Lipid peroxidation induced by DHA enrichment modifies paracellular permeability in Caco-2 cells: protective role of taurine[1]

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Abstract **Dietary enrichment with docosahexaenoic acid (DHA) has numerous beneficial effects on health. However, the intake of high doses of polyunsaturated fatty acids can promote lipid peroxidation and the subsequent propagation of oxygen radicals. The purpose of this study was to evaluate the effect of DHA on lipid peroxidation and tight junction structure and permeability in Caco-2 cell cultures. Moreover, the effects of taurine, a functional ingredient with antioxidant properties, were also tested. Differentiated Caco-2 cell monolayers were maintained in DHA-supplemented conditions with or without added taurine. Incubation with 100 μM DHA increased lipid peroxidation and paracellular permeability, in parallel with a redistribution of the tight junction proteins occludin and ZO-1. Taurine partially prevented all of these effects. The participation of reactive oxygen and nitrogen species in increased paracellular permeability was also examined using various agents that modify the formation of superoxide radical, hydrogen peroxide, nitric oxide, and peroxynitrite. We conclude that hydrogen peroxide and peroxynitrite may be involved in the DHA-induced increase in paracellular permeability and that the protective role of taurine may be in part related to its capacity to counteract the effects of hydrogen peroxide.—** Roig-Pérez, S., F. Guardiola, M. Moretó, and R. Ferrer. **Lipid peroxidation induced by DHA enrichment modifies paracellular permeability in Caco-2 cells: protective role of taurine. *J. Lipid Res.* 2004.** 45: **1418–1428.**

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Dietary supplementation with docosahexaenoic acid (DHA, 22:6 n-3) is an increasing practice as a result of the role described for n-3 PUFAs in several physiological processes, especially in brain, retina, heart, and blood, during postnatal development (1). Several nutritional studies reveal that the intake of these fatty acids by wide communities does not meet their physiological needs, because n-3 PUFAs (eicosapentaenoic acid and DHA) are found in significant amounts only in fish and fish oils and in humans the conversion from their precursor (α-linolenic acid, 18:3 n-3) is not particularly efficient (1). Nevertheless, the intake of PUFAs can also be deleterious in that they promote lipid peroxidation and the subsequent propagation of oxygen radicals (2–4), which are involved in the pathogenesis of various gastrointestinal disorders (5, 6).

The structural integrity of the intestinal epithelium is maintained by intercellular junctions, of which tight junctions are the primary junctions involved in the regulation of paracellular permeability to nutrients, electrolytes, and water. Occludin and ZO-1 are two integral structural components of tight junctions with a crucial role in maintaining these epithelial functions (7). PUFAs have been shown to influence tight junction permeability by modifying either cyclooxygenase and lipooxygenase products (8) or occludin localization (9). Nevertheless, Rao et al. (10) claimed that oxidative stress is the main factor responsible for the loss of epithelial barrier function through tyrosine phosphorylation and redistribution of tight junction proteins.

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Abbreviations: BHT, butylated hydroxytoluene; CAT, catalase; CPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DDC, diethyldithiocarbamate; DEF, deferoxamine; DHA, docosahexaenoic acid; GEN, genistein; iNOS, inducible nitric oxide synthase; L-NIL, L-N^6-(1-iminoethyl)lysine; mBCl, monochlorobimane; MDA, malondialdehyde; $^{\bullet}NO$, nitric oxide; $O_2^{\bullet-}$, superoxide; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid; TEP, 1,1,3,3-tetraethoxypropane; TER, transepithelial electrical resistance; Tiron, 4,5-dihydroxy-1,3-benzene-disulfonic acid; UR, urate.

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To design an optimal nutritional strategy, enough antioxidants have to be provided to counteract oxidation in the organism. Machlin and Bendich (11) pointed out the crucial balance between free radical generation and antioxidant defense as a factor in disease prevention. Reactive oxygen species and reactive nitrogen species (ROS and RNS, respectively) include free radicals (e.g., superoxide, nitric oxide, and hydroxyl radicals) and other reactive species (e.g., hydrogen peroxide, peroxynitrite, and hypochlorous acid) produced in the organism as a result of aerobic metabolism. Excess production of hydrogen peroxide and the free radicals superoxide and nitric oxide ($O_2^{\bullet -}$ and $^{\bullet}NO$, respectively) is related to cell and tissue pathology (12–14). In fact, many of these deleterious effects are attributed to peroxynitrite, a strong biological oxidant that is formed from the reaction between $^{\bullet}NO$ and $O_2^{\bullet -}$ (15, 16).

Some amino acids (e.g., arginine, citrulline, glycine, taurine, and histidine), small peptides (e.g., glutathione and carnosine), and nitrogenous metabolites (e.g., creatine and uric acid) directly scavenge free radicals. The tripeptide GSH is the principal intracellular low-molecular-weight thiol and plays a central role in protecting cells from oxidative and chemical damage. GSH combines with free radicals and their metabolites to form conjugates that are rapidly excreted from the cells (17). Taurine, the most abundant intracellular amino acid, is a functional ingredient with antioxidant properties able to inhibit the expression of inducible nitric oxide synthase (iNOS) in various cell types, thus reducing $^{\bullet}NO$ production (18, 19).

We aimed to evaluate the effects of enriching the intestinal epithelium with DHA on the intestinal epithelial barrier function and to test the capacity of taurine to prevent these effects. To address this issue, lipid peroxidation and enterocyte tight junction structure and permeability were tested in Caco-2 cells enriched with DHA with or without added taurine. Moreover, the participation of ROS and RNS in paracellular permeability regulation was also determined. Cultures of differentiated Caco-2 cells form a highly polarized epithelium with many of the properties of the intestinal villous absorptive cells (20) and constitute an in vitro experimental model, currently used to evaluate intestinal epithelial paracellular permeability (8, 21–23).

MATERIALS AND METHODS

Materials

DMEM, nonessential amino acids, penicillin, streptomycin, L-glutamine, trypsin, FBS, BSA, taurine (cell culture tested), DHA, erucic acid (C22:1 n-9), α-tocopherol (vitamin E), genistein (GEN), superoxide dismutase (SOD) from bovine erythrocytes, catalase (CAT) from bovine liver, butylated hydroxytoluene (BHT), EDTA, 1,1,3,3-tetraethoxypropane (TEP), 2-thiobarbituric acid (TBA), D-glucose, HEPES, 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron), diethyldithiocarbamate (DDC), L-N^6-(1-iminoethyl)lysine (L-NIL), deferoxamine (DEF), urate (UR), GSH, and other chemicals were supplied by Sigma (St. Louis, MO). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) and monochlorobimane (mBCl) were purchased from Molecular Probes (Eugene, OR). D-[2-^{3}H]mannitol (specific activity 30 Ci/mmol) was from ARC (St. Louis, MO). TCA, cyclohexane (spectrophotometric grade), and methanol (analytical grade) were from Panreac (Barcelona, Spain), and paraformaldehyde was from Merck (Darmstadt, Germany). Tissue culture supplies, including Transwells, were obtained from Costar (Cambridge, MA).

