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Effect of γ-Linolenic Acid (GLA) or Docosahexaenoic Acid (DHA) on Tight Junction Permeability in Intestinal Monolayer Cells

Makoto Usami¹, Takako Komurasaki¹, Aki Hanada¹, Kaori Kinoshita¹, Atsushi Ohata¹, Yoshiki Tabuchi¹, Masaki Saitoh², Masashi Nakagawa².

Polyunsaturated fatty acids have been characterized as immunonutrients, however the effect of docosahexaenoic acid (DHA) or γ-linolenic acid (GLA) on intestinal permeability has rarely been reported. Confluent Caco-2 cells on porous filter were used to measure tight junction function by fluorescein sulfonic acid (FS) permeability and transepithelial electrical resistance (TEER). Treatment of 0, 10, 50, 100 µM of DHA or GLA during 24 hours were compared. Then, effects of butylated hydroxytoluene (BHT, antioxidant), H7 (protein kinase C antagonist), or inhibitors of enzymatic degradation to the eicosanoids, indomethacin (cyclooxygenase inhibitor), AA861 (lipoxygenase inhibitor) on DHA or GLA were examined. GLA and DHA enhanced FS permeability to 8.7 and 1.4 fold and lowered TEER to 0.52 and 0.73 fold vs. control with concentration dependently without cell injury (p<0.01-0.05). IND and AA861 enhanced the changes mediated by GLA but did not alter DHA effect. BHT was ineffective. H7 facilitated the changes mediated by GLA, DHA, and eicosapentaenoic acid (EPA). It is indicated that the mechanism to change tight junction permeability via protein kinase C regulation is common but via eicosanoids formation is different among GLA, DHA and EPA. In conclusion, GLA and DHA affect tight junction permeability in intestinal monolayer cells specifically and concentration dependently.

Key Words: tight junction permeability, Caco-2 cell, docosahexaenoic acid, γ-linolenic acid, eicosapentaenoic acid, protein kinase C, indomethacin, AA861

Introduction

Dietary lipids are essential components of every living cells with many important factors for the integrity of bilipid structure of cell membranes, for numerous biologically active compo-
onds via various eicosanoids formation or peroxidation process, and as energy sources. The n-3 series and n-6 series polyunsaturated fatty acids are essential fatty acids and considered as 'immunonutrients'. These polyunsaturated fatty acids have been used in various patients with inflammatory bowel diseases, autoimmune diseases, and under severe surgical stress. 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. 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. However, the influence of polyunsaturated fatty acids on tight junction permeability has rarely been reported.

Recently, we have reported the effect of eicosapentaenoic acid (EPA, C20:5 n-3) to regulate tight junction permeability in a model of intestinal epithelium. Fifty to 200 μM of EPA clearly changes paracellular permeability without cell injury in a concentration dependent fashion. In contrast to EPA, arachidonic acid (AA, C20:4 n-6), the main precursor of eicosanoids, shows slight changes in the same concentration range, and 200 μM of oleic acid (OA, C18:1 n-9) and linoleic acid (LA, C18:2 n-6) does not affect but α-linolenic acid (ALA, C18:3 n-3), the precursor of EPA, enhances tight junction permeability. Both cyclooxygenase (COX) and lipoxygenase (LO) inhibitors partially normalize the changes mediated by EPA, indicating a mechanism via eicosanoids formation from EPA.

In this study, the effects of docosahexaenoic acid (DHA, C22:6 n-3) or γ-linolenic acid (GLA, C18:3 n-6) on tight junction permeability were evaluated. DHA is the main source of n-3 fatty acids as important as EPA. GLA is a recently focused dietary source on its specific biological activity of anti-inflammatory and anti-proliferative properties as reviewed by Fan et al. GLA supplementation studies conducted in humans by Johnson et al. show increased dihomo-γ-linolenic acid (DGLA) level and attenuate the biosynthesis of AA metabolite.

