Intracellular Mechanisms Involved in Docosahexaenoic Acid-Induced Increases in Tight Junction Permeability in Caco-2 Cell Monolayers

Sònia Roig-Pérez, Núria Cortadellas, Miquel Moretó, and Ruth Ferrer

3Departament de Fisiologia, Facultat de Farmàcia and 4Unitat de Microscòpia Electrònica, Serveis Científico-tècnics, Facultat de Medicina, Universitat de Barcelona, Barcelona, Spain

Abstract

We recently showed that enrichment of Caco-2 cells with docosahexaenoic acid (DHA) increases lipid peroxidation and the formation of hydrogen peroxide and peroxynitrite, which disrupt the epithelial barrier function. Studies were designed to test whether the participation of phospholipase C (PLC)/Ca2+/protein kinase C (PKC), cyclooxygenase (COX), and 5-lipooxygenase pathways are involved in mediating the effects of DHA. Paracellular permeability was assessed from D-mannitol flux and transepithelial electrical resistance (TER) in differentiated Caco-2 cell monolayers incubated in control or DHA-enriched conditions (100 μmol/L). The effect of DHA was prevented by U73122 (PLC inhibitor), chelerytrine (PKC inhibitor), and 1-[5-iodonaphtalene-1-sulfonyl]-1H-hexahydro-1,4-diazepine hydrochloride (myosin light chain kinase inhibitor). In contrast, the effect of DHA was enhanced by A23187 (Ca2+ ionophore) and BAPTA-AM (Ca2+ chelator). Indomethacin (COX inhibitor) and AA961 (5-lipooxygenase inhibitor) also prevented the changes in D-mannitol flux induced by DHA, but no effect was detected for TER. Moreover, occludin and ZO-1 immunogold staining microscopy showed that the increase in paracellular permeability was accompanied by the redistribution of both tight junction proteins. We conclude that the disruption of epithelial barrier function by DHA is partly mediated by the PLC/Ca2+/PKC pathway and by the formation of eicosanoids.

Introduction

Dietary supplementation with docosahexaenoic acid [DHA; 22:6(n-3)] is an increasingly common practice, because beneficial effects have been reported, especially in brain, retina, heart, and blood (1). Nevertheless, PUFA can also be deleterious, because they promote lipid peroxidation and the subsequent propagation of oxygen radicals (2,3). We previously reported that, following Caco-2 cell enrichment with DHA, hydrogen peroxide and peroxynitrite disrupt the epithelial barrier function and that taurine has a protective role, counteracting the effect of hydrogen peroxide (4). More recently, we also reported that Caco-2 cells can modulate transepithelial taurine transport and thus attenuate the effect of oxidative stress induced by DHA enrichment (5). We also observed that membrane composition in fatty acids is modified in DHA-enriched Caco-2 cells, which in turn may influence eicosanoid formation (6).

The intestinal epithelium constitutes a permeability barrier that regulates the vectorial transport of ions, solutes, and water. Epithelial barrier function is primarily determined by the tight junctions (TJ), which are complexes formed by transmembrane proteins associated with the cytoskeletal perijunctional actomyosin ring and cytosolic proteins involved in cell signaling and vesicle trafficking (7). PUFA have been shown to modulate TJ permeability by modifying cyclooxygenase (COX) and lipooxygenase (LOX) products (8) or occludin localization (9). Moreover, activation of the phospholipase C (PLC)/Ca2+/protein kinase C (PKC) pathway mediates DHA enrichment effects in diverse cellular processes (10,11). Furthermore, PKC is a key element in the regulation of TJ permeability (12). The results obtained indicate the participation of the PLC/Ca2+/PKC signaling pathway in DHA-induced epithelial barrier disruption and reinforce the contributions of eicosanoids and TJ protein localization in these effects.

