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C18 and C20 Unsaturated Fatty Acids on Tight Junction Permeability in Caco-2 Monolayer Cells

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The purpose of this study is to evaluate C18 and C20 long chain fatty acids on tight junction permeability in a model of intestinal epithelium. Confluent Caco-2 cells on porous filter with double chamber system were used to measure fluorescein sulfonic acid (FS) permeability and transepithelial electrical resistance (TEER). LDH release and ultrastructure were evaluated. Effect of 200 μM eicosapentanoic acid (EPA, C20:5ω-3), arachidonic acid (AA, C20:4ω-6), eicosatrienoic acid (ETA, C20:4ω-9), α-linolenic acid (ALA, C18:3ω-3), linoleic acid (LA, C18:2ω-6), or oleic acid (OA, C18:1ω-9) treatment in the culture medium during 24 hours were compared. Caco-2 cells formed polarized monolayers of columner epithelial cells with long, densely packed microvilli and well developed junctional complexes. Addition of EPA increased FS permeability to 3.0 ± 1.6 and decreased TEER to 0.59 ± 1.2 fold vs. control with concentration dependency without cell injury ($p < 0.01$-0.05). OA, AA or ETA did not change, but ALA decreased tight junction permeability. EPA affects tight junction permeability in intestinal monolayer specifically and concentration dependently.

Key Words: Tight junction permeability, Caco-2 cell, Eicosapentanoic acid, Polyunsaturated fatty acids.

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 ω-3 and ω-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. Long-chain fatty acids can be divided into three families, depending upon the site of the first double bond (ω-3, ω-6, and ω-9). The ω-3 and ω-6 fatty acids are polyunsaturated (PUFA), whereas most of the ω-9 fatty acids are monounsaturated. The parent 18 carbon atom molecule (C18)

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of each class can undergo elongation and further desaturation (ω-3 and ω-9), but the position of the first double bond remains the same when it undergoes these changes. In general, eicosanoids, short-lived lipid-derived mediators, formed from the ω-3 fatty acids are much less potent in causing biological responses than those formed from the ω-6 fatty acids, including stimulation of cytokine production and inflammatory responses (Fig. 1).

Influence of the ω-3 fatty acids on inflammatory bowel disease indicated 50% reduction in steroid use and improved histology,[2] delay of relapse,[3] reduced disease activity and drugs usage.[4] Also, influence of the ω-3 fatty acids on patients under surgical stress indicated reduced wound and major infections, reduced intraabdominal abscess and MOF, and shortening hospital stay in several prospective, randomized studies in burn, trauma and major surgery patients. However, the effect of the ω-3 fatty acids on intestinal permeability is rarely reported.[5]

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]

The purpose of this study is to evaluate the direct effect of long-chain fatty acids in the medium on enterocyte tight

