Control of Intestinal Epithelial Permeability by Lysophosphatidic Acid Receptor 5

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SUMMARY

Bioactive lipid lysophosphatidic acid (LPA) exerts multiple effects, but our understanding of its role in the intestine is limited. Using LPA receptor-deficient mice, we explore the effect of LPA on intestinal permeability and describe a new function of LPA5.

BACKGROUND & AIMS: Epithelial cells form a monolayer at mucosal surface that functions as a highly selective barrier. Lysophosphatidic acid (LPA) is a bioactive lipid that elicits a broad range of biological effects via cognate G protein-coupled receptors. LPA receptor 5 (LPA5) is highly expressed in intestinal epithelial cells, but its role in the intestine is not well-known. Here we determined the role of LPA5 in regulation of intestinal epithelial barrier.

METHODS: Epithelial barrier integrity was determined in mice with intestinal epithelial cell (IEC)-specific LPA5 deletion, Lpar5IEC. LPA was orally administered to mice, and intestinal permeability was measured. Dextran sulfate sodium (DSS) was used to induce colitis. Human colonic epithelial cell lines were used to determine the LPA5-mediated signaling pathways that regulate epithelial barrier.

RESULTS: We observed increased epithelial permeability in Lpar5IEC mice with reduced claudin-4 expression. Oral administration of LPA decreased intestinal permeability in wild-type mice, but the effect was greatly mitigated in Lpar5IEC mice. Serum lipopolysaccharide level and bacterial loads in the intestine and liver were elevated in Lpar5IEC mice. Lpar5IEC mice developed more severe colitis induced with DSS. LPA5 transcriptionally regulated claudin-4, and this regulation was dependent on transactivation of the epidermal growth factor receptor, which induced localization of Rac1 at the cell membrane. LPA induced the translocation of Stat3 to the cell membrane and promoted the interaction between Rac1 and Stat3. Inhibition of Stat3 ablated LPA-mediated regulation of claudin-4.

CONCLUSIONS: This study identifies LPA5 as a regulator of the intestinal barrier. LPA5 promotes claudin-4 expression in IECs through activation of Rac1 and Stat3. (Cell Mol Gastroenterol Hepatol 2021;12:1073–1092; https://doi.org/10.1016/j.jcmgh.2021.05.003)

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Intestinal lumen is lined with epithelial cells forming a barrier that prevents unimpeded movement of solutes and fluid and protects the host from the luminal milieu. Epithelial cells are joined together by a highly organized apical junctional complex, which includes tight junction (TJ) and adherens junction (AJ). TJs are made up of a number of
proteins, including transmembrane claudins, occludin, and junctional adhesion molecules (JAMs). The AJ consists of cadherin adhesion receptors and cytoplasmic proteins associated with them, including catenins and actin filaments. A breach of the intestinal epithelial barrier can elicit a broad range of diseases, including inflammatory bowel disease (IBD), celiac disease, and colorectal cancer.

Although lipids are the major constituents of cell membranes, lipids can also function as a mediator of intercellular and extracellular processes. Among the naturally occurring lipids, lysosphosphatic acid (LPA) has been linked to various pathologic conditions such as cancer, fibrosis, inflammation, and atherosclerosis. LPA signals through 6 distinct G protein-coupled receptors (GPCRs), termed LPA1–LPA6 (encoded by Lpar1–Lpar6 genes in rodents). The expression level of each LPA receptor varies widely among different tissues and cell types. Mouse gastrointestinal (GI) tract expresses at least 5 distinct LPA receptors at various levels, although the locations of each LPA receptor are not known. Bioactive LPA in serum and plasma is mainly produced by autotaxin (ATX), a secreted lysophospholipase D that converts lysophosphatidylcholine to LPA. Elevated level of ATX is associated with intestinal inflammation, and inhibition of ATX is shown to reduce inflammation in the gut and restore intestinal epithelial cell (IEC) differentiation. Orally administered LPA increases tumor incidence in ApcMin/+ mice by stimulating cancer cell proliferation. However, the ability of LPA to modulate cell proliferation and migration is equally critical for the maintenance of the epithelial barrier in the gut. We have shown recently that LPA maintains intestinal epithelial integrity by facilitating wound closure via LPA1-dependent stimulation of IECs. Lpar1−/− mice develop more severe colitis, an effect associated with reduced epithelial mucosa restoration and barrier defect. The expression level of LPA5, also known as GPR92 or GPR93, is relatively high in the GI tract where Lpar5 mRNA is detected in the epithelial cells and lymphocytes. LPA5 differs from LPA1 or LPA2 in that its amino acid sequence shares high homology with the purinergic family of GPCR. In addition, LPA5 has a unique preference for an LPA species with an ether linkage, and it can also be activated by the dietary protein hydrolysate and peptone.

Lpar5 is detected in sensory nerves of the mouse enteric nerve system where it is activated by dietary protein hydrolysate and peptone. The dietary protein peptone can also be activated by the dietary protein hydrolysate and peptone.