To examine whether the tight junction permeability changes induced by DHA or GLA are due to eicosanoids formation, two key enzymes in the generation of eicosanoids, LO and COX, are inhibited with their inhibitors, 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-p-benzoquinone (AA861), and indomethacin, respectively. To evaluate the effect of generated peroxidation products from DHA or GLA, the effect of antioxidant, butylated hydroxytoluene (BHT), was investigated. And, in addition to these mechanisms, the effect of protein kinase C activity changes to modify intracellular signaling by DHA, GLA, or EPA was evaluated using the protein kinase C
antagonist, 1-(5-isooquinolinylsulfonyl)-2-methylpiperazine (H7). Because, tight junction permeability is mediated by protein kinase C and/or cytoskeletal contraction. 

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 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.

Materials and Methods

The methods to prepare Caco-2 cells and to measure tight junction permeability and cellular damage using fluorescein sulphonic acid (FS) permeability, transepithelial electrical resistance (TEER) and lactate dehydrogenase (LDH) resistance using fluorescein sulphonic acid (FS) permeability, transepithelial electrical resistance (TEER) and lactate dehydrogenase (LDH) release are reported in our previous paper. To be brief, Caco-2 cells (Nihon Seiyaku Co., Japan) were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) in 75cm² tissue culture flasks, harvested, and seeded at a density of 6.25 × 10⁵ cells/cm² in Transwell inserts (Costar), 6.5 mm in diameter (surface area 0.33 cm²) and polycarbonate filters with 3.0 µm pore size. Transepithelial electrical resistance (TEER) was 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). Caco-2 cells monolayer was used between day 4 and day 10 postseeding.

Sodium salt of DHA, GLA, and EPA were obtained from Sigma. Fatty acids were dissolved in PBS (-) and stored at -30°C. They were added to the apical and the basal chambers during 24 hours after dilution in the medium without FCS. The concentration of polyunsaturated fatty acids in the medium was indicated in the results. BHT, IND, AA-861, and H7 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 minutes before PUFA administration. For each drug studied, control experiments consisted of administration of the drug solvent (the medium plus 0.1% ethanol) were performed.

Five hundred µg/ml of FS (Molecular Probes) dissolved in the medium without FCS were loaded 40 ml into the apical compartment of the Transwell chambers during 3 hours after removal of 40 ml of culture medium. The concentrations of the FS in the apical and basolateral compartments were assayed. 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. To assess cell membrane integrity, release of the cytosolic enzyme LDH was measured. LDH activity in media samples from the basolateral compartments was determined spectrophotometrically using a single reagent system (LDH-Cytotoxic Test, Wako) on an ELISA reader (Bench-mark 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 mono-layer with 1.0 ml of 0.1% Triton X-100 in PBS followed by centrifugation and assaying the supernatant.

Statistical analysis
Data were expressed as mean±standard deviation (S.D.). One-way factorial analysis of variance (ANOVA) using the Statview statistical software package (version 4.51.1; Abacus Concepts, Berkeley, CA), followed by the Fisher's PLSD 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
Caco-2 cells grown on permeable supports in bicameral chambers under control condition formed polarized monolayers of columnar epithelial cells with typical brush border microvilli on the apical surface, tight junctions at the lateral apical surface, and many desmosomes localized among the length of the interdigitating lateral membranes indicated in our previous report. The Caco-2 monolayers in the control group showed 1243 ± 124 ohm of TEER levels. The permeability to FS was low, 26.9 ± 6.0%, and 2.0 ± 1.3% of total LDH was released. We tested the effect of 10 to 100 mM DHA and GLA during 24 hours on the TEER, FS permeability, and LDH release. Addition of DHA increased FS permeability (p=0.041) and decreased TEER ratio (p=0.001) without LDH change (Fig. 1). GLA also increased FS permeability and decreased TEER with concentration dependently (p=0.001) (Fig. 2). LDH release by GLA was 525.6 ± 705.1% of the control group and 12.6 ± 14.7% of total LDH. The change in tight junction permeability by GLA was more prominent than DHA. BHT, indomethacin and AA861 did not affect the change of tight junction permeability by DHA (data not shown). However their changes by GLA was enhanced by indomethacin and AA861. Addition of BHT did not influence. Addition of indomethacin with GLA enhanced FS ratio, 467.8±477.0 % vs. 136.8 ± 108.0% in the GLA group, (p=0.06), and reduced TEER, 42.4 ± 12.2% vs. 64.6 ± 15.4% in the GLA group (p=0.001). Addition of AA861 with GLA enhanced FS ratio, 459.9±868.8% vs. 141.9 ± 116.6% in the GLA group (n.s.), and reduced TEER, 52.4 ± 18.8% vs. 61.7 ± 16.3% in the GLA group (n.s.). Indome-
DHA and GLA on tight junction in enterocyte monolayer