Cell culture

Caco-2 cells were kindly provided by Dr. David Thwaites from the Department of Physiological Sciences, University of Newcastle upon Tyne (UK). The cells (passages 107–116) were routinely grown in 75 or 150 cm^2 plastic flasks at a density of 5×10^4 cells/cm^2 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 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a modified atmosphere of 5% CO_2 in air. For growth on filters, cells were seeded at a density of 4×10^5 cells/cm^2 onto polycarbonate filters (Transwells; 12 mm diameter) with a pore size of 0.4 μm. Growth medium was replaced twice per week and on the day before the experiment. Cell confluence was confirmed by microscopic observation and transepithelial electrical resistance (TER) determination. All experiments were performed in Transwells, except cell viability and lipid peroxidation evaluation, which were performed in flasks.

Experimental design

After reaching confluence (days 6–7 after seeding), cells were grown for 10–14 additional days in control conditions or in medium supplemented with taurine (10 mM), DHA (10–100 μM), or DHA plus taurine (100 μM and 10 mM, respectively). DHA enrichment was performed according to the methodology described by Dias, Borkowski, and Parsons (24), which allows DHA incorporation into the cells. To evaluate ROS, RNS, and tyrosine phosphorylation involvement in DHA effects and cellular GSH concentration, experiments were also performed after a period of 17–21 days in culture but monolayers were supplemented for only the last 24 h before the experiment. In Transwell experiments, supplementation was performed at the apical side.

DHA and taurine enrichment

DHA was solubilized in the culture medium according to Dias, Borkowski, and Parsons (24). The fatty acid was dissolved in ethanol (containing 4 mg/l BHT) and added, under constant stirring at 56°C, to DMEM containing FBS to achieve a final 3:1 (mol/mol) fatty acid-albumin ratio. The albumin-bound fatty acid solution was filter sterilized by passage through a 0.2 μm membrane filter before adjusting the volume with complete DMEM to the final desired DHA concentration. Taurine was added to the medium and filtered in the same way as the DHA. Control and taurine-maintained cells were exposed to the same final BHT and ethanol concentration as DHA-supplemented medium. The range of DHA concentrations tested (up to 100 μM) are those usually tested in similar fatty acid enrichment experiments in Caco-2 cells (8, 24–26) and are lower than that considered a physiological concentration (800 μM) in similar experimental conditions (27).

The fatty acid composition of the incubation medium was analyzed by gas chromatography according to the method described by Ferrer et al. (28), and the results indicate a 4.5-fold enrichment in DHA.

To further analyze the role of oxidative stress in DHA-induced effects, additional experiments were performed in monolayers maintained for 10–14 days with 100 μM erucic acid (C22:1 n-9) or with 1 mM α-tocopherol added to DHA-enriched medium. Erucic acid was added to the incubation medium in the same way

as DHA. α-Tocopherol was incorporated into the medium dissolved in ethanol and filtered in the same way as the other enriched media.

Cell viability

Cell viability was estimated with ethidium bromide/acridine orange staining as described by Parks et al. (29) and expressed as the percentage of viable cells, normalized to the values obtained in control conditions.

Lipid peroxidation

Dienes and secondary oxidation products. Lipid ultraviolet absorption was used to follow lipid oxidation. Absorbance at 235 nm (mainly attributable to conjugated dienes) and 270 nm (mainly attributable to secondary oxidation products) was measured according to Grau et al. (30), with some modifications. After trypsinization, 200 mg of pelleted cells was resuspended in 1 ml of 0.02% EDTA and 1.6% NaCl aqueous solution. The suspension was mixed with 4 ml of chloroform-methanol (2:1), homogenized for 2 min in a glass with a Teflon pistil, and centrifuged (10 min, 400 *g*). The chloroform phase was filtered through anhydrous sodium sulfate (Whatman number 1), which was washed twice with 2 ml of chloroform. The organic solvent was removed under a nitrogen stream. The extracted lipid fraction was then dissolved in 2 ml of cyclohexane, and absorbance was determined in a double-beam spectrophotometer (Shimadzu UV-160A) at 235 and 270 nm. The spectrophotometric conditions were as follows: spectrum range, 200–300 nm [as suggested by Baron et al. (31)]; scan speed, 480 nm/min and 1 cm quartz cuvettes. The results are expressed as absorbance per microgram of protein. Protein was determined using the Bio-Rad protein assay, with BSA as the standard.

TBA value. Malondialdehyde (MDA) formation was determined as described by Grau et al. (32), with some modifications. After trypsinization, 1 g of pelleted cells was resuspended in 0.5 ml of 0.3% EDTA and gently mixed. A total of 2.5 ml of 0.8% BHT in hexane was then added to the suspension and gently mixed again. Finally, 4 ml of 5% aqueous TCA was added, and the suspension was homogenized in glass with a Teflon pistil, as described above. After centrifugation (5 min, 1,400 *g*), the hexane top layer was discarded and the bottom layer was filtered (Whatman number 1) into a 5 ml volumetric flask and adjusted to the volume with TCA (5% aqueous). An aliquot of 2.5 ml was pipetted into a screw-capped tube and mixed with 1.5 ml of 0.8% aqueous TBA (final pH 0.9). The reaction mixture was then incubated for 30 min at 70°C with gentle agitation, cooled in an ice bath for 5 min, and tempered for 45 min at room temperature. Thereafter, the sample was analyzed by third-derivative spectrophotometry (Shimadzu UV-160A) at 521.5 nm against a blank containing 2.5 ml of 5% aqueous TCA and 1.5 ml of 0.8% aqueous TBA. Spectrophotometric conditions were as follows: spectrum range, 400–650 nm; scan speed, 480 nm/min; derivative difference setting ($\Delta\lambda$), 21 nm. TBA values (expressed as nanograms of MDA per milligram of protein) were calculated on the basis of the calibration curve ($Y = 4.90 \times 10^{-3} + 9.01 \times 10^{-3} X$, where Y is peak height at 521.5 nm expressed in arbitrary units, as printed on the instrument chart, and X is the MDA concentration in nanograms per milliliter of reaction mixture). The calibration curve was constructed as described by Botsoglou (33), using TEP as the MDA precursor. All procedures were performed in attenuated light conditions.

