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PDF issue: 2025-08-15

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(Degree)
博士（保健学）

(Date of Degree)
2005-03-25

(Date of Publication)
2014-07-15

(Resource Type)
doctoral thesis

(Report Number)
甲3387

(URL)
https://hdl.handle.net/20.500.14094/D1003387

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博士論文

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に関する研究

平成17年1月 18日

神戸大学大学院医学系研究科保健学専攻

大 畑　　淳

Title:

Short-Chain Fatty Acids Alter Tight Junction Permeability in Intestinal Monolayer Cells via Lipoxygenase Activation

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Running head: Short-Chain Fatty Acids Induce Lipoxygenase Activation

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ABSTRACT

OBJECTIVE: Involvement of lipoxygenase (LOX) and cyclooxygenase (COX) on cellular differentiation or apoptosis induced by butyrate has been reported recently, however the effect on tight junction (TJ) permeability is not reported. One major activity of butyrate, and to a lesser extent, propionate is to modulate gene transcription via histone acetylation by their histone deacetylase (HDAC) inhibitor activity. In this study, we evaluated the activation of LOX and COX in TJ permeability changes by short-chain fatty acids (SCFAs), butyrate, propionate, and acetate, in intestinal monolayer cells and their possible mechanism by histone acetylation.

METHODS: The effects of LOX and COX inhibitors on TJ permeability and the expression of LOX or COX mRNA induced by SCFAs were investigated in Caco-2 cells using Transwell chambers. The effects of hydroxyeicosatetraenoic acid (HETE, a product of LOX) on TJ permeability were also evaluated. The effects of SCFAs were compared with those of trichostatin A (TSA, HDAC inhibitor).

RESULTS: A LOX inhibitor clearly inhibited the effect of butyrate on TJ permeability, whereas COX inhibitors did not. The LOX and COX inhibitors partially inhibited the effects of propionate, but not of acetate. Butyrate increased LOX mRNA expression, and HETE and TSA mimicked its effect.

CONCLUSION: These results suggest that SCFAs, especially butyrate, induce TJ permeability changes via LOX activation through histone acetylation.

KEYWORDS: Short-chain fatty acids, butyrate, tight junction permeability, lipoxygenase, hydroxyeicosatetraenoic acid, histone acetylation, trichostatin A.

INTRODUCTION

Short-chain fatty acids (SCFAs), predominantly acetate, propionate and butyrate, are the end-products of anaerobic bacterial fermentation of carbohydrates in the colon. SCFAs, especially butyrate, play important roles in the biology of colonocytes by acting as the principal energy source and maintaining the various intestinal environments by preventing epithelial atrophy, increasing sodium absorption, and controlling intestinal peristalsis and pancreatic secretion.[1] Several studies have further demonstrated that butyrate can induce cellular differentiation, growth arrest and apoptosis of colonic epithelial cells in vitro.[2-5] Recently, it has been reported that butyrate induces cellular differentiation or apoptosis via changes in lipoxygenase (LOX) or cyclooxygenase (COX) expression.[5-7] Treatment of Caco-2 cells with butyrate significantly induced 15-LOX expression and shifted the metabolite profile of arachidonic acid (AA) from prostaglandins to 15-hydroxyeicosatetraenoic acid (15-HETE), which was synthesized via the 15-LOX enzyme.[5] In addition, the expression of other isoforms of LOX (5-LOX and 12-LOX) were also up-regulated by butyrate during the differentiation of Caco-2 cells and rat intestinal epithelial cells.[6,7] More recently, it was shown that butyrate down-regulates the expression of GATA-6, a transcriptional factor that is expressed in the gut and exists in the promoter region of 15-LOX, with the up-regulation of 15-LOX expression.[8]

Butyrate was reported to modulate gene transcription via histone acetylation.[9-11] Butyrate induces hyperacetylation of histones by inhibiting histone deacetylation, and is pharmacologically categorized as a histone deacetylase (HDAC) inhibitor. Histones are the core proteins of nucleosomes and the acetylation of nuclear histones is regulated by histone acetyltransferase and HDAC.[12] Binding of transcriptional factors to DNA recruits histone acetyltransferase proteins leading to acetylation of the core histones, enhancement of nucleosomal relaxation, and subsequently induction of transcription. Propionate also inhibits HDAC, although less potently than butyrate, while acetate does not.[13,14] 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.[15] Recently, Mariadason et al reported a microarray analysis of 8,063 genes in colonic epithelial cell maturation, several were induced by butyrate.[16] However, they did not evaluate the expression of LOX or COX.

The gastrointestinal epithelium normally functions as a selective barrier that permits the absorption of nutrients, electrolytes

and water, but restricts the passage from the lumen into the systemic circulation of larger potentially toxic compounds, thus preventing bacterial translocation and systemic infection. It is well established that the gastrointestinal epithelial permeability, especially tight junction (TJ) permeability, of intestinal monolayer cells can be modulated by a number of factors, including tissue pH, adenosine 3'-, 5'-cyclic monophosphate, insulin, insulin-like growth factors, protein kinase C (PKC) activators, nitric oxide and cytokines.[17] Recently, we reported that addition of some polyunsaturated fatty acids (PUFAs) to the medium, namely AA, γ-linolenic acid (GLA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), increased TJ permeability in Caco-2 monolayer cells cultured in a double chamber system as a model of the intestinal epithelium.[18,19] Caco-2 cells grown on permeable supports have been used as an experimental model to measure paracellular permeability, since the cells are highly polarized with a well-formed brush border, and have been shown to express several differentiated markers typical of adult enterocytes and behave like the small intestine.[17] The changes in TJ permeability induced by the PUFAs were mediated via eicosanoid formation and PKC activation.[18,19] On the contrary, SCFAs, especially butyrate and propionate, significantly decreased TJ permeability in a concentration-dependent manner at 48 h in a series of experiments using 0.5-2 mM butyrate, 8-16 mM propionate and 8-32 mM acetate.[20] From these results, we hypothesized that SCFAs change TJ permeability by LOX or COX activation and eicosanoids formation via histone acetylation.

