5-Hydroxyeicosatetraenoic acid and leukotriene D_4 increase intestinal epithelial paracellular permeability

M.J. Rodríguez-Lagunas, C.E. Storniolo, R. Ferrer, J.J. Moreno *

Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, 08028 Barcelona, Spain

A B S T R A C T

The loss of epithelial barrier function plays a crucial role in the pathogenesis of inflammatory bowel disease, with levels of 5-lipoxygenase metabolites being increased in the mucosa of these patients. The objective of this study was to determine the effect of the eicosanoids produced by the 5-lipoxygenase pathway, leukotriene B_4 and D_4, and 5-hydroxyeicosatetraenoic acid on epithelial barrier function. Paracellular permeability was estimated from fluorescein isothiocyanate–dextran fluxes and transepi-thelial electrical resistance in differentiated Caco-2 cells. Our results suggest that leukotriene D_4 and 5-hydroxyeicosatetraenoic acid altered both parameters. Identification of the receptors involved in these changes indicated that cysteinyl-leukotriene receptor 1 participates in the effects of leukotriene D_4. For both eicosanoids, these effects were mediated by activation of the phospholipase C/Ca^{2+}/protein kinase C pathway, in addition to cAMP-independent protein kinase A activation. Furthermore, we observed a correlation between increased paracellular permeability and the redistribution of occludin, and for leukotriene D_4, the disorganization of the subapical actin ring and myosin light chain kinase activation. In conclusion, on the basis of our results, we propose that 5-lipoxygenase pathway metabolites partici-pate in the disruption of epithelial barrier function that is characteristic of inflammatory bowel disease.

1. Introduction

Upon release from cell membranes, arachidonic acid (AA) is metabolized into a range of eicosanoids through three major enzymatic pathways: cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P-450 monooxygenase. The LOX pathway involves the stereospecific enzymes 5-, 12-, and 15-LOX. 5-LOX incorpo-rates molecular oxygen at position C5 of the fatty acid, yielding 5-hydroperoxyeicosatetraenoic acid, which is further metabolized to 5-hydroxyeicosatetraenoic acid (HETE) and to the instable epoxide leukotriene (LT)A_4. The intermediate LTA_4 can be further converted into LTB_4 by LTA_4 hydrolase (Haeggstrom, 2004) or

into cysteinyl-LT (CysLT) LTC_4 by LTC_4 synthase and microsomal glutathione S-transferase, which can conjugate LTA_4 with glutathione yielding LTC_4 (Schroder et al., 2003). Release of LTC_4 into the extracellular milieu and successive amino acid cleavage yields LTD_4 and then LTE_4 (also CysLT) via the action of a dipeptidase.

Mediators of the 5-LOX pathway are released by a variety of cells in response to specific cellular stimuli that result in cell activa-tion. LTs have also been implicated in the pathophysiology of both acute and chronic inflammatory diseases including asthma, arthri-tis, psoriasis, and inflammatory bowel disease (IBD) (Wang and DuBois, 2007). LTs exert their biological effect by binding to distinct receptor subtypes, but which receptors participate in the effect of 5-HETE is not yet known. There are two G-protein-coupled receptors (GPCR) for LTB_4, the high affinity BLT_1 and the low affinity BLT_2 (Yokomizo et al., 2000b). CysLTs bind to at least two distinct receptor subtypes, also belonging to the GPCR family, namely $CysLT_1R$ and $CysLT_2R$ (Matuk et al., 2004). $CysLT_1R$ is thought to mediate bronchospasm, plasma exudation, vasoconstriction, mucus secre-tion, and eosinophil recruitment (El Miedany et al., 2006; Kefalakes et al., 2009). $CysLT_2R$ is less well defined, due to a lack of specific agonists and antagonists, but it is thought to mediate some of the vascular effects attributed to CysLTs (Wang and DuBois, 2007).

The gastrointestinal epithelium is a selective barrier that allows the absorption of nutrients, electrolytes and water, but restricts the passage of larger potentially toxic compounds into the circulation, thereby preventing bacterial translocation and systemic infection. The structural integrity of the epithelium is guaranteed by three adhesion systems: tight junctions (TJs), adherent junctions and desmosomes. Of these, TJs form a selective barrier that restricts paracellular diffusion, being the rate-limiting step for paracellular permeability (PP). Moreover, TJs, which are the most apical inter-cellular junctions, form a barrier between apical and basolateral membrane domains (Shin et al., 2006). TJs are multiprotein com-plexes composed of transmembrane proteins that interact with the actin cytoskeleton and with cytosolic proteins involved in the regulation of signaling cascades. In recent years, various transmembrane proteins of the junctional complex have been identified: occludin, claudins, tricellulin, crumbs, and junctional adhesion molecules, among others. These proteins are associated with the cytoplasmatic plaque, which comprises a wide spectrum of adaptor and scaffold proteins, of which ZO-1, ZO-2, ZO-3, AF6, and cingulin are known to form the nexus with cytoskeletal proteins such as the subapical actin ring (Mitic and Anderson, 1998).

Many lines of evidence indicate that the disruption of TJs and loss of epithelial barrier function play a crucial role in the pathogenesis of gastrointestinal disorders, such as IBD, alcoholic endotoxemia, infectious enterocolitis, celiac disease, and necrotiz-ing enterocolitis (Laukoetter et al., 2006; Oberhuber and Vogelsang, 1998; Pravda, 2005; Rao, 2008). With regard to IBD, the increase in PP in response to injurious factors has been identified as the mechanism responsible for the perpetuation of the inflammatory response (Turner, 2006). Several eicosanoids, such as PGE2, PGD2, and thromboxane B2, 5-, 12- and 15-HETE, as well as LTB4, were found to be increased in the mucosa of IBD patients (Boughton-Smith et al., 1983; Eberhart and Dubois, 1995; Krimsky et al., 2003) and to be involved in this pathology (Wang and DuBois, 2007).

In this regard, we previously observed that the addition of PGE2 to differentiated intestinal Caco-2 cells induces an increase in PP, an effect mediated by the interaction of PGE2 with EP1 and EP4 receptors (Rodriguez-Lagunas et al., 2010). Disease exacerbation has been reported in IBD patients treated with conventional non-steroidal anti-inflammatory drugs or COX-2 inhibitors (El Miedany et al., 2006; Kefalakes et al., 2009; Matuk et al., 2004). For this reason, dual inhibition of the COX and 5-LOX pathways (5-aminosalicylic acid or steroids) is currently used for the treatment of IBD (Krimsky et al., 2003). Blocking only COX path-way might divert the AA cascade to the production of LOX metabo-lites and therefore worsen inflammation. In this context, and given the lack of information about the effect of 5-LOX pathway metabo-lites on the state of TJs, our main objective was to elucidate the effect of 5-LOX pathway metabolites on PP to clarify the mechanism involved in the beneficial effects of dual inhibition in IBD treatment.

2. Materials

DMEM, trypsin, penicillin, and streptomycin were supplied by GIBCO (Paisley, Scotland). Non-essential amino acids, FBS, BSA, PBS, D-glucose, HEPES, Fura-2 acetoxymethylester (Fura-2-AM), fluo-rescein isothiocyanate–dextran (FD-4, average mol wt 3000–5000), U73122, dantrolene, Gö6983, SQ22,536, KT5720, ML-7, ketoprofen, and phalloidin-tetramethylrhodamine B isothiocyanate (TRITC-phalloidin), along with other chemicals, were purchased from
Sigma–Aldrich (St. Louis, MO). 5-HETE, LTD_4, LTB_4, LY171883, Bay u9773, U75302, LY255283, and MK571 were purchased from Cayman (Ann Arbor, MI). Tissue culture supplies, including Transwells, were obtained from Costar (Cambridge, MA).

2.1. Cell culture

Caco-2 cells were provided by the American Type Cell Collec-tion and cultured as previously described (Martin-Venegas et al., 2006). Cells (passages 53–65) routinely grown to approximately 80% confluence were released by trypsinization and subcultured at a density of 4×10^5 cells/cm^2 on polycarbonate filters with a pore size of 0.4 μm (Transwells, 12 mm diameter) for PP experi-ments and at a density of 5×10^4 cells/cm^2 in 12-well clusters for
intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) determination, in 24-well clusters containing coverslips for immunofluorescent microscopy, or in 75 cm^2 flasks for cAMP determination. The medium was replaced every 3 days and on the day before the experiment (19–21 days after seeding).

