and TNF-α (tumour necrosis factor-α)]. SCFAs induced *in vivo* neutrophil migration and increased the release of CINC-2$\alpha\beta$ into the air pouch. These fatty acids increased the number of rolling and adhered cells as evaluated by intravital microscopy. SCFA treatment increased L-selectin expression on the neutrophil surface and L-selectin mRNA levels, but had no effect on the expression of β2 integrin. Propionate and butyrate also increased *in vitro* transmigration of neutrophils. These results indicate that SCFAs produced by anaerobic bacteria raise neutrophil migration through increased L-selectin expression on neutrophils and CINC-2$\alpha\beta$ release.

INTRODUCTION

The SCFAs (short-chain fatty acids) acetate, propionate and butyrate are normally found at high concentrations (70–140 mmol/l) in the human intestine [1,2]. These fatty acids are produced by anaerobic bacterial fermentation. Increased concentrations of SCFAs are observed in inflammatory conditions, such as periodontal disease [3,4], and at sites of anaerobic infection [5,6]. These compounds have been implicated in the initiation and prolongation of these inflammatory processes [7].

In opposition to the view that SCFAs act as potentiating agents of inflammation, these fatty acids have been shown to inhibit the production of pro-inflammatory cytokines by isolated macrophages [8]. Results from our group reinforce the anti-inflammatory effect of these compounds. Propionate and butyrate inhibited LPS (lipopolysaccharide)-stimulated production of cytokines by isolated neutrophils (M.A.R. Vinolo, H.G. Rodrigues, E. Hatanaka and R. Curi, unpublished work). We have also observed a reduction in phagocytosis and production of ROS (reactive oxygen species) by neutrophils after

Key words: anaerobic bacterium, chemotaxis, inflammation, neutrophil, short-chain fatty acid.

Abbreviations: AP-1, activator protein-1; CINC, cytokine-induced neutrophil chemoattractant; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; LFA-1, lymphocyte function-associated antigen-1; LPS, lipopolysaccharide; MIP-1α, macrophage inflammatory protein-1α; NF-κB, nuclear factor κB; ROS, reactive oxygen species; SCFA, short-chain fatty acid; TNF-α, tumour necrosis factor-α; VCAM-1, vascular cell-adhesion molecule-1.

Correspondence: Mr Marco A.R. Vinolo (email mramirez@icb.usp.br).

treatment with butyrate [9]. Therefore pro- and anti-inflammatory properties of SCFAs have been reported.

Migration of neutrophils from the blood stream to inflamed tissue is one of the main steps that takes place in the inflammatory process. Neutrophil rolling along the endothelial cell surface and activation of chemoattractant receptors on the neutrophil membrane, followed by firm adhesion to vessel walls and transmigration through the endothelium, are involved in the neutrophil migration process. Cell-adhesion molecules such as selectins (E-, P- and L-selectins), integrins [LFA-1 (lymphocyte function-associated antigen-1), Mac-1 and CD11c] and their counter-ligand molecules [specific carbohydrates, VCAM-1 (vascular cell-adhesion molecule-1), ICAM-1 (intercellular adhesion molecule-1) and others] play a central role in rolling and adhesion of neutrophils to the endothelium. Owing to the key participation of these molecules in the neutrophil migration process, they have been studied as possible targets for the development of a new class of anti-inflammatory drugs [10].

The effect of SCFAs has been addressed in different steps of the inflammation process, such as the production of cytokines and ROS; however, there is not enough information about the effect of these fatty acids on the migration of neutrophils to inflammatory sites. In the present study, the effect of acetate, propionate and butyrate on neutrophil chemotaxis and migration has been investigated. Experiments were carried out in rats and *in vitro*, and the following parameters were measured: rolling, adherence, transmigration, air pouch influx of neutrophils, production of cytokines [CINC-2$\alpha\beta$ (cytokine-induced neutrophil chemoattractant-2$\alpha\beta$), IL-1β (interleukin-1β), MIP-1α (macrophage inflammatory protein-1α) and TNF-α (tumour necrosis factor-α)] and the expression of adhesion molecules in neutrophils (L-selectin and β2 integrin).

