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Short-chain fatty acids and trichostatin A alter tight junction permeability in human umbilical vein endothelial cells

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Abstract

OBJECTIVE: The short-chain fatty acids (SCFAs), butyrate, propionate, and acetate, produced by bacterial fermentation of dietary fiber, can modulate the transcription of certain genes by inhibiting histone deacetylase (HDAC) in colonocytes and several other cell types *in vitro*. We previously reported that butyrate decreases tight junction permeability by activating lipoxygenase (LOX) in intestinal monolayer cells. Here, we evaluated the effects of SCFAs on tight junction permeability in an endothelial cell culture, and their possible mechanisms of action via HDAC inhibitor activity. We also investigated the factors modulating tight junction permeability.

METHODS: The effects of butyrate, propionate, and acetate on tight junction permeability in human umbilical vein endothelial cells were examined using Transwell chamber cultures. The participations of inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS), estrogen receptor (ER), cyclooxygenase (COX), and LOX in SCFAs' effects were also evaluated. The effects of SCFAs were compared with those of trichostatin A, a typical HDAC inhibitor.

RESULTS: Low concentrations of butyrate and propionate decreased paracellular permeability without inducing cell damage. However, acetate decreased paracellular permeability in a concentration-dependent manner. An ER antagonist attenuated the effects of SCFAs on tight junction permeability. The influences of iNOS, COX, and LOX inhibitors and the expressions of COX and LOX mRNAs were different for each SCFA. Trichostatin A slightly decreased paracellular permeability when used at lower concentrations, but higher concentrations of trichostatin A increased permeability.

CONCLUSION: SCFAs play an important role in the assembly of tight junctions in normal vascular endothelial cells.

KEYWORDS: Short-chain fatty acids, butyrate, tight junction permeability, histone acetylation,

trichostatin A.

Introduction

Tight junctions are the most apical structures in epithelial or endothelial cells. They create a physiological intercellular barrier that helps to maintain distinct tissue spaces, and separate the apical cell membrane from the lateral membrane. Endothelial cells form a barrier between the blood and tissue environments, and control the movement of blood cells, plasma fluid, small molecules, and proteins between the vascular compartment and the extracellular space. The main route of transport of molecules across endothelia is via the intercellular (paracellular) space, and the driving forces include transendothelial hydrostatic and hypertonic gradients [1]. Increased microvascular leakage may be the earliest event in normal and several disease conditions such as angiogenesis, wound healing, atherosclerosis, reperfusion edema, tissue ischemia, tumorigenesis, and diabetic vasculopathy [2]. It is known that endothelial permeability, particularly tight junction permeability, is modulated by a number of factors including tissue pH, adenosine 3′-, 5′-cyclic monophosphate, protein kinase C activators, nitric oxide (NO), estrogen receptor (ER) that is important in the release of NO from the endothelium, and cytokines [1, 3–6].

Fiber is an important nutritional dietary component. A high intake of dietary fiber is associated with improved bowel function in healthy individuals [7]. Many types of dietary fiber are metabolized by anaerobic gut bacteria into short-chain fatty acids (SCFAs) in the intestinal lumen. The major SCFAs produced during fiber fermentation in the gut are butyrate, propionate, and acetate. Butyrate is also present at low levels in many fruits and vegetables, although its richest source is milk fat, and it is recognized as a food component [8]. Tappenden reported that the intravenous (systemic not luminal) administration of SCFAs altered gastrointestinal structure and function, and the expression of early response genes such as c- myc, c- jun, and c-fos [9]. The molar ratio of propionate and acetate in blood is much higher than the butyrate in physiological conditions [10]. A recent report

indicated that the administration of a parenteral mixture of SCFAs was more effective than butyrate alone at increasing the expression of glucose transporter-2 in the colonic mucosa [11]. Thus the importance of SCFAs other than butyrate, such as propionate and acetate is suggested. Conley also reported that plasma butyrate concentration in humans rose to 0.45 mM following the administration of oral tributyrin, a prodrug of butyrate [12]. Furthermore, Intestamin®, a nutritional supplement containing tributyrin that is used for enteral feeding, is commercially available in the EU [13]. All these observations suggest that increasing the concentration of butyrate using newly developed methods of administration affects not only the intestine but a wider range of organs.

Butyrate has been demonstrated to increase the productions of intercellular adhesion molecule-1 and tissue-type plasminogen-activator, and decrease eNOS expression in human umbilical vein endothelial cells (HUVECs) [7, 14, 15]. These *in vitro* effects of butyrate were investigated within 8 to 36 h using a concentration range of 0.5 μM to 5 mM. Butyrate also decreases the expression of ER mRNA in MCF7 breast cancer cells, and upregulates the expression of tight junction proteins (cingulin, ZO-1, and ZO-2) in Rat-1 fibroblasts and the expression of cyclooxygenase (COX)-1 mRNA in human astrocyte cells [16–18].

Butyrate is pharmacologically characterized as a histone deacetylase (HDAC) inhibitor. It induces the hyperacetylation of histones by inhibiting histone deacetylation, and modulates gene transcription via histone acetylation [15, 19]. Propionate, to a lesser extent, also inhibits HDAC whereas acetate does not. The binding of transcription factors to DNA results in the recruitment of histone acetyltransferase proteins leading to the acetylation of histones, enhancement of nucleosomal relaxation, and subsequent induction of transcription. Trichostatin A (TSA), a typical and specific HDAC inhibitor, causes hyperacetylation of chromatin, which in turn leads to the modulation of gene expression, as observed with butyrate.

We previously reported that butyrate and propionate decrease tight junction permeability in

intestinal monolayer cells via the activation of lipoxygenase (LOX) and/or COX, and TSA mimics the effect of butyrate in decreasing tight junction permeability [19]. An *ex vivo* experiment in which SCFAs relaxed precontracted caudal artery strips composed of endothelium, smooth muscle, and other tissues has also been reported [20]. Butyrate affects inducible NO synthase (iNOS), endothelial NOS (eNOS), and ER in several cell types, mediating tight junction permeability [15, 16, 21, 22]. These reports suggest the importance of SCFAs in the modulation of tight junction permeability not only in intestinal monolayer cells but also in endothelial monolayer cells.

Therefore, we hypothesized that SCFAs, butyrate, propionate, and acetate, alter tight junction permeability in endothelial cells. The tight junction permeability characteristics of HUVECs cultured on collagen-treated microporus filters in a double-chamber system were investigated by evaluating the passage of a molecular marker across the cells. This system was established in a complementary *in vitro* model for normal systemic human vascular endothelium studies [23, 24]. Inhibitors of iNOS, eNOS, COX, and LOX and ER antagonist, are the key mediators or mechanisms that alter tight junction permeability in the endothelium. Their activities have been reported to be altered by butyrate, and have been employed to gain a better understanding of the mechanisms of action. We also investigated the effects of TSA on tight junction permeability, compared with that of butyrate, to determine a possible mechanism for the effects of SCFAs mediated via HDAC inhibition.

Materials and Methods

Cells and Reagents HUVECs were purchased from Cambrex Bio Science Walkersville Inc. (Walkersville, MD, USA), and were cultured in EGM-2 medium [the complete medium contained fetal bovine serum (FBS, 2%), hydrocortisone, human fibroblast growth factor-basic, vascular endothelial growth factor, human epidermal growth factor, R3-insulin-like growth factor 1, ascorbic acid, GA-1000 (gentamicin-amphotericin B), and heparin] at 37 °C, in the presence of 5% CO_2. For all experiments, HUVECs were cultured for up to 10 passages.

SCFAs (sodium salts of butyrate, propionate, and acetate) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), dissolved in Ca^{2+}-free phosphate-buffered saline, and stored at – 30 °C.

The following enzyme inhibitors were also purchased from Sigma-Aldrich Co.: TSA (HDAC inhibitor), aminoguanidine hemisulfate (AG; iNOS inhibitor), indomethacin (IND; COX inhibitor), and 2-(12-hydroxydodeca-5,10-diynyl) -3,5,6-trimethyl -*p*- benzoquinone (AA861; LOX inhibitor). Other chemicals used were *L*-N5-1-iminoethyl-ornithine (*L*-NIO, eNOS inhibitor; Calbiochem, San Diego, CA, USA) and ICI182,780 (ICI, ER antagonist; Tocris, Ellisville, MO, USA). TSA, IND, AA861, and ICI were dissolved in ethanol, L-NIO was dissolved in sterile distilled water, and AG was dissolved in EGM-2 without FBS.