2.2. Paracellular permeability

PP was estimated from transepithelial electrical resistance (TER) and transepithelial FD-4 fluxes. After 3 h incubation with the eicosanoids in DMEM in the apical and basolateral compartments, TER was determined at 37 °C using a Millicell-ERS voltohmme-ter (Millipore, Bedford, MA). The results were expressed as Ω cm^2 monolayer surface area. The resistance of the supporting mem-brane was subtracted from all readings before calculations. After TER determination, 1 mg/mL of FD-4 was added to the apical com-partment and after incubation period (1 h at 37 °C), the basolateral medium was removed and fluorescence was determined in a Fluo-rostar Optima (BMG Labtech, Germany) at excitation and emission wavelengths of 485 nm and 544 nm, respectively.

2.3. Western blot analysis

Cells grown in plastic dishes were washed twice with ice-cold PBS, scrapped of into PBS, and pelleted. The pellets were then sonicated in lysis buffer containing 2 mM sodium EDTA, 20 μg/mL aprotinin, 20 μg/mL leupeptin, 20 μg/mL PMSF, 200 μg/mL diethyldithiocarbamic acid, 50 mM Tris–HCl, 150 mM NaCl, 0.5% Igepal CA-630 and 1 mM DTT. After lysis 30 μg of protein from cell lysate was mixed with a reducing buffer containing 0.5 M Tris–HCl, 10% glycerol, 10% SDS, 2% β-mercaptoethanol and 0.5%blue bromopherol and heated at 100 °C for 5 min. Samples were separated by 12% SDS-PAGE gel and blotted for 2 h at voltage of 100 V and constant amperage of 400 mA onto a nitrocellulose mem-brane (Trans-Blot, 0.45 μm pore size, Bio-Rad) using a Mini protean II system (Bio-Rad). A prestained SDS-PAGE protein standard (Bio-Rad) was used as molecular weight marker to check transfer efficiency. Membranes were blocked with 5% non-fat milk power in Tris-buffered saline (TBS) 0.1% Tween-20 (TBS-T20) for 1 h. A rabbit polyclonal antiserum directed against myosin light chain 2 (MLC) or p-MLC (Cell Signaling Technology) was applied at a dilution of 1:1000 for 1 h. Blots were washed several times with TBS-T20 and incubated with goat anti-rabbit antibody at a 1:100,000 dilution for 1 h. Antibody binding was visualized by an enhanced chemical luminescence technique using SuperSignal West Femto Maximun Sensitivity Substrate (Pierce) and Kodak X-OMAT film (Rochester, NY).

2.4. Intracellular Ca^{2+} concentration

$[Ca^{2+}]_i$ was monitored using the selective fluorescent Ca^{2+} indi-cator Fura 2-AM as previously described (Rodriguez-Lagunas et al., 2010). Cells grown on clusters were loaded with 25 μM Fura 2-AM in DMEM for 1 h at 37 °C, then washed in modified Krebs buffer (pH 7.4) at 37 °C and incubated for 1 h at 37 °C to allow Fura 2-AM de-esterification. The monolayers were washed again to ensure the removal of all unloaded indicator, and eicosanoids and inhibitors were added. The fluorescent signal was continuously monitored with excitation wavelengths of 340 and 380 nm and emission at 510 nm using a fluorescent microplate reader (Fluo-rostar Optima, BMG Labtech, Germany) before and after injection of the eicosanoids. Cells were maintained at 37 °C throughout the experiment. At the end of the incubation period, the maximum and minimum intracellular probe fluorescent signals were determined by the addition of cell lysis buffer and 20 mM EDTA in Krebs, respec-tively and [Ca2+]i was calculated following (Grynkiewicz et al., 1985).

2.5. cAMP determination

cAMP determination was performed using a competitive EIA Kit (Cayman, Ann Arbor, MI) following the manufacturer's instructions. Briefly, cells maintained in flasks were incubated for 5, 15, and 30 min at 37 °C with a range of eicosanoid concentrations. They were then incubated for 20 min at room temperature with 2.5 mL of 0.1 N HCl and harvested and homogenized. The homogenate was then centrifuged (1000 × g, 10 min) and the supernatant was assayed following the acetylation procedure (sensitivity 0.1 pM).

2.6. NF□B determination

Cytosolic I□B proteins bind to the NF□B/Rel transcription fac-tor complex to maintain its inactive state. For NF□B to become activated, it must first dissociate from the inhibitor I□B, thereby enabling NF□B to translocate into the nucleus to modulate gene expression. This is induced by phosphorylation of I□B at Ser32 and Ser36 in response to various stimuli. Therefore, NF□B activation was performed by measuring total and phosphorylated I□B using a competitive EIA Kit (eBioscience, San Diego, CA)

2.7. Immunofluorescent staining of TJ proteins

Caco-2 monolayers grown on coverslips were washed gently with PBS and fixed in iced methanol or 3% paraformaldehyde (PF) and 2% sucrose in 0.1 M PBS (pH 7.4) for 15 min at −20 °C. Cells were washed twice for 5 min in 10 mM PBS containing 20 mM glycine (PBS-glycine) and then permeabilized with 0.2% (v/v) Tri-ton X-100 for 10 min at room temperature. Cells were washed twice in PBS-glycine and blocked for 20 min in PBS-glycine contain-ing 1% BSA (incubation solution). Mouse monoclonal anti-occludin (1:500 dilution; Zymed, South San Francisco, CA) and rabbit poly-clonal anti-ZO-1 (1:250 dilution; Zymed) and mouse polyclonal anti-claudin-2 (1:250 dilution; Invitrogen, San Diego, CA) were used as primary antibodies. Cells were incubated with the primary antibodies for 1 h at 37 °C and washed twice in PBS-glycine for 5 min at room temperature. Monolayers were then incubated for 1 h at 37 °C with Alexa dye-conjugated secondary antibodies (1:500 dilution, Molecular Probes, Leiden, The Netherlands). Finally, cells were washed for 10 min at room temperature in PBS, mounted in Mowiol (Calbiochem, San Diego, CA) and examined under an immunofluorescent microscope (BX 41, Olympus, Japan). Images were taken using a 60× (numerical aperture 1.25, phase 3, oil) Olympus UPlanFL N objective. To view the actin subapical ring, coverslips were fixed in paraformaldehyde and permeabilized as described above and incubated with TRITC-phalloidin or direct-labeled claudin-1 and -4 (1:250 dilution; Invitrogen) for 1 h at 37 °C (1:1000 dilution). TJ and cytosolic fluorescence intensity was quan-tified by using the ImageJ software analysis (US National Institutes of Health, Bethesda, MD, USA, http://rsbweb.nig.gov/ij/) along two horizontal axis for each image. The results were expressed as ratio TJ/cytosolic fluorescence.

2.8. Data analysis

The results are expressed as mean ± SE. Data were analyzed by one-way analysis of variance followed by Dunnett's post hoc test using SPSS® software (SPSS Inc. Chicago, IL, USA). $P < 0.05$ was con-sidered to denote significance.

3. Results

We first tested the effect of the representative eicosanoids produced in the intestinal mucosa by the 5-LOX pathway that are involved in IBD initiation and/or perpetuation on PP. 5-HETE and
LTD_4 (Fig. 1) but not LTB_4 (data not shown) induced a significant increase in FD-4 fluxes and a decrease in TER values.

We examined the receptors involved in these effects of LTD_4 and 5-HETE through the capacity of various receptor antagonists to prevent the action of these eicosanoids on FD-4 fluxes and TER. The increase in FD-4 fluxes and the reduction in TER values induced by LTD_4 were prevented by LY171883 and MK 571, both $CysLT_1R$ antagonists (Fleisch et al., 1985; Martin et al., 2001) and by Bay u9773, a non-selective CysLTR antagonist (Nothacker et al., 2000)(Fig. 1A and B). These results suggest that $CysLT_1R$ participates in the effects induced by LTD_4. Since specific $CysLT_2R$ antagonists are not available we cannot rule out the participation of this receptor in these events.