Sucrase activity

Sucrase, a brush border marker enzyme, was assayed according to Dahlqvist (34) and expressed as milli-International Units per milligram of protein.

Paracellular permeability

Paracellular permeability was evaluated by measuring TER and unidirectional apical-to-basal D-mannitol fluxes.

TER. TER was determined as described by Hidalgo, Raub, and Borchardt (35). Monolayers grown in Transwells were gently washed by sequential transfer through four beakers containing 500 ml of modified Krebs buffer (room temperature) containing 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 10 mM D-glucose, and 10 mM HEPES/Tris (pH 7.4). Monolayers were then placed in culture wells containing 1.5 and 0.75 ml of modified Krebs buffer in the basal and apical compartments, respectively, and TER was determined using a Millicell-ERS voltohmmeter (Millipore, Bedford, MA). Results were expressed as ohms per square centimeter of monolayer surface area. The resistance of the supporting membrane in Transwells was subtracted from all readings before calculations.

Unidirectional D-mannitol fluxes. Paracellular permeability was determined according to Thwaites et al. (36). After TER determination, apical medium was replaced by the same volume of modified Krebs buffer containing 0.5 μCi/ml D-[2-^{3}H]mannitol (specific activity 30 Ci/mmol), and cells were incubated for 5 min at 37°C. At the end of the incubation, basal medium was withdrawn and radioactivity was counted in a scintillation counter (Packard 1500 Tri-carb^R).

Pharmacological modification of ROS and RNS concentrations

Several agents were used to determine ROS and RNS involvement in DHA-induced effects on D-mannitol fluxes and TER. The concentrations were selected according to published data. The following agents were tested: SOD (500 U/ml), which catalyzes the conversion of $O_2^{\bullet -}$ to H_2O_2; CAT (1,000 U/ml), which removes H_2O_2; Tiron (100 μM), a nonenzymatic $O_2^{\bullet -}$ scavenger; DDC (10 mM), an inhibitor of SOD; CPTIO (100 μM), a $^{\bullet}$NO scavenger; L-NIL (1 mM), an inhibitor of iNOS; and DEF (1 mM), UR (1 mM), and GSH (10 mM), three peroxynitrite scavengers.

GEN effects

To test the involvement of DHA-induced oxidative stress in tight junction phosphorylation through tyrosine kinase activity, GEN (300 μM), a tyrosine kinase inhibitor, was added to DHA- and DHA plus taurine-enriched media.

Cellular GSH

GSH concentration was determined according to the mBCl assay described by Menconi et al. (37). This reagent specifically forms a stable, strongly fluorescent adduct with GSH in a reaction catalyzed by cellular GSH *S*-transferases. After cell growth on Transwells for 24 h in control, DHA, taurine, and DHA plus taurine conditions, the apical medium was replaced by 200 μl of DMEM (without FBS) containing 1 mM mBCl. The monolayers were then incubated for 20 min at 37°C, the supernatant was discarded, and the monolayers were washed with PBS (pH 7.4) at 37°C. The filters were removed from the inserts, placed into 1 ml of 1% Triton X-100, and microfuged at 10,000 *g* for 5 min at room temperature. The supernatant was assayed spectrofluorometrically at an excitation wavelength of 393 nm (slit width = 5 nm) and an emission wavelength of 474 nm (slit width = 10 nm). Results are expressed as light units per square centimeter.

Occludin and ZO-1 immunofluorescent staining

Caco-2 monolayers grown in Transwells were fixed in 3% paraformaldehyde and 2% sucrose in 0.1 M PBS (pH 7.4) for 15 min at room temperature. Cells were washed for 10 min in 10 mM PBS containing 20 mM glycine (PBS-glycine) and permeabilized with 0.2% Triton X-100 for 10 min at 37°C. Cells were washed

TABLE 1. Cell viability and sucrase activity in the different incubation conditions

Incubation Condition	Cell Viability		Sucrase Activity
	10–14 days	24 h	
	%		*mIU/mg protein*
Control	100.0 ± 1.9b	100.0 ± 1.32a,b	36.3 ± 2.2
Taurine	113.3 ± 1.7a	105.0 ± 1.39a	35.5 ± 3.1
DHA	69.7 ± 3.9d	94.1 ± 1.80b	42.0 ± 1.6
DHA + taurine	84.8 ± 0.7c	98.1 ± 2.86a,b	40.1 ± 3.1

Viability of cells grown for 10–14 days or 24 h and sucrase activity of cells grown for 10–14 days in control conditions or in media enriched with taurine (10 mM), docosahexaenoic acid (DHA; 100 μM), or DHA plus taurine (100 μM and 10 mM, respectively). Viability results are normalized to control values (%). Results are expressed as means ± SE of nine cultures. Mean values with different letters are significantly different ($P < 0.05$).

three times in PBS-glycine and blocked for 20 min in PBS-glycine containing 1% BSA (incubation solution). As primary antibodies, mouse monoclonal anti-occludin (1:500 dilution; Zymed, South San Francisco, CA) and rabbit polyclonal anti-ZO-1 (1:250 dilution; Zymed) were used. Cells were incubated with the antibodies for 1 h at 37°C and washed three times in PBS-glycine for 5 min at room temperature. Monolayers were then incubated for 1 h at 37°C with the secondary antibodies [Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit $F(ab')_2$, 2 μg/ml; Molecular Probes]. Finally, the cells were incubated for 10 min at 37°C in PBS, mounted in Mowiol (Calbiochem, San Diego, CA), and examined with a confocal laser scanning microscope (TCS 4D; Leica Lasertechnik, GmbH). Images were taken using a 63× (numerical aperture 1.3, phase 3, oil) Leitz Plan-Apochromatic objective.

Statistics

Data reported are means ± SE. ANOVA was followed by the Scheffe's multiple comparison test to detect significant differences between treatments. Student's *t*-test was used to compare mean values from two groups. $P < 0.05$ was considered significant.