![Graph of DHA effects on FS permeability and TEER](image1)

Figure 1. Effect of DHA on fluorescein sulfonic acid (FS) permeability and transepithelial electrical resistance (TEER) in Caco-2 monolayer cells. Those values are normalized to those of untreated (control) cells. mean±S.D., n = 9 - 12. P<0.05 with one-way factorial ANOVA. *; p<0.05, **; p<0.01, ***; p<0.001 by Fisher's PLSD multiple comparison test.

![Graph of GLA effects on FS permeability and TEER](image2)

Figure 2. Effect of GLA on fluorescein sulfonic acid (FS) permeability and transepithelial electrical resistance (TEER) in Caco-2 monolayer cells. Those values are normalized to those of untreated (control) cells. mean±S.D., n = 9 - 12. P<0.01 with one-way factorial ANOVA. *; p<0.05, **; p<0.01, ***; p<0.001 by Fisher's PLSD multiple comparison test.

Thiocin itself reduced TEER to 64.5±19.0% and enhanced FS permeability to 345.1±369.1% (p<0.0002 vs. control). AA861 also reduced TEER to 75.5±14.7% (p=1×10^{-6}) and enhanced FS permeability to 197.3±301.8% (p=0.043 vs. control) (Fig. 3). BHT did not change those parameters (data not shown).

Addition of H7, inhibitor of protein kinase C, decreased TEER from 95.8±12.5% in the EPA group to 68.7±16.2% (p=0.0012), from 77.4±23.3% in the DHA group to 47.0±15.1% (p=0.0030), and from 12.3±2.8% in the GLA group to 9.9±0.6% (p=0.073) and enhanced FS ratio, 189.5±77.5% vs. 114.2±37.4% in the EPA group (p=0.023), 497.2±490.1% vs. 179.0±220.7% in the DHA group and 5997.8±2044.0% vs. 4932.3±2766.7% in the GLA group (not significant, NS) (Fig. 4). H7 itself enhanced FS permeability and reduced TEER with concentration dependently (p=4.4×10^{-6}, table 1). H7 did not influence on the
Figure 3. Effect of cyclooxygenase inhibitor, 10 μM of indomethacin (IND), or lipooxygenase inhibitor, 10 μM of 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-p-benzoquinone (AA861), with 100 μM of γ-linolenic acid (GLA) on fluorescein sulfonic acid (FS) permeability and transepithelial electrical resistance (TEER), in Caco-2 monolayer cells. Those values are normalized to those of untreated (control) cells. mean±S.D., n=7-18. *; p<0.05, **; p<0.01 vs. 0 mM of inhibitor.

Figure 4. Effect of protein kinase C inhibitors, 50 μM of H7, with 100 μM of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and γ-linolenic acid (GLA) on 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 mM of inhibitor.

effect of indomethacin and AA861. Those changes in tight junction permeability with PUFAs; EPA, DHA,
DHA and GLA on tight junction in enterocyte monolayer

Table 1. Effects of H7 on FS ratio and TEER

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<th>Conc. (μM)</th>
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<tr>
<td>10</td>
<td>170.0 ± 98.3</td>
<td>74.8 ± 13.7</td>
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<td>338.4 ± 234.1</td>
<td>59.3 ± 19.5</td>
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<tr>
<td>50</td>
<td>348.3 ± 321.0</td>
<td>57.0 ± 19.1</td>
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TEER, FS permeability values are normalized to those of untreated (control) cells (100±6.3%, 100±5.6%, respectively). Values are mean±S.D. (n=6-11). P=0.054 in FS ratio and P=4.4×10^-4 in with one-way factorial ANOVA without interaction.

