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ORIGINAL ARTICLE

Effect of eicosapentaenoic acid (EPA) on tight junction permeability in intestinal monolayer cells

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Abstract—The purpose of this study is to evaluate the effect of C18 and C20 long chain fatty acids on tight junction permeability in a model of intestinal epithelium. *Methods:* Confluent Caco-2 cells on porous filters with double chamber system were used to measure fluorescein sulfonic acid (FS) permeability and transepithelial electrical resistance (TEER). Lactate dehydrogenase release and ultrastructure were evaluated. Effect of 200 μM eicosapentaenoic acid (EPA, C20:5 n-3), arachidonic acid (AA, C20:4 n-6), α-linoleic acid (ALA, C18:3 n-3), linoleic acid (LA, C18:2 n-6), or oleic acid (OA, C18:1 n-9) enrichment in the culture medium during 24 hours were compared. The effect of the cyclooxygenase inhibitor, indomethacin, lipoxygenase inhibitors, NDGA or AA861, and antioxidant, BHT, was evaluated as a mechanism to change tight junction permeability. *Results:* Caco-2 cells formed polarized columner epithelial cells with densely packed microvilli and well developed junctional complexes. Addition of EPA enhanced FS permeability to 3.0 ± 1.6-fold and lowered TEER to 0.59 ± 1.2-fold vs. control with concentration dependency without cell injury ($P < 0.01$–0.05). OA, AA or LA did not change, but ALA enhanced tight junction permeability. Indomethacin and AA861 normalized the changes mediated by EPA. *Conclusions:* EPA affects tight junction permeability in intestinal monolayer cells specifically and concentration dependently via cyclooxygenase and lipoxygenase products. © 2001 Harcourt Publishers Ltd.

Key words: tight junction permeability; Caco-2 cell; eicosapentanoic acid; polyunsaturated fatty acids; indomethaein; AA861; fluorescein sulfonic acid

Introduction

Dietary lipids are essential components of every living cell, being especially important for the integrity of bilipid structures of cell membranes (1). They are also important sources of energy and are precursors for numerous biologically active compounds. Humans can synthesize all of the lipids necessary for good health with the exception of those belonging to the n-3 and n-6 families of long-chain fatty acids. The amount and type of long-chain fatty acids consumed in the diet can profoundly influence biological responses. In general, eicosanoids, short-lived derived mediators, formed from the n-3 fatty acids are much less potent in causing biological responses than those formed from the n-6 fatty acids, including stimulation of cytokine production and inflammatory responses.

The influence of the n-3 fatty acids on inflammatory bowel disease indicated 50% reduction in steroid use and improved histology (2), delayed relapse (3), reduced disease activity and drugs usage (4). Also, the influence of the n-3 fatty acids on patients under surgical stress indicated reduced wound and major infections, reduced intra-abdominal abscess and multiple organ failure, and shortened hospital stay in several prospective randomized studies in burn, trauma and major surgery patients. However, the effect of the n-3 fatty acids on intestinal permeability is rarely reported (5). Shimizu's report indicates that permeation of glycerol is lower in the n-3 rich fat emulsion group which is administered intraperitoneally to rats, not intravenously nor enterally.

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. This characteristic of the intestinal mucosa, which has been referred to as selective permeability, appears to be mediated by the tight junctions ('zonula occludens') surrounding each cell in the epithelial sheet. It is well established that gastrointestinal epithelial permeability can be modulated by a number of factors, including tissue pH, adenosine 3′,5′-cyclic monophosphate, insulin, insulin-like growth factors, activators of protein kinase C, nitric oxide, and cytokines (6). Each of these factors or their combination are final changes of intestinal epithelial cells produced under various diseases or pathological conditions in vivo, i.e., infection, obstruction, circulatory failure, inflammation, or severe illness including sepsis and multiple organ failures. If polyunsaturated fatty acids influence tight junction permeability, administration of polyunsaturated fatty acids could be an effective tool to regulate tight junction permeability changes caused by these disease conditions. Concerning

the effect of fatty acids on tight junction permeability, already, the effect of γ-linolenic acid (GLA) and eicosapentanoic acid (EPA) on tight junction permeability in the human vascular endothelial cell line is reported by Jiang et al. (7). It indicates that GLA and EPA upregulate but linoleic acid (LA) and arachidonic acid (AA) downregulate occludin expression. Lindmark et al. report the absorption enhancement effect of capric acid (C10), medium chain fatty acid, through intracellular regulation of tight junction permeability in Caco-2 cell (8). However, the effect of polyunsaturated fatty acids on enterocyte tight junction permeability has not been reported.