MATERIALS AND METHODS

Reagents

Bovine fetal serum, Hepes, penicillin, RPMI 1640 medium supplemented with L-glutamine, sodium bicarbonate, fatty acids, oyster glycogen and fMLP (*N*-formylmethionyl-leucyl-phenylalanine) were supplied by Sigma. Stock solutions of fatty acids were prepared in PBS and the pH was adjusted to 7.4 with 1 mol/l NaOH.

Animals

Male Wistar rats weighing 180 $\pm$ 20 g were obtained from the Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil. The rats were maintained at 23 °C under a 12/12 h light/dark cycle. Food and water were given *ad libitum*. Animals were anaesthetized with intraperitoneal injection of sodium pentobarbital (65 mg/kg of body weight) for all experimental procedures. The Animal Care Committee of the Institute of Biomedical Sciences approved the experimental procedure of this study.

Air pouch assay and exudate preparation

In order to evaluate the effect of SCFAs on *in vivo* cell migration, the air pouch assay was used. Induction of rat skin air pouches was performed according to the method described by Edwards et al. [11]. Briefly, 20 ml of sterile air (using 0.22 μm fluoropore filters) was insufflated into the subcutaneous tissue of the back trunk of rats under anaesthesia. At 7 days later, an additional 10 ml of sterile air was insufflated and, then, 1 ml of a solution of acetate (25 mmol/l), propionate (12 mmol/l) or butyrate (12 mmol/l) in sterile PBS was injected into the pouch under anaesthesia and aseptic conditions. Negative controls received 1 ml of sterile PBS, and positive controls received 1 ml of sterile PBS plus fMLP (10 nmol/l) through the same route.

At 4 h after the injection of the fatty acids, the animals were killed by decapitation and the inflammatory exudate was collected after washing the cavity with 2 ml of sterile PBS. The suspension was centrifuged at 500 *g* for 10 min at 4 °C. Cells were counted in a Neubauer chamber, and the cytocentrifuged smears were stained with standard May-Grunwald and Giemsa solutions (Sigma). Differential cell counts were performed on 100 cells/slide. Results are expressed as the fractional change in the number of cells that were collected under control conditions (PBS without fMLP). The supernatant was assayed for CINC-2$\alpha\beta$, TNF-α, MIP-1α and IL-1β by ELISA (DuoSet; R&D System), according to the supplier's instructions.

Intravital microscopic assay

Rats were anaesthetized and the mesentery was externalized. After surgery, the animals were kept on a board thermostatically controlled at 37 °C that included a transparent platform on which the tissue to be transilluminated was placed. The preparation was kept moist and warmed by irrigating the tissue with warmed Ringer–Locke solution [154 mmol/l NaCl, 5.6 mmol/l KCl, 2 mmol/l $CaCl_2 \cdot 2H_2O$, 6 mmol/l $NaHCO_3$ and 5 mmol/l glucose (pH 7.2–7.4)] containing 1 % (w/v) gelatin. The rate of the solution outflow on to the exposed tissue was controlled to keep the preparation in continuous contact with a film of liquid. Transilluminated images were obtained by optical microscopy (Axioplan II equipped with 5.0/0.30× plan-neofluar or 10.0/0.25× Achroplan longitudinal distance objectives/numeric aperture and 1.0, 1.25 or 1.60× optovar; Carl Zeiss). The images were captured on a video camera (ZVS, 3C75DE; Carl Zeiss) and were transmitted simultaneously on to a TV monitor and computer. Images obtained on the TV monitor were recorded on video tape. Digitized images were subsequently analysed by using image-analysis software (KS 300; Kontron).