We have shown previously that LPA5 regulates the brush border Na+−/H+ exchanger NHE3 (Slc9A3), which is a major Na+ transporter in the intestine. Oral administration of LPA attenuates intestinal water loss, a hallmark of diarrhea, in an experimental model of diarrhea via activation of NHE3. Despite its abundant expression in the GI tract, the role of LPA5 in IECs is not known beyond the regulation of NHE3. In this study, we investigated the role of LPA5 in regulation of intestinal epithelial barrier function using mice lacking Lpar5 in IECs. Our study demonstrated that loss of LPA5 resulted in epithelial barrier defect, and we have delineated critical signaling pathways that underlie LPA5-dependent regulation of intestinal epithelial barrier.

Results

Dysregulation of Intestinal Epithelial Barrier in the Absence of LPA5

We have reported recently that mice lacking Lpar5 in IECs, Lpar5−/−, do not display a gross morphologic change in the intestine, and the basal intestinal functions assessed by stool frequency and fecal water content are unchanged. However, the comparison of bowel movement does not reveal a subtle change that may affect the intestinal epithelium. Because Lpar5 expression is high in the intestine, we aimed to study whether Lpar5 loss in IECs alters epithelial integrity by examining the epithelial barrier function. We compared intestinal permeability between Lpar5−/− and Lpar5−/− mice by oral administration of fluorescein isothiocyanate-labeled 4 kDa dextran (FD-4). Measuring the fluorescence levels in the serum revealed that FD-4 flux across the intestinal mucosa was significantly elevated in Lpar5−/− mice compared with control Lpar5−/− mice (Figure 1A), indicating a more permeable intestinal epithelium in the absence of LPA5. To determine whether this increase in epithelial permeability is associated with a change in TJ protein expression, we determined mRNA expression levels of several TJ proteins in the mouse colon. Quantification of mRNA levels by quantitative reverse transcription polymerase chain reaction (RT-PCR) showed that claudin-4 (Cldn4) mRNA abundance was reduced in Lpar5−/− mice compared with Lpar5−/− mice, but ZO-1 (TJPN1), occludin (Ocn), JAM-A (F11R), claudin-1 (Cldn1), claudin-2 (Cldn2), and claudin-7 (Cldn7) transcript levels were not statistically different between them (Figure 1B). Western blotting of mucosal lysates from the small intestine and colon confirmed decreased claudin-4 expression in Lpar5−/− mice versus Lpar5−/− mice (Figure 1C). The expression level of E-cadherin, the primary epithelial AJ protein, was not altered by Lpar5 loss. Immunofluorescence (IF) confocal microscopic analysis showed that the membrane expression of claudin-4 of epithelial cells in the small intestine and colon of Lpar5−/− mice was down-regulated (Figure 1D).

Abbreviations used in this paper: AJ, adherens junction; ATX, autotaxin; DSS, dextran sulfate sodium; EGFR, epidermal growth factor receptor; FD-4, fluorescein isothiocyanate-labeled 4 kDa dextran; GEF, guanine nucleotide exchange factor; GI, gastrointestinal; GPCR, G protein-coupled receptor; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IF, immunofluorescence; JAMs, junctional adhesion molecules; LPA, lysophosphatidic acid; LPA5, LPA receptor 5; LPS, lipopolysaccharide; NHE3, Na+/H+ exchanger 3; PBS, phosphate-buffered saline; ROCK, RhoA-associated kinase; RT-PCR, reverse transcriptase polymerase chain reaction; SD, standard deviation; shRNA, short hairpin RNA; Stat, signal transducers and activators of transcription; TJ, tight junction.

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Figure 1. Lpar5 loss dysregulates epithelial barrier in the mouse intestine. (A) FD-4 was orally administered to Lpar5f/f and Lpar5ΔIEC mice, and FD-4 levels in the serum were determined 4 hours later. **P < .01. n = 5. (B) The mRNA levels of TJ proteins in colonic mucosa of Lpar5f/f and Lpar5ΔIEC mice were determined by quantitative RT-PCR. The mRNA expression level of each protein was normalized to β-actin mRNA expression level. **P < .01. n = 5. Data are expressed as mean ± SD. (C) Representative Western blots of junctional proteins in the small intestine (left) and distal colon (right) are shown. Molecular weight (MW) of each protein is shown in parentheses. Comparable results were observed in 3 independent experiments. (D) Confocal IF images for claudin-4 (green), F-actin (red), and nuclei (blue; DAPI) are shown. Representative images from 3 Lpar5f/f and 4 Lpar5ΔIEC mice are shown. Scale bar = 50 μm.
LPA-Mediated Regulation of Epithelial Permeability In Vitro and In Vivo Is Dependent on LPA5