RESULTS

Cell viability and sucrase activity

Enrichment of the incubation medium with DHA resulted in a decrease in cell viability after 10–14 days, and this effect was partially prevented by coincubation with taurine (**Table 1**). Moreover, incubation with taurine alone increased cell viability. Incubation for 24 h with DHA, taurine, and DHA plus taurine did not significantly modify this variable with respect to controls, whereas significant differences were detected between DHA and taurine conditions. Sucrase, a marker of cell differentiation, was not modified after 10–14 days in culture in any of the incubation conditions tested (Table 1).

Lipid peroxidation

Incubation with DHA enhanced the production of dienes (measured in terms of lipid absorbance at 235 nm) and secondary oxidation products (measured in terms of lipid absorbance at 270 nm) (**Fig. 1**). The addition of taurine to DHA-enriched conditions partially prevented the formation of dienes (15.5%) but not that of secondary oxidation products. MDA was not detected in control cells or in cells incubated with taurine (detection limit, 0.029 ng MDA/mg protein). MDA was increased by DHA, and its formation was prevented, in part, by the addition of taurine (**Table 2**). MDA was also analyzed in all of the incubation media but was not detected.

Paracellular permeability

TER and D-mannitol fluxes were determined at several DHA concentrations (10–100 μM). Incubation with taurine alone or with low DHA concentrations (10–50 μM) had hardly any effect, whereas 100 μM DHA significantly increased D-mannitol fluxes (**Fig. 2**) and reduced TER (**Fig. 3**). Therefore, 100 μM DHA is the lowest concentration with significant changes in paracellular permeability assessed, both from TER and D- mannitol fluxes. Cotreatment of cell monolayers with 100 μM DHA plus taurine (10 mM) significantly prevented the effects of DHA, both on D-mannitol fluxes and TER, to values not significantly different from DHA concentrations lower than 100 μM or from controls. The inclusion of 1 mM α-tocopherol in 100 μM DHA-enriched medium (**Table 3**) produced a similar effect to the inclusion of taurine. Monolayers incubated with a monounsaturated fatty acid (erucic acid, 100 μM) showed no effect on D-mannitol fluxes or on TER (Table 3).

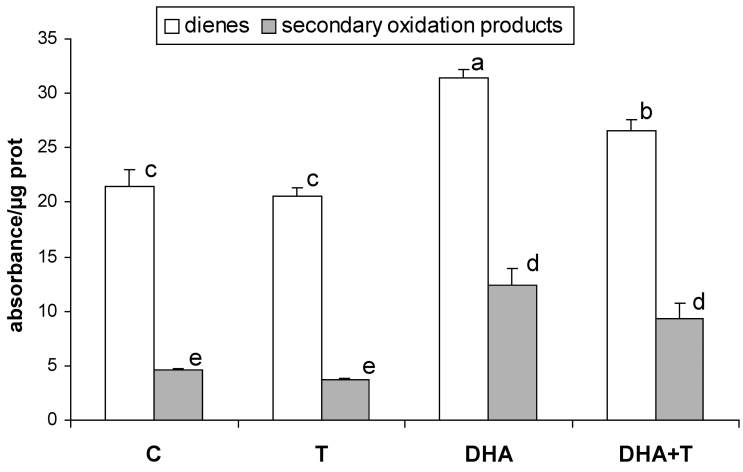

Fig. 1. Conjugated dienes and secondary oxidation products (lipid ultraviolet absorbance at 235 and 270 nm, respectively) of cells grown for 10–14 days after reaching confluence in control conditions (C) or in media enriched with taurine (T; 10 mM), docosahexaenoic acid (DHA; 100 μM), or DHA plus taurine (DHA+T; 100 μM and 10 mM, respectively). Results are expressed as means ± SE of three cultures. Mean values with different letters are significantly different ($P < 0.05$). prot, protein.

TABLE 2. 2-Thiobarbituric acid values in the different incubation conditions

Incubation Condition	Malondialdehyde
	ng/mg protein
Control	Not detected
Taurine	Not detected
DHA	0.335 ± 0.04a
DHA + taurine	0.165 ± 0.02b

2-Thiobarbituric acid values of cells grown for 10–14 days after reaching confluence in control conditions or in media enriched with taurine (10 mM), DHA (100 μM), or DHA plus taurine (100 μM and 10 mM, respectively). Results are expressed as means ± SE of three cultures. Mean values with different letters are significantly different ($P < 0.05$). The detection limit of the analysis was 0.029 ng malondialdehyde/mg protein.

Occludin and ZO-1 immunofluorescent staining

The effects of DHA and DHA plus taurine on occludin and ZO-1 localization in Caco-2 cell epithelial monolayers were analyzed by immunofluorescent staining. Cells cultured in control and taurine conditions showed occludin and ZO-1 only in the plasma membrane and colocalized in the tight junction (**Fig. 4**, horizontal *x-y* sections). DHA treatment resulted in discontinuous staining for occludin and ZO-1 at the cell borders, with adjacent diffuse intracellular staining, granular appearance, and reduced colocalization. The disruption of occludin and ZO-1 distribution induced by DHA was prevented by taurine.

The images of the vertical sections (Fig. 4, *x-z* sections) show that both proteins colocalized at the apical side of the cells. In DHA-incubated monolayers, the images reveal no significant loss of cells and a more disorganized pattern that reverts to the normal state by the inclusion of taurine.

Cellular GSH

GSH was quantified in cells incubated for 24 h with DHA or DHA plus taurine (100 μM and 10 mM, respectively). DHA reduced intracellular GSH by 58%, and the addition of taurine significantly prevented this effect (**Fig. 5**).

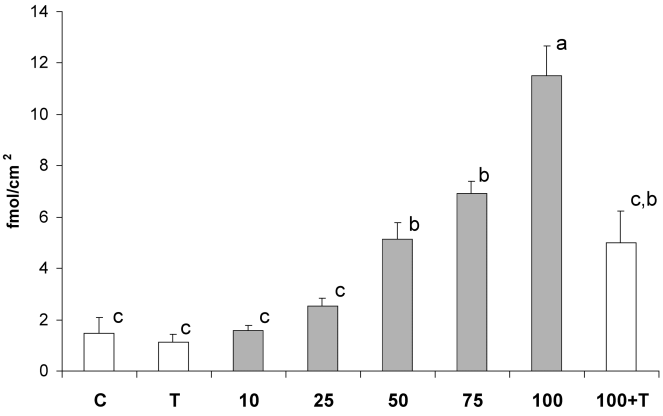

Fig. 2. D-Mannitol (25 nM) apical-to-basal fluxes in monolayers grown in Transwells for 10–14 days after reaching confluence in control conditions (C) or in media enriched with apical taurine (T; 10 mM), different DHA concentrations (10–100 μM; shaded bars), or DHA plus taurine (100+T; 100 μM and 10 mM, respectively). Results are expressed as means ± SE of 7–13 filters. Mean values with different letters are significantly different ($P < 0.05$).