Permeability of HUVEC monolayers Fluorescein isothiocyanate (FITC)-conjugated dextran-10 kDa (Sigma-Aldrich Co.) permeability assays were used to examine paracellular permeability according to a previously described protocol [24]. Because FITC-dextran is hydrophilic and can diffuse between narrow intercellular spaces, it can pass through the tight junction space. Therefore, FITC-dextran is a suitable marker for investigating cultured cell monolayer tight junction permeability [24, 25].

In the present study, we used Transwell multiplates containing cell culture inserts with a

polycarbonate membrane at the lower aperture (pore size, 3.0 μm; diameter, 6.5 mm; Corning International K.K., Tokyo, Japan). Prior to starting the culture, the membrane was coated with type VII collagen (Sigma-Aldrich Co.), and air-dried overnight under UV irradiation in a biohazard ventilator. HUVECs were seeded at a density of 2.0×10^5 cells/cm^2 on the collagen-coated membranes (upper chambers) in the Transwell multiplates. HUVECs were grown to confluence, and formed a monolayer of cells by 4 days after seeding. On the 5th day post-seeding, each SCFA was added to both the upper and lower chambers of the Transwell multiplates at designated final concentrations by diluting in medium (Fig. 1). The HUVEC monolayers were then incubated for 24 h, followed by examination of permeability. The 24-h time point was selected based on the observation that 1 mM butyrate decreased paracellular permeability in a time-dependent manner after 12 h, and no additional decrease was noted at 48 and 72 h in a preliminary experiment. Although higher concentrations of butyrate may increase paracellular permeability, it has been reported that 1.25 to 5 mM butyrate did not affect the proliferation of HUVECs, whereas 10 mM inhibited proliferation at 24 h [7]. Thus, in the present study, paracellular permeability measurements were performed using 0.05 to 5 mM butyrate to avoid the confounding effects of butyrate on cell growth.

The ER-associated signaling pathway has been reported to be involved in the assembly of tight junction molecules [24]. Thus an ER antagonist (ICI182,780) and two inhibitors of cytosolic enzymes (aminoguanidine for iNOS and *L*-NIO for eNOS) were used to investigate how these reagents affect permeability altered by SCFAs. Treatments with the antagonist and inhibitors were performed using the same culture schedule as used for SCFAs alone. A HUVEC monolayer was initially incubated with a single reagent for 30 min without SCFA; the medium was then changed to a freshly prepared medium containing the reagent (ER antagonist or enzyme inhibitors) and a type of SCFA. HUVEC monolayers were cultured for another 24 h (total 24.5 h). Inhibitors of COX and

LOX, IND and AA861, respectively, were also employed because these enzymes are reported to be associated with the function of tight junctions of intestinal monolayer cells [19].

To examine the permeability of HUVEC monolayer tight junctions, 40 μL FITC-dextran (2.0 mg/mL) were added to the upper chamber of the Transwell multiplates (i.e., the same amount of medium was removed), and incubated for 1 h just before harvest. The culture media from both the upper and lower chambers were then collected and analyzed using a spectrophotometer (CytoFluor series 4000; Applied Biosystems, CA, USA). The permeability of the HUVEC monolayers was indicated by the ratio of the concentrations of FITC-dextran in the lower versus the upper chamber.

Immunofluoresent Staining HUVECs were cultured on type VII collagen-coated coverslips for 4 d, and then exposed to butyrate for 24 h. HUVECs were fixed in ice-cold 100% methanol for 15 min at – 30 °C, and then air-dried. Fixed cells were immersed in 1% bovine serum albumin (BSA) in Tris-buffered saline [TBS; 150 mM NaCl, 50 mM Tris-HCl (pH 7.5)] for 1 h. HUVECs were covered with both rabbit anti-occludin (1:100) and mouse anti-ZO-1 (1:100) antibodies in TBS with 1% BSA, the reaction was left to proceed at 4 °C overnight. After incubation, the coverslips were washed 3 times (5 min each) with 50 mM ice-cold Tris-HCl buffer (pH 7.5). Goat anti-rabbit IgG FITC (1:500) and goat anti-mouse IgG TRITC (1:500) were used as secondary antibodies for 1 h at room temperature. Immunostained HUVECs were washed 3 times (5 min each) with 50 mM ice-cold Tris-HCl buffer at 4 °C. Coverslips were mounted on slides with glycerol. Immunostaining patterns of occludin and ZO-1 were observed and photographed using a confocal microscope (LSM510; Carl Zeiss, Oberkochen, Germany).

RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Assay RNA extraction from HUVECs treated with butyrate, propionate, and acetate, and subsequent RT-PCR were performed using commercial kits as described [19]. Transcripts for COX-1, COX-2, 5-LOX, 12-LOX, and 15-LOX were amplified using a commercial PCR protocol (Thermal Cycler Dice, Model TP 600;

TAKARA, Kyoto, Japan). The mRNA expressions of enzymes were standardized against the mRNA expression of the internal standard, glyceraldehyde-3-phosphate dehydrogenase. PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and analyzed densitometrically.

Cell Damage To assess cell membrane integrity, the release of the cytosolic enzyme lactate dehydrogenase (LDH) was measured according to a previously described protocol [19]. LDH activity in the media of the lower chambers was determined spectrophotometrically using an LDH-Cytotoxic Test (Wako Pure Chemical Industries, Osaka, Japan) and a microplate reader (Benchmark Microplate Reader; Bio-Rad Laboratories, Hercules, CA, USA).

Statistical Analysis All numerical data were expressed as means ± standard deviations. The results of the HUVEC monolayer permeabilities for all tests were analyzed using a one-way factorial analysis of variance (ANOVA), followed by a Tukey-Kramer's protected least significant difference (PLSD) multiple comparison test. Finally, the effects of the inhibitors on permeability were analyzed by a *t*-test.

Results

Effects of SCFAs on tight junction permeability

The effects of SCFAs on HUVEC monolayer permeability were investigated. A decrease in the permeability ratio meant the stabilization of the tight junctions in cultured HUVECs, and an increase in the permeability ratio meant the opposite. Low concentrations of butyrate (0.1-2 mM) and propionate (1-16 mM) induced a statistically significant decrease in paracellular permeability ($p < 0.01$), although these two SCFAs became ineffective at higher concentrations (Figs. 1 A and B). On the other hand, acetate decreased paracellular permeability in a concentration-dependent manner ($p < 0.01$; Fig. 1 C).

The release of the cytosolic enzyme LDH after SCFA treatment was measured to determine the cytotoxic effects of high concentrations of SCFAs. LDH was released to 6.0 ± 1.3% in the control group, 6.0 ± 2.3% in the presence of 5 mM butyrate, 7.1 ± 2.2% in the presence of 64 mM propionate, and 6.8 ± 1.5% in the presence of 32 mM acetate. These values implied that even the highest concentrations of SCFAs had no cytotoxic potency on HUVECs.

Immunofluorescent staining of tight junction proteins (occludin and ZO-1)

To investigate the assembly of the tight junction proteins (occludin and ZO-1) of confluent HUVECs, the cells were immunostained. Both rabbit anti-occludin and mouse anti-ZO-1 antibodies showed immunoreactivities along the contours of each HUVEC (Fig. 2), indicating that tight junction molecules are assembled along the coherent cell membrane between adjacent HUVECs. Occludin and ZO-1 in control HUVECs were stained at the endothelial cell borders (Figs. 2 A and C). Although 0.5 mM butyrate decreased paracellular permeability, its immunoreactivity was indistinguishable from that of the control HUVECs (Figs. 2 B and D).

Effects of SCFAs and aminoguanidine (iNOS inhibitor), L-NIO (eNOS inhibitor), or ICI182,780

(ER antagonist) on tight junction permeability

The effects of iNOS and eNOS inhibitors, and an ER antagonist were evaluated using concentrations of SCFAs that decreased permeability (0.5 mM butyrate, 1 mM propionate, and 32 mM acetate) (Fig. 3). Aminoguanidine (10 mM) attenuated the effect of butyrate for paracellular permeability; the permeability ratio shifted from 0.61 ± 0.17 to 0.86 ± 0.35. Similarly, ICI182,780 (1 μM) significantly attenuated the effect of butyrate on permeability ($p < 0.01$); the ratio shifted from 0.61 ± 0.17 to 0.88 ± 0.04. However, L-NIO (100 μM) did not influence the effect of butyrate on permeability.

For both propionate and acetate, ICI182,780 altered the effects of the SCFAs on paracellular permeability; the ratio shifted from 0.68 ± 0.22 to 1.05 ± 0.68 (not significant), and from 0.69 ± 0.17 to 0.80 ± 0.10 ($p < 0.05$), respectively. Neither aminoguanidine nor L-NIO altered the effects of propionate or acetate on permeability (Fig. 3).