To establish a cause-effect relationship between LPA5 and epithelial barrier function, we used 2 IEC lines, SK-CO15 and Caco-2bbe cells. LPA5 expression in these cell lines was assessed by determining Lpar5 mRNA levels by RT-PCR. As in our previous study,27 both lines expressed Lpar1 and Lpar2. Lpar5 mRNA expression was low in SK-CO15 cells or undetectable in Caco-2bbe cells by RT-PCR (Figure 2A). Knockdown of LPA5 in SK-CO15 cells using 2 different short hairpin (sh) RNA (shRNA) specific for LPA5, shLPA5, resulted in >60% decrease in Lpar5 mRNA levels (Figure 2B). To correlate LPA5 expression and epithelial barrier function, we determined transepithelial electrical resistance (TER) in SK-CO15 cells stably expressing HA-LPA5 or shLPA5. HA-LPA5 expression increased TER by more than 75%, whereas knockdown using shRNA resulted in decrease (18.2% by shLPA5-777 and 25.6% by shLPA5-361) in TER (Figure 2C). Consistently, stable expression of HA-LPA5 in Caco-2bbe cells increased TER by 38% (Figure 2D). We next sought to determine whether activation of LPA5 dynamically regulates TER. Cells grown on Transwell inserts were cultured in medium supplemented with 0.5% fetal bovine serum [PBS], and TER was measured hourly for 4 hours. In control SK-CO15/pCDH cells, LPA resulted in a statistically significant increase in TER (Figure 2E). The effect was markedly elevated in cells expressing HA-LPA5 (Figure 2F), whereas knockdown of Lpar5 completely ablated the effect (Figure 2G). We postulated that if LPA indeed regulates epithelial barrier, orally administered LPA should strengthen the epithelial barrier function in the mouse intestine. To test this idea, we gavaged LPA or carrier to Lpar5−/− and Lpar5−IEC mice for 3 days, followed by oral administration of FD-4 to determine intestinal permeability. LPA administration led to a significant decrease in serum FD-4 concentration in Lpar5−/− mice compared with carrier-treated controls (Figure 2H), suggesting that LPA enhances intestinal barrier function in vivo. The difference in serum FD-4 levels between LPA and carrier treatment was significantly reduced in Lpar5−IEC mice. However, a smaller and yet statistically significant difference in FD-4 levels in Lpar5−IEC mice suggests that another LPA receptor, in addition to LPA5, may contribute to LPA-dependent intestinal epithelial barrier function.

Increased Susceptibility to Dextran Sulfate Sodium–Induced Colitis in the Absence of LPA5

Increasing evidence suggests epithelial barrier dysfunction as a significant contributing factor to the pathogenesis of IBD.28,29 To evaluate whether the dysregulation of the epithelial barrier by Lpar5 loss is sufficient to alter intestinal inflammatory disease processes, we used the dextran sulfate sodium (DSS)-induced colitis model. Age and gender matched Lpar5−/− and Lpar5−IEC mice were given 2% DSS in drinking water for 5 days, followed by a 6-day period of recovery with normal water. During the DSS administration, Lpar5−IEC mice developed more severe colitis compared with control mice as evidenced by greater effects on body weight loss, occult blood, and diarrhea (Figure 4A–D). On the last day of the experiment, the disease state in Lpar5−/− mice was close to the baseline, whereas colitis was still active in Lpar5−IEC mice. Determining the expression levels of several proinflammatory cytokines, including tumor necrosis factor-α, interferon-γ, interleukin 6, and interleukin 1β, further corroborated that DSS-induced colitis in Lpar5−IEC mouse colon was exacerbated (Figure 4E). Consistently, histologic analysis revealed increased immune cell infiltration and mucosal damage in Lpar5−IEC mice than in Lpar5−/− mice (Figure 4F and G).

LPA5 Regulates Claudin-4 Expression

In Lpar5−IEC mouse intestine, we observed a significant decrease in claudin-4 expression (Figure 1). To confirm this observation, we determined the mRNA levels of several TJ proteins in SK-CO15 cells expressing HA-LPA5 or shLPA5. In line with the findings in the mouse intestine, there was a LPA5-dependent change in claudin-4 mRNA expression. Specifically, HA-LPA5 expression or Lpar5 knockdown altered claudin-4 mRNA expression without significantly altering mRNA expression levels of ZO-1, JAM-A, occludin, and claudin-1, -2, and -7 (Figure 5A). The LPA5-dependent modulation of claudin-4 expression was confirmed in Caco-2bbe cells expressing HA-LPA5 (Figure 5B). The change in claudin-4 mRNA expression in SK-CO15 cells was mirrored by a similar change in claudin-4 protein expression (Figure 5C). IF confocal microscopy on SK-CO15 cells showed that HA-LPA5 expression increased claudin-4 IF signal levels at the plasma membrane between juxtaposed cells, whereas shLPA5 attenuated the IF signal (Figure 5D). Although the effects of shLPA5 on TER and claudin-4 expression assured the role of LPA5 in epithelial barrier regulation in SK-CO15 cells, we could not detect LPA5 protein expression using anti-LPA5 antibodies, both commercial and homemade, which suggested that LPA5 expression in SK-CO15 cells must be relatively low. For this reason, we

Increased Bacterial Translocation in Lpar5−IEC Mice

Increased epithelial permeability allows infiltration of microorganisms into the intestinal mucosal and peripheral organs. We first determined serum lipopolysaccharide (LPS) levels in Lpar5−/− and Lpar5−IEC mice. Serum LPS concentration in Lpar5−IEC mice was elevated by more than 7-fold versus Lpar5−/− mice (Figure 3A). These results were corroborated by increased bacterial loads in Lpar5−IEC mice. The liver, ileum, and proximal and distal colon of mice were homogenized, and serial dilutions of the homogenates were plated on Agar overnight. Bacterial counts were markedly greater in Lpar5−/− mouse tissues (Figure 3B). In particular, there was more than 20-fold difference between Lpar5−IEC and Lpar5−/− livers, demonstrating increased dissemination of bacteria to peripheral organs in Lpar5−IEC mice.
chose to use SK-CO15 cells that were stably transfected with HA-LPA5 for the following experiments.