The purpose of this study is to evaluate the direct effect of long-chain fatty acids in the medium on enterocyte tight junction permeability. In this experiment, C18 and C20 fatty acids were selected, because C18 fatty acids are dietary source of n-3 or n-6 series of polyunsaturated fatty acids and arachidonic acid (n-6) of C20 fatty acid is the most important of eicosanoid precursors. EPA can be metabolized by cyclooxygenase (COX) to prostaglandin (PG) G_3, which is rapidly metabolized to PGH_3 by PG endoperoxide synthetase and then metabolized to PGD_3, PGE_3, $PGF_3\alpha$, PGI_3, or thormboxan. Alternatively, EPA may be metabolized by lipoxygenase (LOX) in the leukotriene (LT) pathway to 5-hydroperoxyeicosatetraenoic acid (HPETE), which is rapidly metabolized to 5-hydroxyeicosatetraenoic acid (HETE), or LTs, such as LTA_5, LTB_5, LTC_5, LTD_5. and LTE_5 (9). The ability of EPA to introduce tight junction permeability change in Caco-2 monolayer cells may be due to the generation of the reactive eicosanoid products, which may interfere the tight junction permeability. In this experiment, to examine whether the tight junction permeability change induced by EPA is due to eicosanoids, two key enzymes in the generation of eicosanoids, LOX and COX, is inhibited with their inhibitors, nordihydroguaiaretic acid (NGDA), 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-p-benzoquinone (AA861), and indomethacin, respectively. Also, to evaluate the effect of generated peroxidation products from EPA, the effect of antioxidant, butylated hydroxytoluene (BHT), in combination with EPA was investigated.

To address this issue, we used the Caco-2 cell line grown on permeable supports as an experimental model to determine paracellular permeability. Caco-2 cells, derived from transformed human colonic carcinoma cells, have been characterized extensively (10). They have been shown to be highly polarized with a well-formed brush border, express several differentiated markers typical of adult enterocytes and behave like small intestine (7). The Caco-2 cell line has been used previously for studying intestinal permeability in vitro by Menconi (6) and Salzman (11). Kim et al.'s report indicates that permeability values determined in the Caco-2 cell culture model may be a good predictor of the intestinal permeability of peptides in comparison between Caco-2 monolayer and in situ perfusion rat ileum model (12).

Materials and Methods

Caco-2 cells (Nihon Seiyaku Co., Japan) were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum (FCS), 1% L-glutamine, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and sodium bicarbonate (24 mM), in an atmosphere of 5% CO_2 and 100% humidity. All culture media were obtained from Dainippon Seiyaku Co., Japan. Growth medium was replaced twice a week and cell viability was assessed using 0.4% trypan blue solution. Confluent monolayers growing in 75 cm^2 tissue culture flasks were harvested with a solution of 0.25% trypsin-2.65 mM EDTA in PBS (Gibco) and seeded at a density of 6.25×10^5 cells cm^2 in Transwell inserts (Costar), 12 mm in diameter (surface area 1.0 cm^2), and polycarbonate filters with 3.0 μm pore size (Fig. 1).

Transepithelial electrical resistance (TEER) were examined to evaluate structure integrity of the Caco-2 cell monolayer on day 4 postseeding with an electrical resistance system (EVOM-6; World Precision Instruments), equipped with a pair of STX-2 electrode. Probes were placed at the apical and basolateral chamber of three points of the insert and resistance was measured with the voltohmmeter. The relationship of TEER values to Caco-2 monolayer integrity has been investigated by Hidalgo and found to correlate with the presence of well-formed tight junctions (13). Fluid resistance, 122 ± 9 ohms · cm^2, was subtracted to calculate net TEER. The Caco-2 cells were used in experi-

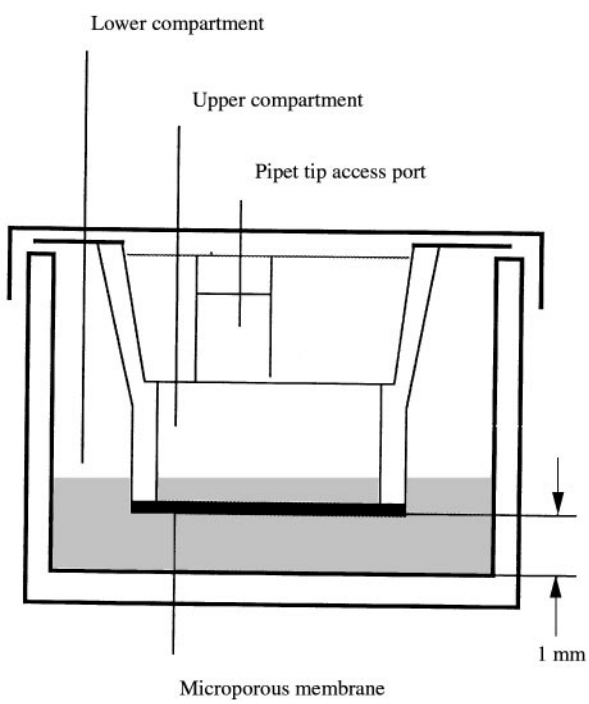

Fig. 1 Transwell bicameral cell culture system.

ments only after the net TEER had risen above 130 ohms · cm^2 (14). Because the Transwell membranes are opaque, it is impossible to evaluate the monolayers directly with phase-contrast microscopy. Caco-2 cells monolayer were used between day 4 and day 10 postseeding.

Sodium salt of eicosapentaenoic acid (EPA, C20:5 n-3), arachidonic acid (AA, C20:4 n-6), α-linolenic acid (ALA, C18:3 n-3), linoleic acid (LA, C18:2 n-6), and oleic acid (OA, C18:1 n-9) were obtained from Sigma. Fatty acids were dissolved in PBS(−) and stored at −30°C. They were added to apical and basal chamber during 24 h after dilution in the medium without FCS. The medium without FCS was selected to make precise fatty acids concentration in the medium without addition of fatty acids containing in the FCS following Lindmark et al.'s study (8) and to keep better integrity of tight junction following our preliminary experimental result. It is indicated that tight junction integrity was decreased with 10% FCS to 10 fold greater fluorescein sulphonic acid permeability (mentioned later), than with 0% FCS.