Table 1 Annealing temperature and sequences of the primers used

Gene	Primer		Annealing temperature
	Sense	Antisense	
β_2-Microglobulin	5′-CTCAGTTCCACCCACCTCAG-3′	5′-GCAAGCATATACATCGGTCTCG-3′	56 °C
L-Selectin	5′-AAATGTGGACATGGGTGGGAAC-3′	5′-CCTTGGACTTCTTGTTGTTGGG-3′	54 °C
β2 Integrin	5′-TGGCACACAAACTTTCCGAGAG-3′	5′-TAGGTGACTTTCAGGGTGTCCG-3′	57 °C

Leucocyte–endothelial interaction

The interaction between leucocytes and vessel walls was evaluated by determining the number of rolling and adhered leucocytes to the post-capillary venule wall (20–30 μm in diameter and 200 μm in length) of the mesentery at 10-min intervals. Three fields were evaluated in each animal after application of 10 μl of fatty acids. Leucocytes moving in the peripheral of the axial stream, in contact with the endothelium, were considered to be rollers [12]. These leucocytes moved sufficiently slowly to be individually visible and were counted as they rolled past a selected point on one side of the vessel during 10 min after the fatty acid addition. The number of leucocytes adhered to the endothelium (stopped at the vessel wall) was determined in the same vascular segment after 10 min of the fatty acid addition.

Expression of adhesion molecules (L-selectin and β2 integrin) evaluated by flow cytometry

In order to estimate L-selectin or β2 integrin expression, leucocytes were collected with EDTA (100 mg/ml) from blood obtained from the abdominal aorta. Neutrophils were isolated using a Histopaque gradient. A 3 ml sample of Histopaque-1077 was placed in a tube and the same volume of whole blood was carefully layered on to the separation medium. The tube was then centrifuged at 400 *g* at 4 °C for 30 min. At the end of the centrifugation, three distinct phases were formed. The upper two phases (plasma, mononuclear cell and Histopaque-1077) were discarded and the lower one, which was rich in neutrophils and erythrocytes, was resuspended in PBS. Erythrocyte lysis was performed using an ammonium chloride solution (0.13 mol/l), and leucocytes were recovered after washing with PBS. Using this methodology for separation, rich neutrophil preparations (> 90 % of leucocytes) were obtained as evaluated by microscopy.

Cells (1.0×10^6) were pre-incubated for 4 h with acetate (25 mmol/l), propionate (12 mmol/l) or butyrate (12 mmol/l) and were then stimulated with fMLP (10 nmol/l during 10 min for L-selectin and 30 min for β2 integrin evaluation). After washing, leucocytes were incubated further for 30 min at 4 °C in the dark with 10 μl of a monoclonal antibody against L-selectin or β2 integrin. Immediately after incubation, cells were analysed in a FACScalibur flow cytometer (Becton Dickinson). Data from 10000 cells were obtained and only morphologically viable neutrophils were considered for analysis.

Expression of L-selectin and β2 integrin mRNA

The effect of SCFAs on mRNA levels of adhesion molecules (L-selectin and β2 integrin) was evaluated in elicited neutrophils. Rat neutrophils were obtained by intraperitoneal lavage with 40 ml of PBS 4 h after the intraperitoneal injection of 10 ml of freshly prepared 1 % (w/v) oyster glycogen (Type II) solution in sterile PBS. The cell suspension was centrifuged at 4 °C (500 *g* for 10 min). The number of viable cells (> 95 % neutrophils) was determined in a Neubauer chamber under an optical microscope by Trypan Blue exclusion. Neutrophils were incubated for 1 or 4 h with acetate (25 mmol/l), propionate (12 mmol/l) or butyrate (12 mmol/l). Total RNA was obtained from 1.5×10^7 neutrophils using TRIzol® reagent (Invitrogen) as described previously [13].

Total RNA (3 μg) was reverse-transcribed to cDNA using the reverse transcriptase *Revertaid*™ M-MuLV. Expression of L-selectin and β2 integrin was evaluated by real-time PCR [14] using Rotor Gene 3000 equipment (Corbett Research) and SYBR Green as the fluorescent dye. The sequence of the primers is shown in Table 1. Evaluation of gene expression was carried out using the method described by Liu and Saint [15], with the β_2-microglobulin gene as an internal control.

