LXA₄ stimulates ZO-1 expression and transepithelial electrical resistance in human airway epithelial (16HBE14o-) cells

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Lipoxin isomer, the LXA₄ (5S,6R,15S-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid), have been reported on leukocyte trafficking via modulation of chemotaxis, adhesion, transmigration, and phagocytic clearance of apoptotic cells (13, 20, 39). In airway epithelial cells, since the first detection of LXA₄ in bronchoalveolar lavage in 1990, an anomalous low production of LXA₄ has been reported in airway of cystic fibrosis (CF) and severe asthmatic patients, which could explain the persistent inflammation in these severe airway diseases (6, 8, 24, 29, 49). In a previous study, we have shown that airway epithelial cells are a biological target for LXA₄. LXA₄ stimulated a rapid and transient intracellular Ca²⁺ mobilization in airway epithelial cells that involved its receptor, the ALX (5). However, very little is known about the role and the mechanism of action of LXA₄ in airway epithelial function.

In the present study, using transepithelial electrical resistance (TER) measurements and immunofluorescence of the ZO-1, we investigated the possible role of LXA₄ in the regulation of tight junction formation.

MATERIALS AND METHODS

Cell culture. The human bronchial epithelial immortalized cell line, 16HBE14o-, was derived from the surface epithelium of mainstream, second-generation bronchi (13a). The 16HBE14o- cells form polarized monolayers with intact tight junctions. These cells were grown in Eagle’s minimal essential medium (BioWhittaker, Walkersville, MD) supplemented with 10% FCS, 1% penicillin G, 1% streptomycin, and 1% l-glutamine. After reaching confluence, the cells were washed twice with a PBS solution and isolated at 37°C, using a trypsin solution (polyvinylpyrrolidone, 0.2% EGTA, and 0.25% trypsin containing 0.02% EDTA). The lung carcinoma A549 cells (European cell culture collection) were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FCS, 1% penicillin G, 1% streptomycin, and 1% l-glutamine. Both cell lines were grown in Corning culture flasks coated with a collagen/fibronectin solution (CFN) at 37°C in a humidified 5% CO₂ atmosphere.

Epithelial cells were plated at a density of 10⁴ to 2 × 10⁵ cells/cm² on CFN-coated 16-well Labtek (Nunc) or on CFN-coated permeable filters (Transwell filters, 0.4-μm pore size; Corning Costar, Cambridge, MA) or on CFN-coated 6-well plates for immunofluorescence experiments, TER measurements, and Western blots, respectively.

Immunofluorescence. For immunofluorescence experiments, 7 days after plating, cell monolayers were fixed for 10 min in 4% paraformaldehyde in PBS, pH 7.5, followed by a 5-min permeabilization in 0.1% Triton X-100 in PBS, and incubated in PBS containing 0.1% BSA. Cells were stained with a monoclonal mouse anti-ZO-1 antibody (1:500; Zymed Laboratories, South San Francisco, CA) and revealed with fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody (Zymed Laboratories). As negative controls, an identical
labeling procedure was carried out without the anti-ZO-1 primary antibody. A microscope LEICA DMRB 2001 coupled with a Roper MicroMax 1300 Y/H camera was used for the classic immunofluorescence studies. MetaFluor software was used for monitoring acquisition and image analyses.

Confocal microscopy. Confocal laser microscopy was performed using a LEICA DMRB 1997 with a ×40 oil-immersion objective and coupled to a CoolSnap HQ Photometrics camera. For confocal acquisition, a spinning Nipkow disk was used. Sequential images were captured as stack images by a time series imaging software MetaFluor with 0.5-µm steps. To facilitate the comparison between preparations, an equal number of horizontal slices with the same vertical depth from apical to basal were acquired under identical exposure parameters. XY planes (parallel to cell monolayer) and XZ/YZ planes (orthogonal to cell monolayer) were exported and processed to Adobe Photoshop.