The concentration of long-chain fatty acids in the medium was indicated in the results. The concentration selected in this experiment followed Jiang et al.'s (7), Chen et al.'s (15) 100 μM, de Jonge et al.'s (16) 244 μM, and Finstad et al.'s (17) 244 μM in vitro studies. Differences among C18 of n-9, n-6, and n-3 unsaturated long chain fatty acids and differences between C20 of n-6 and n-3 unsaturated long chain fatty acids were compared, respectively.

BHT, IND, NDGA, and AA861 were all purchased from Sigma. They were dissolved in ethanol and stocked at −30°C. They were diluted by the medium and added to the Caco-2 cells with the concentration indicated in the results 30 min. before EPA administration. Final concentration of ethanol was 0.1%. The dose chosen were derived from Korn et al.'s (18). Papadogiannakis et al.'s (19), Mastrocola et al.'s (20), and Finstad et al.'s studies (17). For each drug studies, control experiments consisted of administration of the drug solvent (the medium plus 0.1% ethanol) were performed.

Five hundred μg/ml of fluorescein sulphonic acid (FS, Molecular Probes) dissolved in the medium without FCS were loaded 200 μl into the apical compartment of the Transwell chambers during 3 h after removal of 200 μl of culture medium. The concentrations of the FS in the apical and basolateral compartments were assayed after dilution in PBS for fluorescence using a fluorescence spectrophotometer (RF 540, Shimazu, Japan) at an excitation wave length of 492 nm (slit width 2 nm) and an emission wave length of 515 nm (slit width 10 nm) (6). The FS permeability of monolayers was expressed as percent ratio of concentration in the basolateral chambers versus concentration in the apical chamber. FS has small molecular weight, 478, but is not permeable of the cellular membrane in physiological pH due to its lipophobicity. FS passes through tight junction space and used as the paracellular permeability marker (6, 11).

To assess cell membrane integrity, release of the cytosolic enzyme lactate dehydrogenase (LDH) was measured from Caco-2 cells grown on 12-mm Transwell membranes. LDH activity in media samples from the basolateral compartments was determined spectrophotometrically using a single reagent system (LDH-Cytotoxic Test, Wako) on a ELISA reader (Benchmark Microplate Reader, Biorad). Medium LDH levels were expressed as percent levels in control wells. Total cellular LDH in control wells was measured after solubilizing the monolayer with 1.0 ml of 0.1% Triton X-100 in PBS followed by centrifugation and assaying the supernatant.

Caco-2 cell monolayers on Transwell membranes were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 4 h at room temperature for the ultrastructural study by electron microscopy. Specimens were rinsed four times in 10% glucose in 0.1 M sodium cacodylate buffer and postfixed in 0.5% OsO_4 in 0.05 M sodium cacodylate buffer. After dehydration through a graded series of ethanol (35%, 50%, 70%, 96%, and 4 times 100%, 10 min each), intact membranes were embedded in resin (6.3 ml of Epon 812, 3.7 ml of Araldite 502, 13.9 ml of DDSA, 0.3 ml of DMP-30, Nissin EM) by first infiltration in a 1 : 1 mixture of ethanol and resin for 1 h, a 100% resin mixture for one day. Resin was polymerized at 60°C for 2 days. Ultrathin sections cut with a diamond knife were negatively stained with uranyl acetate lead citrate and examined under a transmission electron mircroscope operated at 80 kV (JEM-1220, JEOL) followed Panigrahi et al. (21).

Statistical analysis

Data were expressed as mean ± standard deviation (S.D.). One-way or two-way factorial analysis of variance (ANOVA) using the Statview statistical software package (version 4.51.1; Abacus Concepts, Berkeley, USA), followed by the Scheffe's multiple comparison test was used to identify significant differences among multiple samples. Student's *t*-test was employed to compare mean value from two groups. The statistically significance was assured when P value was less than 0.05.

Results

As shown in Figure 2a and 3a, Caco-2 cells grown on permeable supports in bicameral chambers under control condition formed polarized monolayers of columner epithelial cells. Cells showed typical brush border microvilli on the apical surface, tight junctions at