Effects of SCFAs on COX and LOX

The most effective concentrations of SCFAs on paracellular permeability (0.5 mM butyrate, 1 mM propionate, and 32 mM acetate) were used. Indomethacin (10 μM) combined with butyrate or acetate decreased the paracellular permeability ratio from 0.61 ± 0.17 to 0.55 ± 0.18 ($p < 0.05$) and from 0.69 ± 0.17 to 0.39 ± 0.05, respectively (Fig. 4). Conversely, indomethacin combined with propionate had no effect on paracellular permeability. AA861 (10 μM) attenuated the effects of butyrate (from 0.61 ± 0.17 to 0.85 ± 0.24), propionate (from 0.68 ± 0.22 to 1.09 ± 0.25; $p < 0.05$), and acetate (from 0.69 ± 0.17 to 0.81 ± 0.34).

The expressions of COX-1, COX-2, 5-LOX, 12-LOX, and 15-LOX mRNAs after treatment with SCFAs were evaluated because the effects of SCFAs have been demonstrated to be influenced by COX and LOX inhibitors; as well, butyrate increased the expressions of 5-LOX, 12-LOX, and 15-LOX mRNAs in intestinal monolayer cells [19]. The expression of 12-LOX mRNA did not differ

between the untreated and SCFA-treated cells, although the expression of COX-1 mRNA was slightly decreased in SCFA-treated cells (90% for butyrate; Fig. 5). On the other hand, COX-2 mRNA expression was increased by butyrate (120%; $p < 0.01$), and was decreased by acetate (70%). Propionate did not alter COX-1 and COX-2 mRNA expressions. The expressions of 5-LOX and 15-LOX mRNAs were undetectable in all experimental groups.

Effects of TSA (HDAC inhibitor) on tight junction permeability

The effects of TSA, a typical and specific HDAC inhibitor, on tight junction permeability were assessed to compare the effects with those of butyrate and propionate. TSA slightly decreased paracellular permeability at lower concentrations (0.74 ± 0.28), and at higher concentrations significantly increased the permeability ratio (1.57 ± 0.41, $p < 0.01$; Fig. 6). The LDH value remained at the control value ($6.0 \pm 1.3\%$) with TSA concentrations of 100 ng/mL ($8.1 \pm 1.8\%$) and 400 ng/mL ($7.0 \pm 1.6\%$).

The TSA concentrations to be effective in decreasing and increasing paracellular permeability (40 and 100 ng/mL, respectively) were used to assess the effects of enzyme inhibitors. Aminoguanidine and ICI182,780 attenuated the effect of 40 ng/mL TSA on paracellular permeability from 0.84 ± 0.19 to 1.29 ± 0.53 and to 1.39 ± 0.68, respectively ($p < 0.05$; Fig. 7). In contrast, L-NIO had no effect on permeability.

As with 100 ng/mL TSA, both aminoguanidine and ICI182,780 increased paracellular permeability from 1.24 ± 0.33 to 1.96 ± 0.66 ($p < 0.05$) and to 1.53 ± 0.23 (not significant), respectively (Fig. 7). However L-NIO, indomethacin, and AA861 combined with 100 ng/mL TSA did not influence permeability.

As for the effects of 40 ng/mL TSA on tight junction permeability, the results in Fig. 7 ($n = 24$, $p < 0.01$) are more reliable than those in Fig. 6 ($n = 6$, N.S. due to the larger sample size and statistically significance).

Discussion

This study presents three novel findings. First, we showed that the SCFAs, butyrate, propionate, and acetate, decreased tight junction permeability in HUVECs. In the case of butyrate and propionate, the decreased effect was biphasic while with acetate, the effect was dose-dependent. Second, the decreasing effects and mechanisms were different among the SCFAs. An ER antagonist attenuated the effects of the SCFAs. Furthermore, the influences of iNOS, COX, and LOX inhibitors and the expressions of COX and LOX mRNAs were elucidated for each SCFA, and an interesting finding was that butyrate altered tight junction permeability via ER and COX. Third, TSA, a typical HDAC inhibitor, increased tight junction permeability when used at high concentrations, but decreased tight junction permeability when used at lower concentrations, these effects were also observed with butyrate. Overall, these results support the hypothesis that SCFAs alter tight junction permeability in HUVECs, suggesting that SCFAs may have the potential to protect barrier properties in cases of increased microvascular leakage that lead to several disease conditions as described in the introduction.

Both butyrate and propionate decreased paracellular permeability when used at low concentrations. By contrast, acetate decreased paracellular permeability in a concentration-dependent manner. The significant different effects exerted by these common SCFAs on tight junction permeability in this *in vitro* model suggest that the effects and the mechanisms of SCFAs on cellular responses are diverse. The most effective concentration of butyrate (0.5 mM) to decrease paracellular permeability was lower than that of propionate (1 mM) and acetate (32 mM). These values are consistent with a previous *ex vivo* study, which indicated that the potency of butyrate (0.8 mM) to change vascular permeability was greater than that of propionate (1.8 mM) and acetate (2 mM), using rat caudal artery strips [20]. Although the most effective concentrations of butyrate and propionate in the present study were similar to those in the aforementioned *ex vivo* study, the

negative effect of 2 mM acetate on tight junction permeability requires further analysis. Each tight junction is composed of a transmembrane protein and sub-membranous anchorage proteins, occludin and ZO-1, respectively. Our immunofluorescent double-staining analysis of occludin and ZO-1 indicated the composition of the tight junction. Butyrate did not significantly increase the amounts of occludin and ZO-1, but significantly decreased tight junction permeability as evaluated by the passage of a molecular marker. It has been reported that butyrate does not affect the expression of ZO-1 in HeLa and CHO cells [17]. As well, tight junction permeability is increased without a significant effect on the amounts of ZO-1 and occludin in the blood cerebrospinal-fluid barrier [26]. Thus, it is suggested that permeability is not always inversely correlated with the conformation of tight junction proteins.

The results of our study indicated that an ER antagonist attenuates the decreased paracellular permeability mediated by butyrate and acetate, suggesting that butyrate and acetate decrease tight junction permeability via the ER in HUVECs. This result was in accordance with the study by deFazio et al. who demonstrated a decreased expression of ER in MCF7 breast cancer cells in response to butyrate [16]. Furthermore, it has been reported that 17-beta-estradiol decreases tight junction permeability biphasically via eNOS through the ER in HUVECs [1, 6, 24].

NO has been demonstrated to alter tight junction permeability in endothelial cells [1, 4]. Similarly, our results indicated that iNOS inhibitors attenuated the effects of butyrate, but not those of propionate and acetate. Several studies have indicated that iNOS activity is altered by butyrate in various cells and by various stimulations; however, the results have been inconsistent and have been found to depend on the experimental conditions [21, 22]. To the best of our knowledge, no other study has reported the effects of SCFAs on iNOS activity in endothelial cells under normal conditions. It has been reported that 2 μM butyrate downregulates eNOS protein and mRNA expression after 18 h in HUVECs [15]. However, our unexpected result, i.e., eNOS inhibitor did not

influence the effect of SCFAs, suggests that butyrate in the present system, may regulate tight junction permeability independent of eNOS.

We previously reported COX- and/or LOX-mediated tight junction permeability changes in intestinal monolayer cells, in which butyrate decreased permeability through LOX activation via histone acetylation. Propionate also altered tight junction permeability through LOX and COX activations, but acetate did not [19]. However, in the present study, we demonstrate that the decrease in tight junction permeability induced by butyrate was enhanced in the presence of COX inhibitor, and the effect of propionate was recovered with LOX inhibitor. This result suggests that the modulation effect of butyrate on tight junction permeability was enhanced by the combination of COX inhibitor, indomethacin, and butyrate. A similar observation that indomethacin enhanced the effect of butyrate has been reported by Galfi et al. using butyrate-mediated proliferation inhibition [27], however the exact mechanism has not been elucidated. The result of LOX inhibitor was not consistent with the results for LOX mRNA expression after propionate activation. The difference might depend on the incubation time. LOX mRNA was detected at 24 h in the present study using the permeability assay. However 12-LOX mRNA was detected at 72 h in porcine aortic endothelial cells [28], and a change in 12-LOX mRNA at 72 h would be expected. The undetectable expressions of 5-LOX and 15-LOX mRNAs are consistent with previous studies [29, 30]. Furthermore, eicosanoid formation may explain the regulation of tight junction permeability by SCFAs in HUVECs; however, the mechanism is probably different to that which we observed using intestinal monolayer cells. It has also been reported that 17-beta-estradiol increases COX-2 mRNA via ER, but raloxifene, a selective ER modulator, increases COX-2 mRNA expression independent of the classical pathway for ER in endothelial cells [31, 32]. Therefore, the relationship between ER, COX, and SCFAs with respect to tight junction permeability requires further analysis.