Figure 5. Summary of tight junction permeability in Caco-2 monolayer cells. ↑ indicates enhancement, ↓ indicates suppression, → indicates no change, ↑↑ indicates multiplication effect.

As for the mechanism of DHA and GLA on tight junction permeability, firstly peroxide production is postulated. However, BHT, an antioxidant, does not show any effect to modulate PUFAs effect in this experiment and the tight junctions by changing paracellular permeability measured using lipophobic small molecule, FS, permeability and TEER. The effect of the same concentration of three PUFAs in both FS ratio and TEER was in the order of GLA, EPA and DHA in combination with this result and our recent report. ⁹

Discussion

This study is the first report to demonstrate the effect of both DHA and GLA on a tight junction function in a model of intestinal mucosa. Both DHA and GLA clearly exerted an effect on

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Temperature of PUFAs solution is controlled strictly in a refrigerator in this experiment to prevent peroxide production in accordance with Cepinska et al's control data. Secondy, participation of eicosanoids formation is evaluated. Our result indicates that no participation of eicosanoids production by DHA is shown using both inhibitors in accordance with Reinboth et al's recent report showing that DHA does not generate docosanoid. They indicate eicosanoids production from DHA by retroconversion to EPA in retina, but negative results of this DHA experiments are not in accordance with positive results in eicosanoids formation in our EPA experiment. GLA's effect on tight junction permeability was not inhibited but enhanced with both indomethacin and AA861 on the contrary of our report indicating inhibition of EPA effect by indomethacin and AA861. If the permeability change by GLA is mediated by the production of prostaglandin (PG)E, one of the 1 series prostanoid produced from DGLA that is an elongation product of GLA, the change should be inhibited by COX inhibitor, indomethacin. And to elucidate the possibility of 15-(S)-hydroxy-8,11,13-eicosatrienoic acid (15 HETRe) produced from DGLA via 15-LO on the change of tight junction permeability, further experiments using other LO inhibitor except AA861 that indicates weak effect on 15-LO, or NDGA that indicates strong effect on tight junction permeability in our previous experiment, should be considered to use. Our previous data indicating no effect of AA on tight junction permeability, the main precursor of eicosanoids generation, in the concentration range between 100 and 200 \( \mu \text{M} \), suggests lower possibility of the 2 series of pro-stanoids and thromboxane produced from AA and the 4 series of leukotrienes.

Increased paracellular permeability by indomethacin is in accordance with Nagase et al's report using urinary lactulose/rhamnose secretion measurement after oral administration in normal volunteer. However, they indicate decreased permeability by PGE\(_1\), analogue administration in the same report on the contrary of this result. PGE\(_1\) has various effects on biological activity inducing vascular circulation system in vivo, and our result showing enhanced tight junction permeability in vitro is controversial but may not be contradictory.

Following Lindmark et al's possible intracellular pathways activated by medium chain fatty acids (C10 or C12) leading to regulate tight junction permeability including activation of phospholipase C, diacylglycerol, then protein kinase C, our results of different PUFAs might have a similar intracellular signaling pathway. Several reports indicate effect of phospholipase C and protein kinase C activity changes due to PUFAs in various cells and stimulations. They are direct effect of dietary EPA and DHA on phospholipase C inhibition in neutrophils in Sperling et al's study, suppression of macrophage protein...
kinase C activity with 100 μM of EPA and DHA in Tappia et al's in vitro study(19), translocation of protein kinase C subfractions with 30 μM of DHA in Huang et al's in vitro study using HL-60 cells(15), and the effect of protein kinase C suppression via 15-HETE production with 30 μM of GLA in Cho et al's in vitro study using epidermal cells(12). These results are in accordance with our findings that participation of protein kinase C is common phenomena as the mechanism of tight junction permeability changes by these three different PUFAs; GLA, EPA and DHA.

In conclusion, GLA and DHA affect tight junction permeability in intestinal monolayer. It is indicated that the mechanism to change tight junction permeability via protein kinase C regulation is common but via eicosanoids formation is different among GLA, DHA and EPA.

References