Quantification of fluorescence intensity. As already done by others, quantification of the immunofluorescence intensity has been possible in our system (3, 43, 54). An important preliminary step for the quantification was to establish an appropriate primary antibody dilution. To obtain a fluorescence intensity proportional to the number of ZO-1 molecules, different dilutions of the anti-ZO-1 antibody have been tested, and the saturating antibody concentration has been estimated. During acquisition, to avoid saturation of the images and false negative results, and to be able to compare the fluorescence intensities between differently treated cell preparations, in each set of experiments, the auto-scale parameters obtained for the stronger staining were used for all preparations. To obtain quantitative data, for each condition, three images of ZO-1 labeling were acquired (at the same exposure duration) in three different sections of the cell monolayer preparation. For each acquisition, 10 regions of interest (ROI) were selected at the cell-to-cell contact, and 10 others were selected into the cytoplasm area. The intensity of fluorescence was measured in each ROI and divided by the area of the ROI. The average values of the fluorescence intensity measured at the cell-to-cell contact and within the cytoplasm were calculated. Finally, the mean fluorescence intensities were calculated from n independent experiments.

Western blotting. For Western blot analyses, 7 days after plating, cells were rinsed with PBS (BioWhittaker) and extracted in lysis buffer (10 mM Tris-HCl, pH 7.4, 30 mM sodium pyrophosphate, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2% sodium dodecyl sulfate, 20 mM β-glycerol phosphate, 100 µM orthovanadate, and 1 mM dithiothreitol) containing protease inhibitors (0.1 M phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml aprotinin). Samples were normalized for protein content with the BCA protein assay kit (Pierce Chemical). Protein samples were fractionated on an 8% sodium dodecyl sulfate polyacrylamide gel followed by a transfer at 475 mA during 2 h onto an Immobilon-P membrane (Millipore). Blots were blocked with blocking buffer (3% BSA, 0.05 M Tris-HCl, 0.15 M NaCl, 0.1% Triton X-100) before being probed with monoclonal antibody against ZO-1 (1:400), a polyclonal antibody against occludin (1:400) (both from Zymed Laboratories). Monoclonal antibody against anti-β-actin (1:5,000, Sigma) was used for standardization. Horseradish peroxidase-conjugated anti-mouse antibodies, anti-mouse IgG peroxidase conjugate (1:3,000), and anti-rabbit IgG peroxidase conjugate (1:5,000, Sigma) were used as secondary probes. The blots were developed with an enhanced chemicluminescence kit (Amersham Life Sciences, Arlington Heights, IL). The densitometric analysis of immunoblots was performed by using Kodak 1D Image Analysis Software.

TER measurement. The 16HBE14o- cells were seeded in the upper chamber of a Transwell tissue culture plate (12-mm diameter, 0.4-µm pore size, Costar) and allowed to reach confluence. The basolateral and apical sides of the filters were exposed to LXA4 when indicated. The TER of cells grown on filters was measured every day, with an epithelial voltohmeter (Endohm; World Precision Instruments, Sarasota, FL). To study the rapid effect of LXA4 on the TER, the voltohmeter was coupled to an A/N converter (World Precision Instruments), and the TER measurement was monitored using Powerlab software (Chart for Windows, v4.0 ADInstruments) with an acquisition frequency of 1 every 0.5 s. The background electrical resistance attributed to fluid and a blank Transwell filter were subtracted from the measured TER. The TER measurements were normalized by the area of the monolayer and given as Ω·cm².

Statistical analysis. Statistical analyses were performed using Microsoft Excel. Data were expressed as means ± SE of n experiments. The Student’s t-test was used, with differences considered as significant for P < 0.05.

RESULTS

Basal tight junction formation in airway epithelial cells. The basal level of tight junction protein components was investigated using the Western blotting technique in two different human airway epithelial cell types: the 16HBE14o- and A549 cell lines. As illustrated in Fig. 1A, the ZO-1 protein was expressed in both airway epithelial cell lines. A higher level of ZO-1 was detected in A549 cells. Occludin was mainly detected in 16HBE14o- cells.

The ZO-1 localization was studied by ZO-1 immunostaining in these two cell lines, 7 days after plating cells to confluence on collagen/fibronectin-coated permeable filters or on coated glass. The 16HBE14o- cells grown on permeable filters appeared smaller (planar view) and higher (orthogonal view) than cells grown on glass. However, the ZO-1 immunostaining was seen as a continuous line on the 16HBE14o- cells at the cell-to-cell contact in both culture conditions. In A549 cells, the staining appeared to be diffuse in the cytosol with no staining at the plasma membrane (Fig. 1B).