Transmigration assay

Adult male rats were killed by decapitation without anaesthesia. Neutrophils were obtained by intraperitoneal lavage, 4 h after the intraperitoneal injection of 10 ml of sterile 1 % (w/v) oyster glycogen (Type II) solution in PBS. This treatment induces a substantial migration of neutrophils (> 95 %) to the intraperitoneal cavity with little contamination by monocytes. Neutrophil chemotactic responses were tested using 96-well disposable chemotactic plates (Neuroprobe), according to the manufacturer's instructions. Briefly, flat-bottomed chambers were filled with the chemotactic agent fMLP (10 nmol/l) in PBS containing 0.01 % albumin or with PBS containing 0.01 % albumin only. Chemotactic membranes with a pore size of 5 μm were fixed to the

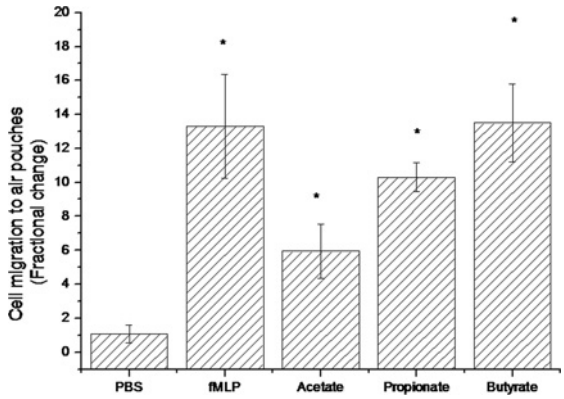

Figure 1 Neutrophil influx into air pouches after injection of PBS, SCFAs or fMLP

Values are means ± S.E.M. of six animals per group and are presented as the fractional change (treatment value/control value). $^*P < 0.05$ compared with control (PBS).

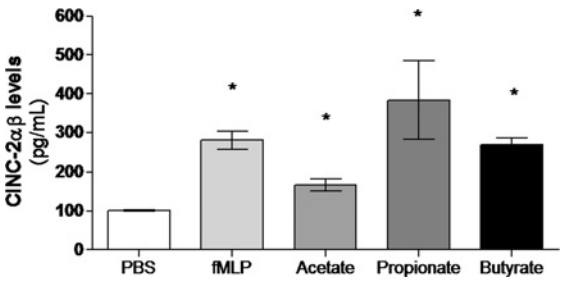

Figure 2 CINC-2$\alpha\beta$ release into air pouches after injection of PBS, SCFAs or fMLP

Values are means ± S.E.M. of six animals per group. $^*P < 0.05$ compared with control (PBS).

Table 2 Effect of SCFAs on neutrophil rolling and adherence

Rolling and adherence of neutrophils in mesenteric venules were analysed after topical application of PBS, SCFAs or fMLP. Values are means ± S.E.M. of five animals per group. $^*P < 0.05$ compared with control (PBS).

Treatment	Rolling (% change)	Adherence (% change)
Control	98 ± 10	91 ± 5
Acetate		
25 mmol/l	170 ± 21*	144 ± 21*
Propionate		
12 mmol/l	90 ± 9	99 ± 8
24 mmol/l	170 ± 14*	135 ± 6*
Butyrate		
12 mmol/l	92 ± 19	114 ± 10
24 mmol/l	146 ± 23*	129 ± 24*
fMLP		
10 mmol/l	155 ± 24*	207 ± 14*

filter seat and a suspension of neutrophils (2.5×10^6 cells/ml), pre-treated for 4 h with SCFAs, was added to the top of each well. The chamber assembly was incubated in a humidified 5% CO_2 atmosphere at 37°C for 60 min. After incubation, neutrophils were counted in a Neubauer chamber. The chemotactic factor fMLP (10 nmol/l) was used as a positive control for migration. Results were normalized by the number of cells that transmigrated under control conditions (PBS without fMLP).