Decreased claudin-4 mRNA expression by Lpar5 loss in the mouse intestine implied that LPA5 regulates claudin-4 at the level of transcription. To substantiate this notion, we determined claudin-4 mRNA and protein expression in cells treated with LPA. LPA increased claudin-4 mRNA in SK-CO15 cells, and the effects were elevated in SK-CO15/HA-LPA5 cells (Figure 6A, upper). As expected, similar changes in claudin-4 protein expression were observed in these cells (Figure 6A, lower). In Caco-2bbe cells lacking endogenous expressed LPA5, claudin-4 expression was not affected by LPA, but LPA had a robust effect on claudin-4 mRNA and protein expression in Caco-2bbe/HA-LPA5 cells (Figure 6B).

LPA5-Dependent Regulation of Claudin-4 Is Mediated via Transactivation of Epidermal Growth Factor Receptor

The activation of NHE3 by LPA5 involves transactivation of the epidermal growth factor receptor (EGFR), which results in the activation of RhoA/RhoA-associated kinase (ROCK) and MEK-ERK pathways.23,24 To explore whether these signaling pathways regulate LPA-dependent claudin-4
suggest that the LPA5-EGFR-ERK signaling causes the 
EGFR, or MEK (Figure 8A). Targeting of YFP-Rac1 was blocked by the inhibition of Rac1, resulting in the appearance of YFP-Rac1 on the cell membrane (Figure 8C). The latter results indicated that the LPA5-EGFR-MEK-ERK cascade modulates Rac1 activity. To confirm the role of the MEK-ERK pathway on Rac1, we transiently expressed YFP-Rac1 in SK-CO15/HA-LPA5 cells and determined cellular distribution of YFP-Rac1. In the resting cells, YFP-Rac1 fluorescence signal was diffusely distributed in the cytoplasm, although some distinct fluorescent puncta were also visible (Figure 8C, Control - upper lane). LPA treatment resulted in the appearance of YFP-Rac1 on the cell membrane (Figure 8C, LPA - upper lane). This cell membrane targeting of YFP-Rac1 was blocked by the inhibition of Rac1, EGFR, or MEK (Figure 8C, lower 3 lanes). These results suggest that the LPA5-EGFR-ERK signaling causes the movement of Rac1 to the cell junction. However, Rac1 is not a transcription factor, and it is unlikely that Rac1 directly modulates claudin-4 transcription.

**LPA-Induced Regulation of Claudin-4 Is Dependent on Rac1 and Stat3**

Rac1 was initially discovered for its ability to stimulate the polymerization of actin filaments, but Rac1 has been shown to have distinct roles in the regulation of gene transcription.21 Among the transcription factors that are regulated by Rac1, the signal transducers and activators of transcription (STATs) are known to regulate cell permeability, and Rac1 can bind and activate Stat3.22 Because phosphorylation and translocation of STAT to the nucleus are regulated by various growth factors including LPA, we determined whether LPA5 regulates Stat3 by determining phosphorylation of Stat3. LPA increased phosphorylation at S727 (p-S727-Stat3). On the other hand, the phosphorylation level at Y705 (p-Y705) was relatively high under basal conditions, and it was not significantly altered by LPA (Figure 9A). LPA-induced p-S727 was inhibited by U0126 and NSC23766 (Figure 9A), indicating that the MEK-ERK-Rac1 signaling is necessary for Stat3 activation. Moreover, inhibition of Stat3 with Stattic, a small molecule inhibitor of Stat3, ablated up-regulation of claudin-4 mRNA and protein by LPA (Figure 9B and C). Stattic also inhibited LPA-induced increase in TER (Figure 9D), indicating that LPA5 regulates epithelial barrier function by Stat3-dependent induction of claudin-4.