No specific receptors have been identified for 5-HETE to date. Nevertheless, it has been reported that HETEs can interact with the LTB_4 receptor, BLT_2 (Yokomizo et al., 2001). To test the role of these receptors in 5-HETE-induced barrier disruption, we used U75302 and LY255283 as selective BLT_1 and BLT_2 receptor antago-nists, respectively (Yokomizo et al., 2000a), as well as Bay u9773 as a non-selective CysLTR antagonist. The results indicate that none of these antagonists was able to prevent the effects of 5-HETE on PP (Fig. 1C and D).

Some eicosanoids such as LTD_4 and 5-HETE are able to induce PGE_2 production in intestinal epithelial cells (Di Mari et al., 2007; Ohd, 2000; Ohd et al., 2000). Given that PGE_2 disrupts PP (Martin-Venegas et al., 2006; Rodriguez-Lagunas et al., 2010), we must consider that the increase in PP induced by LTD_4 or 5-HETE might be attributable instead to the COX metabolites induced by these eicosanoids. To test this hypothesis, we tested the effect of keto-profen, a COX inhibitor (Sanchez and Moreno, 1999), on PP effects induced by LTD_4/5-HETE. The addition of ketoprofen did not signif-icantly modify the increase in FD-4 fluxes induced by LTD_4 (Fig. 1A) or 5-HETE (Fig. 1C) although the TER values did not differ signifi-cantly from control values.

Next, we addressed the participation of several signaling path-ways in the disruption of PP by eicosanoids. The results revealed that the increase in FD-4 fluxes induced by LTD_4 (Fig. 2A) was pre-
vented by U73122, a phospholipase C (PLC) inhibitor (Smith et al., 1990); dantrolene, an inhibitor of intracellular Ca^{2+} release from the endoplasmic reticulum (Van Winkle, 1976); Gö6983, a pan protein kinase (PK) C inhibitor (Gonzalez-Mariscal et al., 2008); KT5720, a PKA inhibitor (Doherty et al., 1995) and ML7, a myosin light chain kinase (MLCK) inhibitor (Park et al., 2002). However, SQ22,536, an adenylate cyclase inhibitor (Harris et al., 1979), had no effect. The TER values showed a similar profile, with the exception of that for SQ22,536, which did not differ significantly from control condi-tions (Fig. 2B). In the case of 5-HETE, the effect on FD-4 fluxes was reverted by U73122, dantrolene, Gö6983, and KT5720, but not by SQ22,536 or ML7 (Fig. 2C). Again, TER values (Fig. 2D) matched the profile of FD-4 fluxes, with the exception of that for SQ22,536.

We further examined the participation of myosin light chain inLTD4-treated cells. As observed on western blot analysis of MLC phosphorylation (Fig. 3), there is a significant increase in the ratio p-MLC/MLC in cells treated with LTD4 but not with 5-HETE. Fur-thermore when cells are treated with ML7 in the presence of LTD4, the ratio significant reverts to control conditions.

Our results also show the capacity of LTD4 and 5-HETE to increase [Ca2+]i, an effect that was prevented by U73122 and dantrolene (Fig. 4). The increase in [Ca2+]i was also prevented when Ca2+ was withdrawn from the incubation media. Regarding cAMP levels, PGE2 increased cAMP, which reached the highest concentra-tion after 5 min of incubation. However, neither LTD4 nor 5-HETE modified this variable at either of the concentrations tested (0.1 and 1

The phosphorylation of I⊠B at Ser32 and Ser36 allows the dis-

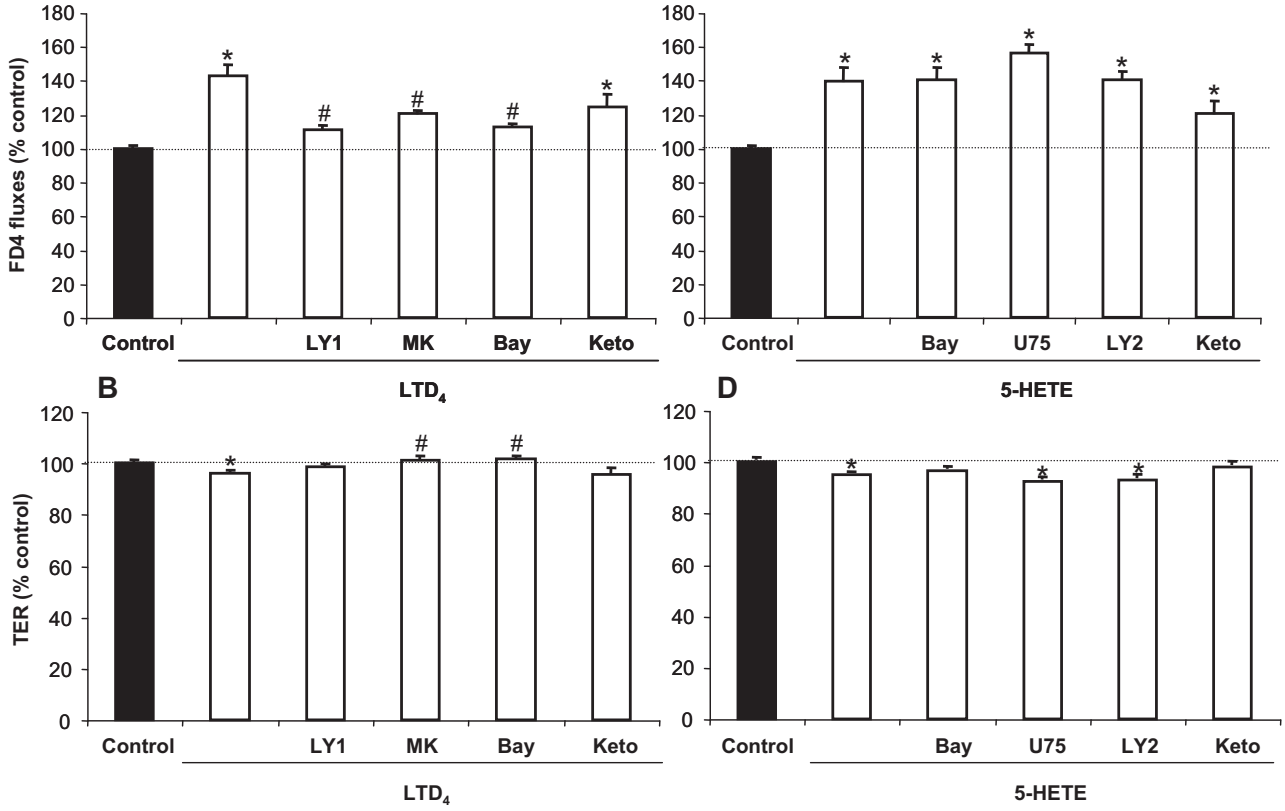

Fig. 1. Effect of LT receptor antagonists or COX inhibitor on epithelial barrier disruption induced by LTD_4 or 5-HETE. FD-4 fluxes (A and C) and TER (B and D) were determined in differentiated Caco-2 cell monolayers, as described in Section 2. Cells were incubated for 3 h with LTD_4 (A and B) (0.1 μM) and LTD_4 plus LY171883 (LY1, 25 μM), MK 571 (MK, 25 μM), Bay u9773 (Bay, 1 μM), or ketoprofen (Keto, 5 μM), or 5-HETE (C and D) (0.1 μM) and 5-HETE plus Bay u9773 (Bay, 1 μM), U75302 (U75, 5 μM), LY255283 (LY2, 50 μM), or ketoprofen (Keto, 5 μM) in the apical and basolateral compartments. Results are expressed as the percentage of FD-4 fluxes and TER values obtained in control conditions (0.33 ± 0.01 ng/μL and $1720.65 \pm 68.49\ \Omega\ cm^2$, respectively). Data are means ± SE of $n = 6$–10 experiments. $^*P < 0.05$ vs. control and $^\#P < 0.05$ vs. LTD_4 (A and B) or 5-HETE (C and D).

sociation of the complex IκB-NFκB/Rel, thereby enabling NFκB to translocate into the nucleus to modulate gene expression. We wished to know whether NFκB is activated by LTD_4 or 5-HETE, and for this purpose IκB phosphorylation was determined. The results show that neither LTD_4 nor 5-HETE were able to increase the amount of phosphorylated IκB with respect to control conditions in any of the incubation periods assayed (5 min, 15 min, and 3 h), whereas TNFα, which was used as a positive control, was able to increase IκB phosphorylation (Table 1).