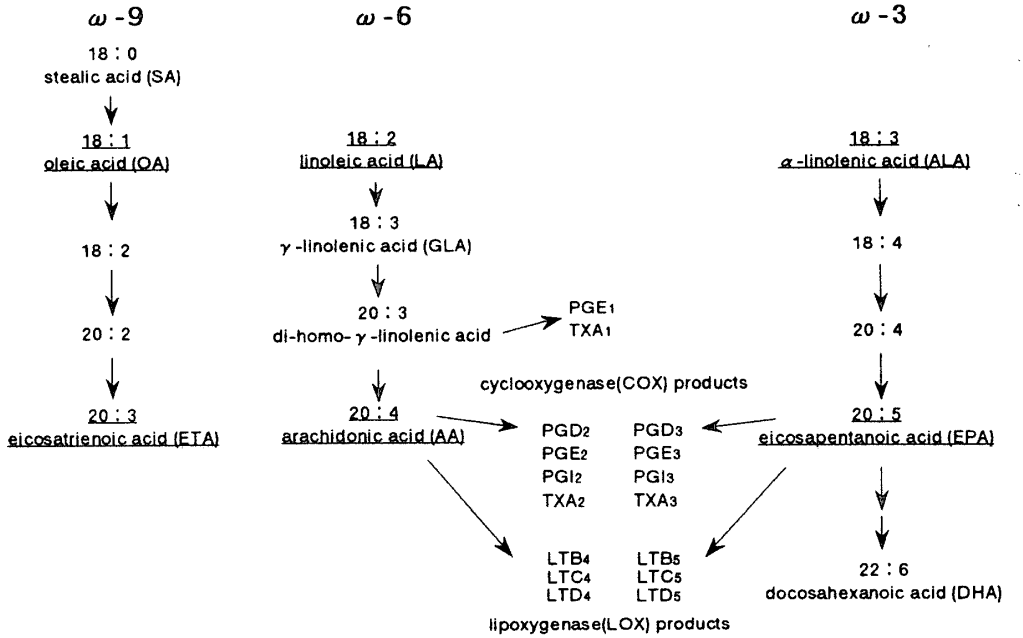

Figure 1. Metabolic map of unsaturated fatty acids.

junction permeability. In this experiment, C18 and C20 fatty acids were selected, because C18 fatty acids are dietary source of ω-3 or ω-6 series of PUFAs and arachidonic acid (ω-6) of C20 fatty acid is the most important of eicosanoid precursors (Fig. 1). 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. 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] Salzmen,[8] and Dodane.[9]

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 placed 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/ml in Transwell inserts (Costar), 12 mm in diameter (surface area 1.0 cm^2), and polycarbonate filters with 3.0 μm pore size (Fig. 2).

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 point of the insert and resistance was measured with the voltohmmeter. The relationship of TEER values to Caco-2 monolayer integrity has been investigated by Hidalgos and found to correlate with the presence of well-formed tight junctions.[10] Fluid resistance, 122 ± 9 ohms·cm^2, was subtracted to calculate net TEER. The Caco-2 cells were used in experiments only after the net TEER had risen above 130 ohms·cm^2.[11] 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

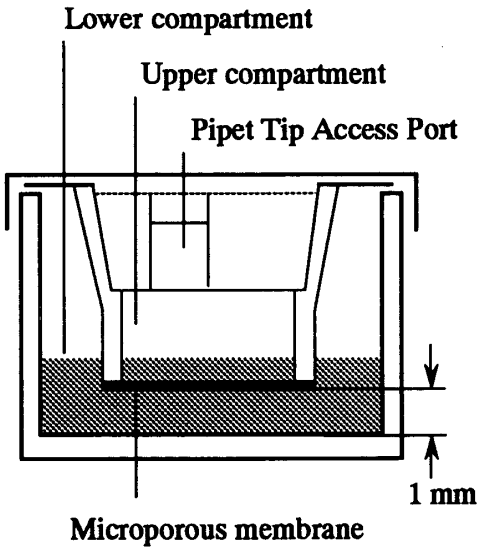

Figure 2. Transwell bicameral cell culture system.

postseeding.

Sodium salt of eicosapentanoic acid (EPA, C20:5ω-3), arachidonic acid (AA, C20:4ω-6), eicosatrienoic acid (ETA, C20:4ω-9), α-linolenic acid (ALA, C18:3ω-3), linoleic acid (LA, C18:2ω-6), and oleic acid (OA, C18:1ω-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 hours after dilation in medium without FCS. Concentration of long chain fatty acids in the medium was indicated in the results. Three series of experiments, difference among C18 of ω-9, ω-6, and ω-3 unsaturated long chain fatty acids and difference among C20 of ω-9, ω-6, and ω-3 unsaturated long chain fatty acids were compared. Then concentration dependency of EPA was evaluated.

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 hours 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.[6,8]

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.