If confocal microscopy of SK-CO15/HA-LPA5 cells showed that LPA induced translocation of Stat3 from the cytoplasm to the nucleus (Figure 10A, upper lane). Surprisingly, in addition to its nuclear expression, Stat3 was visible along the edge of juxtaposed cells. The localization of Stat3 at the nucleus and cell membrane was blocked by U0126 and NSC23766 (Figure 10A, middle and lower lanes), further suggesting that Rac1 activity is necessary for the activation of Stat3 by LPA. To determine whether LPA regulates the interaction between Rac1 and Stat3, Stat3 and YFP-Rac1 cellular localization was analyzed. In cells under
Figure 4. Lpar5 loss increases the severity of colitis induced by DSS. Lpar5<sup>ff</sup> and Lpar5<sup>DIEC</sup> mice were given 2% DSS for 5 days, followed by 6 days of recovery. Each day the mice were weighed (A), and their stools were collected to determine the Hemoccult scores (B) and diarrhea scores (C, D). Mean disease activity indices are shown. n = 8. **P < .01, ***P < .001 versus Lpar5<sup>ff</sup>. (E) Expression levels of tumor necrosis factor-α, interferon-γ, interleukin 6, and interleukin 1β mRNA in mouse colon isolated on day 1 (before DSS), day 5 (end of DSS), and day 11 (end of recovery) were determined and normalized to β-actin mRNA levels. Data are expressed as mean ± SD. *P < .05. **P < .01. (F) Representative images of Swiss roll mounts of whole mouse colon collected from Lpar5<sup>ff</sup> and Lpar5<sup>DIEC</sup> mice treated with 2% DSS for 5 days are shown. Scale bars = 200 μm. (G) Histologic damage index scores from Swiss roll mounts of colon after DSS treatment. n = 3. *P < .05.
basal conditions, both Stat3 and YFP-Rac1 were diffusely distributed in the cytoplasm (Figure 10B, left column). LPA induced the appearance of Stat3 in the nucleus and on the cell membrane, where it co-localized with YFP-Rac1 (Figure 10B, right column). However, the induction of nuclear and membranous appearance of Stat3 was rapid, and we could not discern which event occurred first. Nonetheless, the presence of Stat3 at the cell membrane suggested that LPA induces the interaction between Rac1 and Stat3, in line with a previous study that activated Rac1 forms a complex with Stat3 in COS-1 cells. To confirm their interaction, we performed co-immunoprecipitation of YFP-Rac1 and Stat3. Co-immunoprecipitation of Stat3 with YFP-Rac1 under basal condition suggested that Rac1 and Stat3 interact without LPA treatment (Figure 10C). The interaction of YFP-Rac1 with Stat3 under basal conditions was not surprising because transfected YFP-Rac1 partially retained its activity without LPA treatment (Figure 8B). Importantly, LPA stimulated co-immunoprecipitation of Stat3 with YFP-Rac1 (Figure 10C), indicating that LPA promotes the Rac1-Stat3 interaction. However, we were surprised that we could not observe increased co-immunoprecipitation of p-S727-Stat3 with YFP-Rac1 in response to LPA. These results suggest that LPA induces the interaction of Rac1 with Stat3, and this interaction precedes phosphorylation of Stat3 at S727.

Discussion

Our previous studies have demonstrated the role of LPA5 in the regulation of NHE3, which plays a major role in sodium and fluid absorption in the intestine. However,
Lpar5 is highly expressed in mature enterocytes, and cellular signaling by LPA5 is expected to have a broad range of effects based on general functions of other LPA receptors. In the current study, we show that Lpar5 loss in IECs dysregulates epithelial barrier, resulting in increased paracellular permeability in the mouse intestine. *Lpar5*DIEC mice had increased serum FD-4 level compared with *Lpar5f/f* mice, suggesting increased leakiness of the intestinal epithelial monolayer in *Lpar5*DIEC mice. In addition, we found elevated serum LPS level and increased bacterial translocation into the intestinal mucosa and the liver of *Lpar5*DIEC mice. These results suggest that loss of Lpar5 compromises innate immunity by disrupting the intestinal epithelial barrier function.

IECs express multiple forms of claudin that have different expression patterns within the GI tract.34 Claudin-4, which was the first claudin shown to regulate the TJ permeability in cultured cells, is highly expressed in the villus epithelial cells in the intestine of rats and mice.35–37 Claudin-1 and claudin-2 expression is more restricted to the crypt base in the small intestine and colon.36 Claudin-7 is roughly evenly distributed along the crypt-villus axis, but its expression is greater on the basolateral surface of the epithelial cell than on the apical membrane.37,38 We observed that Lpar5 loss specifically led to decreased claudin-4 expression in both mouse intestine and colonic epithelial cell lines. In contrast, claudin-1, claudin-2, or claudin-7 expression was not altered by Lpar5 loss. It is noteworthy that the current findings differ from our previous study of Lpa1, where *Lpar1* loss in mice resulted in decreased expression of claudin-2, -4, and -7.15 Cellular distribution of LPA5 or other LPA receptors in native intestinal tissue is not known, but HA-LPA5 expressed in Caco-2bbe cells preferentially partitioned to the apical surface over the basolateral surface.23 This differs from LPA1, which localizes to both apical and basolateral membranes.14 Therefore, the differences in the expression patterns of LPA5 versus LPA1 within the intestinal epithelium and along the crypt-villus axis are likely to influence the expression of a specific claudin.

LPA and precursors of LPA, such as phosphatidic acid, are present in a variety of food products.39,40 Orally administered LPA has been linked to biological effects, including wound healing, electrolyte balance, and cancer in the GI tract.14,22,39,41,42 A recent study showed that LPA can mitigate villus blunting and restore apical transporter expression in the small intestine of myosin 5B-deficient mice, a rodent model of microvillus inclusion disease.43 Our current study demonstrated that orally delivered LPA can stimulate the epithelial barrier function in vivo, and LPA5 plays a major role in manifesting this effect. Interestingly, we found that LPA was able to decrease FD-4 flux in *Lpar5*DIEC mice, albeit by a lesser extent compared with control mice. We postulate that the residual effect on LPA-dependent epithelial permeability is mediated by LPA1. Lpar1-deficient mice have delayed wound healing and decreased epithelial barrier function.14,15 Because LPA1 is expressed in the intestine of *Lpar5*DIEC mice, it is likely that LPA1 mediates LPA-induced epithelial permeability in these mice.