Materials and Methods

Materials. DMEM, nonessential amino acids, penicillin, streptomycin, t-glutamine, trypsin, fetal bovine serum, bovine serum albumin (BSA),
DHA, U73122, d-glucose, HEPES, 1-[5-iodonaphthalene-1-sulfonyl]-1H-hexahydro-1,4-diazepine hydrochloride (ML7), chelerythrine, A23187, indomethacin, 2-(12-hydroxydocosa-5,10-dienyl)-3,5,6-trimethyl-p-benzoquinone (AA861), thio-carbohydrazide, and BHT along with other chemicals were supplied by Sigma. BAPTA-AM and diocanoylglycerol (diC8) were purchased from Molecular Probes. D-[2-3H] mannotol (specific activity 30 Ci/mmol) was from ARC. Tissue culture supplies, including Transwells, were obtained from Costar.

Cell culture. Caco-2 cells were kindly provided by Professor David Thwaites at the School of Cell and Molecular Biosciences, University of Newcastle-upon-Tyne (UK). The cells (passages 107–116) were routinely cultured in 75- or 150-cm² plastic flasks at a density of 5 × 10⁴ cells/cm² and cultured in DMEM supplemented with 4.5 g/L D-glucose, 1% (v:v) nonessential amino acids, 2 mM L-glutamine, 10% (v:v) heat-inactivated fetal bovine serum, 100 kU/L penicillin, and 100 µg/mL streptomycin at 37°C in a modified atmosphere of 5% CO₂ in air. For growth on filters, cells were seeded at a density of 4 × 10⁴ cells/cm² onto polycarbonate filters (Transwells, 12-mm diameter) with a pore size of 0.4 µm. Experiments were performed in monolayers maintained for 17–21 d in control conditions or in DHA-supplemented medium (100 µmol/L) for 24 h before the experiment. In the experiments performed to examine the participation of the PLC/Ca²⁺/PKC pathway, cells were incubated for only 1 h with DHA and the various pharmacological agents tested to minimize their effect on cell viability. DHA was solubilized in ethanol containing 4 mg/mL BHT to prevent oxidation and bound to BSA, as described elsewhere (4). Supplementation was performed at the apical side of the Transwell. Growth medium was replaced twice per week and on the day before the experiment.

Paracellular permeability. Transepithelial electrical resistance (TER) and D-mannitol flux were determined as previously described (4). Briefly, monolayers grown in Transwells were gently washed by sequential transfer through 4 beakers containing 500 mL of modified Krebs buffer (room temperature) containing (mmol/L): NaCl, 137; KCl, 5.4; CaCl₂, 2.8; MgSO₄, 1.0; NaH₂PO₄, 0.3; d-glucose, 10; and HEPES/Tris, 10 (pH 7.4). Monolayers were then placed in culture wells containing 1.5 and 0.75 mL modified Krebs buffer in the basal and apical compartments, respectively, and TER was determined by a Millicell-ERS voltohmmeter (Millipore). Results are expressed as cm² of monolayer surface area. The resistance of the supporting membrane in Transwells was subtracted from all readings before calculations. After TER determination, apical medium was replaced by the same volume of modified Krebs containing 0.5% (w/v/L) D-[2-3H]mannotol and cells were incubated for 5 min at 37°C. At the end of the incubation, basal media were withdrawn and radioactive activity was counted in a scintillation counter (1500 Tri-Carb, Packard). In the case of COX and 5-LOX inhibition experiments, indomethacin and AA861 were added 30 min before DHA supplementation.

Electron microscopy. Monolayers grown on filters were fixed in 2.5% glutaraldehyde in 0.1 mol/L PBS (pH 7.4) for 2 h at 4°C and subsequently washed in 0.1 mol/L PBS. The samples were postfixed in OsO₄ (1% in 0.1 mol/L PBS) containing 0.8% potassium ferricyanide, dehydrated in acetone, and further embedded in Spurr’s embedding medium (13). Ultra-thin sections (60 nm, Ultracut) were stained with uranyl acetate and lead citrate, following Reynolds (14), and examined in a Hitachi 600 AB electron microscope operating at 75 kV. To enhance the contrast of cellular lipid droplets, electron microscopy samples were processed following the methodology of OTO described by Seligman et al. (15). Briefly, after conventional fixation with OsO₄, ultra-thin sections were exposed to a 1% aqueous solution of thio-carbohydrazide for 1 h at 50°C, washed several times in distilled water for 5 min at 50°C, and again exposed to OsO₄ vapor for 1 h.