Electron microscopy for ultrastructural study, 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 hours at room temperature. 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 hour, 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 microscope operated at 80 kV (JEM-

1220, JEOL).[9]

Statistical analysis

Data were expressed as mean ± standard deviation (S.D.) and 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 3, Caco-2 cells grown on permeable supports in bicameral chambers under control condition formed polarized monolayers of columner epithelial cells. Cells had long, densely packed microvilli in the apical side and well developed junctional complexes, including tight junctions and many desmosomes localized among the length of the interdigitating lateral membranes (Fig. 4).[6]

TEER levels were 370 ± 142 ohm·cm^2 on the fourth to tenth day old cells indicating enough integrity of this monolayer system.[11] The Caco-2 monolayers showed low permeability to FS with only 0.6 ± 0.7% of the small molecular dye crossing the monolayer during the 3 hours test period. FS clearances in untreated (control) cells were 492 ± 142 nanoliters/cm^2/hour in accordance with Menconi' report.[6] The activity of LDH, a cytosolic enzyme released when cells are injured, was evaluated in the medium. Caco-2 cells in control group indicated under 12% of total cellular LDH. Correlation between TEER and FS permeability, and correlation between TEER and LDH levels indicated negative relationship with statistical significance (data not shown) in the preliminary time course study.

We first tested the effect of C18 unsaturated fatty acids on the TEER, FS permeability, and LDH release. Figure 4 indicated that OA did not change TEER or FS permeability. LA decreased TEER ratio to 71.9 ± 3.7%, increased FS ratio to 108.5 ± 164.5%, and increased LDH ratio to 151.1 ± 38.4%. ALA decreased TEER ratio to 58.0 ± 10.5%, more than LA ($p < 0.01$). ALA highly increased FS permeability to 259.9 ± 127.6%, with the increase of LDH level to 182.5 ± 68.1% ($p<0.01$).

Then, the effect of C20 unsaturated fatty acids on tight junction permeability was evaluated (Fig. 5). ETA and AA decreased TEER ratio ($p < 0.01$). FS permeability ratio in the EPA group increased with statistical significance to 255.2 ± 194.1% ($p<0.05$), but FS ratio in the AA and ETA group did not increase. LDH ratio in the EPA group were lower than that in the ETA or AA group without statistical significance.

Concentration dependency of EPA on tight junction permeability clearly indicated decrease in TEER with increase in FS permeability in Figure 6. 50 μM of EPA decreased TEER ratio and increased FS ratio ($p < 0.01$) and those effects were prominent in the addition of 200 μM of EPA. LDH level in 50 or 100 μM EPA was the same as the control level.

Ultrastructural analysis in the EPA group was indicated in figure 3 and 7. Mitochondria were prominent and no overall changes in the polarity of epithelial cells or structure of nucleus. Phagosomes, lysosomes, and fat droplets were prominent. The tight junction and desmosomes suggested to con-