The purpose of this study was to evaluate the effects of LOX and COX inhibitors on SCFAs-induced TJ permeability changes, cellular differentiation, and LOX and COX mRNA expression in Caco-2 monolayer cells. The direct effects of three species of HETE, namely 5-HETE, 12-HETE and 15-HETE, which are the eicosanoids produced by 5-LOX, 12-LOX and 15-LOX activation, respectively, on TJ permeability were investigated. Regarding the possible mechanism of SCFAs, we also investigated the effect of TSA on TJ permeability in comparison with butyrate.

MATERIALS AND METODS

Cells and Reagents Caco-2 cells (Dainippon Pharmaceutical Co., Osaka, Japan) were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM). Sodium salts of butyrate, propionate and acetate were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). SCFAs were dissolved in PBS(-) and stored at -30 °C . 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-p-benzoquinone (AA861, a LOX inhibitor), indomethacin (a non-selective COX inhibitor), N-[2-(cyclohexyloxy)-4-nitro-phenyl]methanesulfonamide (NS398, a COX-2-selective inhibitor) and trichostatin A (TSA, a typical and specific HDAC inhibitor), all purchased from Sigma, were dissolved in ethanol. Three species of HETE (5(S)-HETE, 12(S)-HETE and 15(S)-HETE) were obtained from Cayman Chemical Co. (Michigan, USA), and dissolved in DMEM without fetal calf serum (FCS) after evaporating the ethanol under nitrogen.

Measurement of TJ Permeability Transepithelial electrical resistance (TEER) and fluorescein sulfonic acid (FS; Molecular Probes, Eugene, OR, USA) permeability assays were performed as described previously.[18,19] Briefly, Caco-2 cells were seeded at a density of 6.25×10^5 cells/cm^2 in Transwell inserts (Corning International K.K., Tokyo, Japan), that were 6.5 mm in diameter (surface area, 1.0 cm^2) and contained polycarbonate filters with a 3.0 μm pore size. TEER was examined to evaluate the structural integrity of the Caco-2 monolayer cells on d 4 postseeding, and was performed using an electrical resistance system (EVOM; World Precision Instruments, Sarasota, FL, USA). The Caco-2 monolayer cells were used between d 5 and d 12 postseeding. SCFAs were added to the apical and basal chambers in the Transwell plates within 48 h after dilution in medium. One mM butyrate and 16 mM propionate showed prominent effects without cytotoxicity in a previous report, and hence these concentrations were used in the current study.[20] The concentrations of all reagents in the medium are indicated in the results. The LOX and COX inhibitors were pre-incubated for 30 min before SCFAs addition.

Forty microliters of FS (500 μg/ml) was loaded into the apical compartments of the Transwell plates for 3 h after removing 40 μl of the culture medium. The concentrations of FS in the apical and basolateral compartments were then assayed. The FS permeability was expressed as the percent ratio of the concentration in the basolateral chamber *versus* that in the apical chamber. FS has a small molecular weight, 478 Da, but is unable to permeate the cellular membrane at physiological pH due to its lipophobicity.

and therefore has to pass through the TJ space.

RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Assay After exposure to SCFAs and TSA for 24 h, total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Tokyo, Japan). Confluent Caco-2 cells grown on 6-well tissue culture plates were washed twice with PBS(-), lysed in guanidine isothiocyanate solution, extracted with phenol/chloroform, and precipitated with isopropanol. The RNA pellets were washed with 75% ethanol and dissolved DEPC-treated water. The RNA concentration was determined by the absorbance at 260 nm (Gene Quant Pro; Amersham Bioscience Corp., Tokyo, Japan). Cellular RNA was reverse-transcribed into cDNA using the Superscript™ first-strand synthesis system for RT-PCR (Invitrogen). The total volume of each RT reaction was 21 μl and contained the following: 0.5 μg total RNA, 25 ng/μl oligo(dT)$_{12\text{-}18}$ primer, 0.5 mM dNTP mixture, DEPC-treated water, RT buffer, 5 mM $MgCl_2$, 10 mM DTT, 20 U/μl RNase OUT, 2.5 U/μl SuperScript™ II, and 0.1 U/μl RNase H. The thermocycler protocol for the RT phase was performed according to the manufacturer's protocol.

After the RT reaction, the reaction solution was diluted to a final volume of 105 μl with DEPC-treated water, and 5 μl was used for PCR. The total volume of each PCR reaction was 100 μl and contained the following: PCR buffer without Mg^{2+}, 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 2.5 U *Taq* DNA polymerase, and 0.5 μM of each primer. The sequences of the oligonucleotide primers, purchased from Sigma Genosys, are shown in Table 1. The amplifications of COX-1, -2,[21] glyceraldehyde-3-phosphate dehydrogenase (GAPDH),[21] and 5-, 12-, 15-LOX[22] were performed according to the following profiles: 94°C for 1 min, 54°C for 2 min, 72°C for 3 min (10 min in the final cycle) for 30 cycles; 94°C for 1 min, 54°C for 2 min, 72°C for 3 min for 26 cycles; and 94°C for 45 s, 62°C for 45 s, 72°C for 1 min for 30 cycles, respectively. The final PCR products were separated in 2% agarose gels and visualized with ethidium bromide.

Measurement of Cellular Differentiation The cellular differentiation of the Caco-2 monolayer cells was examined by measuring the alkaline phosphatase (ALP) activity, which has been well characterized as a marker for absorptive cell differentiation.[23,24] Cells grown on 10 cm^2 culture dishes were exposed to SCFAs at various concentrations for 72 h after dilution in medium containing 10% FCS. The cells were then harvested, washed in PBS(-), and stored in 1 ml of homogenization buffer (2 mM Tris-HCl, 50 mM mannitol and 1 mM phenylmethylsulfonyl fluoride in distilled H_2O, pH 7.4). Before the assay, the pellets

were homogenized for 1 min and Triton X-100 was added to final concentration of 1%. The protein content was determined spectrophotometrically using a DC protein assay kit (Bio-Rad Laboratories, Tokyo, Japan). The ALP activity of the cell homogenates was measured using p-nitrophenyl phosphate (Wako Pure Chemical Industries, Osaka, Japan) as a substrate. After 15 min at 37°C, the reaction was terminated by adding 5 ml of 0.02 N NaOH, and the absorbance at 405 nm was measured using a spectrophotometer. Calibration was performed using a p-nitrophenol standard solution. The enzyme activity was expressed as mU/mg protein.