Intestinal epithelial barrier dysfunction is a major cause of the development and progression of IBD, and the association between epithelial dysfunction and DSS-induced colitis has been demonstrated.44–46 Administration of 2% DSS, which is in a low range used in similar experiments, exacerbated colitis in *Lpar5*DIEC mice. In particular, weight loss and diarrhea were much greater in *Lpar5*DIEC mice. Diarrhea is one of the most common symptoms of IBD, and NHE3 has been shown to be down-regulated in patients with IBD.47,48 A positive correlation between reduced NHE3 expression and increased severity of colitis has been
Because LPA₅ regulates NHE3, it is possible that Lpar5 loss reduces NHE3 expression or function that may have increased diarrhea in DSS-treated Lpar5⁻/⁻ mice. A recent study of NHE3 regulation in Lpar5⁻/⁻IECs showed that basal NHE3 mRNA expression was decreased in Lpar5-decient IECs, although no significant change in NHE3 activity was observed. On the other hand, LPA-mediated activation of NHE3 or fluid absorption was completely absent in these mice. It is not yet known whether the lack of LPA-mediated NHE3 regulation is sufficient to cause more severe diarrhea in the setting of IBD or infectious diseases. Because dysregulation of NHE3 is a frequent cause of diarrhea, the lack of NHE3 stimulation by LPA and dietary peptone may attenuate NHE3-dependent fluid absorption and hence increases fluid loss in the gut, resulting in more severe diarrhea. However, this possibility remains to be experimentally evaluated.

The majority of extracellular LPA is generated by ATX, which is elevated in inflamed mucosa of human and mouse intestine. Excess amounts of LPA generated within the damaged tissues can trigger proinflammatory and pro-angiogenic responses by immune cells, fibroblasts, or cancer cells. Therefore, inhibition of ATX mitigates inflammation, as demonstrated in rodent models of IBD. Because of the potential benefit of ATX inhibition to treat chronic inflammation, the current findings of LPA₅-dependent regulation of intestinal epithelial barrier and the protection from DSS-induced colitis by Lpar5 loss in IECs appear paradoxical. In the intestine, ATX is expressed by endothelial venules and B cells, and it seems unlikely...
Figure 8. LPA₅ activates Rac1 activity via an ERK-dependent mechanism. (A) Phosphorylation of ERK by LPA was determined in presence or absence of NSC23766. (B) Rac1 activity was determined as described in Materials and Methods. n = 3. Data are expressed as mean ± SD. **P < .01. (C) Representative confocal IF images of SK-CO15/HA-LPA₅ cells transiently transfected with YFP-Rac1 are shown. Cells were treated with carrier (left) or LPA (right) for 10 minutes in presence or absence of NSC23766, AG1478, or U0126. YFP-Rac1 (green), F-actin (red), and nuclei (blue) are shown. Scale bar = 10 μm.
that ATX-derived LPA can access LPA5 at the apical side of the epithelium. Hence, we speculate that orally administered LPA primarily acts on apically expressed LPA receptors such as LPA1 and LPA5 to maintain epithelial barrier and fluid homeostasis in the GI tract.

The transactivation of EGFR by various GPCR agonists, including LPA, thrombin, and endothelin-1, was first reported by the Ullrich group. An important aspect of EGFR transactivation is that it provides a mechanism that enables GPCR agonists to activate the MEK-ERK signaling pathway. As expected, LPA5 regulates the MEK-ERK pathway via EGFR transactivation. EGFR is generally thought to be located in the basolateral membrane of polarized epithelial cells, but others have demonstrated apically located EGFR. Previous studies have shown the presence of EGFR on both apical and basolateral sides of monolayers formed from primary canine oxyntic epithelial cells, but apical EGFR and not basolateral EGFR regulates epithelial barrier to gastric acid. In Caco-2bbe cells, EGFR is expressed on both apical and basolateral membranes, but the regulation of NHE3 by LPA5 is observed only when apical EGFR is stimulated. Although we did not specifically compare the role of apical vs basolateral EGFR in claudin-4 regulation, we infer from our previous study that apically located EGFR mediates claudin-4 regulation by LPA.

The Rho GTPase family constitutes important effectors of LPA receptors. LPA causes actin cytoskeletal rearrangement through activation of RhoA, which results in barrier dysfunction in endothelial cells and migration of cancer cells. We have shown recently that LPA mediates mouse colonic epithelial YAMC cell migration through a Rac1-dependent mechanism. On the other hand, LPA inhibits RhoA in YAMC cells, and RhoA inactivation results in spreading and migration of fibroblasts. In Caco-2bbe cells, LPA5 regulates RhoA independent of the MEK-ERK pathway. On the contrary, we found in the current study that LPA-induced stimulation of Rac1 activity was mediated via the MEK-ERK pathway. The latter observation is in line with previous studies demonstrating the upstream regulatory role of the MEK-ERK signaling in Rac activation. NSC23766, which specifically inhibits Rac1 activation through interfering its binding to a Rac1-specific guanine nucleotide exchange factor (GEF), blocked targeting of YFP-Rac1 to cell-cell junction by LPA. It is not
known which GEF is responsible for Rac1 activation by LPA5. LPA is known to regulate the Rac-specific GEF Tiam1, which regulates Rac1 activity via its recruitment to epithelial junction.62–64 A future study is needed to determine whether LPA5 regulates Tiam1 and whether Tiam1 activates Rac1 in the context of epithelial barrier regulation.