Immunogold staining was performed in ultra-thin cryosections as described by Tokuyasu (16) with some modifications. Transwell filters were fixed in 1% formaldehyde in 0.1 mol/L PBS (pH 7.4) for 1 h at room temperature. Monolayers were then embedded in 10% gelatin, infused with 2.1 mol/L sucrose at 4°C, and rapidly frozen in liquid nitrogen. Ultra-thin sections were obtained from frozen samples in an FC-4E low-temperature sectioning system (Reichert-Jung) at −110°C using glass knives with tungsten, collected on formvar-filmed gold grids, and washed in 0.1 mol/L PBS (pH 7.4). The samples were then incubated for 10 min in 0.1 mol/L PBS (pH 7.4) containing 2% gelatin for 3 min in 0.1 mol/L PBS (pH 7.4) containing 0.02 mol/L glycine and thereafter for 1 min in 0.1 mol/L PBS (pH 7.4) containing 0.1% BSA (PBS-BSA, incubation solution). As primary antibodies, mouse monoclonal anti-occludin (1:50 dilution) or rabbit polyclonal anti-ZO-1 (1:25 dilution, Zymed) were used. Cells were incubated for 1 h at room temperature with the antibody and washed 3 times in PBS-BSA for 5 min. Monolayers were incubated for 1 h with goat anti-mouse IgG (BBInternational) or goat anti-rabbit IgG (BBInternational), both coupled to 10-nm gold particles (1/25 dilution), for occludin and ZO-1 detection, respectively. The samples were then washed in PBS and distilled water and incubated for 10 min with 1.8% methyl cellulose containing 0.4% uranyl acetate. Finally, the grids were picked up with a loop and dried. Control grids were prepared following the same procedures but omitting the primary antibody. The samples were examined with a Hitachi 600 AB electron microscope, operating at 75 kV, using a small objective aperture to obtain the best contrast. Sample processing and observation was carried out at the Serveis Científicote cèntrics of the Universitat de Barcelona.

Statistics. Results are expressed as mean ± SEM. ANOVA was followed by the Scheffé multiple comparison test to detect significant differences between treatments using the SPSS 241 software. The homogeneity of variance was tested with Levene’s test. Student’s t test was used to compare the means of 2 groups. Differences with P < 0.05 were considered significant.

Results

The effect of the different pharmacological agents used to investigate the participation of the PLC/Ca²⁺/PKC pathway in DHA-induced changes in TJ permeability was first tested in control cells that were not treated with DHA. U73122 (PLC inhibitor) and ML7 [myosin light chain kinase (MLCK) inhibitor] did not affect D-mannitol flux (Fig. 1A) or TER (Fig. 1B). Chelerythrine (PKC inhibitor) did not affect D-mannitol flux, but it increased TER. In contrast, BAPTA (membrane-permeable Ca²⁺ chelator), diC8 [diacylglycerol (DAG) analogue], and to a lesser extent A23187 (Ca²⁺ ionophore) increased paracellular permeability (P < 0.05 vs. control). DHA increased D-mannitol flux and reduced TER (Fig. 1). These effects were prevented by the addition of U73122. A23187 did not prevent the effect on D-mannitol flux but restored TER to values not different from control and DHA conditions. BAPTA did not prevent the effects of DHA, although D-mannitol flux was lower and TER was higher in the presence of DHA than in control cells. In DHA-enriched cells, diC8 further increased paracellular permeability. Chelerythrine and ML7 did not modify D-mannitol flux but prevented the effects of DHA on TER.

The participation of eicosanoids in the DHA-induced effect on paracellular permeability was investigated with indomethacin (COX inhibitor) and AA861 (5-LOX inhibitor). The effect induced after 24-h incubation with DHA did not significantly differ from that after 1-h incubation (Table 1). The addition of indomethacin or AA861 had no effect in control cells, but both reagents protected D-mannitol flux from the effects of DHA.