Finally, we studied the contribution of TJ proteins and cytoskele-tal actin to the increase in PP induced by these eicosanoids. TJ protein immunofluorescent staining in control conditions showed occludin and ZO-1 located mainly at the cell border (Fig. 6A). Treatment with the eicosanoids that disrupted barrier function,
namely LTD_4 and 5-HETE, resulted in a redistribution of occludin with adjacent diffuse intracellular staining and a granular appearance. However, neither LTD_4 nor 5-HETE had a significant effect

on ZO-1 location. Morphological assessment of subapical actin showed the characteristic perijunctional ring in control conditions. Treatment with LTD_4 but not 5-HETE induced complete disorganization of the actin belt. In control monolayers, claudin-4 was predominantly present at TJs and a weak cytoplasmic localization was also observed. However, in response to either 5-HETE or LTD_4, claudin-4 markedly dissociated from the TJ to form protein clumps, being this effect more pronounced in LTD_4-treated cells. The localization of claudin-1 and claudin-2 was also assessed but no difference was found with respect to control monolayers (data not shown). Moreover, the images show that PLC and MLCK inhibitors were able to prevent the redistribution of occludin, actin and claudin-4 in LTD_4-treated cells (Fig. 6B). In the case of 5-HETE-treated cells, the addition of PLC inhibitor also prevented occludin and claudin-4 redistribution (data not shown)

Table 1
Effect of 5-HETE and LTD_4 on NFκB pathway.

	5 min	15 min	3 h
Control	100 ± 10.52	100 ± 7.59	100 ± 17.74
5-HETE	73.60 ± 10.27	86.28 ± 14.82	95.51 ± 13.30
LTD_4	102.09 ± 15.75	82.80 ± 23.27	95.10 ± 13.82
TNFα	259.12 ± 20.15*	285.42 ± 36.29*	284.64 ± 26.68*

Changes in phosphorylated IκB levels were determined in differentiated Caco-2 cell monolayers as described in Section 2. Cells were incubated for 5 min, 15 min, or 3 h in the presence of the eicosanoids (1 μM) or TNF-α as a positive control (100 ng/mL) and the results are expressed as the % with respect to control conditions. Data are mean ± SE of $n = 4$–6 experiments.

* $P < 0.05$ vs. control.

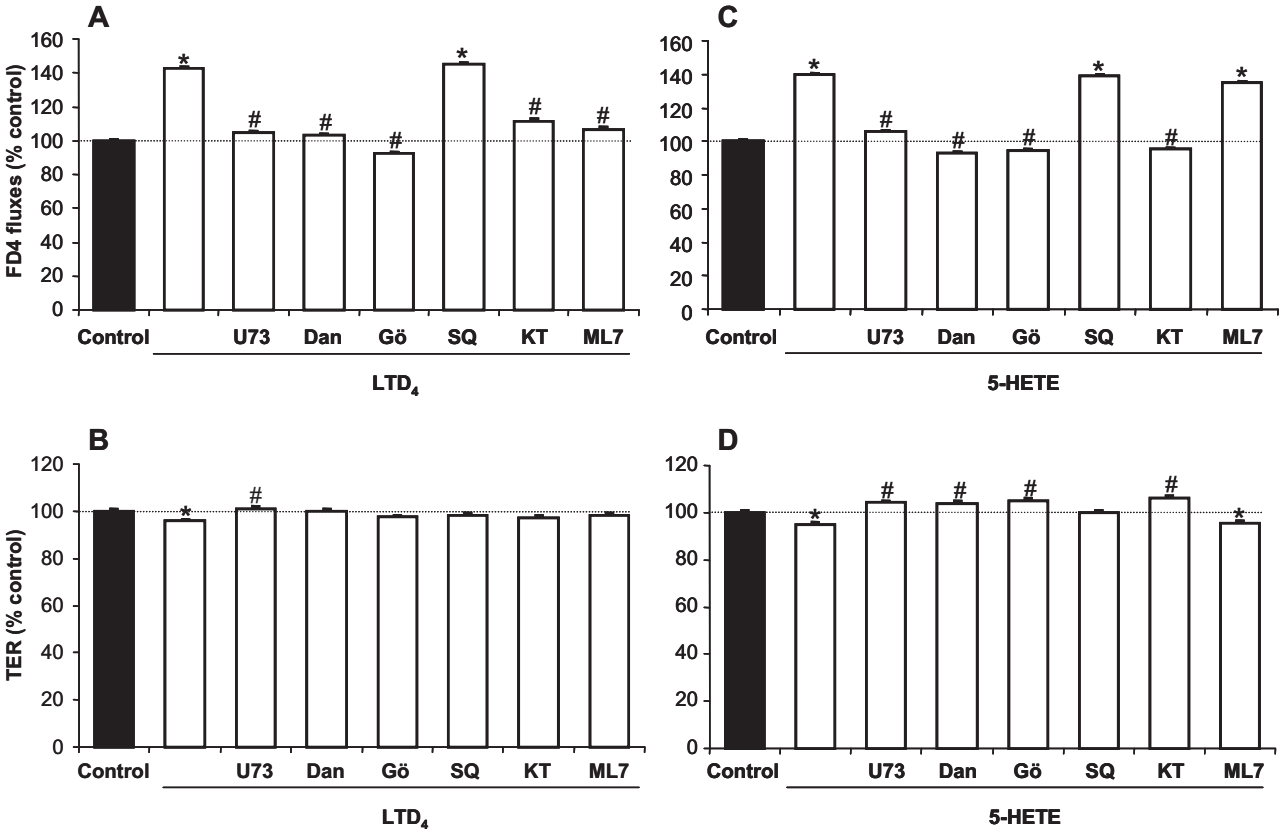

Fig. 2. Cell signaling pathways involved in the effects exerted by LTD_4 and 5-HETE on PP. FD-4 fluxes (A and C) and TER (B and D) were determined in differentiated Caco-2 cell monolayers, as described in Section 2. Cells were incubated for 3 h with LTD_4 (A and B) (0.1 μM) or 5-HETE (C and D) (0.1 μM) plus U73122 (U73, 0.1 μM), dantrolene (Dan, 50 μM), Gö6983 (Gö, 1 μM), SQ22,536 (SQ, 10 μM), KT5720 (KT, 1 μM), or ML7 (0.05 μM) in the apical and basolateral compartments. Results are expressed as the percentage of FD-4 fluxes and TER values obtained in control conditions (0.42 ± 0.04 ng/μL and $1750.40 \pm 89.81\ \Omega\,cm^2$, respectively). Data are means ± SE of $n = 6$–10 experiments. $^{*}P < 0.05$ vs. control and $^{\#}P < 0.05$ vs. LTD_4 (A and B) or vs. 5-HETE (C and D).

4. Discussion

Increased PP of the intestinal epithelium is now believed to be involved in the pathophysiology of a variety of gastrointestinal disorders (Clayburgh et al., 2004). In IBD, altered PP increases the entrance of pro-inflammatory stimuli to the underlying immune cells, thereby triggering further cytokine-induced changes to TJs and a vicious cycle of mucosal barrier dysfunction and activation of the mucosal immune response (Barbara, 2006; Bruewer et al., 2006; Mankertz and Schulzke, 2007). On the basis of previous