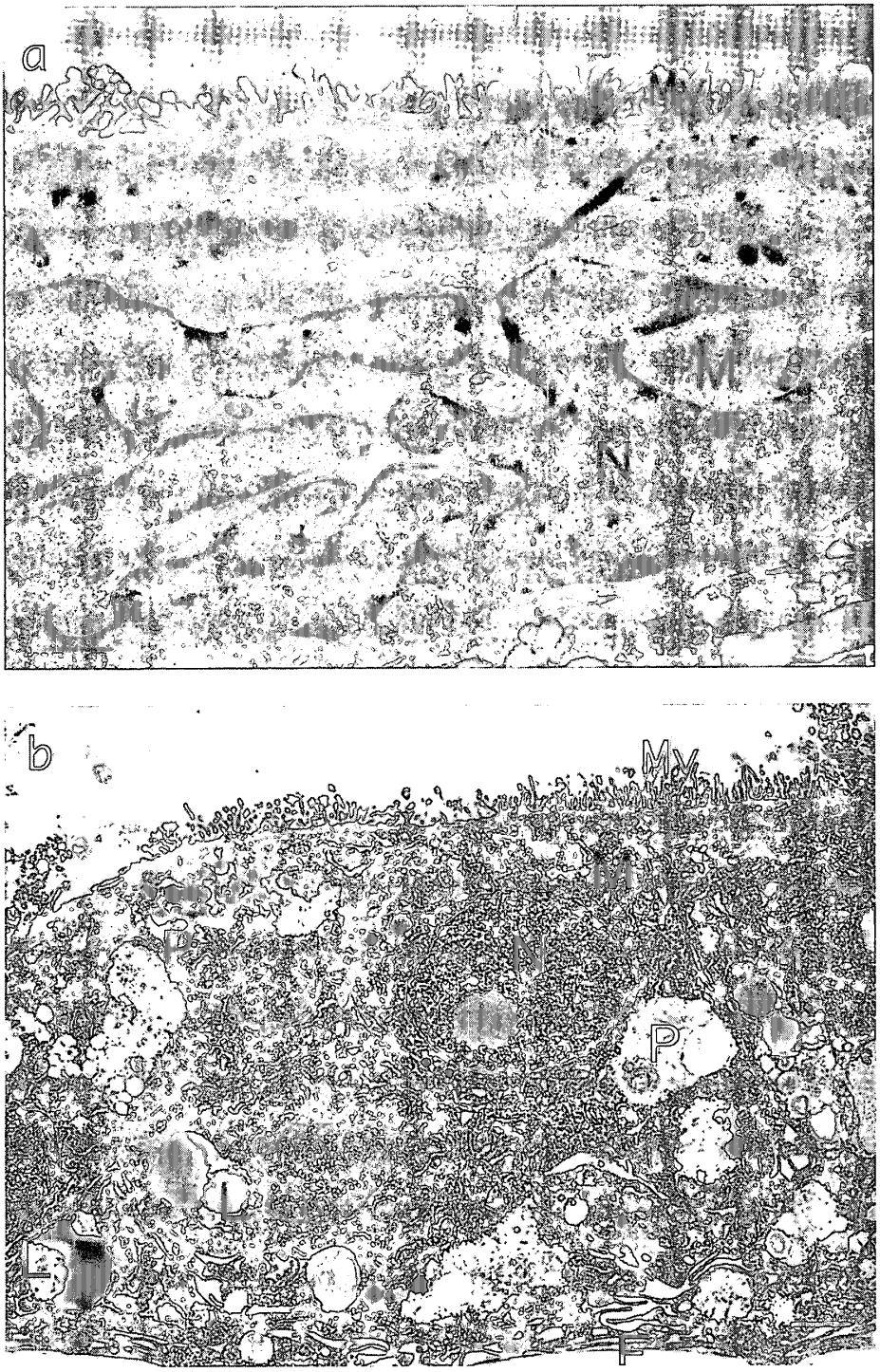

Figure 3. Transmission electron micrograph of the Caco-2 cell monolayer incubated for 24 hours in the medium with 0 (control) or 200 μM EPA on 7 - 10 days postseeding. In control cells (a), cells grown on Transwell filters (F) formed polarized monolayers of columnar epithelial cells. Cells had long, densely packed microvilli (Mv) and well-developed cell to cell junctional complexes (see Fig. 7). 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, lysosomes, and fat droplets were prominent. Microvilli appeared same as the control. Scale bar, lower left in a is 1 μm and lower right in b is 2 μm.