Measurement of Cellular Damage To assess cell membrane integrity, release of the cytosolic enzyme lactate dehydrogenase (LDH) was measured.[18] LDH activity in media samples from the basolateral chambers was determined spectrophotometrically using a single reagent system (LDH-Cytotoxic Test, Wako Pure Chemical Industries, Osaka, Japan) on an ELISA reader (Benchmark Microplate Reader, Bio-Rad Laboratories, Hercules, CA, USA).

Statistical Analysis The data were expressed as the mean ± standard deviation (S.D.). The results for all the tests were evaluated by one-way factorial analysis of variance (ANOVA), followed by Fisher's protected least significant difference (PLSD) multiple comparison test to identify significant differences among multiple samples. Statistical significance was seen when the p value was less than 0.05.

RESULTS

Effects of SCFAs with AA861 (LOX Inhibitor), Indomethacin (Non-Selective COX Inhibitor) or NS398 (COX-2-Selective Inhibitor) on TJ Permeability

One mM butyrate, 16 mM propionate and acetate were prominent effects on TJ permeability without cytotoxicity in our previous report.[20] And over 5 mM butyrate was toxic to the cells, so we used the their concentrations in this study. Addition of 1 mM butyrate (Fig. 1) or 16 mM propionate (Fig. 2A) increased the TEER ratio to 185.9 ± 77.7 ($p<0.01$) and 134.0 ± 12.4 ($p<0.01$), but decreased the FS permeability ratio to 38.7 ± 24.1 ($p<0.01$) and 81.8 ± 26.0 ($p<0.05$), respectively, indicating a "tightening" of the TJ. The effects of butyrate on both the TEER and FS permeability ratios were stronger than those of propionate. On the other hand, the addition of acetate had no significant effects on the TEER and FS permeability ratios (Fig. 2B).

Addition of 10 μM AA861 with 1 mM butyrate decreased the TEER ratio from 185.9 ± 77.7 to 131.1 ± 31.5 (Fig. 1A, $p<0.01$), and increased the FS permeability ratio in a concentration-dependent manner from 38.7 ± 24.1 to 113.1 ± 68.7 ($p<0.05$). AA861 alone increased the TEER ratio and decreased the FS permeability ratio (Fig. 1A, $p<0.05$). Indomethacin or NS398 alone and the addition of these inhibitors with 1 mM butyrate did not change the TEER or FS permeability ratios (Fig. 1B, C).

Addition of AA861 with 16 mM propionate decreased the TEER ratio from 134.0 ± 12.4 to 119.0 ± 13.9 (Fig. 2A, $p<0.05$), and increased the FS permeability ratio from 81.8 ± 26.0 to 123.4 ± 44.5 ($p<0.05$). Indomethacin with 16 mM propionate also decreased the TEER ratio to 107.2 ± 9.2 (Fig. 2A, $p<0.01$), and increased the FS permeability ratio to 130.0 ± 40.6 ($p<0.01$). NS398 showed similar effects to indomethacin.

Addition of AA861with 16 mM acetate did not change the TEER or FS permeability ratios. Indomethacin with 16 mM propionate also had no effect on the TEER ratio, but increased the FS permeability ratio from 88.3 ± 18.1 to 136.4 ± 36.8 (Fig. 2B, $p<0.01$).

Expressions of 5-LOX, 12-LOX, 15-LOX, COX-1 and COX-2 mRNAs after Treatment with SCFAs or TSA

The time courses for 15-LOX and COX-2 mRNA expressions after treatment with butyrate have been reported and both mRNA expressions were observed after 10 to 72 h.[5] Consistent with that report, the expressions of these mRNAs were observed

after 24 h in this study (Fig. 3A). The expressions of 5-LOX, 12-LOX and 15-LOX were undetectable in the control group. Butyrate and TSA significantly increased the expressions of all LOX mRNAs. Propionate slightly increased 5-LOX and 12-LOX, whereas acetate did not. The expression of COX-2 mRNA was not changed in either the control cells or SCFAs- and TSA-treated cells. The expression of COX-1 mRNA was undetectable in all experimental groups (data not shown).

Effects of HETE Compounds (Products of LOX) and TSA (HDAC Inhibitor) on TJ Permeability

5-HETE, 12-HETE and 15-HETE all increased the TEER ratio (Table 2, $p<0.01$) and decreased the FS permeability ratio ($p<0.01$) in a concentration-dependent manner.

Addition of TSA at 100-200 ng/ml increased the TEER ratio (Table 3, $p<0.01$) and decreased the FS permeability ratio ($p<0.01$), while 400 ng/ml had no effect. Butyrate showed no statistically significant change in the TEER ratio at 24 h but increased it at 48 h in a time course experiment (Fig. 3B). TSA increased the TEER ratio at 48 h, similar to butyrate.

Effects of TSA with AA861 or NS398 on TJ Permeability

Neither AA861 nor NS398 modulated the effects of 200 ng/ml TSA on the TEER and FS permeability ratios (Fig. 4). Furthermore, the inhibitors had no effect on a lower concentration (100 ng/ml) of TSA (data not shown).

Effect of Butyrate and TSA on Cellular Differentiation

The ALP activity was 22.0 ± 9.3 mU/mg protein in control cells after 6 d. Butyrate and TSA increased the ALP activity to 708% and 298% in a concentration-dependent manner, respectively (Fig. 5A, $p<0.01$). The effect of butyrate on cellular differentiation was stronger than that of TSA. AA861 alone did not change the ALP activity (Fig. 5B). Addition of AA861 with 1 mM butyrate significantly decreased the ALP activity from 546% to 186% ($p<0.01$), whereas AA861 did not modulate the effect of TSA.

Cellular Damage

Total LHD was released to 1.8 ± 1.3% in the control group, 3.1 ± 1.1% in three SCFAs groups, 1.6 ± 1.6% in inhibitor groups, 1.2 ± 0.2% in three HETE groups, 1.7 ± 0.3% in TSA groups, and 3.7 ± 1.5% in SCFAs or TSA plus inhibitor groups, showing that these levels were less than that of cytotoxicity (data not shown).