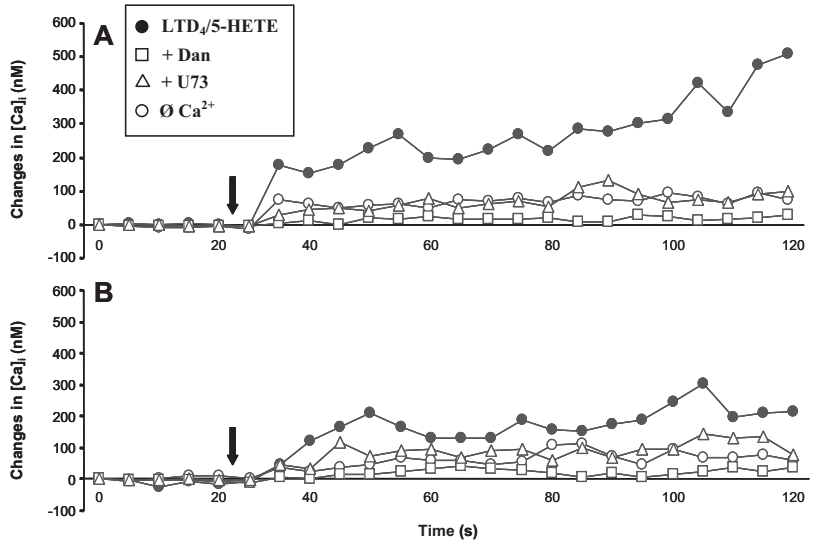

Fig. 4. Changes in $[Ca^{2+}]_i$ induced by LTD_4 and 5-HETE. $[Ca^{2+}]_i$ were determined in differentiated Caco-2 cell monolayers using Fura-2 AM, as described in Section 2. Cells were incubated for 120 s in the presence of LTD_4 (1 μM) (A) or 5-HETE (1 μM) (B) (●) plus dantrolene (□, 50 μM) or U73122 (△, 0.1 μM), or in the absence of extracellular Ca^{2+} (○). The arrow indicates the injection of LTD_4 or 5-HETE. Inhibitors were pre-incubated for 30 min. Each plot corresponds to a representative profile obtained for $n = 3$ experiments.

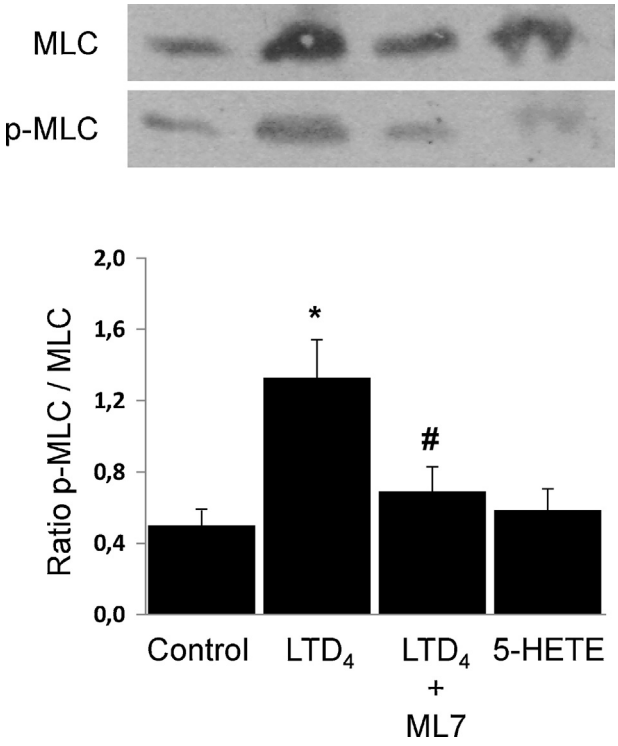

Fig. 3. Western blot of p-MLC in the presence of LTD_4. Cells were incubated with LTD_4 (0.1 μM) ± ML7 (0.05 μM) or 5-HETE (0.1 μM) and MLC and p-MLC were determined using specific antibodies. Values are mean ratio p-MLC/MLC ± SE ($n = 4–5$). $^*P < 0.05$ vs. control, $^\#P < 0.05$ vs. LTD_4.

findings indicating that the AA cascade is activated in intestinal mucosa in IBD (Boughton-Smith et al., 1983; Eberhart and Dubois, 1995; Krimsky et al., 2003), we hypothesized that eicosanoids could be involved in the regulation of intestinal epithelial barrier function in the above-mentioned pathophysiological processes. In this regard, we previously reported that the enhancement of PGE_2 levels increases PP in differentiated Caco-2 cell cultures (Martin-Venegas et al., 2006). Given that 5-LOX metabolites are also increased in the mucosa of IBD patients, and that 5-LOX pathway inhibition is effective in the clinical treatment of IBD, we studied the effect of these eicosanoids on intestinal PP. The addition of representative 5-LOX pathway metabolites such as LTD_4 and 5-HETE, at concentrations reached in the inflamed intestinal mucosa (Wardle et al., 1993; Zijlstra and Wilson, 1991; Zijlstra et al., 1992), disrupt barrier function, while LTB_4 had no effect.

To study the cell signaling involved in the effect of LTD_4 and 5-HETE on intestinal PP, the first step was to identify the receptors involved. LTD_4 binds to $CysLT_1$ and $CysLT_2$, both of which are

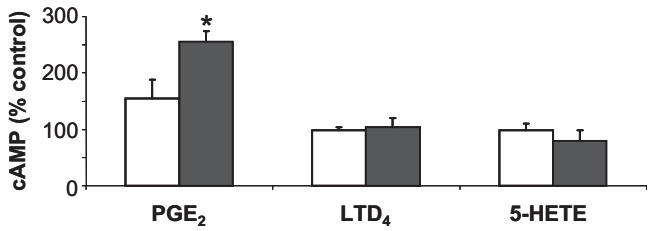

Fig. 5. Effect of LTD_4 and 5-HETE on intracellular cAMP concentration. cAMP was determined in differentiated Caco-2 cell, as described in Section 2. Cells were incubated for 5 min with LTD_4 and 5-HETE at two concentrations (0.1 μM, white bars and 1 μM black bars). PGE_2 was used as a positive control. Results are expressed as the percentage of cAMP values obtained in control conditions (22.59 ± 1.64 nM). The data are means ± SE of $n = 3–5$ experiments. $^*P < 0.05$ vs. control.

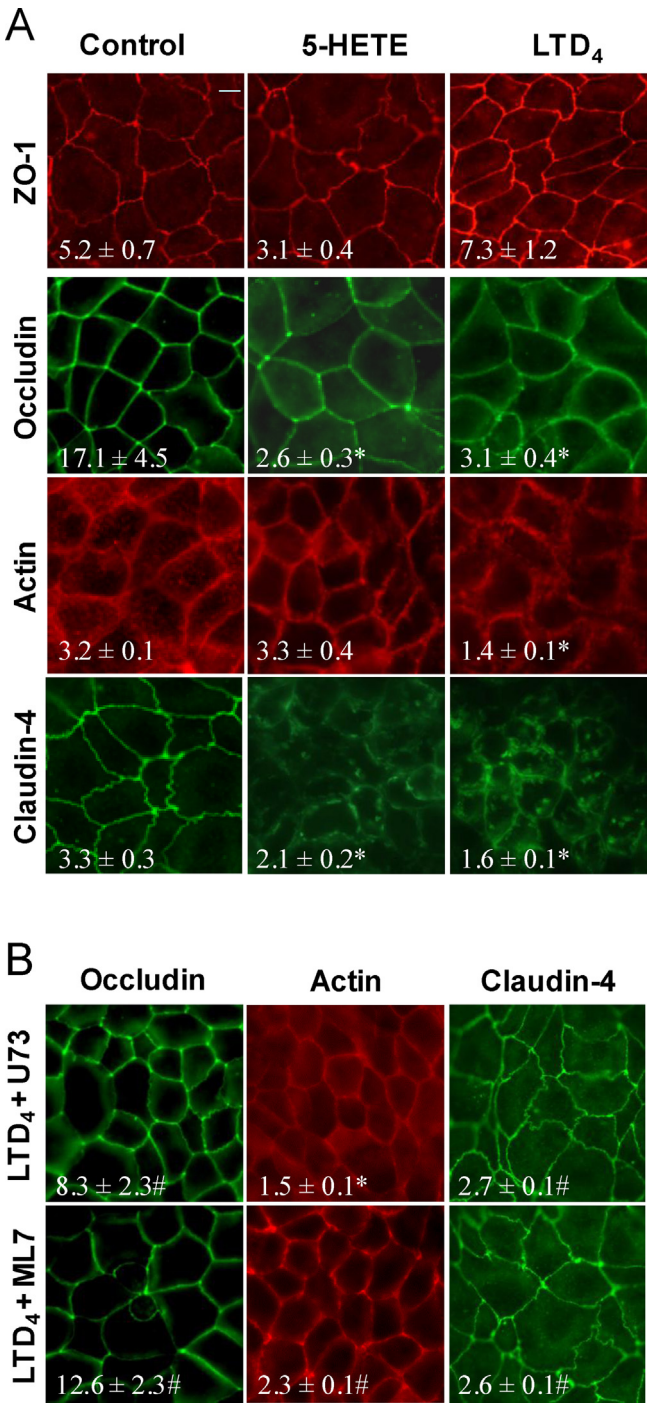

Fig. 6. Changes in TJ proteins induced by eicosanoids. Fluorescent analysis was performed in cells incubated for 3 h with different eicosanoids (0.1 μM) and inhibitors using specific ZO-1, occludin and claudin-4 antibodies and TRITC-phalloidin, as described in Section 2. In each case, a representative x–y image of sections close to the apical cell side is shown. (A) ZO-1, occludin, perijunctional actin and claudin-4 distribution in cells treated with LTD_4 and 5-HETE. (B) Effect of U-73122 (U73, 0.1 μM) or ML7 (0.05 μM) on occludin, perijunctional actin and claudin-4 distribution in LTD_4-treated cells. All the images are shown at the same magnification. White numbers indicated on each image corresponds to the ratio TJ/cytosolic fluorescence intensity. The results are means ± SE of $n = 2$ horizontal axis from 3 images. $^*P < 0.05$ vs. control, $^\#P < 0.05$ vs. LTD_4.