LPA often mediates its proinflammatory or oncogenic effects through transcriptional regulation of Myc, Stat, nuclear factor kappa B, and β-catenin.65–68 We found that inhibition of Stat3 by Stattic attenuated LPA-mediated claudin-4 transcriptional activation, demonstrating that Stat3 is a transcriptional factor regulating claudin-4 expression. Stat3 is mostly latent in the cytoplasm until activated through receptor-mediated phosphorylation.69 Y705 phosphorylation is generally regarded as a dominant actuator of Stat3 signaling, but Y705 was phosphorylated under basal conditions, and LPA did not significantly alter p-Y705 in SK-CO15 cells. Interestingly, cell-cell adhesion can induce p-Y705-Stat3, and it is thought to confer differentiated epithelial morphology of cancer cells.70,71 Therefore, it is possible that the polarized epithelial morphology of SK-CO15 and Caco-2bb cells maintains phosphorylation at Y705. Instead of phosphorylating Stat3 at Y705, LPA increased phosphorylation at S727. Phosphorylation at S727 (p-S727) has been documented to be associated with Stat3 activation,72,73 although others have shown a negative role of p-S727 on Stat3.74,75

It has been shown previously that activated Rac1 complexes with Stat3.33 In addition, Stat3 activation by Ca2+-dependent cell-cell adhesion has been suggested.70 Our findings that LPA promoted co-immunoprecipitation of Rac1 and Stat3 and their co-localization at the cell membrane are in line with these earlier studies. However, we were not able to detect increased Rac1-p-S727-Stat3 interaction or the
presence of p-S727-Stat3 at the cell junction. Although this failure could have been due to differences in antibodies used against total Stat3 versus p-S727-Stat3, it also suggests that membrane-targeted Rac1 recruits Stat3 and not p-S727-Stat3.

One potential concern in the current study is the use of pharmacologic inhibitors despite their broad usage to target their respective proteins. Like all pharmacologic inhibitors, some of the inhibitors used here show off-target effects. On the other hand, other studies have confirmed the specificity of these inhibitors by comparing with gene knockdown. Likewise, we have previously demonstrated the identical effect of AG1478 and EGFR knockdown on LPA₅-mediated ERK activation in Caco-2bbe cells. Another potential limitation of the current study is the use of intestinal cell lines SK-CO15 and Caco-2bbe for the mechanistic insight into LPA₅ function. Although these cell lines are used widely as model IECs and decreased claudin-4 expression by Lpar5 loss was recapitulated in these cell lines, it is unlikely that these cells have kept all the features of native colonic epithelial cells. With these limitations in mind, we suggest that claudin-4 regulation by LPA₅ is a 3-step process. First, LPA-LPA₅ signaling initiates activation of EGFR-MEK-ERK, which stimulates the translocation of Rac1 to the cell membrane. Second, Rac1 recruits and interacts with Stat3 at the cell membrane where Stat3 is phosphorylated at S727. Third, activated Stat3 rapidly dissociates from Rac1 and translocates to the nucleus to transcribe claudin-4.

In summary, our study demonstrates that LPA enhances the epithelial barrier function via LPA₅. The absence of LPA₅ results in increased epithelial permeability, which allows bacterial translocation across the intestinal luminal membrane. LPA₅ regulates the epithelial barrier by transcriptionally modulating claudin-4 via a mechanism requiring Rac1 and Stat3. Together with our previous studies on LPA₅ facilitating epithelial wound healing in the intestine, the present study suggests a potential benefit of using LPA to enhance intestinal epithelial barrier integrity.

Materials and Methods

Cell Culture and Plasmids

SK-CO15 and Caco-2bbe human colonic epithelial cells were grown on plastic dishes or Transwell inserts (Corning, Tewksbury, MA) for 7 days after confluence before all assays.

Chemicals and Antibodies

LPA (18:1; 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate) was purchased from Avanti Polar Lipids (Alabaster, AL) and prepared according to the manufacturer’s instructions. For in vitro study, LPA was used at the final concentration of 10 μmol/L in PBS containing 0.1% bovine serum albumin, unless otherwise specified. An equal volume of PBS containing 0.1% bovine serum albumin was added as a control. All chemicals, including FD-4, AG1478, U0126, NSC23766, and Y27632, were obtained from Sigma-Aldrich or EMD Millipore (Billerica, MA). The following commercial antibodies were used: rabbit anti-claudin-1, rabbit anti-claudin-4, rabbit anti-occludin, and rabbit anti-ZO-1 (Thermo Fisher Scientific, Waltham, MA); goat anti-E-cadherin and mouse anti-Rac1 (R&D Systems, Minneapolis, MN); mouse anti-β-actin (Sigma-Aldrich); and rabbit anti-Stat3 and rabbit anti-p-Stat3 (Cell Signaling, Danvers, MA).

Animals

Generation of Lpar₅ff mice and Lpar₅f/dIEC (Lpar₅ff/Vil-cre) mice was previously reported. Littermates were used in all experiments. Genotypes were determined by PCR using the following primers: Vil-Cre forward, CAA GCC TGG CTC GAC GGC C and reverse, GCC GAA CAT CCT CAG GTT CT; loxP forward, CCA GCC AGA GAG AGG TG and reverse, TGG CCT CAG AAG TG ATT TGC TC. Experiments with animals were performed under approval by the Institutional Animal Care and Use Committees of the Atlanta Veterans Administration Medical Center and Emory University (Atlanta, GA) and in accordance with the NIH’s Guide for the Care and Use of Laboratory Animals.