Statistical analysis

Statistical analysis was carried out by comparing the control group with the SCFA-treated groups. ANOVA with a Dunnett's test (Graph Pad Prism 4.0 Software) was used for comparison between the treated groups and the controls. The significance level was set at $P < 0.05$.

RESULTS

The number of cells that migrated to the air pouches was determined 4 h after the injection of the SCFAs, the positive control (fMLP) or PBS. Cells collected from the pouches consisted mainly of neutrophils (> 90%). fMLP treatment increased leucocyte migration to the air pouches in comparison with PBS (1.98×10^6 cells compared with 0.15×10^6 respectively). Significant cell influx into the pouches was induced by the SCFAs. Cell migration was increased 6-, 10- and 13- fold for acetate, propionate and butyrate respectively (Figure 1). The amount of cytokines (CINC-2$\alpha\beta$, IL-1β, TNF-α and MIP-1α) released into air pouches was also determined. No effect of the SCFAs on IL-1β, TNF-α and MIP-1α release was observed (control values, 514 ± 11 pg/ml for IL-1β, 956 ± 51 pg/ml for TNF-α and 77 ± 30 pg/ml for MIP-1α). However, CINC-2$\alpha\beta$ release into the pouches was increased 1.7-, 3.8- and 2.7-fold by acetate, propionate and butyrate respectively (Figure 2). No difference was found between the effect of fMLP, a classical neutrophil chemoattractant agent [16], and that of propionate or butyrate on cell migration (Figure 1) or CINC-2$\alpha\beta$ release (Figure 2). No difference in cell migration was observed between fMLP alone or fMLP plus SCFAs (results not shown).

In order to evaluate the effect of SCFAs on leucocyte–endothelial cell interactions, these fatty acids were topically applied on to mesentery venules. Rolling and adherence of leucocytes to endothelium was evaluated using transilluminated images obtained by optical microscopy. The analyses were made 10 min after SCFA, PBS or fMLP application. The number of rolling cells was counted during 10 min. Acetate (25 mmol/l), propionate (24 mmol/l) and butyrate (24 mmol/l) significantly increased the number of rolling and adhered cells compared with the control group (Table 2).

The stimulatory effect of the fatty acids on neutrophil migration led us to examine L-selectin and $\beta 2$ integrin expression on the neutrophil surface. These adhesion

Table 3 Effect of SCFAs on expression of L-selectin and $\beta 2$ integrin on neutrophil surface

L-selectin and $\beta 2$ integrin expression was evaluated by flow cytometry after pre-treatment (4 h) with PBS or SCFAs. fMLP was used as a positive control. Values are means $\pm$ S.E.M. of the fractional changes (treatment value/control value) of three experiments. $^*P < 0.05$ compared with control (PBS).

Treatment	$\beta 2$ Integrin	L-Selectin
Control	1.0	1.0
Acetate (25 mmol/l)	1.14 ± 0.07	$1.51 \pm 0.23^*$
Propionate (12 mmol/l)	1.21 ± 0.10	$1.41 \pm 0.23^*$
Butyrate (12 mmol/l)	1.08 ± 0.09	$1.41 \pm 0.17^*$
fMLP (10 nmol/l)	$1.90 \pm 0.18^*$	$1.89 \pm 0.19^*$

molecules are expressed on leucocytes and play a key role in leucocyte rolling and adhesion to the endothelium. Neutrophils were pre-incubated for 4 h in the presence of the SCFAs. Cells were then stimulated with fMLP and the abundance of L-selectin and $\beta 2$ integrin on the neutrophil surface was evaluated by flow cytometry. L-Selectin expression on the surface of neutrophils pre-treated with SCFAs for 4 h was higher than in control cells (PBS) not treated with fMLP (Table 3). Stimulation of neutrophils with fMLP also increased the expression of L-selectin (Table 3). The stimulatory effect of SCFAs on L-selectin was not observed when neutrophils were incubated for 1 h (results not shown). $\beta 2$ integrin expression was increased with fMLP treatment, but no effect of the SCFAs was observed (Figure 3).