DISCUSSION

This is the first report to demonstrate that SCFAs, especially butyrate and propionate, decrease TJ permeability in Caco-2 intestinal monolayer cells via the activation of three isoforms of LOX and metabolites of AA produced by the LOX pathway, through histone acetylation due to SCFAs-mediated HDAC inhibition. Our findings indicate that butyrate up-regulated 5-LOX, 12-LOX and 15-LOX mRNA expressions, and that the LOX inhibitor completely normalized the concentration-dependent effect of butyrate on paracellular permeability. In addition, hydroxy derivatives of AA (5-HETE, 12-HETE and 15-HETE) produced by three isoforms of LOX (5-LOX, 12-LOX and 15-LOX) clearly decreased TJ permeability in a concentration-dependent manner, similar to the effects of butyrate. The LOX inhibitor attenuated the butyrate-induced increase in ALP activity, suggesting that LOX activated by butyrate induces cellular differentiation and controls TJ permeability. Moreover, TSA, a typical and specific HDAC inhibitor, decreased TJ permeability with the same time-dependency as butyrate, and up-regulated the three LOX isoform mRNA expressions. Regarding the COX activity, butyrate did not alter COX-2 mRNA expression and COX-1 mRNA was undetectable both with and without butyrate. This suggests that butyrate alters TJ permeability independently of COX activity.

Besides butyrate, our results also show that propionate acts in a similar manner to butyrate, but is less effective, as indicated by the slight up-regulation of 5-LOX and 12-LOX mRNA expressions due to propionate and inhibition of the propionate effect on TJ permeability by the LOX inhibitor. Propionate did not change the COX-2 mRNA expression, while indomethacin, a non-specific COX inhibitor, attenuated the decreased paracellular permeability mediated by propionate. Acetate did not affect TJ permeability or LOX mRNA expression. The apparently different effects exerted by the common SCFAs on TJ permeability in this in vitro model suggest that the effects of SCFAs on cellular responses at the molecular level are diverse.

The changes in LOX activity and the effects of LOX metabolites on the differentiation and apoptosis induced by butyrate have recently been reported.[5,7] It has been hypothesized that activation of LOX mRNA expression and/or the three LOX metabolites (5-HETE, 12-HETE and 15-HETE) regulate TJ permeability in intestinal monolayer cells. The increased LOX mRNA expressions induced by butyrate in the current study are consistent with previous reports demonstrating that 5-LOX mRNA expression was up-regulated 78-fold in Caco-2 cells at 8 d after addition of 2 mM butyrate, as measured by RT competitive multiplex PCR,[6] and

that 15-LOX and 12-LOX mRNA expressions were significantly increased as early as 24 h after 5 mM butyrate addition in Caco-2 cells and rat intestinal epithelial cells.[5,7] These reports also indicated that 12-HETE and 15-HETE levels in the cells were significantly increased by addition of butyrate.[5,6] Following these reports, it is suggested that up-regulation of LOX mRNA in our results will lead to increased HETE productions, although we did not measure the LOX metabolites. However, these reports did not present any data showing a direct effect on 15-HETE. In Caco-2 cells, HETE was well taken up and metabolized in an early time frame.[25,26]

Our results clearly demonstrate suppression of TJ permeability by the three HETE in a concentration-dependent manner. These results suggest that the mechanisms for altering TJ permeability after butyrate addition operate via these LOX products through activation of LOX mRNA expression. Our results are supported by a previous report indicating that 15-HETE increased corneal epithelial TJ resistance, as evaluated with an Ussing chamber.[27] Our results indicate that 5-HETE and 12-HETE also decreased TJ permeability. However, 5-HETE may not be directly linked to the butyrate-induced change in TJ permeability, because 5-HETE is not increased by butyrate due to the lack of butyrate-induced up-regulation of the 5-LOX activating protein, FLAP, which acts in 5-LOX metabolite synthesis.[6] There is some evidence that 5-LOX has other functions related to signal transduction that are independent of LT synthesis.[28-30] Thus, the butyrate-induced increases in 5-LOX mRNA and protein seem rather to be a response to the TJ permeability changes in Caco-2 monolayer cells.

AA861 is a commercially available inhibitor of LOX. Grimminger et al reported that 10 μM AA861 clearly inhibited LOX metabolites including HETE in vivo.[31] However there is no publication concerning the effects of AA861 and/or LOX inhibitors on TJ permeability. The result showing that AA861 alone increased the TEER ratio and decreased the FS permeability ratio is not consistent in our series of experiments.[18] The mechanism to decrease TJ permeability by AA861 is not determined yet in our laboratory. Further studies in the mechanism of AA861 will be required.

Regarding the COX activity changes produced by SCFAs, regulation of COX-2 mRNA expression in Caco-2 cells by butyrate has previously been reported.[6,7] One of these reports indicated that butyrate slightly up-regulated COX-2 mRNA, although the change was not as marked as that for LOX mRNA,[6] while the other reported that butyrate decreased COX-2 mRNA expression.[7] From these contradictory reports regarding the regulation of COX-2 mRNA expression by butyrate and our results indicating no

change in COX-2 mRNA expression with the lack of influence of two different COX inhibitors (indomethacin and NS398) on the effects of butyrate, the activities of COX and its metabolites do not appear to participate in the effects of butyrate on TJ permeability. However, the significant inhibition of the propionate effect on TJ permeability by indomethacin is presently under evaluation.

Histone acetylation by SCFAs was hypothesized to be a possible mechanism for altering TJ permeability. Butyrate and propionate induce hyperacetylation of histones by inhibiting histone deacetylation, and thereby modulate gene transcription.[9-11,13,14] We found that 100-200 ng/ml of TSA, a typical and specific HDAC inhibitor, significantly decreased TJ permeability at 48 h, as observed with butyrate. In addition, our data showing up-regulation of LOX mRNA expression by TSA is in accordance with a previous report.[10] These results suggest that the effects of SCFAs on TJ permeability change and that the expressions of LOX mRNAs may be modulated by gene transcription via histone acetylation. However, LOX inhibitor did not modulated the effect of TSA in our experiment. The discrepancy between butyrate and TSA effects by the LOX inhibitor has yet to be determined. Mariadason et al reported that the time points for the alteration of histone H4 acetylation and the pattern of gene expression indicated marked difference for the both agents.[16] It is suggested that TSA might decrease TJ permeability separately from LOX activity. The precise mechanism should be analyzed in a future study.