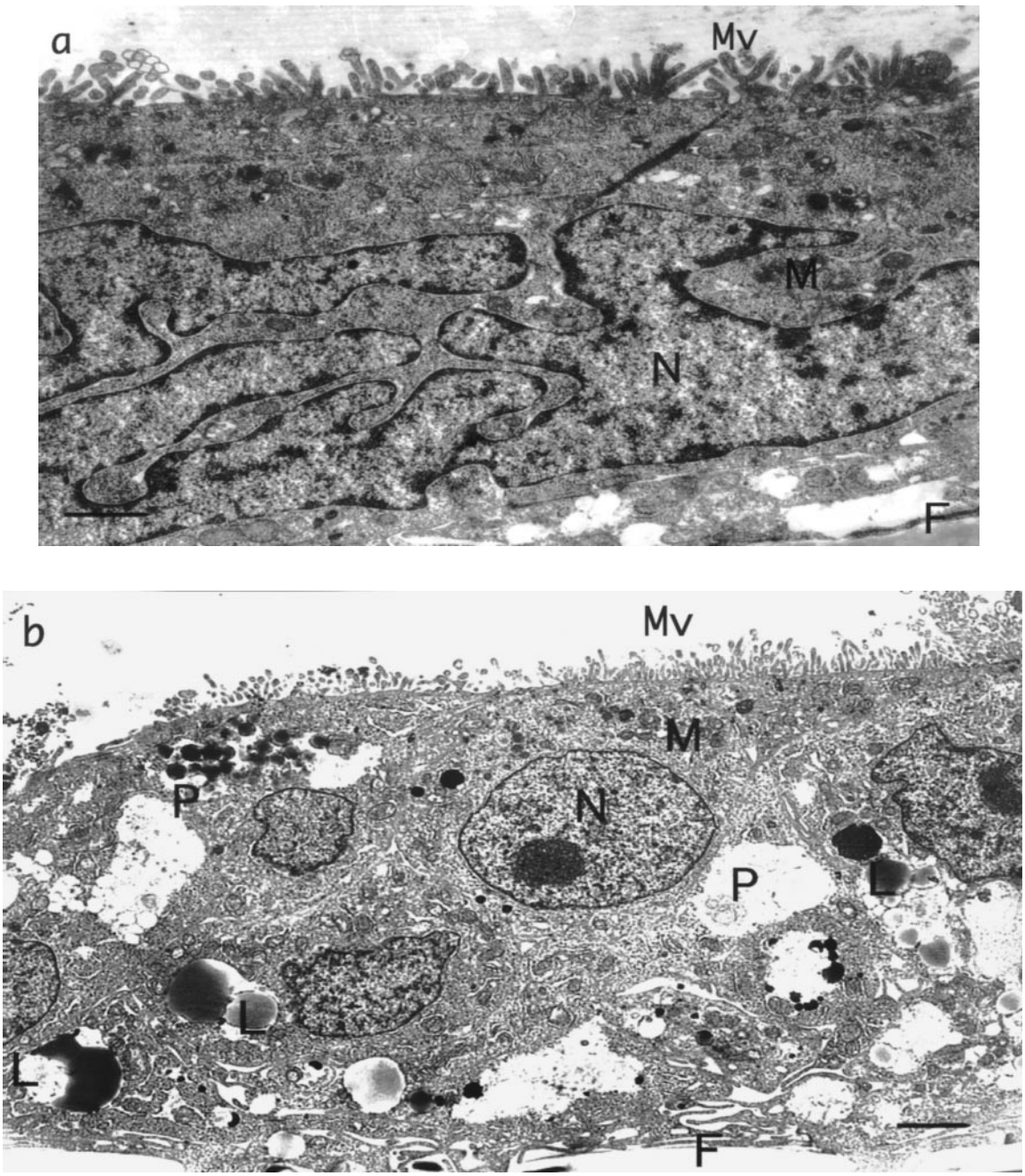

Fig. 2 Transmission electron micrograph of the Caco-2 cell monolayer incubated for 24 h in the medium with 0 (control) or 200 μM EPA on 7–10 days postseeding. In control cells (a), cells grown on Transwell filters (F) formed polarized monolayers of columnar epithelial cells. Cells had long, densely packed microvilli (Mv) and well-developed cell to cell junctional complexes (see Fig 3). Mitochondria (M) were prominent in both above and below the nucleus (N). After a 24 h incubation with EPA (b), no overall changes in the polarity of epithelial cells or structure of nucleus. Phagosomes (P), lysosomes (L), and fat droplets were prominent. Microvilli appeared same as the control. Scale bar, lower left in a is 1 m and lower right in b is 2 μm.

the lateral apical surface, and many desmosomes localized among the length of the interdigitating lateral membranes (Fig. 3) same as Menconi et al. indicated (6). Caco-2 cells contained lipid droplets in the basal part of their cell bodies.

The Caco-2 monolayers in the typical untreated (control) group showed $370 \pm 142\ \text{ohm} \cdot \text{cm}^2$ of TEER levels on the fourth-to-tenth day old cells indicating enough integrity of this monolayer system as Cruz et al. study (14). The permeability to FS was low with only $0.6 \pm 0.7\%$ of the small molecular dye crossing the monolayer during the 3 h test period. FS clearances were 492 ± 142 nanoliters/cm^2/h in accordance with Menconi's report (6). The activity of LDH, a cytosolic enzyme released when cells are injured, was $4.5 \pm 1.6\%$ of total cellular LDH. Correlation between TEER and FS permeability, and correlation between TEER and LDH levels indicated negative relationship with statistical significance in the preliminary time course study (data not shown).