The functional integrity of the tight junction was evaluated by the measurement of the TER of the airway epithelial cell monolayers. The 16HBE14o- cells reached a maximum TER of 867 ± 38 Ω·cm² (n = 3) 7–10 days after plating, whereas the TER of A549 cells did not increase over a similar time period in culture (Fig. 1C).

LXA4 effect on the daily TER increase. The effect of LXA4 has been tested on the TER daily increase of 16HBE14o- cells grown on permeable filters over 10 days. To investigate the effect of LXA4 on tight junction formation rather than cell proliferation, which occurs during the first days after plating the cells, the exposure to LXA4 started when cells had reached a TER of 200 Ω·cm². The LXA4 (100 nM) significantly stimulated the daily TER increase by 25% on average compared with untreated controls (n = 3, P < 0.01). However, LXA4 did not affect the TER of A549. As shown on Fig. 2, the stimulatory effect of LXA4 (100 nM) on TER was already significant the day after starting the treatment (day 1). The LXA4 effect on the TER increase was inhibited when cells were exposed to 10 nM boc-2, the ALX/FPR1 antagonist peptide (n = 3, P < 0.01). The boc-2 alone did not have a significant effect on TER (n = 3, P > 0.05).

LXA4 effects on ZO-1, occludin, and claudins expression. The effect of LXA4 was tested on ZO-1, occludin, claudin-1, and claudin-4 expression in 16HBE14o- cells using Western blotting. As illustrated in Fig. 3, A and B, 2 days of exposure of confluent 16HBE14o- cell monolayers to LXA4 stimulated the expression of ZO-1, occludin, and claudin-1. Densitometric analysis demonstrated that the degree of the stimulatory effect of LXA4 was dependent on the LXA4 concentration. For cells treated with 100 nM LXA4, the ZO-1 signal was six times
higher than the control, the occludin signal was four times higher than the control, and the claudin-1 signal doubled from the control (Fig. 3B). LXA₄ did not significantly affect claudin-4 expression (Fig. 3B).

**ZO-1 localization.** To test the effect of LXA₄ on the localization of ZO-1, we investigated the effect of LXA₄ exposure on ZO-1 staining in 16HBE14o- cells grown on permeable filters, using immunofluorescence techniques. In the absence of the first antibody, unspecific fluorescence was not detected in either control or LXA₄-treated cell preparations (data not shown). As shown in Fig. 3B, after 2 days of treatment with LXA₄ (100 nM), the amount of ZO-1 immunoreactive signal increased in the overall preparation and was more pronounced at the plasma membrane. The quantitative analysis of the ZO-1 immunoreactive signal measured at the cell-to-cell contact markedly increased between not-treated and LXA₄-treated cells (n = 4, P < 0.005), whereas no significant change of the staining was detected in the cytosol.

**Rapid effect of LXA₄ on TER.** The effect of LXA₄ on 16HBE14o- cell tight junction formation was also investigated for short-term exposure. The 16HBE14o- cells grown on permeable filters during 10 days had a mean TER value of 611 ± 40 Ω·cm² (n = 22). Exposure of the 16HBE14o- cell monolayers to either apical or basolateral LXA₄ (100 nM) stimulated a TER increase of 14.5 ± 0.6 Ω·cm² within 1.7 ± 0.3 min (Fig. 4A). This increase was small but significant compared with the TER variation measured in the same interval of time without treatment (P < 0.005) and was completely inhibited by boc-2 treatment (P < 0.005). In addition, since we have previously shown that LXA₄ stimulated Cl⁻ secretion in the 16HBE14o- cells, we used the Na-K-2Cl cotransporter inhibitor, bumetanide, to inhibit the transepithelial Cl⁻ conductance and then had a more specific estimation of the contribution of the paracellular permeability to the TER (5). Basolateral stimulation with bumetanide (10⁻⁴ M) alone stimulated a TER increase of 12.4 ± 3.0 Ω·cm² (n = 16) (Fig. 4B). After bumetanide, apical and basolateral exposure to LXA₄ stimulated an additive TER increase of 35 ± 3.0 Ω·cm² that reached a sustained value after 1.8 ± 0.2 min (Fig. 4B).