expressed in Caco-2 cells (Magnusson et al., 2007; Nielsen et al., 2005). Our results indicate for the first time that the LTD_4–$CysLT_1R$ interaction participates in the regulation of intestinal barrier function. In this regard, LTD_4–$CysLT_1R$ was recently reported to be involved in the regulation of other intestinal epithelial functions,

such as cell survival and proliferation (Paruchuri et al., 2006) and to participate in plasma protein extravasation in inflammatory conditions (Beller et al., 2004).

Specific HETE receptors have not been identified to date. However, it has been reported that 12-(S) and 15-(S)-HETE bind to LTB_4 receptor, BLT_2 (Yokomizo et al., 2001). However a BLT_2 receptor antagonist (LY255283) did not affect the increased PP induced by 5-HETE. Moreover, given that BLT_1 is expressed in Caco-2 cells (Ihara et al., 2007), a specific antagonist of the high affinity BLT_1 receptor (U75302) was used to rule out the participation of this LTB_4 receptor in these events, and again no effect was observed. In addition, the results obtained with a non-selective CysLTR antagonist (Bay u9773) indicate the lack of participation of these receptors. Therefore, no candidate receptor involved in PP regulation has thus far been identified for 5-HETE.

It has recently been reported that LTD_4 and 5-HETE induce the expression of COX-2, and consequently PGE_2 production (Di Mari et al., 2007; Krimsky et al., 2003). Therefore, it is possible that the effects of these eicosanoids on PP may be due indirectly to an increase in PGE_2 production. Considering that we previously demonstrated that PGE_2 induces the disruption of epithelial barrier function (Rodriguez-Lagunas et al., 2010), we assayed whether COX inhibition reverses the effect of LTD_4 and 5-HETE on PP. Our results suggest that the formation of PGE_2 does not play a pivotal role in the effects exerted by LTD_4 and 5-HETE on intestinal epithelial barrier function.

The mechanism by which 5-HETE and LTD_4 induce epithelial barrier disruption was further studied by characterization of the intracellular signaling pathways involved. According to recent data, $CysLT_1R$ activates a G protein that induces PLC activation and consequently diacylglycerol and inositol trisphosphate (IP_3) release. Thus, LTD_4 coupled to $CysLT_1R$ results in an increase in $[Ca^{2+}]_i$ and PKC activation in various cell types (Profita et al., 2008; Singh et al., 2010; Suzuki et al., 2008; Woszczek et al., 2008). Our results revealed that the increase in PP induced by LTD_4 was prevented by the inhibition of PLC, by the reduction of Ca^{2+} release from the endoplasmic reticulum, and by PKC inhibition. Moreover, we also observed that the enhancement of $[Ca^{2+}]_i$ induced by LTD_4 was prevented by PLC inhibition as well as by the inhibition of Ca^{2+} release from intracellular stores, and by the withdrawal of extracellular Ca^{2+}. Therefore, our results suggest that intracellular and extracellular Ca^{2+} participate in these events. Furthermore, we should also consider that PKA inhibition prevented the increase in PP induced by LTD_4 and 5-HETE, although cAMP levels were not modified by either eicosanoid. Even though PKA is commonly activated by cAMP, some cAMP-independent mechanisms of activation have been reported in various cell types including epithelial cells (Howe, 2004; Kohr et al., 2010; Niu et al., 2001). NFκB activation mediates the effects of several stimuli on epithelial barrier disruption in intestinal Caco-2 cell cultures (Al-Sadi, 2009). Moreover, it should also be considered that NFκB-dependent PKA activation has been previously described (Gambaryan et al., 2010). In this regard, NFκB activation was also evaluated as a possible way of cAMP-independent PKA activation in LTD_4/5-HETE-treated cells. As phosphorylated IκB levels were not increased we suggest that the NFκB transcription factor is not a downstream target of either LTD_4 or 5-HETE. Therefore, other cAMP and NFκB-independent PKA activation pathways should be further considered as it has been described before in different cell types (Kohr et al., 2010; Niu et al., 2001).

The delocalization of occludin and ZO-1 from TJs is associated with epithelial barrier dysfunction and increased epithelial permeability (Harhaj and Antonetti, 2004). Our findings revealed a change in occludin and actin distribution, while ZO-1 was not modified when cells were incubated with LTD_4. Similarly, despite the dramatic redistribution of TJ proteins following IFN-γ exposure, it has been reported that ZO-1 is only minimally affected and that most of it remains at the TJ (Bruewer et al., 2003). Recently, the pathophysiological relevance of claudins in the intestine has been highlighted as claudin-2 expression has been described to be elevated in colon epithelium of patients suffering from IBD (Amasheh et al., 2011; Prasad et al., 2005). In contrast, the expression of claudin-4 was reduced and this protein was redistributed (Prasad et al., 2005). In general, overexpression of claudin-4 localized within the TJ has been associated to an improvement in epithelial barrier function (Suzuki and Hara, 2009; Vreeburg et al., 2012). For the moment, little information is available on claudin redistribution induced by 5-LOX pathway derived eicosanoids. Although, the redistribution of claudin-4 without effect on claudin-1 and claudin-2 observed in microorganism-induced intestinal inflammation is in agreement with our data (Hering et al., 2011; Lejeune et al., 2011).

The presence of cytosolic occludin is associated with protein internalization by endocytosis. In the case of barrier loss induced by TNF-α, occludin internalization has been described as a MLCK-dependent process (Schwarz et al., 2007). In this regard, previous studies indicate that MLCK is crucial for the regulation of intestinal TJ permeability. Thus, the increase in PP induced by several proinflammatory cytokines, short chain fatty acids, ethanol, and extracellular Ca^{2+} is mediated by an increase in MLCK activity (Al-Sadi et al., 2008; Ma et al., 1999; Ohata et al., 2005; Shen et al., 2006; Ye et al., 2006). In fact, MLCK expression is significantly enhanced in the mucosa of IBD patients (Blair et al., 2006). Our results indicate that the increase in PP induced by LTD_4 correlates with the redistribution of TJ proteins (mainly occludin) and the contraction of the perijunctional actin ring through MLCK activation and subsequent increase in MLC phosphorylation. In contrast, the effects induced by 5-HETE on occludin redistribution may be mediated by a mechanism other than that induced by LTD_4. In this case, the contraction of the actin subapical ring through MLCK activation and MLC phosphorylation is not involved.

On the basis of our findings, we can conclude that LTD_4 effects on TJ permeability are mediated by the interaction with $CysLT_1R$, which activates the PLC-Ca^{2+}-PKC and MLCK pathways. Moreover, cAMP-independent PKA activation may also be involved in these events. Furthermore, these events are accompanied by the redistribution of occludin and claudin-4 and the contraction of the perijunctional actin ring. Regarding the effects of 5-HETE on PP, we did not identify any receptor nevertheless, a common intracellular signaling pathway to LTD_4 can be predicted, excluding the redistribution of the subapical actin ring. Interestingly, our findings provide a highly plausible explanation for the negative effect of COX inhibition on PP in IBD attributable to the enhancement of 5-LOX pathway eicosanoids synthesis.