ROS and RNS involvement in DHA-induced changes on paracellular permeability

The roles of $O_2^{\bullet -}$, H_2O_2, $^{\bullet}NO$, and peroxynitrite as mediators of the DHA-induced increase in epithelial permeability were tested in cells incubated for 24 h with DHA or with DHA plus taurine in the presence of agents able to modify ROS and RNS concentrations. The incubation time was reduced to 24 h to avoid the possible effects of these agents on cell viability. CAT, Tiron, L-NIL, and GSH partially prevented the effects of DHA on D-mannitol fluxes, whereas CPTIO and UR completely prevented them. SOD and DDC had no effect and DEF enhanced DHA effects (**Fig. 6A**). For SOD, L-NIL, DEF, GSH, and UR, D-mannitol fluxes were well correlated with changes in TER (Fig. 6B), whereas an increase for DDC and no effect for the other agents tested were detected.

In the presence of taurine (**Fig. 7A**), CAT, L-NIL, CPTIO, and UR significantly surpassed the preventive effects of taurine on D-mannitol fluxes, attaining control values except for CPTIO. Regarding TER, only the inclusion of GSH improved taurine effects, whereas the effects of CAT, DDC, and UR did not differ from that of control or DHA plus taurine, and DEF showed no preventive effect of taurine.

GEN effects

To determine the involvement of DHA-induced oxidative stress on tyrosine kinase activity, GEN, a tyrosin kinase inhibitor, was tested. As shown in Fig. 6, the inclusion of GEN in DHA-enriched medium prevented, in part, the changes in D-mannitol fluxes and prevented, completely, the changes in TER induced by the fatty acid. In the presence of taurine (Fig. 7), the inclusion of this agent had no additional effect on D-mannitol fluxes and surpassed the preventive effect of taurine on TER.

DISCUSSION

We have investigated in Caco-2 cell monolayers the effects of DHA on cellular oxidation and tight junction structure and permeability and the role of taurine, a functional ingredient with many physiological functions including antioxidant properties (38), in the prevention of these effects. Our results show that incubation of monolayers with DHA stimulated the formation of dienes (lipid absorbance at 235 nm), secondary oxidation products (lipid absorbance at 270 nm), and MDA, which was partially prevented by taurine. These results are consistent with the observations of Pasantes-Morales and Cruz (39) that high concentrations of taurine (25 mM) can decrease MDA formation in rod outer segments and with those of Hwang, Hour, and Cheng (40), who observed that dietary enrichment with oxidized fish oil increases MDA formation, which is also prevented by taurine.

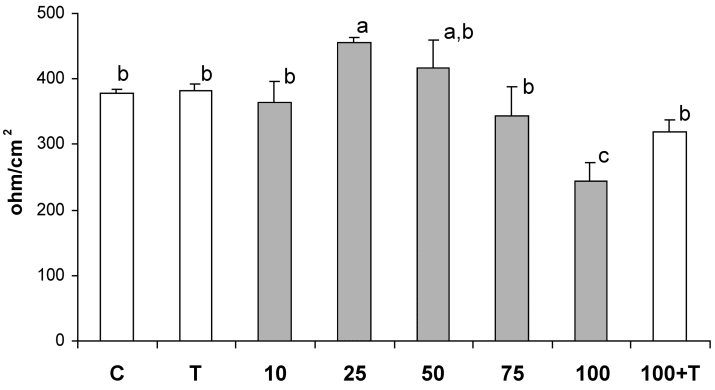

Fig. 3. Transepithelial electrical resistance (TER) of monolayers grown in Transwells for 10–14 days after reaching confluence in control conditions (C) or in media enriched with apical taurine (T; 10 mM), different DHA concentrations (10–100 μM; shaded bars), or DHA plus taurine (100+T; 100 μM and 10 mM, respectively). Results are expressed as means ± SE of 7–39 filters. Mean values with different letters are significantly different ($P < 0.05$).

Nano et al. (26) and Fybe and Abbey (41) attributed to MDA the cytotoxic effect of n-3 PUFAs on Caco-2 cells and J774 macrophages, respectively. The decrease in cell viability detected in the present study in DHA-enriched conditions is in the range of the results obtained by other authors with cell cultures (3, 26). The addition of taurine increased the viability of control and DHA-incubated cells, which can be related to a direct effect of this amino acid on cell viability or to the capacity of this amino acid to reduce the formation of lipid peroxidation products, mainly MDA. This result is in agreement with the dose-dependent increase in the viability of human lymphoblastoid cells incubated with taurine observed by Gaull, Wright, and Tallan (42), attributed to the capacity of taurine to reduce the production of ROS.

D-Mannitol fluxes were found to increase with DHA concentration in the incubation medium. However, the expected inverse correlation between changes in D-mannitol fluxes and TER was observed only for the highest DHA concentration tested (100 μM) and for cultures incubated with DHA plus taurine. In this sense, a slight but significant increase in TER, without changes in D-mannitol fluxes, was detected for 25 μM DHA. Moreover, the effect on D-mannitol fluxes was higher than on TER (100 μM DHA, 7.8- and 1.54-fold versus control, respectively). The lowest sensitivity of TER was also detected in experiments designed to determine the involvement of ROS and RNS on the DHA-induced increase in paracellular permeability. To explain this apparently functional dissociation that occurs in some experimental conditions, Matter and Balda (43) have suggested that these two 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 tight junction ion selectivity, whereas D-mannitol permeability is a variable taken over a longer period of time that allows the quantification of slow diffusion across tight junctions and the determination of the size selectivity of the paracellular diffusion barrier.

The results showing similar changes in D-mannitol fluxes and TER after either 10–14 days or 24 h of incubation with DHA (Figs. 2 and 3 vs. Fig. 6) rule out the possibility that the increase in paracellular permeability is attributable to dead cells in the monolayer, because cell viability after 24 h was not affected by fatty acid inclusion. Moreover, incubation of the cells with taurine alone increased cell viability, which was not reflected in D-mannitol fluxes or in the TER results.