**Rapid effects of LXA₄ on ZO-1 localization.** Using confocal microscopy techniques, we investigated the rapid effect of LXA₄ on ZO-1 localization. As illustrated in Fig. 4C, treatment with LXA₄ (100 nM) for 10 min increased the immunostaining of ZO-1. The increased ZO-1 staining was more pronounced at the cell-to-cell contact. The quantitative analysis of the ZO-1 immunoreactive signal measured at the cell-to-cell contact indicated a significant increase (n = 10, P < 0.01) between untreated and treated cells for 10 min with LXA₄ (100 nM), whereas a significant difference was not detected for the immunoreactive signal in the cytoplasm of cells treated or not treated with LXA₄.

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**Fig. 1.** Zonula occludens-1 (ZO-1) expression and transepithelial electrical resistance (TER) of 2 different human bronchial cell lines. A: typical Western blots of total cell lysates from confluent 16HBE14o- and A549 cell monolayers using MAb anti-ZO-1, anti-occludin, and anti-β-actin for standardization. B: immunofluorescence of ZO-1 in 16HBE14o- and A549 cell monolayers. The cells were grown for 7 days on coated glass coverslips and fixed with paraformaldehyde before staining. The same camera, lens, and exposure parameters were used (7 s, ×63). C: mean TER ± SE of 3 independent experiments as a function of time expressed as the number of days after plating the airway epithelial cells on coated permeable filters.

**Fig. 2.** Effect of lipoxin A₄ (LXA₄) on the daily TER increase of 16HBE14o-cells. A typical set of experiments showing the mean TER increase of 16HBE14o-cells as a function of the number of days after the beginning of LXA₄ treatment. The TER increases were measured in 4 different conditions: not-treated cells (control), cells treated with LXA₄ (100 nM) in the apical and basolateral solution, cells treated with LXA₄ (100 nM) and boc-2 (10 nM), and cells treated with boc-2 alone (10 nM) (n = 3, P < 0.05). The LXA₄ treatment started 2 days after plating the cells on a permeable filter, when the TER had reached 200 Ω·cm². For each condition, the mean TER obtained the treatment starting day has been subtracted from the mean TER measured the following days (*P < 0.05).
with LXA₄ (n = 10, P > 0.05) (Fig. 4D). Orthogonal views indicated that LXA₄ rapidly stimulated expression of ZO-1 at the most apical junction of the cells (Fig. 4C). Data are representative of results of 10 independent experiments.

**Role of PKC and intracellular [Ca²⁺]**. The role of PKC activity was investigated using the PKC₁₋₃₁, a pseudosubstrate PKC inhibitor, and the Go₆-6976 PKC and PKC₁ inhibitor. Both PKC inhibitors significantly decreased the ZO-1 staining and inhibited its increase induced by LXA₄ at the plasma membrane (n = 7, P > 0.05) (Fig. 5). However, in BAPTA-AM-loaded cells, the stimulation of ZO-1 membrane staining by LXA₄ was inhibited by 47% (n = 7, P < 0.05) compared with LXA₄ treatment alone (Fig. 5).

**DISCUSSION**

One of the consequences of chronic airway inflammation and/or infection in pulmonary diseases is bronchial epithelial shedding and the breakdown of epithelium cohesion (25, 27). However, the cellular mechanisms by which tight junction formation and disruption are regulated are not well understood. In CF and severe asthmatic patients, changes in the production levels of the endogenous lipid mediator, the LXA₄, in the...
and under perfusion of apical LXA4 (100 nM) perfused from 22 independent 16HBE14o- cells. The difference of ZO-1 location between A549 cells might be related to the tight junction function and could suggest a role for occludin in modulating the subcellular distribution of tight junction components resulting in junction formation. The ability of 16HBE14o- cells to form tight junctions and the location of ZO-1 expression justified the use of the study of airway epithelial cells tight junction regulation, and, as employed by others, the use of TER measurement as an indicator of the tight junction functional integrity (12, 18).