The results obtained by flow cytometry led us to evaluate L-selectin and $\beta 2$ integrin mRNA expression after 1 and 4 h of pre-incubation with the fatty acids. No effect of the SCFAs on L-selectin and $\beta 2$ integrin mRNA expression was observed after 1 h incubation (results not shown); however, there was a significant increase in L-selectin mRNA levels by 2.0-, 2.2- and 3.2-fold in comparison with controls after incubation of neutrophils with acetate, propionate or butyrate respectively, for 4 h. $\beta 2$ integrin mRNA expression was not affected by the treatments with the fatty acids.

Previous studies have shown that the SCFAs induce chemotaxis of neutrophils [17]. Therefore we examined neutrophil chemotactic responses after pre-treatment (4 h at 37°C) with acetate (10 and 25 mmol/l), propionate (4, 8 and 12 mmol/l) and butyrate (4, 8 and 12 mmol/l). Our results indicated that 25 mmol/l acetate (Figure 4A), 12 mmol/l propionate (Figure 4B) and 4, 8 and 12 mmol/l butyrate (Figure 4C) enhanced neutrophil chemotaxis. The values obtained were higher than those observed in response to vehicle and were equivalent to neutrophil migration induced by fMLP, which was used as the positive control. However, pre-treatment of neutrophils with SCFAs did not interfere with the fMLP effect (Figure 4).

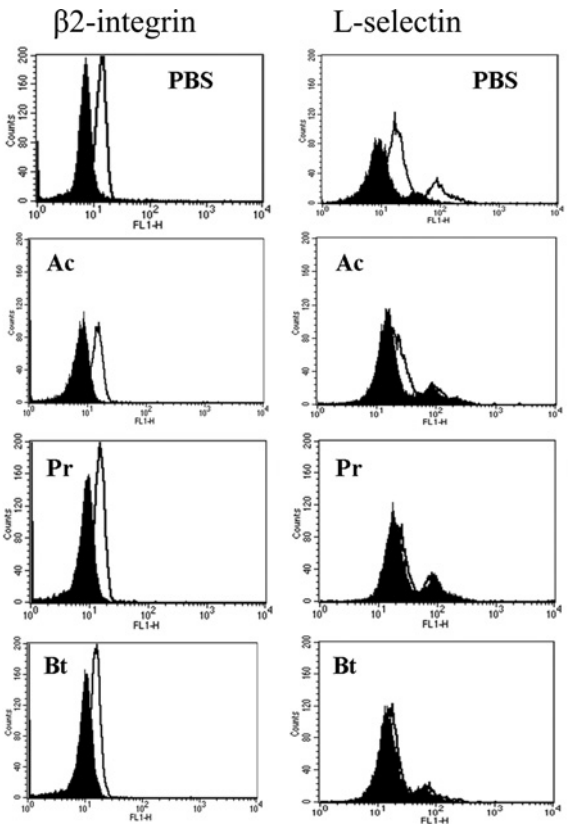

Figure 3 Representative traces from flow cytometric analyses of $\beta 2$ integrin and L-selectin expression on the membrane surface

Open trace, cells stimulated with fMLP; closed trace, cells not stimulated with fMLP. Ac, acetate; Pr, propionate; Bt, butyrate.

DISCUSSION

Our findings support the hypothesis that SCFAs interfere with a key step in the inflammatory process, namely neutrophil migration. SCFAs induced neutrophil migration *in vivo*, and this effect was associated with an increased production of the chemoattractant cytokine CINC-2$\alpha\beta$ and the increased expression of L-selectin on the neutrophil surface. These fatty acids also increased *in vitro* transmigration of neutrophils. These results indicate that SCFAs produced by anaerobic bacterial fermentation increase neutrophil migration, exacerbating the inflammatory process.