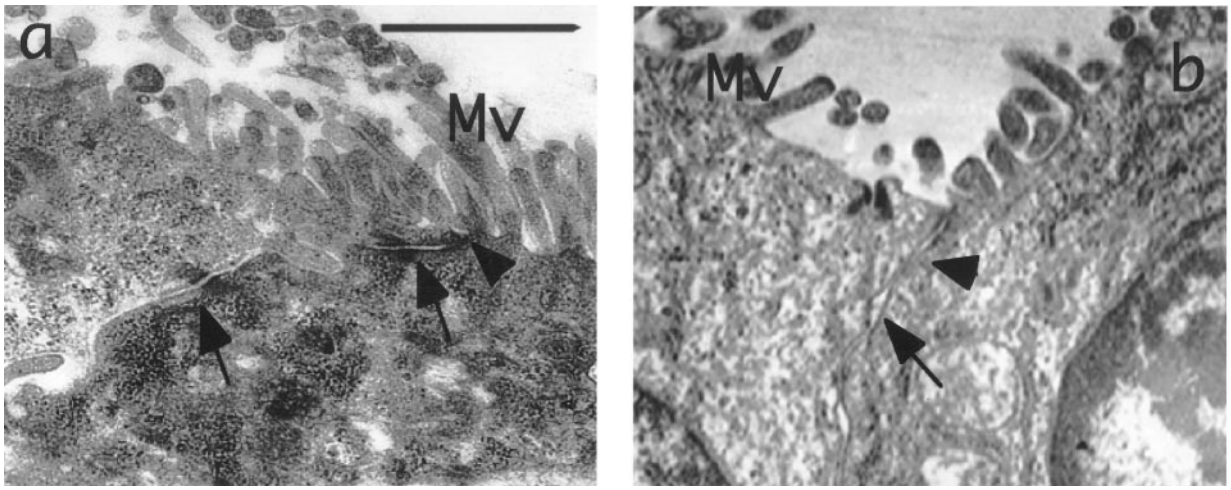

Fig. 3 Transmission electron micrographs showing microvilli (Mv) and junctional complex in apical region of control and EPA-treated Caco-2 cells. In control cells (a), the tight junction (arrowhead) and desmosome (arrow) are intact. In cells exposed to 200 μM of EPA for 24 h (b), the tight junction (arrow head) and desmosome (arrow) suggested to contain less electron-dense material. Scale Bar (a, upper right) = 1 μm.

We tested the effect of 200 μM C18 unsaturated fatty acids, OA, LA, and ALA, during 24 h on the TEER, FS permeability, and LDH release. OA did not change TEER or FS permeability shown in Table 1. TEER ratio in the LA group was lower than that in the control group ($P<0.01$ vs control). ALA lowered TEER more than LA ($P<0.01$) and enhanced FS permeability ($P<0.01$ vs control). ALA enhanced and LA slightly enhanced tight junction permeability. Both ALA and LA increased LDH activity ($P<0.05$–0.01 vs control), but the increased level was under 10% of the total LDH (Table 2).

Then, the effect of C20 unsaturated fatty acids on tight junction permeability was evaluated between the 50 and 200 μM range (Table 2, 3, 4). Addition of 100 or 200 μM of AA lowered TEER ($P<0.05$–0.01 vs control) but did not change FS permeability. Addition of 50 μM of AA lowered FS permeability without a change in TEER. In comparison with AA, concentration dependency of EPA on tight junction permeability clearly indicated reduction in TEER with enhancement in FS permeability in Tables 3 and 4 ($P<0.0005$). Fifty μM of EPA reduced TEER ($P<0.01$ vs control) and enhanced FS permeability. Those effects were prominent in the higher concentration of EPA. LDH level with addition of 200 μM of EPA was 1.25-fold of the control level, but it was under 9% of total LDH.

Ultrastructure of Caco-2 cells in the EPA group is indicated in Figures 2b and 3b. Mitochondria were prominent and there were no overall changes in the polarity of epithelial cells or structure of nucleus. Phagosomes, lysosomes, and lipid droplets were prominent. Some of these lysosomes were filled with an electron-opaque or electron-dense material of more or less homogeneous appearance. The tight junction and desmosomes appeared to contain less electron-dense material. Glycogen was not prominent. Few cells indicated apoptosis with apoptotic bodies with condensed nuclei peripherally.

Table 1 Effects of 200 μM of OA, LA, and ALA on Tight Junction Permeability

Parameters	OA	LA	ALA
TEER	102.4 ± 7.2	71.9 ± 3.7[ab]	58.0 ± 10.5[abd]
FS Permeability	86.1 ± 36.1	180.5 ± 164.5[c]	259.9 ± 127.6[ab]

TEER, FS permeability values are normalized to those of untreated (control) cells (100 ± 4%, 100 ± 11.4%, respectively). Values are mean ± S.D. ($n=10\sim12$). $P<0.01$ for TEER and FS permeability with one-way factorial ANOVA.
[a] $P<0.01$ vs control.
[b] $P<0.01$, [c] $P\pm0.05$ vs OA.
[d] $P<0.01$ vs LA.

Table 2 Effects of 200 μM of C18 and C20 Long Chain Fatty Acids on LDH Activity in the Medium

LCFA	C18	LDH activity	C20	LDH activity
$n-9$	OA	109.9 ± 9.5	—	—
$n-6$	LA	151.1 ± 38.4[a]	AA	169.1 ± 87.1[a]
$n-3$	ALA	182.4 ± 68.1[bc]	EPA	125.9 ± 20.8[a]

LDH activity values are normalized to those of untreated (control) cells (100 ± 6%). Values are mean ± S.D. ($n=10\sim14$). $P<0.01$ for C18 and C20 fatty acids with one-way factorial ANOVA.
[a] $P<0.05$, [b] $P<0.0$ vs control.
[c] $P<0.01$ vs OA.