Regarding the effects of SCFAs on HDAC activity, butyrate and propionate inhibit HDAC,

but acetate does not. We found that TSA increases tight junction permeability, but decreases it at lower concentrations in accordance with butyrate and propionate. Butyrate, propionate, and TSA, at the higher concentration ranges examined in this study, affected tight junction permeability without causing cell damage. These results agreed with an earlier study indicating that cell viability is not decreased in the presence of 5 mM butyrate and 1 μM (303 ng/mL) TSA after 24 h in HUVECs [7, 33]. These biphasic effects are considered to be characteristic of HDAC inhibitor activity since the biphasic effects of butyrate or TSA on thyroid hormone receptor in neuroblastoma N2A cells, or on the expression of 5′transforming growth (TG)3′ interacting factor in skin fibroblasts, have been reported [34, 35]. Estradiol has also been shown to biphasically affect tight junction permeability [1, 6, 24], and it is reported that estrogen hormones recruit mechanisms controlling histone acetylation to bring about their effects in the uterus [36]. The effects of TSA on tight junction permeability were affected by iNOS inhibitor and an ER antagonist. Since the results for TSA were similar to the results for butyrate, we suggested that butyrate decreases tight junction permeability via iNOS and ER through histone acetylation. Arts, using the same concentration ranges and incubation times as those used in the present study, has reported that 0.3–3 mM butyrate or 0.1–1 μM (30–303 ng/mL) TSA acetylate histone H4 affected HUVECs over 16 to 24 h [37]. The effect of TSA was not the same as those of butyrate or propionate, the most remarkable effect involved an increase of tight junction permeability by TSA, which could be decreased by butyrate or propionate. Inducible NOS inhibitor significantly attenuated the effect of TSA, but did not significantly affect butyrate effects. It has been reported that the effects of butyrate sometimes differ from those of TSA [21, 38], indicating that butyrate suppresses iNOS expression via mechanisms independent of histone acetylation as reported in colon cancer cells [21].

We demonstrated the positive effects of SCFAs on tight junction permeability in HUVECs, and that the possible mechanisms involve ER and COX for butyrate, LOX for propionate, and ER

for acetate. A similar inhibition of propionate's effect on tight junction permeability by a LOX inhibitor was observed in Caco-2 intestinal monolayer cells in our earlier report [19]. Provided adequate systemic concentrations of SCFAs can be optimized, the present results could be applied to future approaches to protect barrier properties in cases of increased microvascular leakage leading to several disease conditions. In a human study, the plasma concentrations of butyrate, propionate, and acetate were reported to be 1–3 μM, 4–5 μM, and 100–150 μM, respectively [39], which are lower than those found in the present study. There is some evidence that tributyrin, a triglyceride with butyrate molecules esterified at the 1, 2, and 3 positions, administered orally to patients can produce plasma butyrate concentrations ranging from 0 to 0.45 mM [12]. Thus, plasma butyrate concentrations may be dependent upon the method of administration, and this needs to be further analyzed. In an *ex vivo* study, Nutting et al. reported that SCFAs relaxed rat caudal artery strips composed of endothelial and smooth muscle cells and connective tissues [20]. The current study using normal endothelial cells without any stimulation differs from the aforementioned study, in which the cells were precontracted with various stimulants: KCl, phenylephrine, arginine vasopressin, and prostaglandin $F_{2\alpha}$. However, both our results and those of Nutting et al. [20] suggest that SCFAs are important for permeability. SCFAs may be a crucial element not only for colonocytes but also for endothelial cells. Although investigation is still required to determine the precise mechanisms of action, these data suggest that SCFAs are involved in the assembly of tight junctions in normal vascular endothelial cells.

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References

[1] Cho MM, Ziats NP, Pal D, Utian WH, Gorodeski GI. Estrogen modulates paracellular permeability of human endothelial cells by eNOS- and iNOS-related mechanisms. Am J Physiol 1999;276:C337-C349.

[2] Varma S, Breslin JW, Lal BK, Pappas PJ, Hobson RW, Duran WN. p42/44MAPK regulates baseline permeability and cGMP-induced hyperpermeability in endothelial cells. Microvasc Res 2002;63:172-178.

[3] Menconi MJ. Acidosis induces hyperpermeability. Am J Physiol 1997;272:G1007-G1021.

[4] van Hinsbergh WM. Endothelial permeability for macromolecules. Mechanistic aspects of pathophysiological modulation. Arterioscler Thromb Vasc Biol 1997;17:1018-1023.

[5] Dewi BE, Takasaki T, Kurane I. In vitro assessment of human endothelial cell permeability: effects of inflammatory cytokines and dengue virus infection. J Virol Methods 2004;121:171-180.

[6] Hayashi T, Yamada K, Esaki T, Kuzuya M, Satake S, Ishikawa T, et al. Estrogen increases endothelial nitric oxide by a receptor-mediated system. Biochem Biophys Res Commun 1995;214:847-855.

[7] Miller SJ, Zaloga GP, Hoggatt AM, Labarrere C, Faulk WP. Short-chain fatty acids modulate gene expression for vascular endothelial cell adhesion molecules. Nutrition 2005;21:740-748.

[8] Davis T, Kennedy C, Chiew YE, Clarke CL, deFazio A. Histone deacetylase inhibitors decrease proliferation and modulate cell cycle gene expression in normal mammary epithelial cells. Clin Cancer Res 2000;6:4334-4342.

[9] Tappenden KA, McBurney MI. Systemic short-chain fatty acids rapidly alter gastrointestinal structure, function, and expression of early response genes. Dig Dis Sci 1998;43:1526-1536.

[10] Roy CC, Kien CL, Bouthillier L, Levy E. Short-chain fatty acids: Ready for prime time?. Nutr

Clin Prac 2006;21:351-66.

[11] Drozdowski LA, Dixon WT, McBurney MI, Thomson AB. Short-chain fatty acids and total parenteral nutrition affect intestinal gene expression. JPEN 2002;26:145-50.

[12] Conley BA, Egorin MJ, Tait N, Rosen DM, Sausville EA, Dover G, et al. Phase I study of the orally administered butyrate prodrug, tributyrin, in patients with solid tumors. Clin Cancer Res 1998;4:629-634.

[13] Schroeder J, Alteheld B, Stehle P, Cayeux MC, Chiolero RL, Berger MM. Safety and intestinal tolerance of high-dose enteral antioxidants and glutamine peptides after upper gastrointestinal surgery. Eur J Clin Nutr 2005;59:307-310.

[14] Kooistra T, van den Berg J, Tons A, Platenburg G, Rijken DC, van den Berg E. Butyrate stimulates tissue-type plasminogen-activator synthesis in cultured human endothelial cells. Biochem J 1987;247:605-612.

[15] Rossig L, Li H, Fisslthaler B, Urbich C, Fleming I, Forestermann U, et al. Inhibitors of histone deacetylation downregulate the expression of endothelial nitric oxide synthase and compromise endothelial cell function in vasorelaxation and angiogenesis. Circ Res 2002;91:837-844.

[16] deFazio A, Chiew YE, Donoghue C, Lee CS, Sutherland RL. Effect of sodium butyrate on estrogen receptor and epidermal growth factor receptor gene expression in human breast cancer cell lines. J Biol Chem 1992;267:18008-18012.

[17] Bordin M, D'Atri F, Guillemot L, Citi S. Histone deacetylase inhibitors up-regulate the expression of tight junction proteins. Mol Cancer Res 2004;2:692-701.

[18] Taniura S, Kamitani H, Watanabe T, Eling TE. Transcriptional regulation of cyclooxygenase-1 by histone deacetylase inhibitors in normal human astrocyte cells. J Biol Chem 2002;277:16823-16830.

[19] Ohata A, Usami M, Miyoshi M. Short-chain fatty acids alter tight junction permeability in

intestinal monolayer cells via lipoxygenase activation. Nutrition 2005;21:838-847.

[20] Nutting CW, Islam S, Daugirdas JT. Vasorelaxant effects of short chain fatty acid salts in rat caudal artery. Am J Physiol 1991; 261: H561-H567.