Given the involvement of eicosanoids in the disruption of the homeostasis of intestinal barrier function in inflammatory processes such as IBD (Ferrer and Moreno, 2010), our findings provide a valuable basis on which to perform research into the interrelation between 5-LOX pathway activation, PP changes, and the initiation/perpetuation of IBD. Moreover, these findings may be useful to understand the effectivity of dual COX/LOX inhibitors and for the future development of new diagnostic tools and therapeutic strategies for IBD.

Acknowledgments

This study was supported by the following Grants: BFU2007-61727/BFI (Ministerio de Ciencia y Tecnología), 2005SGR0269 and 2009SGR0438 (Generalitat de Catalunya).

References

Al-Sadi R. Mechanism of cytokine modulation of epithelial tight junction barrier. Frontiers in Bioscience 2009;14:2765.

Al-Sadi R, Ye D, Dokladny K, Ma TY. Mechanism of IL-1beta-induced increase in intestinal epithelial tight junction permeability. Journal of Immunology 2008;180:5653–61.

Amasheh S, Fromm M, Gunzel D. Claudins of intestine and nephron – a correlation of molecular tight junction structure and barrier function. Acta Physiologica (Oxford) 2011;201:133–40.

Barbara G. Mucosal barrier defects in irritable bowel syndrome. Who left the door open? American Journal of Gastroenterology 2006;101:1295–8.

Beller TC, Maekawa A, Friend DS, Austen KF, Kanaoka Y. Targeted gene disruption reveals the role of the cysteinyl leukotriene 2 receptor in increased vascular permeability and in bleomycin-induced pulmonary fibrosis in mice. Journal of Biological Chemistry 2004;279:46129–34.

Blair SA, Kane SV, Clayburgh DR, Turner JR. Epithelial myosin light chain kinase expression and activity are upregulated in inflammatory bowel disease. Laboratory Investigation 2006;86:191–201.

Boughton-Smith NK, Hawkey CJ, Whittle BJ. Biosynthesis of lipoxygenase and cyclooxygenase products from [14C]-arachidonic acid by human colonic mucosa. Gut 1983;24:1176–82.

Bruewer M, Luegering A, Kucharzik T, Parkos CA, Madara JL, Hopkins AM, et al. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. Journal of Immunology 2003;171:6164–72.

Bruewer M, Samarin S, Nusrat A. Inflammatory bowel disease and the apical junctional complex. Annals of the New York Academy of Sciences 2006;1072:242–52.

Clayburgh DR, Shen L, Turner JR. A porous defense: the leaky epithelial barrier in intestinal disease. Laboratory Investigation 2004;84:282–91.

Di Mari JF, Saada JI, Mifflin RC, Valentich JD, Powell DW. HETEs enhance IL-1-mediated COX-2 expression via augmentation of message stability in human colonic myofibroblasts. American Journal of Physiology – Gastrointestinal and Liver Physiology 2007;293:G719–28.

Doherty CM, Tarchala SM, Radwanska E, De Jonge CJ. Characterization of two second messenger pathways and their interactions in eliciting the human sperm acrosome reaction. Journal of Andrology 1995;16:36–46.

Eberhart CE, Dubois RN. Eicosanoids and the gastrointestinal tract. Gastroenterology 1995;109:285–301.

El Miedany Y, Youssef S, Ahmed I, El Gaafary M. The gastrointestinal safety and effect on disease activity of etoricoxib, a selective cox-2 inhibitor in inflammatory bowel diseases. American Journal of Gastroenterology 2006;101:311–7.

Ferrer R, Moreno JJ. Role of eicosanoids on intestinal epithelial homeostasis. Biochemical Pharmacology 2010;80:431–8.

Fleisch JH, Rinkema LE, Haisch KD, Swanson-Bean D, Goodson T, Ho PP, et al. LY171883, 1-less than 2-hydroxy-3-propyl-4-less than 4-(1H-tetrazol-5-yl) butoxy greater than phenyl greater than ethanone, an orally active leukotriene D4 antagonist. Journal of Pharmacology and Experimental Therapeutics 1985;233:148–57.

Gambaryan S, Kobsar A, Rukoyatkina N, Herterich S, Geiger J, Smolenski A, et al. Thrombin and collagen induce a feedback inhibitory signaling pathway in platelets involving dissociation of the catalytic subunit of protein kinase A from an NFkappaB–IkappaB complex. Journal of Biological Chemistry 2010;285:18352–63.

Gonzalez-Mariscal L, Tapia R, Chamorro D. Crosstalk of tight junction components with signaling pathways. Biochimica et Biophysica Acta 2008;1778:729–56.

Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. Journal of Biological Chemistry 1985;260:3440–50.

Haeggstrom JZ. Leukotriene A4 hydrolase/aminopeptidase, the gatekeeper of chemotactic leukotriene B4 biosynthesis. Journal of Biological Chemistry 2004;279:50639–42.

Harhaj NS, Antonetti DA. Regulation of tight junctions and loss of barrier function in pathophysiology. International Journal of Biochemistry and Cell Biology 2004;36:1206–37.

Harris DN, Asaad MM, Phillips MB, Goldenberg HJ, Antonaccio MJ. Inhibition of adenylate cyclase in human blood platelets by 9-substituted adenine derivatives. Journal of Cyclic Nucleotide Research 1979;5:125–34.

Hering NA, Richter JF, Krug SM, Gunzel D, Fromm A, Bohn E, et al. Yersinia enterocolitica induces epithelial barrier dysfunction through regional tight junction changes in colonic HT-29/B6 cell monolayers. Laboratory Investigation 2011;91:310–24.

Howe AK. Regulation of actin-based cell migration by cAMP/PKA. Biochimica et Biophysica Acta 2004;1692:159–74.

Ihara A, Wada K, Yoneda M, Fujisawa N, Takahashi H, Nakajima A. Blockade of leukotriene B4 signaling pathway induces apoptosis and suppresses cell proliferation in colon cancer. Journal of Pharmacological Sciences 2007;103:24–32.

Kefalakes H, Stylianides TJ, Amanakis G, Kolios G. Exacerbation of inflammatory bowel diseases associated with the use of nonsteroidal anti-inflammatory drugs: myth or reality? European Journal of Clinical Pharmacology 2009;65:963–70.

Kohr MJ, Traynham CJ, Roof SR, Davis JP, Ziolo MT. cAMP-independent activation of protein kinase A by the peroxynitrite generator SIN-1 elicits positive inotropic effects in cardiomyocytes. Journal of Molecular and Cellular Cardiology 2010;48:645–8.

Krimsky M, Yedgar S, Aptekar L, Schwob O, Goshen G, Gruzman A, et al. Amelioration of TNBS-induced colon inflammation in rats by phospholipase A2 inhibitor. American Journal of Physiology – Gastrointestinal and Liver Physiology 2003;285:G586–92.

Laukoetter MG, Bruewer M, Nusrat A. Regulation of the intestinal epithelial barrier by the apical junctional complex. Current Opinion in Gastroenterology 2006;22:85–9.

Lejeune M, Moreau F, Chadee K. Prostaglandin E2 produced by entamoeba histolytica signals via EP4 receptor and alters claudin-4 to increase ion permeability of tight junctions. American Journal of Pathology 2011;179:807–18.

Ma TY, Nguyen D, Bui V, Nguyen H, Hoa N. Ethanol modulation of intestinal epithelial tight junction barrier. American Journal of Physiology 1999;276:G965–74.

Magnusson C, Ehrnstrom R, Olsen J, Sjolander A. An increased expression of cysteinyl leukotriene 2 receptor in colorectal adenocarcinomas correlates with high differentiation. Cancer Research 2007;67:9190–8.

Mankertz J, Schulzke JD. Altered permeability in inflammatory bowel disease: pathophysiology and clinical implications. Current Opinion in Gastroenterology 2007;23:379–83.

Martin V, Sawyer N, Stocco R, Unett D, Lerner MR, Abramovitz M, et al. Molecular cloning and functional characterization of murine cysteinyl-leukotriene 1 (CysLT(1)) receptors. Biochemical Pharmacology 2001;62:1193–200.

Martin-Venegas R, Roig-Perez S, Ferrer R, Moreno JJ. Arachidonic acid cascade and epithelial barrier function during Caco-2 cell differentiation. Journal of Lipid Research 2006;47:1416–23.