Here, we report for the first time that LXA4 stimulated ZO-1, occludin, and claudin-1 expression into tight junctions in human airway epithelial 16HBE14o- cells. However, claudin-4 expression was not significantly affected by LXA4. The effects of LXA4 on cytoskeleton organization, which is associated with its role on chemotaxis, have been reported in non-epithelial cell types such as monocytes, macrophages, and endothelial cells (7, 32, 33, 47). Furthermore, we have shown that LXA4 also stimulated the TER daily increase. The enhanced TER measured under LXA4 treatment is the result of an increase in paracellular electrical resistance since the transcellular resistance is decreased due to enhanced Cl− secretion, as previously reported (5). Therefore, in the present study, the stimulatory effect of LXA4 on the total transepithelial resistance underestimated the increase in the paracellular junctional resistance. One simple explanation is that the raise in paracellular electrical resistance is the consequence of stimulation of tight junction components expression and tight junction assembly by LXA4. However, this result cannot exclude that LXA4 might enhance the TER via a more complex mechanism involving other tight junction molecules and/or adherent junctions regulation.

We also found that a short-term exposure to LXA4 rapidly enhanced the ZO-1 staining at the cell-to-cell contact and produced a fast increase of TER. The more pronounced effect of LXA4 on the ZO-1 staining at the cell-to-cell contact compared with the cytoplasm suggested that LXA4 stimulated the rapid translocation of ZO-1 at the membrane and also excluded the possible effect of LXA4 in discovering the antibody epitope and therefore producing an artifact. The ZO-1 rapidly translocated at the membrane must come from the cytoplasmic pool. However, ZO-1 staining in the cytosol was not significantly decreased after LXA4 because the staining changes per surface unit was below the detection threshold of the immunofluorescent signal.

Airway has been reported (6, 8, 24, 41, 49). In the present study, using multiple approaches, we provide evidence for a novel role of the anti-inflammatory molecule LXA4 in stimulating tight junction formation.

Tight junctions are formed by molecular species including ZO-1, claudins, and occludins that assemble through protein-protein interactions. In our study, the 16HBE14o- cells were able to form monolayers of high TER. In contrast, as already reported, the alveolar A549 cells did not generate a TER under any of the cell culture conditions employed in our study (22). However, the A549 cells expressed a high level of ZO-1, but diffusely in the cytosol, which is not its functional location, and expressed very low levels of occludin compared with 16HBE14o- cells. The difference of ZO-1 location between these two cell lines and the defective expression of occludin in the airway has been reported (37, 52), cAMP (1, 26, 28), and kinases (2, 11, 36). Since in non-epithelial cells, the role of LXA4 on PKC activation has been reported, we investigated a possible PKC signaling pathway in the effect of LXA4 on tight junctions (10, 21, 30, 46). Our results suggested that a PKC activity was implicated in the stimulatory effects of LXA4 on ZO-1 translocation into the membrane of airway epithelial cells. Several studies have shown that PKC activation, which is associated with its translocation from the cytosol to the membrane, is also involved in the regulation of tight junctions and could be considered as a part of the tight junction complex. Ligand-specific activation of distinct PKC isoforms can exert paradoxical effects in epithelial monolayers: either increasing or decreasing permeability due to tight junction regulation. Some PKC isoforms participate in a tight junction disruption mechanism (9, 17, 48). In contrast, in our study, both PKC inhibitors used abolished the
stimulatory effect of LXA4 on ZO-1 staining at the plasma membrane, indicating the involvement of PKC signaling and intracellular Ca\(^{2+}\) in the effect of LXA4 on ZO-1. In MDCK, PKC inhibition antagonized the TER increase during a calcium switch (50). In addition, known agonists of PKC rapidly stimulated the TER and the translocation of tight junction proteins from the cytoplasm to cell-cell contact in TMK-1 gastric cancer cells and in T84 colonic epithelia (56, 57).