There is evidence that occludin plays a key role in epithelial barrier function (44, 45) and that ZO-1 binding to occludin is required for the maintenance of tight junction integrity (7). In control and taurine conditions, colocalization of occludin and ZO-1 in the tight junctions showed the typical honeycomb pattern described in epithelia (9, 46). DHA incubation increased the paracellular permeability, and this was accompanied by a loss of occludin and ZO-1 immunoreactivity in the tight junction and an increase in cytosolic signal, probably reflecting the release of these junctional proteins to the cytosol, as observed by Roche et al. (9) and Usami et al. (8) after Caco-2 cell enrichment with PUFAs. Although oxidative stress can cause tight junction disruption as a consequence of the dissociation of the occludin-ZO-1 complex (10), the images of cells incubated with DHA obtained in the present study showing slight occludin and ZO-1 colocalization in the cytosol support the view that these proteins are rather released to the cytosol-forming complexes.

Several hypotheses have tried to explain the effects of PUFAs on paracellular permeability, such as changes in fatty acid membrane composition, which in turn modifies

TABLE 3. D-Mannitol fluxes and TER of cells grown in the different incubation conditions

Incubation Condition	D-Mannitol Fluxes	TER
	%	
Control	100 ± 28.1a	100 ± 1.45a
DHA	573 ± 50.8b	62.4 ± 4.48b
Erucic acid	142 ± 17.7a	89.9 ± 2.28a
DHA + taurine	204 ± 17.3a	84.6 ± 4.35a
DHA + α-tocopherol	174 ± 23.1a	96.7 ± 2.7a

D-Mannitol (25 nM) apical-to-basal fluxes and transepithelial electrical resistance (TER) in monolayers grown in Transwells for 10–14 days after reaching confluence in control conditions or in media enriched with DHA (100 μM), erucic acid (100 μM), DHA plus taurine (100 μM and 10 mM, respectively) and DHA plus α-tocopherol (100 μM and 1 mM, respectively). The results are normalized to control values (%) and expressed as means ± SE of three filters. Mean values with different letters are significantly different ($P < 0.05$).

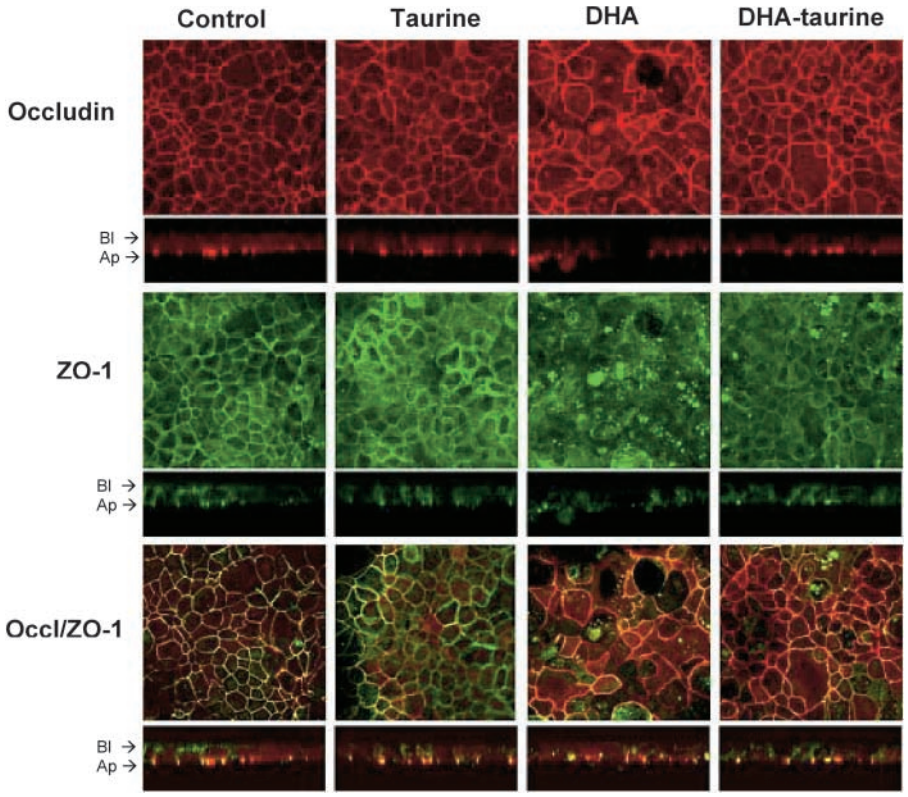

Fig. 4. Confocal analysis of monolayers grown in Transwells for 10–14 days after reaching confluence in control conditions or in media enriched with apical taurine (10 mM), DHA (100 μM), or DHA plus taurine (DHA-taurine; 100 μm and 10 mM, respectively) and stained for occludin and ZO-1. Shown are the en face (*x-y*) sections and the corresponding vertical (*x-z*) sections. Arrows in the *x-z* sections indicate the apical (Ap) and basolateral (Bl) membranes of the cells.

protein interactions (9), changes in eicosanoid production, protein kinase C activity (8, 25), or increased oxidative stress (10, 47–49). However, the correlation between fatty acid enrichment, lipid peroxidation, and epithelial barrier function has not been considered to date.

We have previously reported (50) that the culture conditions assayed here (10–14 days, 100 μM DHA) result in a 16.3-fold DHA enrichment of the Caco-2 cell brush border membrane. This is at the expense of n-6 PUFAs and without changes in the polyunsaturated versus saturated fatty acid ratio (50). Calderon et al. (51) described no changes in tight junction permeability after the modification of brush border membrane fatty acid composition. Erucic acid, structurally similar to DHA but less susceptible to lipid peroxidation, was also tested. The lack of effect of this fatty acid on D-mannitol fluxes and TER supports the hypothesis that oxidative stress is involved in the DHA-induced increase in paracellular permeability. In addition, the capacity of α-tocopherol, the main antioxidant of the cell membrane (52), to prevent the DHA-induced increase in paracellular permeability confirms the role of oxidative stress in tight junction regulation and the antioxidant properties attributed to taurine. We next investigated the involvement of ROS and RNS in the changes in paracellular permeability induced by DHA. The decrease observed in intracellular endogenous GSH after 24 h of DHA incubation, and the protective effect of taurine, suggest the involvement of ROS and/or RNS generation in these experimental conditions.