Several possible modes of action other than LOX activation also need to be addressed, because butyrate has a multitude of potential effects on cells. SCFAs have been reported to reduce paracellular permeability in a Caco-2 cell model of the colonic epithelium, possibly as a manifestation of the promotion of a more differentiated phenotype.[23] Our data indicating that butyrate induced cellular differentiation with decreased TJ permeability are in accordance with that report. It is well established that TJ permeability is regulated by various factors, including insulin-like growth factor,[17] and SCFAs or TSA have been reported to alter insulin-like growth factor-binding protein secretion in Caco-2 cells.[32] Recently, we revealed other possible mechanisms for the alteration of TJ permeability by SCFAs via intracellular signaling pathways such as PKC and/or cytoskeletal contraction by myosin light chain kinase activation.[20] From these results, we suggest that SCFAs may regulate TJ permeability through several different pathways.

SUMMARY

We reveled that SCFAs, especially butyrate, increased LOX mRNA and LOX inhibitor attenuated the effect of butyrate on TJ permeability. HETE and TSA mimicked the effect suggesting that butyrate induce TJ permeability changes associated with the up-regulation of LOX activity via modulation of gene transcription by histone acetylation.

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Figure Legends

Figure 1. Effects of AA861 (LOX inhibitor), indomethacin (IND, non-selective COX inhibitor) or NS398 (COX-2-selective inhibitor) with 1 mM butyrate on FS permeability and TEER. AA861 attenuates the effect of butyrate (A), whereas IND (B) and NS398 (C) do not. The values are normalized to those of untreated (control) cells. Values are the mean ± S.D. (n = 6-22). $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$ vs. C. * $p<0.05$, ** $p<0.01$ vs. 0 μM of inhibitors. NS, not significant.

Figure 2. Effects of AA861 (LOX inhibitor) or indomethacin (IND, non-selective COX inhibitor) with 16 mM propionate or acetate on FS permeability and TEER. AA861 and IND both attenuate the effects of propionate (A), but do not alter the effects of acetate (B). The values are normalized to those of untreated (control) cells. Values are the mean ± S.D. (n = 9-11). $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$ vs. C. * $p<0.05$, ** $p<0.01$ vs. 0 μM of inhibitors. NS, not significant.

Figure 3. Comparison of the effects of SCFAs and trichostatin A (TSA). (A) 5-LOX, 12-LOX, 15-LOX and COX-2 mRNA expressions in Caco-2 cell monolayers. Total RNA was purified, converted to cDNA, and amplified with specific primers. The RT-PCR products were separated in 2% agarose gels and visualized with ethidium bromide. The data represent one of three separate experiments with similar results. Lane 1, control; lane 2, 16 mM acetate; lane 3, 16 mM propionate; lane 4, 1 mM butyrate; lane 5, 200 ng/ml TSA. (B) Time courses of TEER for butyrate and TSA. The values are normalized by those at 0 h. Values are the mean ± S.D. (n=4). Closed circles and open squares indicate 1 mM butyrate and 200 ng/ml TSA, respectively. $^{\#\#}$ $p<0.01$ vs. 0 h.

Figure 4. Effects of AA861 (LOX inhibitor) or NS398 (COX-2-selective inhibitor) with 200 ng/ml trichostatin A (TSA) on FS permeability and TEER. The values are normalized to those of untreated (control) cells. Values are the mean ± S.D. (n=6-12). $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$ vs. C. NS, not significant.

Figure 5. Effects of butyrate and TSA on cellular differentiation in Caco-2 monolayer cells. (A) Enhanced ALP activity after

treatment with butyrate or TSA. (B) AA861 (LOX inhibitor) attenuates the induction of ALP activity in butyrate-treated cells. The enzyme activity is expressed as mU/mg protein. Values are the mean ± S.D. (n=6-12). $^{\$}$ $p<0.01$, $^{\#\#}$ $p<0.01$ vs. C. ** $p<0.01$ vs. 0 μM of inhibitors.

Table 1. Oligonucleotides for 6 target genes

mRNA species	sense	anti-sense	Reference
COX-1	5'-TGCCCAGCTCCTGGCCCGCCGCTT-3'	5'-GTGCATCAACACAGGCGCCTCTTC-3'	21
COX-2	5'-TTCAAATGAGATTGTGGGAAAAT-3'	5'-AGATCATCTCTGCCTGAGTATCTT-3'	21
GAPDH	5'-CCACCCATGGCAAATTCCATGGCA-3'	5'-TCTAGACGGCAGGTCAGGTCCACC-3'	21
5-LOX	5'-ATCAGGACGTTCACGGCCGAGG-3'	5'-CCAGGAACAGCTCGTTTTCCTG-3'	22
12-LOX	5'-TGGACACTGAAGGCAGGGGCT-3'	5'-GGCTGGGAGGCTGAATCTGGA-3'	22
15-LOX	5'-GCCAAGGGGCTGGCCGACCT-3'	5'-TGGTGGGGATCCTGTGCGGGGCA-3'	22

Table 2. Effects of HETEs on FS permeability and TEER.

	Concentration (μM)	FS permeability ratio	TEER ratio
5-HETE	5	95.2±20.9	105.4±7.4
	10	66.6±10.7	138.8±12.8
	20	64.1±22.2 *	150.3±16.2 *
12-HETE	5	102.3±4.0	87.4±2.8
	10	64.9±10.0	123.7±14.3
	20	43.8±5.2 *	145.6±9.2 *
15-HETE	5	95.5±21.1	107.1±15.2
	10	72.1±12.4	117.2±7.7
	20	46.2±17.5 *	155.9±17.1 *

Fluorescein sulfonic acid (FS) permeability and transepithelial electrical resistance (TEER) The values are normalized to those of untreated (control) cells (100 ± 10.6%, 100 ± 9.8%, respectively). Values are the mean ± S.D. (n = 6 - 11). * $p<0.01$ for FS permeability and TEER ratios with one-way factorial ANOVA without interaction.