Addition of antioxidant, 10 μM of BHT, did not change TEER or FS permeability of itself nor influenced EPA effect on TEER or FS permeability in Figure 4. Indomethacin, a COX inhibitor, reduced TEER, 81.3 ± 16.4% at 10 μM, ($P<0.01$ vs control) and enhanced FS permeability to 174.0 ± 270.8%. Addition of 10 μM of indomethacin with 200 μM of EPA normalized the enhanced FS permeability by EPA, 107.5 ± 85.2% versus 237.2 ± 159.0% in the EPA group, ($P<0.05$),

however, did not change the reduced TEER by EPA. Ten μM of NDGA, a LOX inhibitor, remarkably reduced TEER, 30.0±3.8%, and enhanced FS ratio, 2862.6±600.8%, ($P<10^{-7}$, Fig. 5). This effect of NDGA did not influence the EPA effect on TEER or FS permeability. Ten μM of AA861, also a LOX inhibitor, lowered TEER ($P<0.01$ vs control) and enhanced FS permeability. Ten μM of AA861 normalized the enhancement of FS permeability, 476.3± 727.8% by the addition of 200 μM of EPA, to 91.1± 43.8% (not significant). In this set of AA861 experiment, measured FS permeability values were used due to the large S.D. of normalized data in the EPA group.

Table 3 Effects of AA and EPA on TEER

Concentration (μM)	AA	EPA
50	92.9±11.2	84.4±6.6
100	88.5±15.0	81.5±13.8
200	87.2±16.7	69.1±13.6

TEER values are normalized to those of untreated (control) cells (100±13%). Values are mean±S.D. ($n=9\sim18$). $P<0.0005$ between AA and EPA and $P<0.05$ among concentrations with two-way factorial ANOVA without interaction.

Table 4 Effects of AA and EPA on FS Permeability

Concentration (μM)	AA	EPA
50	48.8±46.2	116.8±82.2
100	110.1±62.3	178.5±135.6
200	90.5±67.0	255.2±194.1

FS permeability values are normalized to those of untreated (control) cells (100±51%). Values are mean±S.D. ($n=9\sim18$). $P<0.0005$ between AA and EPA and $P<0.05$ among concentrations with two-way factorial ANOVA without interaction.

Discussion

Tight junctions between adjacent epithelial cells create a physiological intercellular barrier which maintains distinct tissue spaces and separate the apical from the lateral plasma membranes (7). Tight junctions are important structures in controlling the diffusion of molecules, such as toxic compounds and drugs, through the intestine.

This study is the first report to demonstrate an effect of C18 and C20 long chain fatty acids on tight junction function in a model of intestinal mucosa. Only EPA of the n-3 series C20 essential fatty acids clearly exerted an effect on the tight junctions by changing paracellular permeability measured using TEER and permeability of lipophobic small molecules. The reason that ALA, C18 of n-3 series ployunsaturated fatty acid, also shows the same effect of EPA is explained by desaturation and elongation of ALA. EPA is formed from ALA in a short time, during 4 h, in Caco-2 cells (15). Caco-2 metabolizes

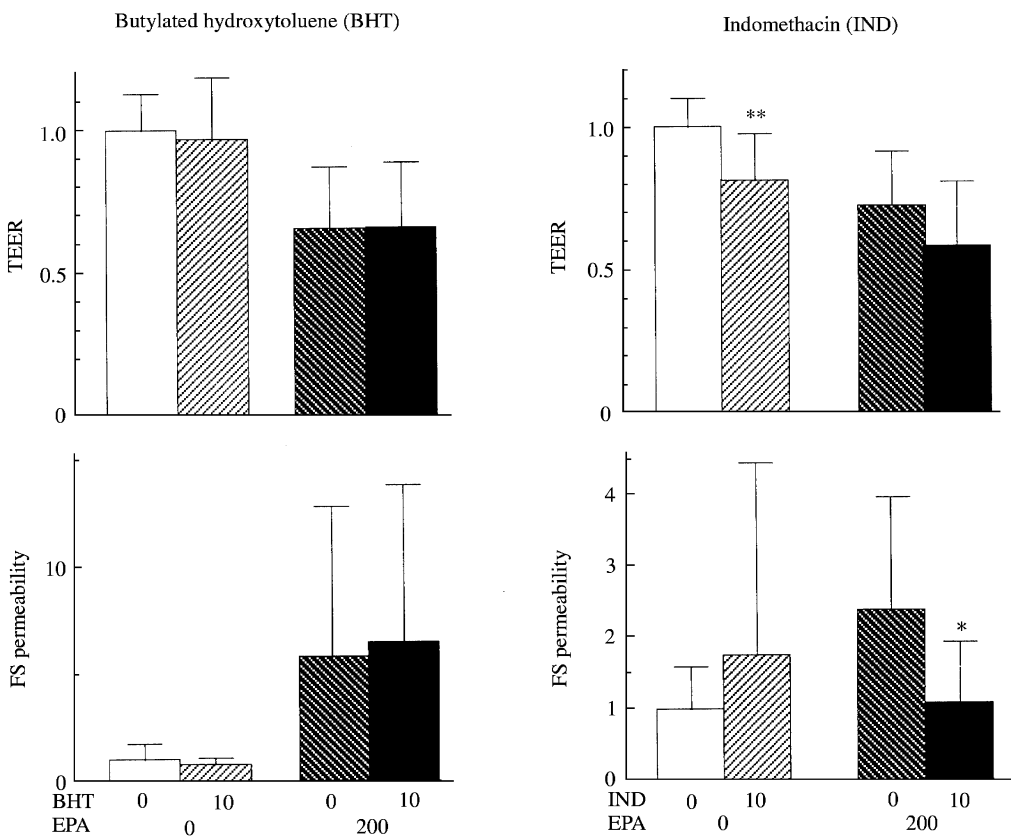

Fig. 4 Effect of antioxidant, 10 μM of butylated hydroxytoluene (BHT), or cyclooxygenase inhibitor, 10 μM of indomethacin (IND), with 200 μM of eicosapentaenoic acid (EPA) on transepithelial electrical resistance (TEER), fluorescein sulfonic acid (FS) permeability in Caco-2 monolayer cells. Those values are normalized to those of untreated (control) cells. mean±S.D., $n=8$–18. *; $P<0.05$, **; $P<0.01$ vs 0 μM of inhibitor.