Matuk R, Crawford J, Abreu MT, Targan SR, Vasiliauskas EA, Papadakis KA. The spectrum of gastrointestinal toxicity and effect on disease activity of selective cyclooxygenase-2 inhibitors in patients with inflammatory bowel disease. Inflammatory Bowel Diseases 2004;10:352–6.

Mitic LL, Anderson JM. Molecular architecture of tight junctions. Annual Review of Physiology 1998;60:121–42.

Nielsen CK, Campbell JI, Ohd JF, Morgelin M, Riesbeck K, Landberg G, et al. A novel localization of the G-protein-coupled $CysLT_1$ receptor in the nucleus of colorectal adenocarcinoma cells. Cancer Research 2005;65:732–42.

Niu J, Vaiskunaite R, Suzuki N, Kozasa T, Carr DW, Dulin N, et al. Interaction of heterotrimeric G13 protein with an A-kinase-anchoring protein 110 (AKAP110) mediates cAMP-independent PKA activation. Current Biology 2001;11:1686–90.

Nothacker HP, Wang Z, Zhu Y, Reinscheid RK, Lin SH, Civelli O. Molecular cloning and characterization of a second human cysteinyl leukotriene receptor: discovery of a subtype selective agonist. Molecular Pharmacology 2000;58:1601–8.

Oberhuber G, Vogelsang H. Gastrointestinal permeability in celiac disease. Gastroenterology 1998;114:226.

Ohata A, Usami M, Miyoshi M. Short-chain fatty acids alter tight junction permeability in intestinal monolayer cells via lipoxygenase activation. Nutrition 2005;21:838–47.

Ohd. Leukotrienes induce cell-survival signaling in intestinal epithelial cells. Gastroenterology 2000;119:1007.

Ohd JF, Wikstrom K, Sjolander A. Leukotrienes induce cell-survival signaling in intestinal epithelial cells. Gastroenterology 2000;119:1007–18.

Park JK, Lee SO, Kim YG, Kim SH, Koh GY, Cho KW. Role of rho-kinase activity in angiotensin II-induced contraction of rabbit clitoral cavernosum smooth muscle. International Journal of Impotence Research 2002;14:472–7.

Paruchuri S, Mezhybovska M, Juhas M, Sjolander A. Endogenous production of leukotriene D4 mediates autocrine survival and proliferation via $CysLT_1$ receptor signalling in intestinal epithelial cells. Oncogene 2006;25:6660–5.

Prasad S, Mingrino R, Kaukinen K, Hayes KL, Powell RM, MacDonald TT, et al. Inflammatory processes have differential effects on claudins 2, 3 and 4 in colonic epithelial cells. Laboratory Investigation 2005;85:1139–62.

Pravda J. Radical induction theory of ulcerative colitis. World Journal of Gastroenterology 2005;11:2371–84.

Profita M, Sala A, Bonanno A, Siena L, Ferraro M, Di Giorgi R, et al. Cysteinyl leukotriene-1 receptor activation in a human bronchial epithelial cell line leads to signal transducer and activator of transcription 1-mediated eosinophil adhesion. Journal of Pharmacology and Experimental Therapeutics 2008;325:1024–30.

Rao R. Oxidative stress-induced disruption of epithelial and endothelial tight junctions. Frontiers in Bioscience 2008;13:7210–26.

Rodriguez-Lagunas MJ, Martin-Venegas R, Moreno JJ, Ferrer R. PGE2 promotes Ca^{2+}-mediated epithelial barrier disruption through EP1 and EP4 receptors in Caco-2 cell monolayers. American Journal of Physiology – Cell Physiology 2010;299:C324–34.

Sanchez T, Moreno JJ. Ketoprofen S(+) enantiomer inhibits prostaglandin production and cell growth in 3T6 fibroblast cultures. European Journal of Pharmacology 1999;370:63–7.

Schroder O, Sjostrom M, Qiu H, Stein J, Jakobsson PJ, Haeggstrom JZ. Molecular and catalytic properties of three rat leukotriene C(4) synthase homologs. Biochemical and Biophysical Research Communications 2003;312:271–6.

Schwarz BT, Wang F, Shen L, Clayburgh DR, Su L, Wang Y, et al. LIGHT signals directly to intestinal epithelia to cause barrier dysfunction via cytoskeletal and endocytic mechanisms. Gastroenterology 2007;132:2383–94.

Shen L, Black ED, Witkowski ED, Lencer WI, Guerriero V, Schneeberger EE, et al. Myosin light chain phosphorylation regulates barrier function by remodeling tight junction structure. Journal of Cell Science 2006;119:2095–106.

Shin K, Fogg VC, Margolis B. Tight junctions and cell polarity. Annual Review of Cell and Developmental Biology 2006;22:207–35.

Singh RK, Gupta S, Dastidar S, Ray A. Cysteinyl leukotrienes and their receptors: molecular and functional characteristics. Pharmacology 2010;85:336–49.

Smith RJ, Sam LM, Justen JM, Bundy GL, Bala GA, Bleasdale JE. Receptor-coupled signal transduction in human polymorphonuclear neutrophils: effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness. Journal of Pharmacology and Experimental Therapeutics 1990;253:688–97.

Suzuki S, Takeuchi K, Ishinaga H, Basbaum C, Majima Y. Leukotriene D4 upregulates MUC2 gene transcription in human epithelial cells. Pharmacology 2008;81:221–8.

Suzuki T, Hara H. Quercetin enhances intestinal barrier function through the assembly of zonula [corrected] occludens-2, occludin, and claudin-1 and the expression of claudin-4 in Caco-2 cells. Journal of Nutrition 2009;139:965–74.

Turner JR. Molecular basis of epithelial barrier regulation: from basic mechanisms to clinical application. American Journal of Pathology 2006;169:1901–9.

Van Winkle WB. Calcium release from skeletal muscle sarcoplasmic reticulum: site of action of dantrolene sodium. Science 1976;193:1130–1.

Vreeburg RA, van Wezel EE, Ocana-Calahorro F, Mes JJ. Apple extract induces increased epithelial resistance and claudin 4 expression in Caco-2 cells. Journal of the Science of Food and Agriculture 2012;92:439–44.

Wang D, DuBois RN. Measurement of eicosanoids in cancer tissues. Methods in Enzymology 2007;433:27–50.

Wardle TD, Hall L, Turnberg LA. Inter-relationships between inflammatory mediators released from colonic mucosa in ulcerative colitis and their effects on colonic secretion. Gut 1993;34:503–8.

Woszczek G, Chen LY, Nagineni S, Kern S, Barb J, Munson PJ, et al. Leukotriene D(4) induces gene expression in human monocytes through cysteinyl leukotriene type I receptor. Journal of Allergy and Clinical Immunology 2008;121. pp. 215.e1–221.e1.

Ye D, Ma I, Ma TY. Molecular mechanism of tumor necrosis factor-alpha modulation of intestinal epithelial tight junction barrier. American Journal of Physiology – Gastrointestinal and Liver Physiology 2006;290:G496–504.

Yokomizo T, Kato K, Hagiya H, Izumi T, Shimizu T. Hydroxyeicosanoids bind to and activate the low affinity leukotriene B4 receptor, BLT2. Journal of Biological Chemistry 2001;276:12454–9.

Yokomizo T, Kato K, Terawaki K, Izumi T, Shimizu T. A second leukotriene B(4) receptor, BLT2. A new therapeutic target in inflammation and immunological disorders. Journal of Experimental Medicine 2000a;192:421–32.

Yokomizo T, Masuda K, Kato K, Toda A, Izumi T, Shimizu T. Leukotriene B4 receptor. Cloning and intracellular signaling. American Journal of Respiratory and Critical Care Medicine 2000b;161:S51–5.

Zijlstra FJ, van Dijk AP, Garrelds IM, Ouwendijk RJ, Wilson JH. Species differences in the pattern of eicosanoids produced by inflamed and non-inflamed tissue. Agents and Actions 1992, Spec No: C73-5.

Zijlstra FJ, Wilson JH. 15-HETE is the main eicosanoid present in mucus of ulcerative proctocolitis. Prostaglandins Leukotrienes and Essential Fatty Acids 1991;43:55–9.