Caco-2 cells maintained in culture for 21 d formed a polarized columnar epithelium with well-developed microvilli (Fig. 2A). DHA-incubated cells had larger and more numerous lipid inclusions at the basal pole (Fig. 2B), a more disorganized cellular pattern and greater dilatations at the intercellular spaces (Fig. 2C,D). Immunogold localization of occludin and ZO-1 in the TJ (Fig. 3) shows a reduction in the number of gold particles for both proteins in DHA-treated cells. Moreover, the images...
also revealed gold particles corresponding to occludin farther from the TJ in DHA-incubated cells.

**Discussion**

The participation of the PLC/Ca\(^{2+}\)/PKC pathway in the regulation of paracellular permeability was tested in control and DHA-enriched cells. PLC cleaves phosphatidylinositol 4,5-bisphosphate into DAG and inositol 1,4,5-triphosphate (IP\(_3\)), and the interaction of IP\(_3\) with its receptors at the endoplasmic reticulum results in an increase in intracellular Ca\(^{2+}\) concentration. DAG, alone or with Ca\(^{2+}\), can then activate PKC (12). The results indicate that neither D-mannitol flux nor TER was affected in control cells by the PLC inhibitor, U73122. Nevertheless, the increase in paracellular permeability induced by DHA was prevented by U73122, which indicates PLC activation. Aires et al. (11) also reported that DHA activates the PLC/IP\(_3\) pathway and conventional and novel PKC \(\gamma\) and \(\delta\) isoforms, which are involved in apoptosis of monocytic leukemia U937 cells. Similarly, Padma and Das (10) observed that eicosapentaenoic acid [EPA; 20:5(n-3)] and DHA activate PLC and PKC in macrophage tumor cells. They also found that activated PKC intensifies the prooxidant state by increasing NADPH oxidase and inducing superoxide anion generation. In contrast, Awad et al. (17) reported that PLC activity in HT-29 cells is not affected by membrane fatty acid composition or by the increase in the unsaturation index induced by EPA or DHA membrane enrichment. Supporting the participation of PLC in the regulation of TJ permeability, Lindmark et al. (18) found that incubation of Caco-2 cells with capric acid induces an increase in paracellular permeability mediated by an increase in PLC activity.

In control and DHA-treated cells, the expected inverse correlation between changes in D-mannitol flux and TER was not always observed. To explain this apparent functional dissociation that occurs in some experimental conditions, Matter and Balda (19) have suggested that these 2 variables may not measure the same characteristics of transepithelial permeability. Hence, TER is an instantaneous measurement of ionic conductivity that reflects epithelial integrity as well as TJ ion selectivity, whereas D-mannitol permeability is a variable measured over a longer period of time that allows the quantification of slow diffusion across TJ and the determination of the size selectivity of the paracellular diffusion barrier.

Previous studies have shown the importance of Ca\(^{2+}\) on TJ formation and epithelial cell polarization (20–22), but there is controversy about the role of Ca\(^{2+}\) in TJ disassembly and barrier function maintenance. Ca\(^{2+}\) depletion enhances TJ permeability in differentiated Caco-2 cells and in confluent Madin-Darby canine kidney cells (23,24), but increased intracellular Ca\(^{2+}\) by

**TABLE 1** Effect of COX and 5-LOX inhibition on DHA-induced changes in D-mannitol flux and TER in Caco-2 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>D-mannitol flux,fmol/cm(^2)</th>
<th>TER, (\Omega/cm^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.0 ± 0.5(^a)</td>
<td>385 ± 9.2(^a)</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>1.3 ± 0.3(^b)</td>
<td>383 ± 14.6(^b)</td>
</tr>
<tr>
<td>AAB81</td>
<td>2.8 ± 1.6(^b)</td>
<td>409 ± 12.9(^b)</td>
</tr>
<tr>
<td>DHA</td>
<td>8.4 ± 1.0(^b)</td>
<td>330 ± 16.5(^b)</td>
</tr>
<tr>
<td>DHA + indomethacin</td>
<td>1.4 ± 0.7(^b)</td>
<td>331 ± 10.1(^b)</td>
</tr>
<tr>
<td>DHA + AAB81</td>
<td>1.6 ± 0.5(^b)</td>
<td>340 ± 5.2(^b)</td>
</tr>
</tbody>
</table>

\(^1\) Results are expressed as mean ± SEM, \(n = 4-6\) monolayers. Means in a column without a common letter differ, \(P < 0.05\).