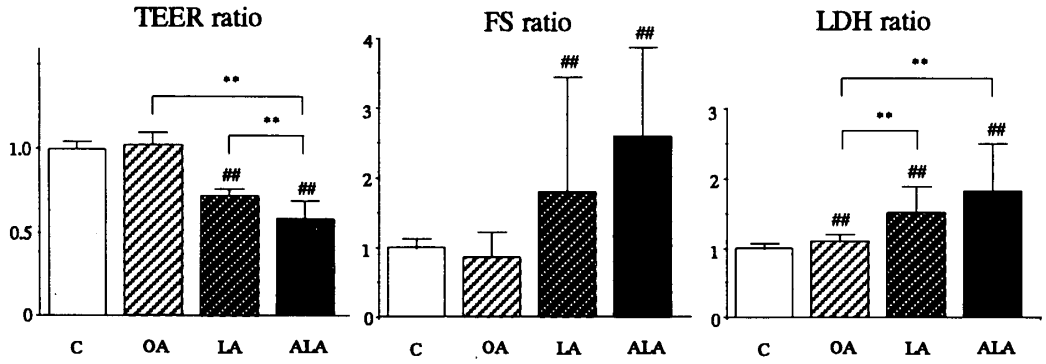

Figure 4. Effect of OA, LA, ALA (200 μM) on transepithelial electrical resistance (TEER) ratio, fluorescein sulfonic acid (FS) permeability ratio, LDH concentration ratio in Caco-2 monolayer cells. Those values are normalized to those of untreated (control) cells. mean ± S.D., n = 10 − 12. **; p<0.01, # #; p<0.01 vs. C

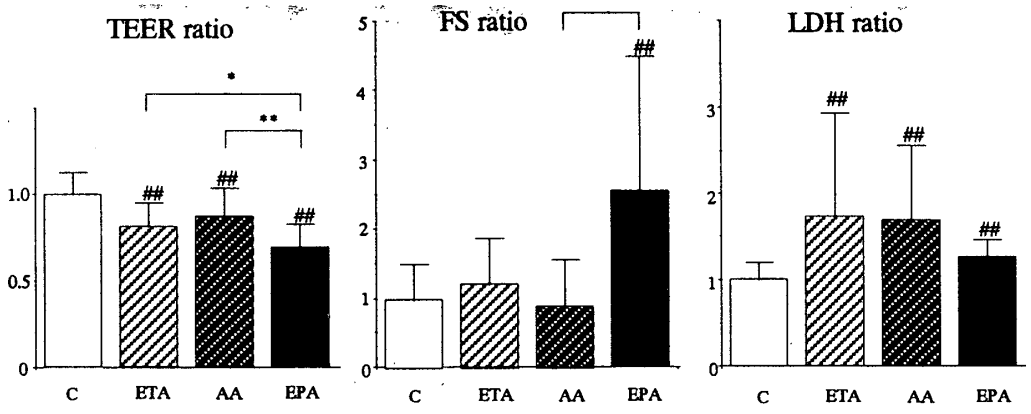

Figure 5. Effect of ETA, AA, EPA (200 μM) on transepithelial electrical resistance (TEER) ratio, fluorescein sulfonic acid (FS) permeability ratio, LDH concentration ratio in Caco-2 monolayer cells. Those values are normalized to those of untreated (control) cells. mean ± S.D., n = 9 − 18. *; p<0.05, **; p<0.01, # #; p<0.01 vs. C

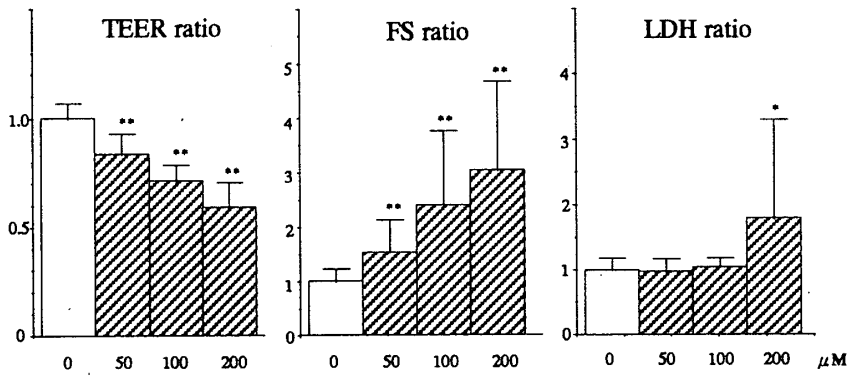

Figure 6. Effect of EPA on transepithelial electrical resistance (TEER) ratio, fluorescein sulfonic acid (FS) permeability ratio, LDH concentration ratio in Caco-2 monolayer cells. Those values are normalized to those of untreated (control) cells. mean ± S.D., n = 9 − 18. *; p<0.05, **; p<0.01 vs. C