The Go-6976 selectively inhibits PKC\(\alpha\) and PKC\(\beta\) but does not inhibit the activity of PKC\(\delta\), \(\epsilon\), or \(\zeta\). Our results suggested that a calcium-dependent PKC activity was involved in the rapid effect of LXA4 on ZO-1 at cell-to-cell contact. Thus, we investigated specifically the role of intracellular Ca\(^{2+}\) on the effect of LXA4 on tight junction formation. We found that the stimulatory effect of LXA4 on ZO-1 expression required intracellular Ca\(^{2+}\) changes since BAPTA-AM used as a chelator of intracellular Ca\(^{2+}\) inhibited the effect of LXA4 on ZO-1 expression at the plasma membrane. In MDCK, the role of intracellular Ca\(^{2+}\) in regulation of tight junction has been reported. It was also reported that intracellular Ca\(^{2+}\) appears to be necessary for the dissociation of tight junction-cytoskeletal complexes, thus permitting functional tight junction reassembly (51, 52, 55). The role of an intracellular Ca\(^{2+}\) signal in the regulation of tight junction is consistent with other reports indicating that an external Ca\(^{2+}\) switch (increase of external Ca\(^{2+}\) after its depletion) produced a significant rise in intracellular Ca\(^{2+}\) and that chelation of intracellular Ca\(^{2+}\) during the Ca\(^{2+}\) switch markedly attenuated the development of TER.

**Fig. 5.** Effect of PKC and intracellular Ca\(^{2+}\) on ZO-1 localization in 16HBE14o- cells. Immunofluorescence illustrating the involvement of PKC signaling and intracellular Ca\(^{2+}\) in the effect of LXA4 on ZO-1. A: immunofluorescence illustrating the inhibitory effect of the PKC inhibitors 19–31 (200 nM) and Go-6976 (100 nM) and BAPTA-AM (10 \(\mu\)M, 40 min). Cells were not treated (top left) or treated with LXA4 (100 nM) for 10 min (top right) or treated with the inhibitor alone for 40 min (bottom left) or preincubated with the inhibitor for 40 min and treated afterwards with LXA4 (100 nM) for 10 min (4.5-s exposure, \(\times\)40). Bar = 20 \(\mu\)m. For each image, a zoom is presented. B: means \pm SE fluorescence intensities in arbitrary units (AU) measured at the cell-to-cell contact and within the cytoplasm in the 6 different conditions illustrated above (\(n = 7\), **\(P < 0.01\)).
Finally, this finding is coherent with our previous report showing the rapid effect of LXA4 on Ca2+ mobilization from intracellular Ca2+ stores (5).

We have previously shown, using RT-PCR, that the 16HBE14o- cells express mRNA of the LXA4 receptor, whereas A549 cells do not express the receptor (5). The LXA4 receptor is a G protein-coupled receptor, also referred to as formyl peptide receptor-like 1 (FPRL1) (45). Using an anti-FPRL1 antibody, we confirmed that the 16HBE14o- cells express the LXA4 receptor at a much higher level than in A549 cells (data not shown). Furthermore, the LXA4 stimulatory effect on TER was abrogated by the use of a competitive antagonist of the LXA4 receptor (boc-2) indicating the involvement of this receptor in the LXA4 regulation of tight junction resistance.

Tight junctions are responsible for the selective regulation of transport of inflammatory cells through the paracellular pathway (14). The evidence of a role for LXA4 in stimulating tight junctions assembly suggests that, in the airway of CF and severe asthmatic patients, the decreased LXA4 levels could favor the transmigration of inflammatory cells via disrupted tight junctions, in addition to other consequences on the inflammatory process. Epithelial cohesion and more specifically tight junction integrity also determine the quality of the fence between external environment and the milieu interieur. In intestinal diseases, it was suggested that the bacterial invasion and destruction of the epithelium is primarily due to migration of leukocytes, particularly polymorphonuclear PMN that destroy cohesion of the epithelial barrier (40). In airways, it was also reported that invasion of epithelial cells in culture by *Burkholderia multivorans* strains was reduced when cells were grown as tight monolayers compared with unpolarized cells (16). Another study reported that epithelial cells in monolayers with tight junctions were entirely resistant to PAO1-induced apoptosis. In contrast, cell lines that do not form tight junctions were susceptible, with 50% of the population apoptotic after 6 h of exposure to PAO1 (42). This suggests that tight junctions are a potential therapeutic target for intervention in diseases where their integrity has been compromised and reassembly is required.

In conclusion, we provide evidence for a novel effect of LXA4 on tight junction structure and function in human airway epithelial cells. Our results suggest that, in addition to the stimulation of ZO-1, occludin, and claudin-1 expression, LXA4 also enhances tight junction formation from existing intracellular pools via a mechanism involving the LXA4 receptor AXL/FPRL1 and calcium and PKC signaling pathway.

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