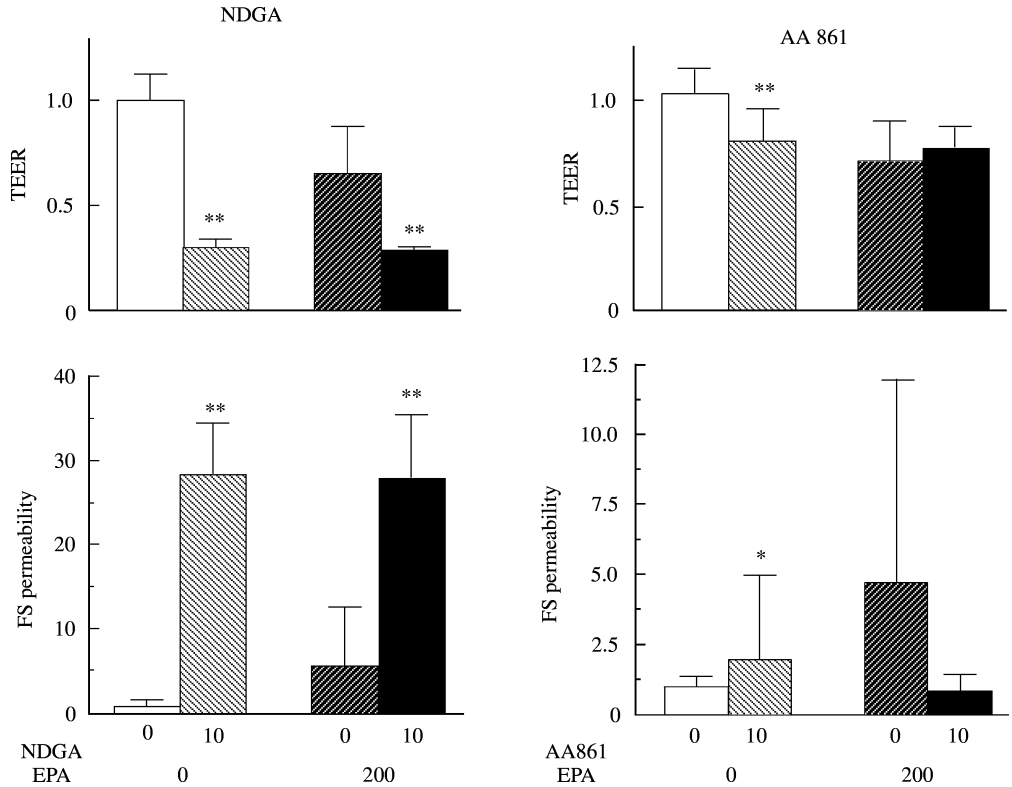

Fig. 5 Effect of lipoxygenase inhibitors, 10 μM of nordihydroguaiaretic acid (NGDA) or 10 μM of 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-p-benzoquinone (AA861), with 200 μM of eicosapentaenoic acid (EPA) on transepithelial electrical resistance (TEER), fluorescein sulfonic acid (FS) permeability in Caco-2 monolayer cells. Those values are normalized to those of untreated (control) cells. mean ± S.D., $n = 8$–18. *; $P < 0.05$, **; $P < 0.01$ vs 0 μM of inhibitor.

large amount of ALA to EPA but not to DHA. Because, the conversion of 22 : 5 to 22 : 6 (DHA) requiring the action of either Δ^4 desaturase or by alternative pathways does not occur in the Caco-2 cell line (15). Explanation of the slight but significant effect of LA, C18 of n-6, is that Caco-2 metabolizes small amount of LA to GLA (C18 : 3 n-6). Recently, we have been accumulating the result that GLA also shows a positive effect on the tight junction permeability in the series of experiment using Caco-2 monolayer cells (data not shown).

Results of ultrastructural analysis, in accordance with the slight increase of LDH release in the EPA group, reveal that this change in tight junction permeability is due to functional changes with intact nucleus, organelles, cell polarity, and microvilli and is not originated from cytotoxicity. The observation of few apoptotic cells in the EPA group is in accordance with Chiu et al.'s report showing induction of apoptosis in human leukemic HL-60 cells by 40 μM of EPA during 24 h (22). Quantitative analysis will be required to evaluate apoptosis in Caco-2 cells in a future study. Increased lysosomes and lipid vacuoles in the EPA group is in accordance with Darimont et al.'s ultrastructural analysis with addition of 100 μM palmitic acid in Caco-2 cells (23). Differentiated Caco-2 cells possess large amounts of liver type fatty acid-binding protein in the cytosol and metabolize long chain fatty acids.