SCFAs induce morphological changes and cell polarization, Ca^{2+} release and actin cytoskeleton remodelling in incubated neutrophils [18,19]. These effects involve activation of GPR43 (G-protein-coupled receptor 43), an SCFA membrane receptor [17,20]. Activation of this receptor, which is coupled to $G_{i/o}$ and G_q proteins, has been shown to increase intracellular Ca^{2+} concentrations and to induce neutrophil chemotaxis [17]. In agreement with

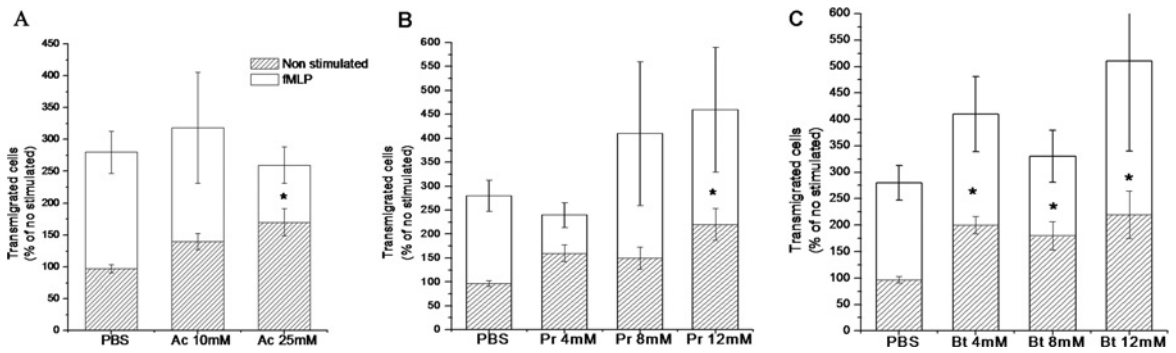

Figure 4 Effect of acetate (A), propionate (B) and butyrate (C) on neutrophil transmigration

Values are means ± S.E.M. of four experiments performed in duplicate $^{*}P < 0.05$ compared with control (PBS). Ac, acetate; Pr, propionate; Bt, butyrate.

these observations, pre-treatment with acetate, propionate or butyrate increased neutrophil migration *in vitro*.

Confirming the effect observed *in vitro*, SCFAs increased the migration of neutrophils to the air pouch, and the number of rolling and adhered cells, as observed by intravital microscopy. The interaction between endothelial cells and neutrophils plays a key role in the recruitment of these latter cells to the inflamed tissue. Previous studies have shown the effect of SCFAs on this interaction, but the focus of these studies was the endothelial cells [21,22] and conflicting results have been reported. Zapolska-Downar et al. [21] have shown that pre-treatment of HUVECs (human umbilical vein endothelial cells) with butyrate inhibits the expression of VCAM-1 and ICAM-1 induced by TNF-α and IL-1β, suggesting an anti-inflammatory effect of this fatty acid. However, Miller et al. [22], using the same cells, showed that butyrate increased the expression of ICAM-1 and E-selectin, but had no effect on VCAM-1 expression. In the present study, we have shown that SCFAs increase neutrophil surface expression of L-selectin, but not of β2 integrin.

L-Selectin, an adhesion molecule constitutively expressed on leucocytes, together with E- and P-selectins expressed on endothelial cells, is responsible for the initial step of tethering and rolling of leucocytes on the endothelium. The expression of this adhesion molecule on the membrane surface is controlled by transcriptional regulation and shedding. SCFAs augmented L-selectin surface expression, an effect at least in part related to an increase in mRNA expression. This effect, together with increased E-selectin expression, as shown by Miller et al. [22], may be responsible for the enhanced leucocyte rolling activity observed *in vivo* in the presence of the SCFAs.

Expression of β2 integrins, of which the most important for neutrophil adhesion during acute inflammatory responses are Mac-1 (macrophage-1) and LFA-1 [23], were not modified by the SCFAs. However, an increased number of adhered leucocytes on the endothelium was observed in the intravital microscopic assay. This effect may result from the increased number of rolling neutrophils and/or the increased expression of the endothelial ligand ICAM-1, as shown previously by Miller et al. [22].