[21] Sasahara Y, Mutoh M, Takahashi M, Fukuda K, Tanaka N, Sugimura T, et al. Suppression of promoter-dependent transcriptional activity of inducible nitric oxide synthase by sodium butyrate in colon cancer cells. Cancer Lett 2002;177:155-161.

[22] Laubach VE, Garvey EP, Sherman PA. High-level expression of human inducible nitric oxide synthase in Chinese hamster ovary cells and characterization of the purified enzyme. Biochem Biophys Res Commun 1996;218:802-807.

[23] Schleger C, Platz SJ, Deschl U. Development of an in vitro model for vascular injury with human endothelial cells. Altex 2004;21:12-19.

[24] Ye L, Martin TA, Parr C, Harrison GM, Mansel RE, Jiang WG. Biphasic effects of 17-beta-estradiol on expression of occludin and transendothelial resistance and paracellular permeability in human vascular endothelial cells. J Cell Physiol 2003;196:362-369.

[25] Hashida R, Anamizu C, Yagyu-Mizuno Y, Ohkuma S, Takano T. Transcellular transport of fluorescein dextran through an arterial endothelial cell monolayer. Cell Struct Funct 1986;11:343-349.

[26] Shi LZ, Zheng W. Early lead exposure increases the leakage of the blood-cerebrospinal fluid barrier, in vitro. Hum Exp Toxicol 2007;26:159-167.

[27] Galfi P, Neogrady Z, Amberger A, Margreiter R, Csordas A. Sensitization of colon cancer cell lines to butyrate-mediated proliferation inhibition by combined application of indomethacin and nordihydroguaiaretic acid. Cancer Detect Prev 2005; 29: 276-285.

[28] Patricia MK, Natarajan R, Dooley AN, Hernandez F, Gu JL, Berliner JA, Rossi JJ, Nadler JL, Meidell RS, Hedrick CC. Adenoviral delivery of a leukocyte-type 12 lipoxygenase ribozyme inhibits

effects of glucose and platelet-derived growth factor in vascular endothelial and smooth muscle cells. Circ Res 2001; 88: 659-665.

[29] Fiorucci S, Distrutti E, Mencarelli A, Morelli A, Laufor SA, Cirino G, et al. Evidence that 5-lipoxygenase and acetylated cyclooxygenase 2-derived eicosanoids regulate leukocyte-endothelial adherence in response to aspirin. Br J Pharmacol 2003;139:1351-1359.

[30] Lee YW, Kuhn H, Kaiser S, Hennig B, Daugherty A, Toborek M. Interleukin 4 induces transcription of the 15-lipoxygenase I gene in human endothelial cells. J Lipid Res 2001;42:783-791.

[31] Tamura M, Deb S, Sebastian S, Okamura K, Bulun SE, Estrogen up-regulates cyclooxygenase-2 via estrogen receptor in human uterine microvascular endothelial cells. Fertil Steril 2004;81:1351-1356.

[32] Oviedo PJ, Hermenegildo C, Tarin JJ, Cano A. Raloxifene promotes prostacyclin release in human endothelial cells through a mechanism that involves cyclooxygenase-1 and -2. Fertil Steril 2005;83:1822-1829.

[33] Wang J, Mahmud SA, Bitterman PB, Huo Y, Slungaard A. Histone deacetylase inhibitors suppress TF-kappaB-dependent agonist-driven tissue factor expression in endothelial cells and monocytes. J Biol Chem 2007;282:28408-28418.

[34] Yusta B, Ortiz-Caro J, Bed G, Pascual A, Aranda A. Regulation of thyroid hormone receptor and c-erbA mRNA levels by butyrate in neuroblastoma (N2A) and glioma (C6) cells. J Neurosci Res 1990;27:1-9.

[35] Rombouts K, Niki T, Greenwel P, Vandermonde A, Wielant A, Hellemans K, et al. Trichostatin A, a histone deacetylase inhibitor, suppresses collagen synthesis and prevents TGF-beta(1)-induced fibrogenesis in skin fibroblasts. Exp Cell Res 2002;278:184-197.

[36] Gunin AG, Kapitova IN, Suslonova NV. Effects of histone deacetylase inhibitors on estradiol-induced proliferation and hyperplasia formation in the mouse uterus. J Endocrinol

2005;185:539-549.

[37] Arts J, Lansink M, Grimbergen J, Toet KH, Kooistra T. Stimulation of tissue-type plasminogen activator gene expression by sodium butyrate and trichostatin A in human endothelial cells involves histone acetylation. Biochem J 1995;310:171-176.

[38] Robe PA, Jolois O, N'Guyen M, Princen F, Malgrange B, Merville MP, Bours V. Modulation of the HSV-TK/ganciclovir bystander effect by n-butyrate in glioblastoma: correlation with gap-junction intercellular communication. Int J Oncol 2004;25:187-192.

[39] Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, et al. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J Biol Chem 2003;278:25481-25489.

Figure Legends

Fig. 1. Effects of SCFAs on tight junction permeability. Monolayers were incubated for 24 h with the indicated concentrations of SCFAs, butyrate (A), propionate (B), and acetate (C), and permeability was examined. The values were normalized against those for untreated (control) cells. Values represent means ± standard deviation (n = 6–12). $^{\#}$ $p < 0.01$ versus control for tight junction permeability using a Tukey-Kramer's PLSD multiple comparison test. SCFAs, short-chain fatty acids.

Fig. 2. Immunofluorescent staining of occludin and ZO-1 in HUVECs. Confluent monolayers were exposed to 0.5 mM butyrate for 24 h. After treatment, the monolayers were incubated with primary antibodies against occludin and ZO-1, followed by FITC- or TRITC-conjugated secondary antibodies. A, occludin, control; B, occludin, butyrate; C, ZO-1, control; D, ZO-1, butyrate. HUVECs, human umbilical vein endothelial cells; ZO-1, zonula occludens; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.

Fig. 3. Effects of AG (iNOS inhibitor, 10 mM), *L*-NIO (eNOS inhibitor, 100 μM), or ICI (ER antagonist, 1 μM) with SCFAs on tight junction permeability. A monolayer was initially incubated with the inhibitor for 30 min without SCFAs. The medium was then changed to a medium containing the inhibitor and the SCFAs, butyrate (A), propionate (B), or acetate (C), for 24 h. The values were normalized against those for untreated (control) cells. Values represent means ± standard deviation (n = 6–24). $^{\#\#}$ $p < 0.01$ versus control and * $p < 0.05$, ** $p < 0.01$ versus no inhibitor using a *t*-test . SCFAs, short-chain fatty acids; AG, aminoguanidine hemisulfate; iNOS, inducible nitric oxide synthase; *L*-NIO, *L*-N5-1-iminoethyl-ornithine; eNOS, endothelial nitric oxide synthase; ICI,

ICI182,780; ER, estrogen receptor.

Fig. 4. Effects of IND (COX inhibitor, 10 μM) or AA861 (LOX inhibitor, 10 μM) with SCFAs on tight junction permeability. A monolayer was initially incubated with the inhibitor for 30 min without SCFAs. The medium was then changed to a medium containing the inhibitor and SCFAs, butyrate (A), propionate (B), or acetate (C), for 24 h. The values were normalized against those forthe untreated (control) cells. Values represent means ± standard deviation (n = 4–20). $^{\#\#}$ $p < 0.01$ versus control and * $p < 0.05$ versus no inhibitor using a *t*-test . SCFAs, short-chain fatty acids; IND, indomethacin; COX, cyclooxygenase; AA861, 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-*p*-benzoquinone; LOX, lipoxygenase.

Fig. 5. Expressions of COX-1, COX-2, 5-LOX, 12-LOX, and 15-LOX mRNAs with SCFAs in HUVECs. Total RNA was purified, converted to cDNA, and then amplified using specific primers. The RT-PCR products were separated on 2% agarose gels and visualized with ethidium bromide. The data represent one of three separate experiments with similar results. Lane 1, untreated (control); lane 2, 0.5 mM butyrate; lane 3, 1 mM propionate; lane 4, 32 mM acetate. The values were normalized against those for the control cells. Values represent means ± standard deviation (n = 3). $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ versus control using a *t*-test. HUVECs, human umbilical vein endothelial cells; SCFAs, short-chain fatty acids; COX, cyclooxygenase; LOX, lipoxygenase.

Fig. 6. Effects of TSA on tight junction permeability. A monolayer was incubated for 24 h with the indicated concentrations of TSA. The values were normalized against those for the untreated (control) cells. Values represent means ± standard deviation (n = 6–14). $^{\#}$ $p < 0.01$ versus control for tight junction permeability using a Tukey-Kramer's PLSD multiple comparison test. TSA,

trichostatin A.