\(^2\) D-mannitol flux and TER were measured in Caco-2 cells maintained in control conditions for 17–21 d or in DHA-enriched medium for the last 24 h before the experiment in the presence of indomethacin (10 \(\mu\)mol/L) or AAB81 (10 \(\mu\)mol/L).
A23187 has the same effect in T-84, Madin-Darby canine kidney, and Caco-2 cells (25–27). Similarly, our results obtained in control cells indicate that A23187 and BAPTA increased D-mannitol flux and reduced TER. As for DHA effects, PLC involvement led us to expect that this fatty acid would also increase intracellular Ca\(^{2+}\) concentration. In this sense, the capacity of (n-3) PUFA to induce Ca\(^{2+}\) mobilization from endoplasmic reticulum has already been described (11). Treatment of human colon adenocarcinoma cells (SW620 cells) with DHA increases cytosolic Ca\(^{2+}\), an effect that has also been described in other tissues (28–31). The results showing no significant differences between control and DHA-enriched cells in the presence of A23187 support this hypothesis. Moreover, the reduction of intracellular Ca\(^{2+}\) induced by BAPTA was so pronounced that the addition of DHA to the cultures treated with BAPTA apparently ameliorated paracellular permeability, thus also suggesting the capacity of DHA to increase intracellular Ca\(^{2+}\).

The activation of PLC, as is suggested for DHA, increases DAG formation and intracellular Ca\(^{2+}\) concentration, which in turn can activate PKC. This protein kinase consists of a family of Ser-/Thr-specific kinases that includes 12 known isozymes that can be classified into 3 subfamilies: conventional, novel, and atypical isoforms. Conventional isoforms are both Ca\(^{2+}\) and DAG dependent, novel isoforms are Ca\(^{2+}\) independent but DAG dependent, and atypical PKC isoforms are both Ca\(^{2+}\) and DAG independent (12). Contradictory results were reported on the contribution of PKC to the regulation of epithelial barrier function, but it is generally accepted that conventional isozymes participate in TJ disassembly whereas novel isoforms regulate TJ formation (32). We have observed that chelerythrine, a general PKC inhibitor (33), increases TER values in control cells and prevents the effects of DHA on TER. Furthermore, in control cells, diC8 increases D-mannitol flux and reduces TER, which was accentuated with DHA enrichment. Therefore, the association of increased intracellular Ca\(^{2+}\) levels with PKC activation, already described in the regulation of TJ permeability (12), may be involved in the effect of DHA. In this sense, several authors have described the capacity of fatty acids to modulate PKC activity directly or via PLC activation (10,34–36). Since generalized PKC activation has been described to induce colitis and its inhibition reduces the protection against the development of this inflammatory process (37), the results of the present study suggest that high dietary DHA intake can contribute to epithelial barrier impairment through the PKC pathway.

Oxidative stress is also linked to signaling pathways involved in the regulation of TJ permeability. Oxidants increase intracellular Ca\(^{2+}\) levels (38) and activate PKC, which in turn is inhibited by antioxidants (39). In rat hepatocytes, Sato et al. (40) reported that oxidative stress induced by hydrogen peroxide increases intracellular Ca\(^{2+}\) concentration by a mechanism mediated by PLC activation. Moreover, we previously observed that the effect of DHA enrichment on epithelial barrier disruption was mediated by the formation of hydrogen peroxide (4), thus suggesting PLC/Ca\(^{2+}\)/PKC activation, either through changes in membrane composition or through the formation of reactive oxygen and nitrogen species. In epithelial cells, TJ dysfunction induced by oxidative stress is also associated with Tyr phosphorylation of occludin and ZO-1, their dissociation from the apical cytoskeleton, and redistribution from the junctional area (41). Furthermore, we previously described the involvement of Tyr phosphorylation in epithelial barrier disruption induced by DHA (4).