In rats, an important family of chemokines is termed CINC [24]. CINC-2 is one of the chemokines within this family. There is one CINC-2 gene, with CINC-2α and CINC-2β arising by alternative RNA splicing [25]. Neutrophils are both a source and a target of CINC-2. *In vitro*, rat CINC has been shown to be a potent neutrophil chemoattractant agent [24], and *in vivo* it mediates neutrophil accumulation in different models of inflammation by increasing the emigration and activation of neutrophils [26,27]. Nakagawa et al. [28] have shown that LPS-stimulated rat macrophages produce CINC-2α as a major chemoattractant agent for neutrophils. In the present study, SCFAs enhanced the release of CINC-2$\alpha\beta$ into the air pouch. This effect can account, at least in part, for the increased migration of neutrophils to the air pouch. Other studies have shown that these fatty acids increase the production of chemokines by different cell types, such as colonic epithelial cell lines [29,30], cervical cells (HeLa) [31] and a microglial cell line (N9) [32]. CINC-2$\alpha\beta$ expression is regulated by transcription factors such NF-κB (nuclear factor κB) and AP-1 (activator protein-1) [25]. Activation of both NF-κB [31,33] and AP-1 [34] is modulated by SCFAs. Thus the increased release of CINC-2$\alpha\beta$ possibly occurs due to an enhancement of the activation of these transcription factors by SCFAs.

Concentrations of SCFAs used in the present study (acetate up to 25 mmol/l, propionate up to 24 mmol/l and butyrate up to 24 mmol/l) are higher than those described by Niederman et al. [3] for gingival crevices in periodontal subjects. However, other studies in which SCFAs were measured in culture filtrates of anaerobic bacteria or at the sites of anaerobic bacterial infection have shown higher concentrations of these fatty acids than the ones used in the present study. Filtrates from *Porphyromonas gingivalis*, *Prevotella loescheii* and *Fusobacterium nucleatum* cells contain from 0.6–19.1 mmol/l propionate and 13.3–26.8 mmol/l butyrate [4]. Rotstein et al. [35]

also measured concentrations of SCFAs in filtrates of *Bacteroides fragilis* and found concentrations higher than 40 mmol/l. Concentrations of SCFAs in excess of 30 mmol/l have also been measured in clinical abscesses [36]. Mills et al. [6], with the aim of determining whether foot-rot-causing bacteria produced SCFAs in clinically relevant quantities, measured acetate, propionate and butyrate in cultured anaerobic bacteria. Concentrations as high as 25.3, 25.4 and 10.4 mmol/l for acetate, butyrate and propionate respectively, after incubation for 72 h were found [6].

In spite of the fact that SCFAs had a pro-inflammatory effect in our present experimental conditions, anti-inflammatory actions of these fatty acids have also been observed in other conditions. The inconsistencies of the results can be associated with the use of different concentrations of SCFAs, pH and cell types. Stempelj et al. [37] have shown that butyrate increases the expression of iNOS (inducible NO synthase) and NO production in intestinal epithelial cells. However, this fatty acid inhibited NO production by macrophages and intestinal myofibroblasts [37]. Huuskonen et al. [32] reported opposite effects depending on the cell type. Butyrate and propionate potentiated LPS-induced production of NO and IL-6 in N9 microglial cells, but inhibited LPS-induced responses in rat primary microglia, as in cultured hippocampal slices and neural co-cultures of microglial cells, astrocytes and cerebellar granule neurons [32].

In the present study, we have shown that SCFAs increase neutrophil migration to the inflammatory site, contributing to the initiation of the inflammation process. Taking into account the results of the studies mentioned above and the fact that, at the infection site *in vivo*, different SCFAs are produced achieving together much higher concentrations than the ones used in our present study, the results shown are clinically relevant.

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