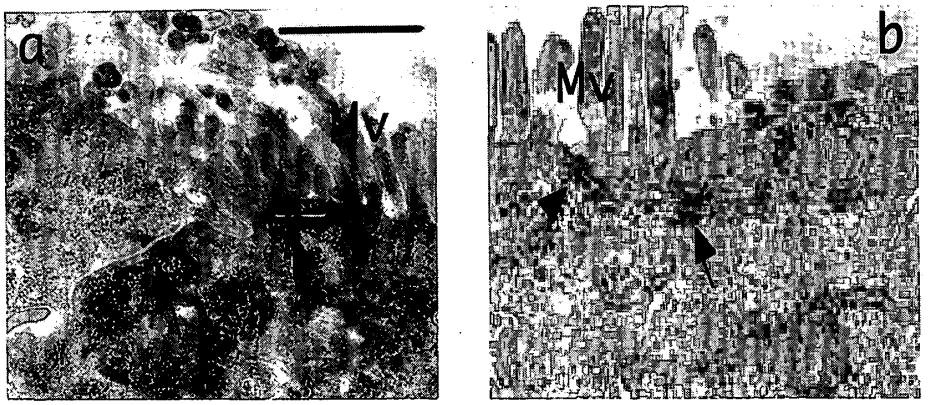

Figure 7. Transmission electron micrographs showing microvilli and junctional complex in apical region of control and EPA-treated Caco-2 cells. In control cells (a), the tight junction (arrowhead) and desmosome (arrow) are intact. In cells exposed to 200 μM of EPA for 24 hours (b), the tight junction (arrow head) and desmosome (arrow) suggested to contain less electron-dense material. Scale Bar (a, upper right) = 1 μm.

tain less electron-dense material.

Discussion

Tight junctions between adjacent epithelial cells create a physiological intercellular barrier which maintains distinct tissue spaces and separates the apical from the lateral plasma membranes.[12] Tight junctions are important structures in controlling the diffusion of molecules, such as drugs, through the intestine.

This study is the first report showing a regulatory effect of PUFAs on tight junction function in a model of intestinal mucosa. EPA of the ω-3 series essential fatty acids clearly exerted an effect on the tight junctions, by changing TEER and the paracellular permeability by lipophobic small molecules. The reason why ALA also showed the same effect of EPA is explained by desaturation and elongation of ALA. EPA is formed in a short time, 4 hours, in Caco-2 cells observed by Chen et al.[13] Caco-2 metabolizes large amount of ALA to EPA but not to DHA. Explanation of slight but significant effect of LA is that Caco-2 metabolizes small amount of LA to γ-linolenic acid (GLA). Recently, we have been accumulating the result that GLA also shows the effect on the tight junction permeability in the series of experiment (data not shown).

PUFAs 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] In this study, AA, the most important of eicosanoid precursors producing prostaglandins, thromboxans, and leukotriens, does not affect the tight junction permeability. However, eicosanoids produced from EPA may have the effect. In the series of experiments, coadministartion of indomethacin, a cyclooxygenase blocker, have no effect. But, coadministration of 2,3,5-trimethyl-6-(12-hydoxy-5,10-dodecadinyl)-1,4-benzoquinone (AA861), lipoxygenase blocker, blocked the increase in FS % permeation rate by EPA to the control level, suggesting participation of 5 series of leukotriens

on tight junction permeability (data not shown).

The regulatory effect of PUFAs, EPA or GLA, on tight junction function indicated in this study is in accordance with the effect of PUFAs in endothelial cell culture reported by Jiang,[12] however, the direction of the effect of PUFAs is opposite. Those effects vary with different experimental settings and cell types shown by Lindmerk.[14] In fact, we also have the results showing that EPA decreases FS permeability in the different experimental setting. Future in vivo study is required to elucidate this discrepancy. And, the effect of protein kinase C activation by PUFAs would be evaluated using this experimental models. Because, PUFA is postulated to modify intracellular signaling and several reports are published recently indicating PKC activation on tight junction function.[1,15,16]

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

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