Table 3. Effects of TSA on FS permeability and TEER.

Concentration (ng/ml)	FS permeability ratio	TEER ratio
100	47.2±10.2 *	175.8±56.5 *
200	40.4±15.0 *	222.5±111.2 *
400	106.4±35.6	123.6±33.4

Fluorescein sulfonic acid (FS) permeability and transepithelial electrical resistance (TEER) The values are normalized to those of untreated (control) cells (100 ± 6.6%, 100 ± 4.9%, respectively). Values are the mean ± S.D. (n = 9 - 10). * $p<0.01$ vs. C for FS permeability and TEER ratios with Fisher's PLSD multiple comparison test.

Fig. 1

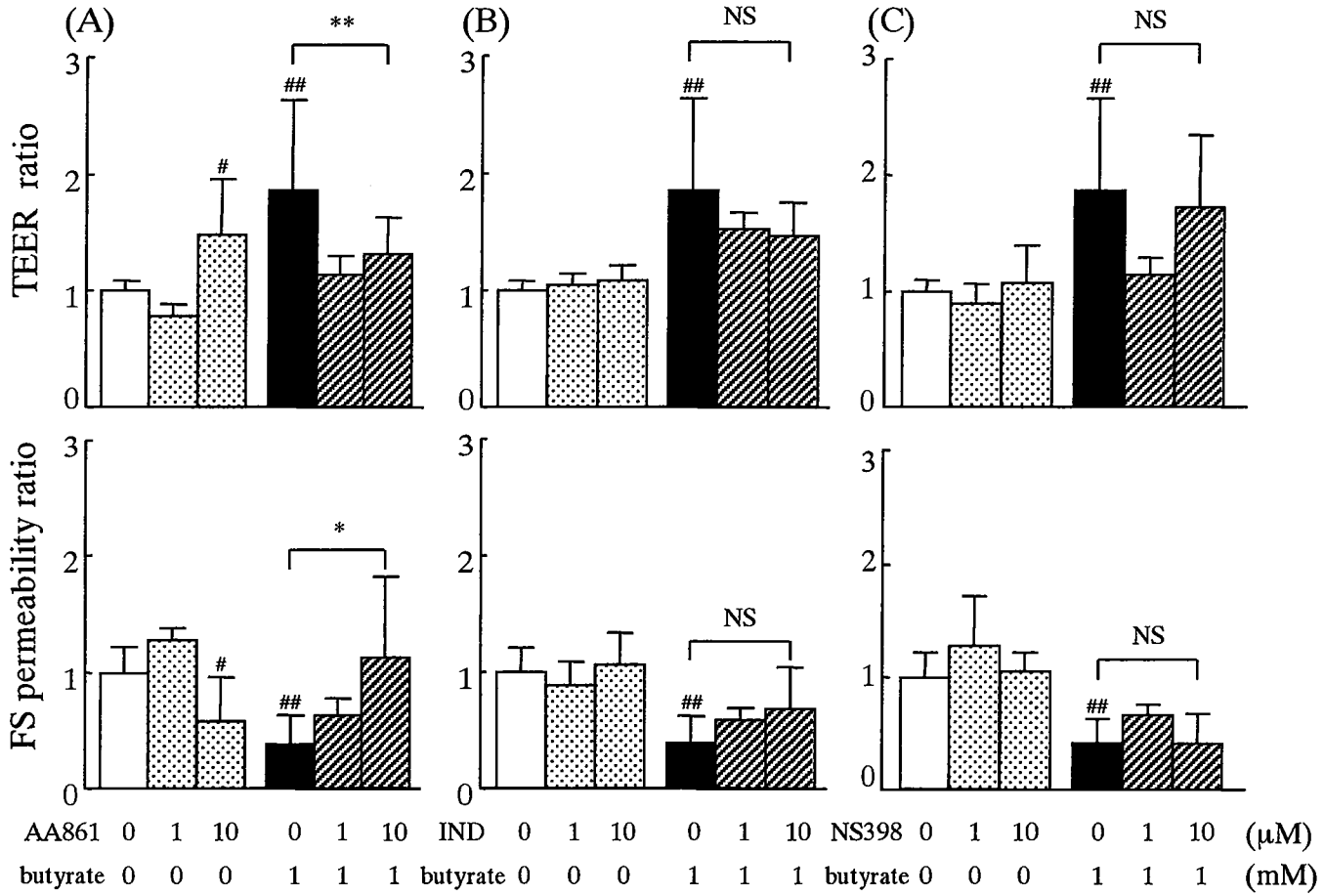

(A)
(B)
(C)
**
NS
NS
TEER ratio
FS permeability ratio
3
2
1
0
##
#
*
NS
AA861 0 1 10 0 1 10
butyrate 0 0 0 1 1 1
IND 0 1 10 0 1 10
butyrate 0 0 0 1 1 1
NS398 0 1 10 0 1 10
butyrate 0 0 0 1 1 1
(μM)
(mM)