Fig. 7. Effects of AG (iNOS inhibitor, 10 mM), *L*-NIO (eNOS inhibitor, 100 μM), ICI (ER antagonist, 1 μM), IND (COX inhibitor, 10 μM), or AA861 (LOX inhibitor, 10 μM) with TSA on tight junction permeability. A monolayer was initially incubated with the inhibitor for 30 min without TSA. The medium was then changed to a medium containing the inhibitor and TSA for 24 h. The values were normalized against those for the untreated (control) cells. Values represent means ± standard deviation (n = 4–24). $^{\#\#}$ $p < 0.01$ versus control and * $p < 0.05$ versus no inhibitor using a *t*-test . TSA, trichostatin A; AG, aminoguanidine hemisulfate; iNOS, inducible nitric oxide synthase; *L*-NIO, *L*-N5-1-iminoethyl-ornithine; eNOS, endothelial nitric oxide synthase; ICI, ICI182,780; ER, estrogen receptor; IND, indomethacin; COX, cyclooxygenase; AA861, 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-*p*-benzoquinone; LOX, lipoxygenase.

Fig. 1

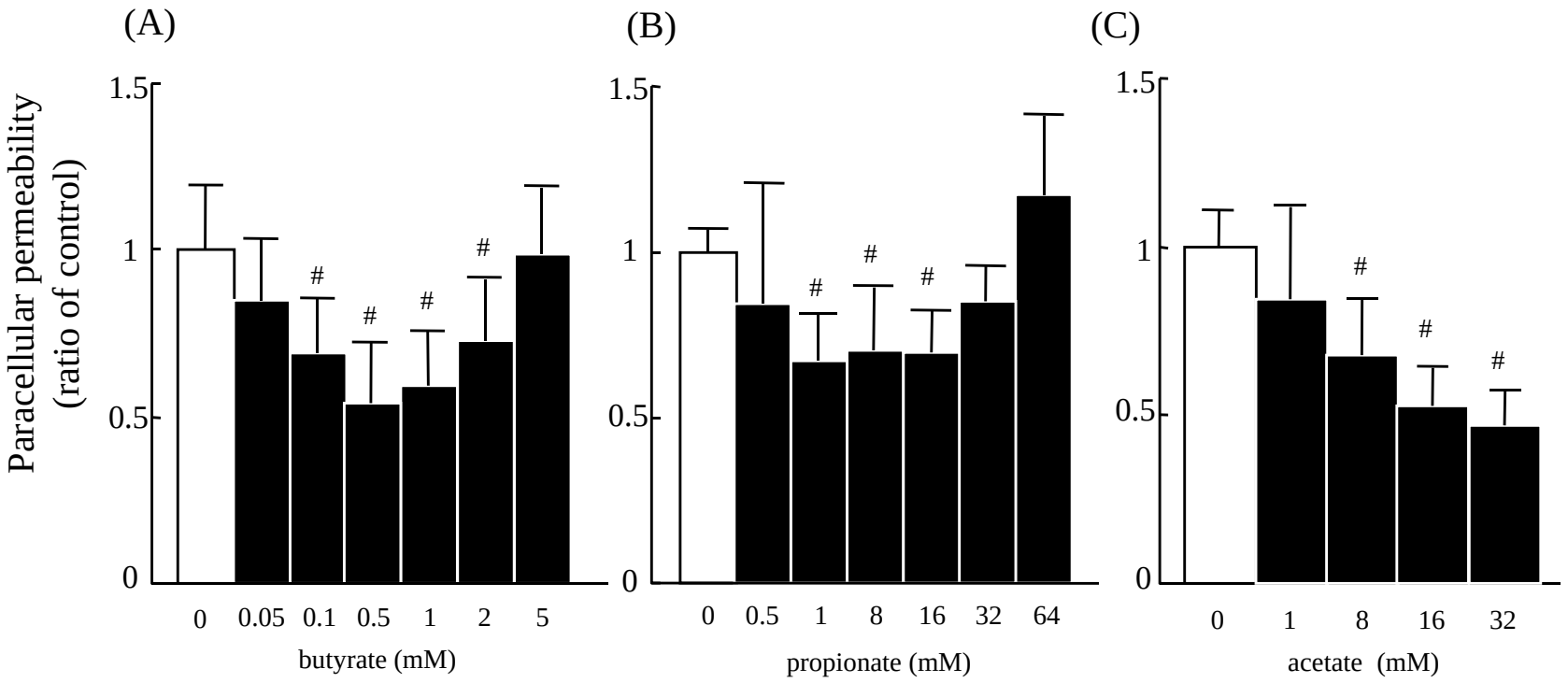

(A)
(B)
(C)
Paracellular permeability
(ratio of control)
1.5
1
0.5
0
#
0 0.05 0.1 0.5 1 2 5
butyrate (mM)
0 0.5 1 8 16 32 64
propionate (mM)
0 1 8 16 32
acetate (mM)