The addition of SOD, which catalyzes the conversion of $O_2^{\bullet -}$ into H_2O_2, did not afford any protection against the

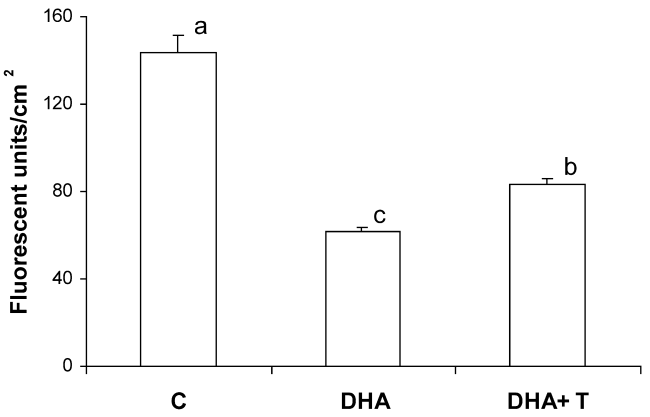

Fig. 5. GSH values in monolayers maintained in Transwells for 10–14 days after reaching confluence and incubated for 24 h before the experiment in control conditions (C) or in media enriched with apical DHA (100 μM) or DHA plus taurine (DHA+T; 100 μM and 10 mM, respectively). Results are expressed as means ± SE of nine filters. Mean values with different letters are significantly different ($P < 0.05$). GSH levels in taurine-enriched conditions were not different from control values (138 ± 20, n = 2 filters; $P \geqslant 0.05$).

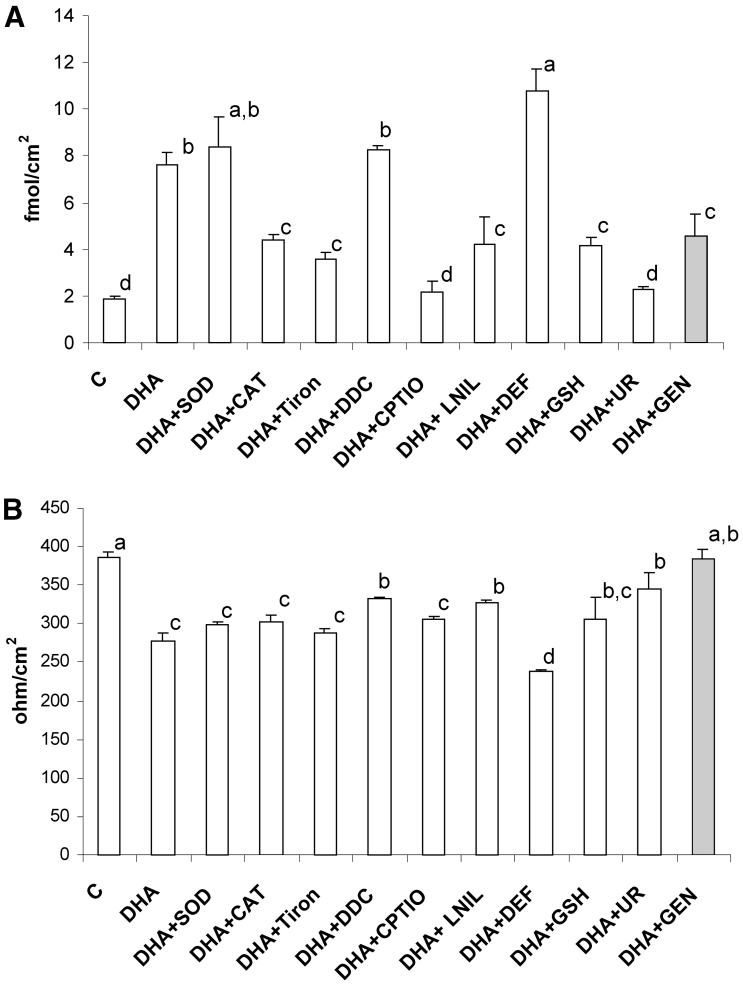

Fig. 6. D-Mannitol (25 nM) apical-to-basal fluxes (A) and TER (B) of monolayers maintained in Transwells for 10–14 days after reaching confluence and incubated for 24 h before the experiment in control conditions (C) or in media enriched with apical DHA (100 μM) in the absence or presence of superoxide dismutase (SOD; 500 U/ml), catalase (CAT; 1,000 U/ml), 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron; 100 μM), diethyldithiocarbamate (DDC; 10 mM), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO; 100 μM), L-N^6-(1-iminoethyl)lysine (L-NIL; 1 mM), deferoxamine (DEF; 1 mM), GSH (10 mM), urate (UR; 1 mM), or genistein (GEN; 300 μM). Results are expressed as means ± SE of four to six filters. Mean values with different letters are significantly different ($P < 0.05$).

loss of barrier integrity induced by DHA. The lack of SOD effects may be attributable to the fact that this enzyme is restricted to the extracellular environment (53). Radi et al. (54) demonstrated that added SOD does not inhibit peroxynitrite effects and suggested that peroxynitrite is formed in regions not readily accessible to externally added SOD. In contrast, Tiron, a low-molecular-weight nonenzymatic $O_2^{\bullet-}$ scavenger, and DDC, an inhibitor of endogenous SOD, attenuated the increase in D-mannitol fluxes and prevented the TER reduction induced by DHA, respectively. Moreover, CAT, which enzymatically removes H_2O_2, significantly prevented the increase in D-mannitol fluxes induced by DHA. These results suggest that H_2O_2 and $O_2^{\bullet-}$ (as substrate for H_2O_2 formation) may play an important role in the DHA-induced increase in paracellular permeability and support the observation that the paracellular permeability increase in the Caco-2 cell monolayer induced by oxidative stress is mainly attributable to H_2O_2 (12).

The rapid reactions of $^{\bullet}NO$ with free radicals is one of the major routes for RNS formation. The best understood is the reaction with $O_2^{\bullet-}$ to form peroxynitrite (55–57). $^{\bullet}NO$ behaves as a potent inhibitor of the lipid peroxidation chain reaction by scavenging propagatory lipid peroxyl radicals (58). In the presence of $O_2^{\bullet-}$, however, $^{\bullet}NO$ forms peroxynitrite, a powerful oxidant capable of initiating lipid oxidation (58). Peroxynitrite is a major mediator of tissue injury in conditions associated with enhanced $^{\bullet}NO$ and $O_2^{\bullet-}$ production (59, 60). This RNS has a short half-life but can diffuse across cell membranes and cause tissue injury (61). In this way, Moreno et al. (62) observed that dietary enrichment with fish oil increased $^{\bullet}NO$ and $O_2^{\bullet-}$ formation in rat macrophages.