Fig. 2

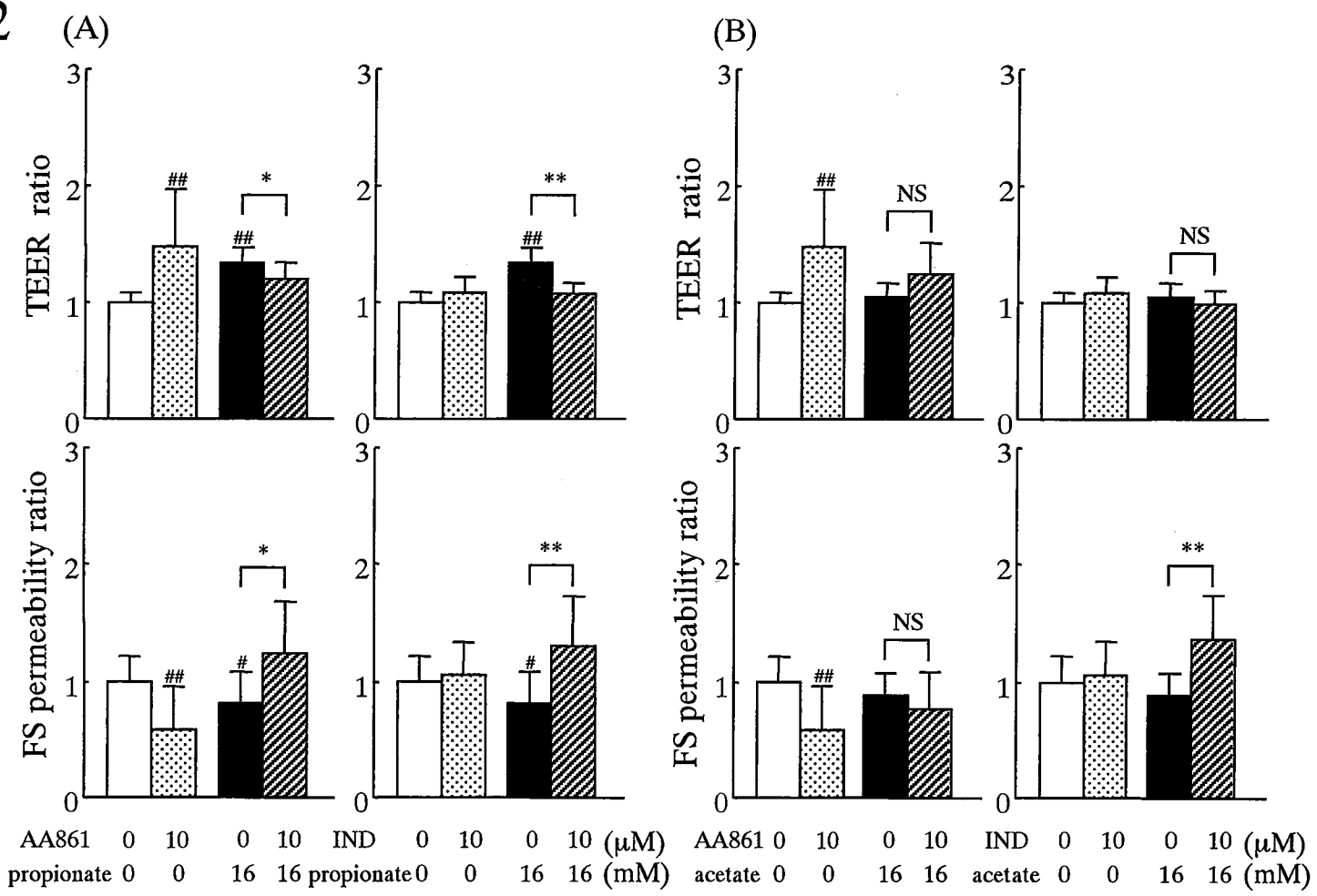

(A)
(B)
TEER ratio
FS permeability ratio
0
1
2
3
##
#
*
**
NS
AA861 0 10 0 10 IND 0 10 0 10 (μM)
propionate 0 0 16 16 propionate 0 0 16 16 (mM)
AA861 0 10 0 10 IND 0 10 0 10 (μM)
acetate 0 0 16 16 acetate 0 0 16 16 (mM)

Fig. 3

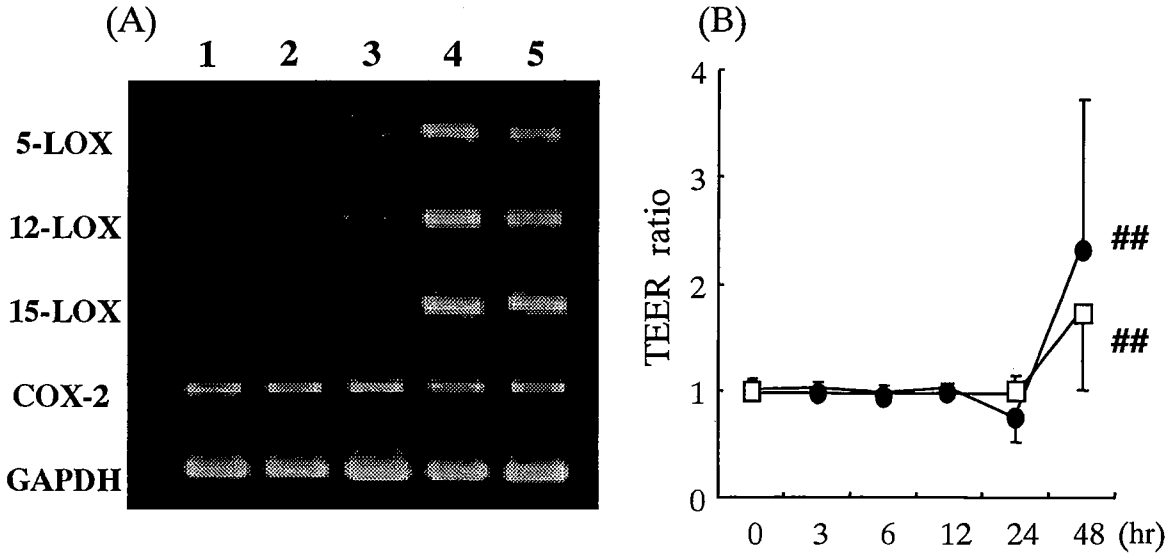

(A)
1
2
3
4
5
5-LOX
12-LOX
15-LOX
COX-2
GAPDH
(B)
TEER ratio
0
1
2
3
4
##
##
0
3
6
12
24
48
(hr)