Fig. 2

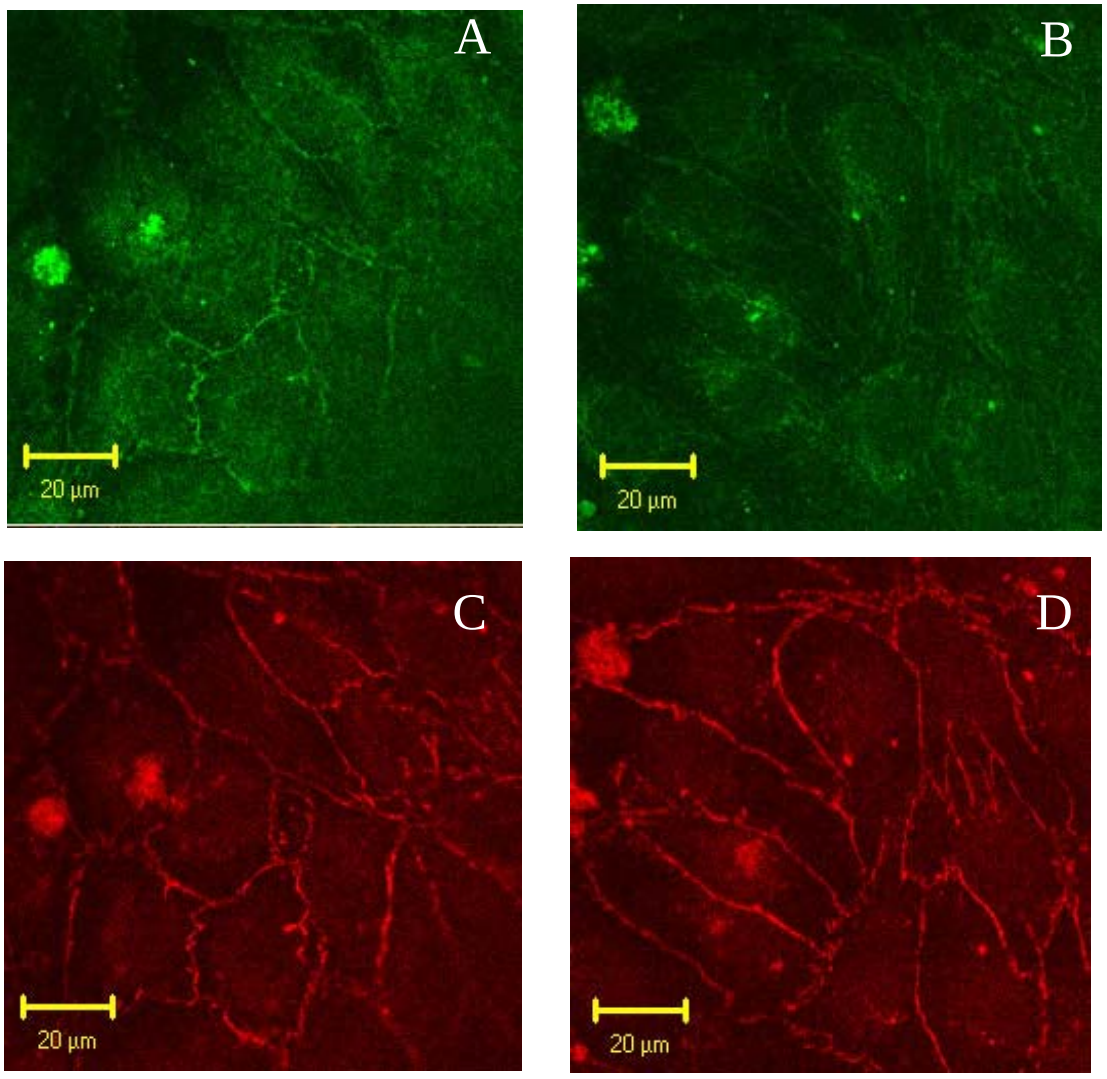


Fig. 3

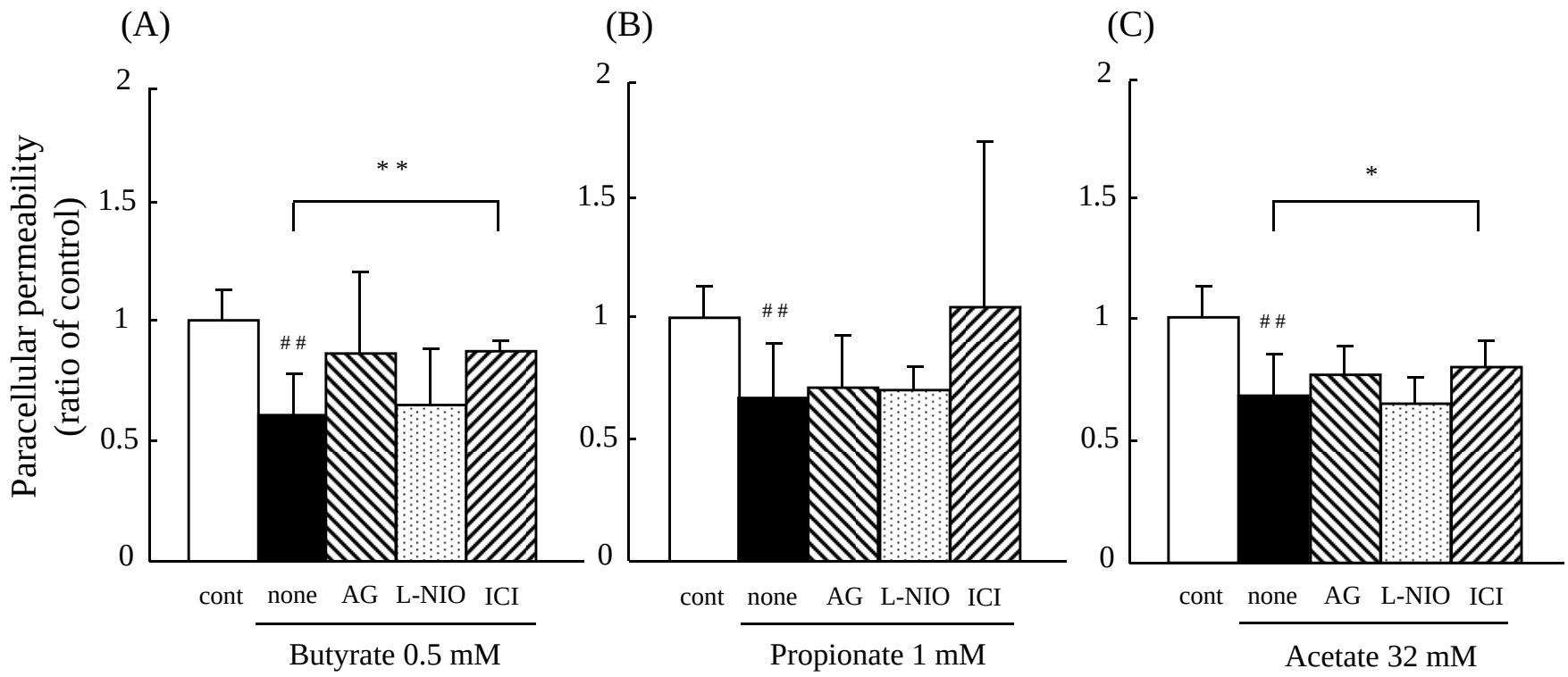

(A)
(B)
(C)
Paracellular permeability
(ratio of control)
2
1.5
1
0.5
0
* *
*
##
cont
none
AG
L-NIO
ICI
Butyrate 0.5 mM
Propionate 1 mM
Acetate 32 mM

Fig. 4

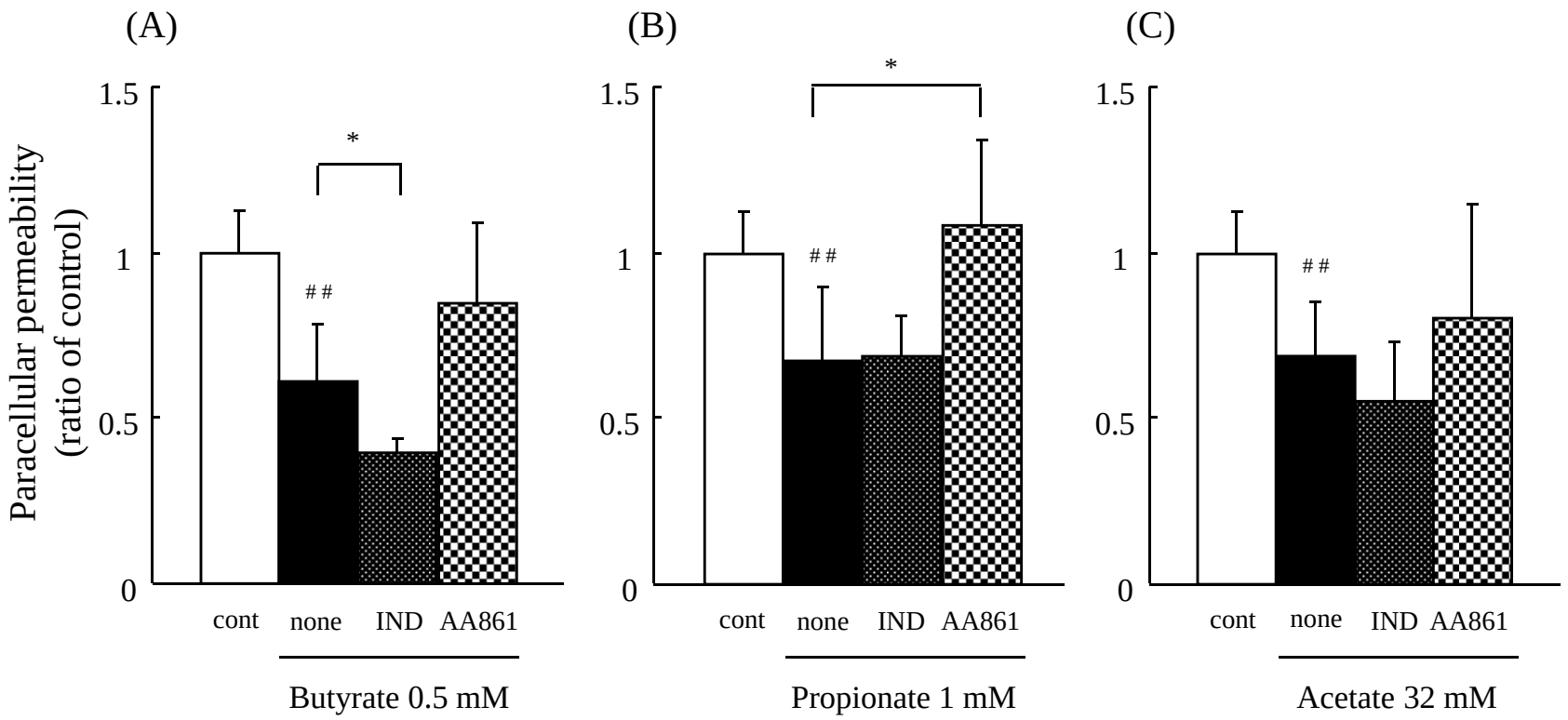

(A)
(B)
(C)
Paracellular permeability
(ratio of control)
1.5
1
0.5
0
*
##
cont
none
IND
AA861
Butyrate 0.5 mM
Propionate 1 mM
Acetate 32 mM