Changes of fatty acid composition in the membrane phospholipid in Caco-2 cells under enrichment of media with various fatty acids are reported by Dias et al. (24). They indicate that AA in the cell membrane of 7 days culture with 100 μM EPA supplement is 0.67-fold and 1.03-fold, and EPA in the same fraction is 51-fold and 60-fold of the control culture, respectively in phosphatydylethanolamine and phosphatydylcholine membrane phospholipid composition. Following their results, the difference in membrane phospholipid composition in our experiment is considered to come mainly from high level of EPA and is not due to low level of AA.

As for the mechanism of EPA on tight junction permeability, peroxide and/or eicosanoid production from EPA is postulated.

The oxidant injury to the epithelium has been implicated in the pathogenesis of various gastrointestinal disorders and the direct cytotoxic effect of n-3 fatty acid hydroperoxides on Caco-2 cells is reported by Cepinskas et al. (25). They show that cytotoxicity in Caco-2 cells is observed with addition of over 200 μM of peroxidized fish oil generated by air oxidation of MaxEPA fish oil for 5 days. Our results showing that the oxide did not change tight junction permeability indicated by the negative result in coadministration of BHT, antioxidant, with the EPA solution of well

controlled condition are in accordance with Cepinskas's control date.

Polyunsaturated fatty acids are incorporated into cellular phospholipids and greatly influence eicosanoid production, membrane fluidity, the formation of receptors, binding of ligands to their receptors, and the activation of intracellular signaling pathway (1). AA is the most important eicosanoid precursors producing prostaglandins, thromboxans, and leukotriens. AA shows usually 10- to 100-fold more biological potency for inducing cell response compared to n-3 series fatty acids. However, high concentration of AA did not affect tight junction permeability in this study. It is suggested from the reduction of FS permeability by 50 μM AA without TEER change that AA has the opposite effect from EPA. AA in lower concentration may reduce paracellular permeability in spite of less effectiveness than EPA. Further studies in lower concentration range of AA will be required.

The result that the enhanced tight junction permeability by EPA was reversed by AA861, an inhibitor of the LOX, suggests that participation of 5 series of LTs formed from EPA on tight junction permeability in the intestinal monolayer. But, the effect of 5 series of LTs on intestinal monolayer or intestinal permeability has not been reported. Concerning LTB_4, Caco-2 cells produce LTB_4 with addition of OA, LA, and AA during 7 days culture reported by Dias et al. (26) and with addition of trinitrobenzene sulfonic acid reported by Stratton et al. (27). However, other researchers, Cortese et al., report that Caco-2 cells are devoid of the 5-LOX messenger RNA (28). And also, AA supplementation showed slight effect in our experiment. Participation of LTB_4 or PGE_2 on tight junction permeability in intestinal monolayer cells is considered to have lower possibility, even though Mion et al. report the effect of LTB_4 and LTD_4 on intestinal permeability in the rat vascular perfusion model indicating intestinal permeability changes as a result of an intensive vasocongestion of the mucosal capillaries with LTs (29). From these contradictory reports, participation of LTs in this biological response should be analyzed in a future study.

Caco-2 cells normally express barely detectable levels of COX-1 and COX-2 mRNA and protein following Tsujii's report (30). Even induced COX-2 is not resistant to indomethacin in Caco-2 cells reported by Stratton et al. (28). Our results indicating partial participation of indomethacin on the EPA effect on tight junction permeability with positive result in FS permeability and negative result in TEER and a slight effect of AA are in accordance with their reports.

The result that both indomethacin and AA861, independent of EPA, lower TEER and enhance FS permeability suggests their direct effect on tight junction permeability in intestinal monolayer culture or the effect of eicosanoids produced from fatty acids of control cells. In vivo experiment by Mion et al. indicates that intravascular perfusion of higher concentration of indomethacin, 1.25 to 2.5 mM, increases intestinal permeability with increased inflammational changes in histology, but the direct effect of COX inhibitor or LOX inhibitor on tight junction permeability has not reported (27). The most prominent effect of NDGA on tight junction permeability in this series of experiments, is not similar to the effect of EPA. The result showing that the increase in FS ratio by NDGA was over 4-fold above EPA and that NDGA was not effective to normalize changes by EPA suggest the difference in the mechanism on tight junction between NDGA and EPA. We are aware that caution is required in the interpretation of NDGA effect. NDGA is widely used LOX inhibitor, but inhibits Ca^{2+} channel currents independently of LOX inhibition at the concentration greater than 10 μM in pituitary cells (18). This NDGA effect is suggested due to the Ca^{2+}-dependent intracellular function of tight junction permeability reported recently in culture cells (8).

Jiang et al. also reported the regulatory, 'to enhance', effect of EPA or GLA on tight junction function in endothelial cell culture (7). However, their results are contradictory. Effects of various factors affecting tight junction permeability in monolayer cells vary with different experimental settings and cell types shown Lindmark (8). In fact, we also have preliminary results showing that EPA lowered FS permeability with no change of TEER in the different experimental setting using the media with 10% FCS and enhanced tight junction integrity described in the material and method section. Future in vivo studies are required to elucidate this discrepancy. And, in addition to the mechanism by eicosanoids produced from polyunsaturated fatty acids, the effect of protein kinase C activation by polyunsaturated fatty acids would be evaluated using this experimental models, because, polyunsaturated fatty acids are postulated to modify intracellular signaling and several reports have been published indicating protein kinase C activation on tight junction (1, 8).

In conclusion, we have presented evidence that EPA affects tight junction permeability in intestinal monolayer specifically and concentration dependently via COX and LOX products.

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