L-NIL, an iNOS inhibitor, and CPTIO, a scavenger of $^{\bullet}NO$, attenuated the increase in D-mannitol fluxes. L-NIL also attenuated the reduction in TER induced by DHA, suggesting the involvement of $^{\bullet}NO$ formation. Salzman et al. (23) demonstrated that $^{\bullet}NO$ by itself induces the hyperpermeability to hydrophilic macromolecules in monolayers of Caco-2 cells by dilating tight junctions. Banan et al. (63) have also shown that chemical agents that are injurious to the intestinal tract (e.g., ethanol, H_2O_2) upregulate iNOS and that the activity of iNOS leads to intracellular increases in $^{\bullet}NO$ and peroxynitrite that can directly damage cellular proteins. The formation of peroxynitrite in cells incubated with DHA was predictable from the results indicating the contribution of $O_2^{\bullet-}$ and $^{\bullet}NO$ on DHA effects. The prediction would be confirmed by data showing that two peroxynitrite scavengers, GSH and UR, prevented the DHA-induced effects on D-mannitol fluxes and

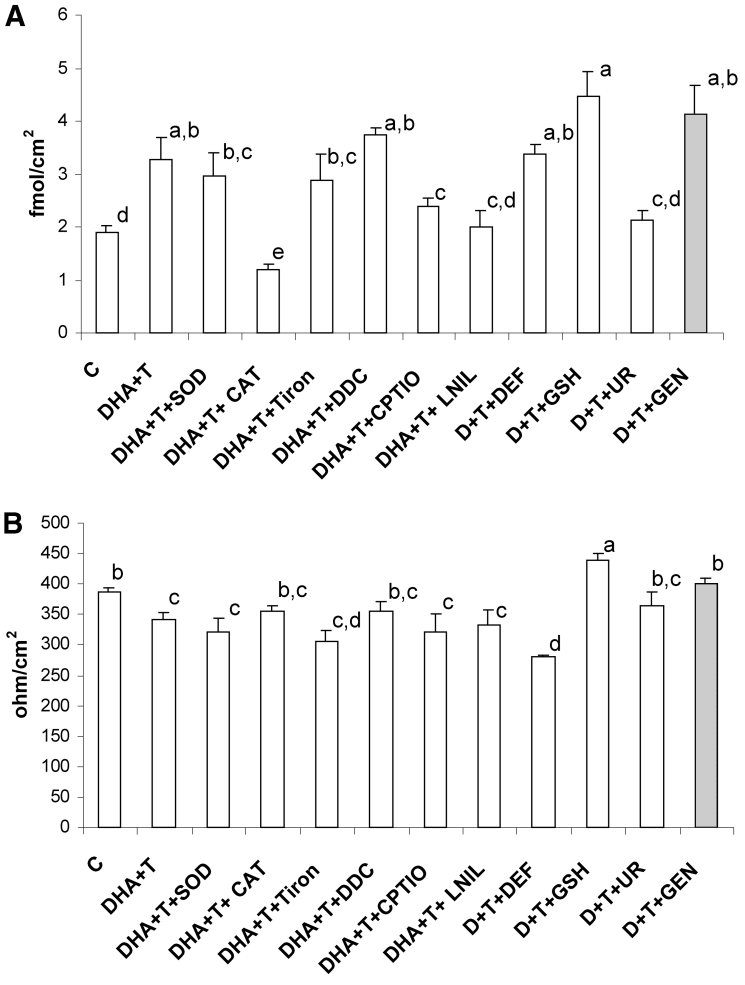

Fig. 7. D-Mannitol (25 nM) apical-to-basal fluxes (A) and TER (B) of monolayers maintained in Transwells for 10–14 days after reaching confluence and incubated for 24 h before the experiment in control conditions (C) or in media enriched with apical DHA plus taurine (DHA+T; 100 μM and 10 mM, respectively) in the absence or presence of SOD (500 U/ml), CAT (1,000 U/ml), Tiron (100 μM), DDC (10 mM), CPTIO (100 μM), L-NIL (1 mM), DEF (1 mM), GSH (10 mM), UR (1 mM), or GEN (300 μM). Results are expressed as means ± SE of four to six filters. Mean values with different letters are significantly different ($P < 0.05$).

TER. Therefore, these RNS may also mediate the disruption of the epithelial barrier induced by DHA.

Cells incubated with DEF in the presence of DHA showed a further increase in paracellular permeability. This may be attributable to the fact that this agent is both a peroxynitrite scavenger and a Fe^{2+} chelator, and Fe^{2+} can convert H_2O_2 to $OH^{\bullet}$, which does not seem to be involved in paracellular permeability modulation in Caco-2 cells (12). However, in the presence of DHA and DEF, the addition of taurine significantly reduced D-mannitol fluxes and increased TER (DEF in Fig. 6 vs. Fig. 7; $P < 0.05$), which supports the involvement of H_2O_2 in DHA-induced hyperpermeability and reveals the capacity of taurine to reduce the availability of H_2O_2. Moreover, in cells incubated with DHA plus taurine, CAT, L-NIL, CPTIO, and UR exerted an additional protective effect on D-mannitol fluxes, thus supporting the role of H_2O_2 and peroxynitrite as the main mediators of DHA-induced hyperpermeability.

Several studies in epithelial and endothelial cells demonstrate that tyrosine kinase agonists and tyrosine phosphatase inhibitors affect phosphotyrosine levels of ZO-1 and ZO-2 in correlation with the altered permeability and redistribution of tight junction proteins (44, 64, 65). Rao et al. (10, 12, 66) demonstrated that oxidative stress increases Caco-2 cell paracellular permeability by a mechanism involving protein tyrosine phosphorylation. In these experiments, GEN, a tyrosine kinase inhibitor, prevented oxidant-induced disruption of the occludin-ZO1 complex. The present results, showing a similar GEN effect, implicate tyrosine phosphorylation in DHA-induced changes in tight junction structure and permeability and reinforce the hypothesis that these effects are mediated by oxidative stress.

In conclusion, DHA supplementation of Caco-2 cells enhances cellular lipid peroxidation and the redistribution of tight junction proteins, which increases paracellular permeability. Moreover, H_2O_2 and peroxynitrite may mediate DHA-induced effects on paracellular permeability, although direct effects of $O_2^{\bullet-}$ and $^{\bullet}NO$ cannot be ruled out. Finally, taurine prevents this effect owing in part to its ability to counteract the effects of H_2O_2.

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