Fig. 5

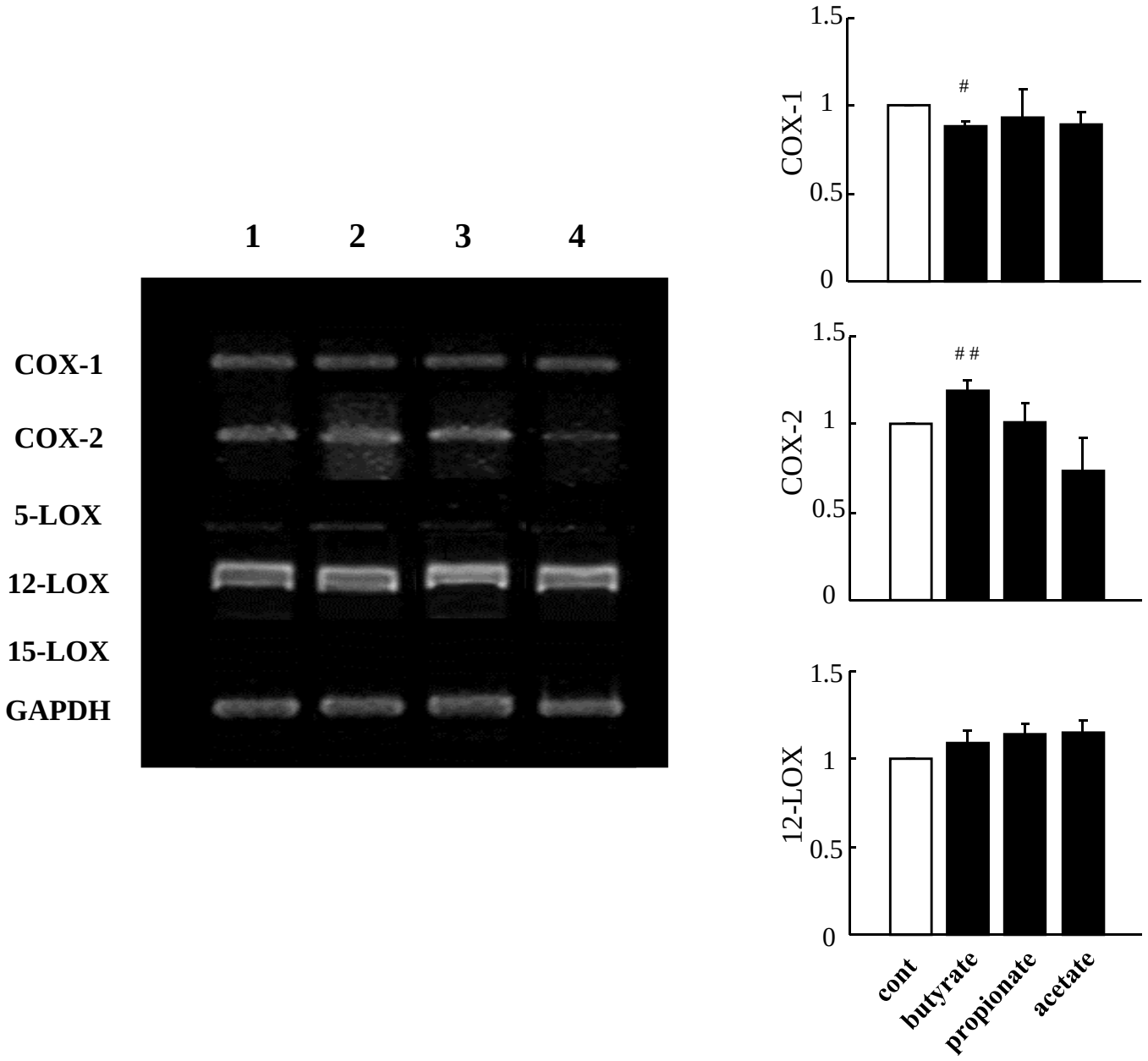

1
2
3
4
COX-1
COX-2
5-LOX
12-LOX
15-LOX
GAPDH
1.5
1
0.5
0
#
COX-1
1.5
1
0.5
0
##
COX-2
1.5
1
0.5
0
12-LOX
cont
butyrate
propionate
acetate

Fig. 6

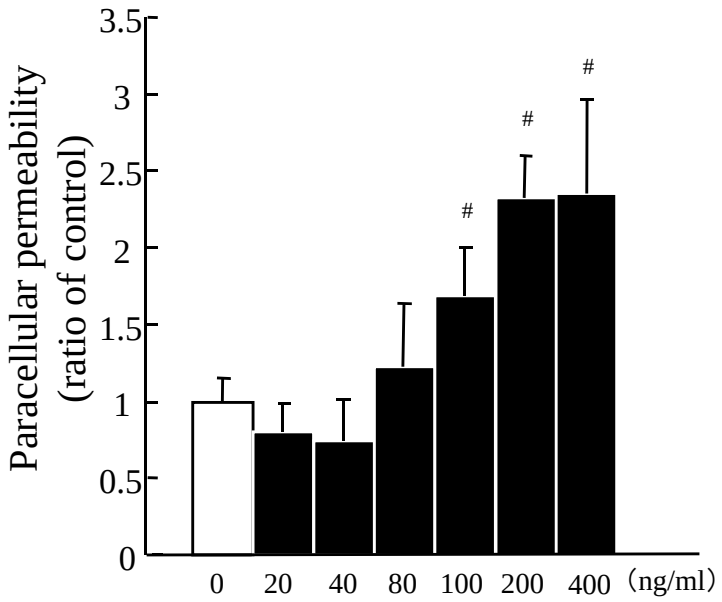
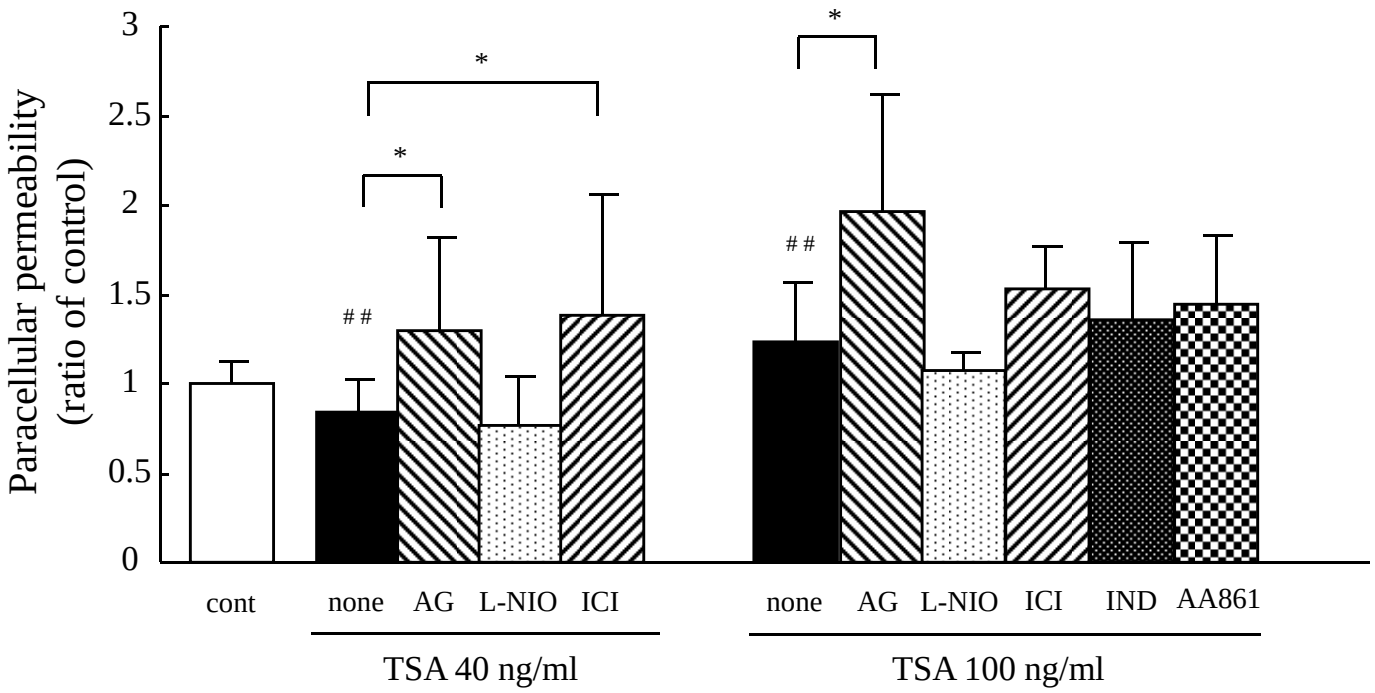

Paracellular permeability
(ratio of control)
3.5
3
2.5
2
1.5
1
0.5
0
#
#
#
0
20
40
80
100
200
400
(ng/ml)

Fig. 7


Paracellular permeability
(ratio of control)
3
2.5
2
1.5
1
0.5
0
*
*
*
##
##
cont
none
AG
L-NIO
ICI
none
AG
L-NIO
ICI
IND
AA861
TSA 40 ng/ml
TSA 100 ng/ml