Fig. 4

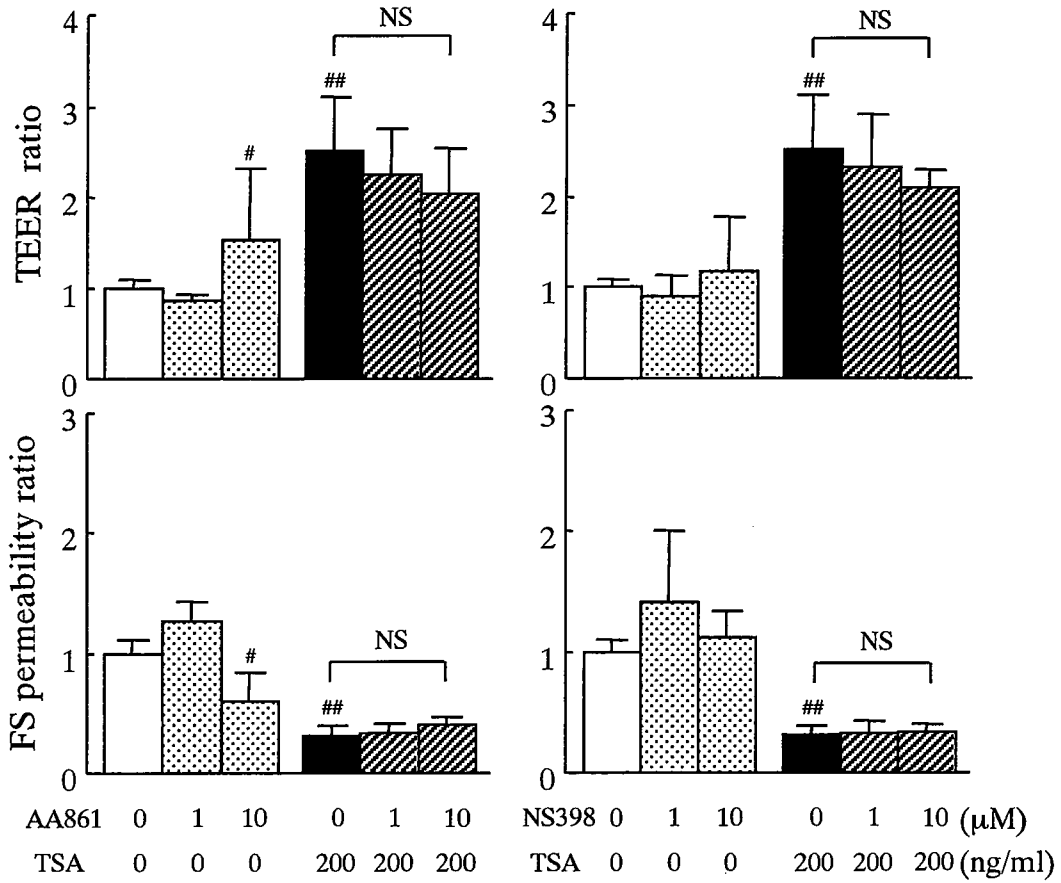
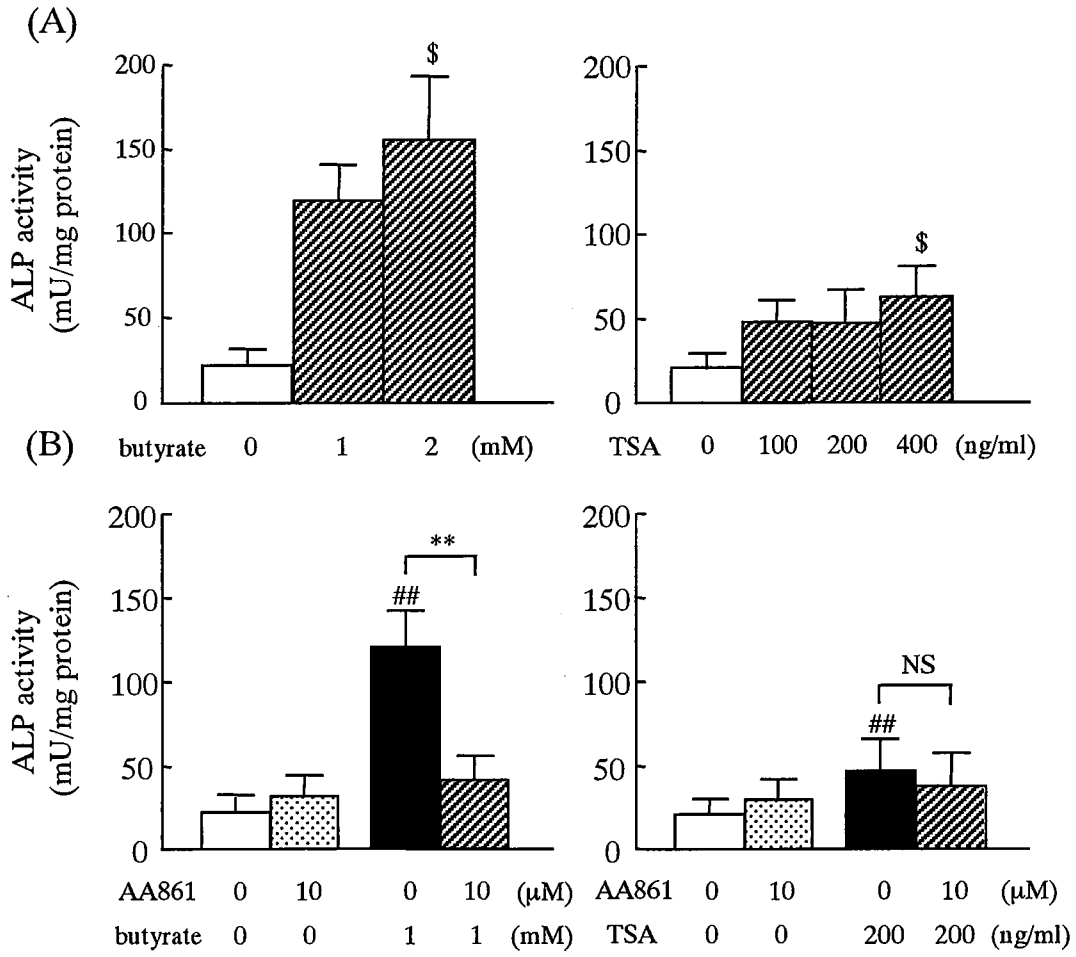

TEER ratio
FS permeability ratio
NS
##
#
AA861 0 1 10 0 1 10
TSA 0 0 0 200 200 200
NS398 0 1 10 0 1 10 (μM)
TSA 0 0 0 200 200 200 (ng/ml)

Fig. 5


(A)
ALP activity (mU/mg protein)
200
150
100
50
0
$
butyrate 0 1 2 (mM)
TSA 0 100 200 400 (ng/ml)
(B)
**
##
NS
AA861 0 10 0 10 (μM)
butyrate 0 0 1 1 (mM)
TSA 0 0 200 200 (ng/ml)