We have reported that COX-2 activity and therefore the formation of PGE\(_2\) is reduced during Caco-2 cell differentiation (42). Moreover, the addition of PGE\(_2\) to differentiated cells increased paracellular permeability. Therefore, no effect of COX inhibition was expected in differentiated control cells. In DHA-treated cells, the data revealed the capacity of indomethacin and AA861 to prevent the effect of this fatty acid on D-mannitol flux. DHA enrichment results in the formation of PGE\(_3\), TXA\(_3\), and
LTB₄ instead of the PGE₂, TXA₂, and LTB₄ formed from arachidonic acid (43). The eicosanoids produced from (n-3) PUFA generate less proinflammatory activity than those derived from arachidonic acid (44). Nevertheless, the prevention of DHA effects exerted by indomethacin and AA861 indicates that the eicosanoids from DHA may also contribute to epithelial barrier dysfunction. In fact, preliminary results obtained in our laboratory (R. Ferrer, unpublished data) showed that PGE₃ can increase paracellular permeability but to a lesser extent than PGE₂ (42). Chen and Nilsson (45) found a significant increase in 22:5(n-3) incorporation after membrane EPA enrichment, which was explained as the capacity of the cells to limit the accumulation of EPA (an eicosanoid precursor) by converting it to a less active fatty acid such as 22:5(n-3). In contrast, Usami et al. (46) argued that the formation of eicosanoids cannot explain the mechanism of TJ regulation by DHA. However, they studied paracellular permeability in undifferentiated Caco-2 cells, which show higher COX-2 expression and activity than differentiated cultures, as we have previously described (42).

There is a direct relationship between the amount of occludin expressed and TER values (47). Moreover, highly phosphorylated occludin on Ser and Thr residues is selectively concentrated at the TJ (48,49). In mammalian cells, the overall phosphorylation of occludin is reduced during TJ disassembly triggered by a switch to low Ca²⁺ concentration or treatment with phorbol esters (50). Clarke et al. (51) associated the role of PKC with the activation of Ser/Thr phosphatases, thus reducing occludin phosphorylation. Moreover, the activation MLCK induces the phosphorylation of the regulatory myosin light chain II and thus the contraction of the subapical acto-myosin ring, leading to an increase in paracellular permeability (12). Internalization of TJ proteins by an actomyosin-dependent process is a common mechanism for modification of TJ permeability in response to physiological and physiopathological stimuli (52). Ca²⁺ depletion causes internalization of both adherens and TJ proteins by clathrin-mediated endocytosis (53), which requires MLC phosphorylation by MLCK (23). Therefore, our results of immunogold staining showing the appearance of cytosolic occludin far from the TJ (thus suggesting the internalization of this protein) are consistent with PKC activation and regulatory myosin light chain II phosphorylation in cells treated with DHA. Similar results were previously obtained in immunofluorescence occludin and ZO-1 localization for cultures maintained for 10 d with DHA (4).

Our results confirm previous data suggesting the involvement of the PLC/Ca²⁺/PKC pathway in TJ regulation and demonstrate the contribution of this signaling pathway, as well as the arachidonic acid cascade, to TJ disassembly induced by DHA enrichment. Moreover, the role of oxidative stress induced by DHA in the activation of the PLC/Ca²⁺/PKC pathway and TJ protein internalization should also be considered.

**Acknowledgments**

M.M. and R.F. designed the research (project conception, development of overall research plan, and study oversight). S.R.P. and N.C. conducted the microscopic research and S.R.P. conducted the experiments on paracellular permeability and the statistical analysis. S.R.P., N.C., and R.F. wrote the paper and M.M. and R.F. had primary responsibility for final content. All authors read and approved the final manuscript.