Intestinal Permeability In Vivo

Intestinal permeability was determined using FD-4 as a mucosal tracer flux marker as described previously. Briefly, FD-4 in PBS (100 mg/mL; Sigma-Aldrich) was administered by gastric gavage at a final dose of 60 mg/100 g body weight. Four hours later, mice were euthanized, and blood samples were collected by cardiac puncture. Blood was centrifuged at 5000 rpm for 10 minutes, and serum was diluted in an equal volume of PBS. Fluorescence intensity in the serum was measured at an excitation of 485 nm and an emission of 525 nm (485/528 nm) using a Synergy 2 (BioTek, Winooski, VT) plate reader. Concentrations of FD-4 were tabulated against a standard curve.

Oral Administration of LPA

Male mice of 9 weeks of age were pretreated either with LPA (150 μL of 300 μmol/L stock) or PBS by gavage daily for 3 days. On day 3, mice were given oral administration of FD-4, and intestinal permeability was determined.
**Measurement of Serum LPS**

LPS in the serum was determined by using a Pierce Chromogenic Endotoxin Quant kit according to the manufacturer’s instruction (Thermo Fisher Scientific).

**Measurement of Bacteria Loads**

Mice were euthanized by using isoflurane and cervical dislocation. The liver was perfused by injecting 10 mL PBS into the hepatic portal vein. The perfused liver was removed and stored on ice. The ileum and colon were removed. Sections of the liver, ileum, proximal colon, and distal colon were excised and weighted. After homogenizing, serial dilutions of the homogenates were plated on Agar plates, and plates were incubated at 37°C for 24–36 hours.

**DSS-Induced Colitis**

Eight- to 12-week-old male mice (n = 16 per strain) were permitted free access to 2% DSS (w/v; mol wt, 36,000–50,000; Affymetrix, Inc, Santa Clara, CA) in drinking water for 5 days to induce acute colitis. Half of the mice were euthanized at the end of DSS treatment, and the remaining half were given normal water for the next 6 days to recover. The body weight of each mouse was measured and recorded daily. Assessment of stool consistency and the presence of occult blood by a guaiac test (Hemoccult Sensa; Beckman Coulter, Fullerton, CA) were determined daily for each mouse. The disease activities (diarrhea, occult blood, and weight loss) were quantified as we described previously. Mice were euthanized by using isoflurane and cervical dislocation on day 5 or 11. Colon was removed, flushed with chilled (4°C) Ca²⁺ and Mg²⁺ free PBS, and fixed in RNAlater (Thermo Fisher Scientific) for RNA isolation. Whole colon tissues were fixed in 10% buffered formalin overnight for histologic analysis. Paraffin-embedded sections were stained with H&E for microscopic assessment of colitis.

**Histology**

For each animal, a histologic examination was performed on 3 samples of the distal colon. Histologic parameters were quantified in a blinded manner (MW and YH) using the scores previously published. Three independent parameters used were severity of inflammation (0–3: none, slight, moderate, severe); depth of injury (0–3: none, mucosal, mucosal and submucosal, transmural); and crypt damage (0–4: none, basal one-third damaged, basal two-thirds damaged, only surface epithelium intact, entire crypt and epithelium lost). The score of each parameter was multiplied by a factor reflecting the percentage of tissue involvement (×1: 0%–25%, ×2: 26%–50%, ×3: 51%–75%, ×4: 76%–100%), and these values were summed to obtain a total score.

**In Vitro Permeability Measurement**

SK-CO15 cells and Caco-2 cells were grown on Transwell inserts with 0.4-μm pore size (Corning). TER of a monolayer was measured using an epithelial V-Ω meter (World Precision Instruments, Inc, Sarasota, FL). Resistance of cells on filter was calculated by subtracting the resistance of the membrane plus medium from the resistance of the membrane plus medium plus cells. Each experiment was measured in triplicate, and the average value was taken. This value was then multiplied by the area of the Transwell membrane (0.9 cm²) to obtain a final value in Ω × cm².

**Western Immunoblot and Immunoprecipitation**

Lysate from mouse intestinal tissues or cultured cells was prepared, and Western blotting was performed as previously described. For immunoprecipitation of YFP-Rac1, SK-CO15 cells were lysed in cold lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 5 mmol/L glycerophosphate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L Na₂EDTA, 1 mmol/L EGTA, 1 mmol/L Na₃VO₄, 1 mmol/L NaF, 10 mmol/L leupeptin, 1% Triton X-100, protease inhibitors mixture, and 2.5 mmol/L N-ethylmaleimide). Protein concentration was determined by bichinonic acid assay (Sigma-Aldrich). Equal amounts of cell lysates (typically 500 μg) were incubated overnight with anti-green fluorescent protein antibody. The immunocomplex was purified by incubating with protein G-Sepharose beads for 1 hour, followed by 2 washes in lysis buffer and 1 wash in PBS. Immunocomplexes were eluted from the beads in 2× Laemmli buffer, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotted with anti-Stat3 or anti-pS727-Stat3 antibody.

**Quantitative RT-PCR**

Total RNA was extracted from colon mucosal scrapes or cultured cells using the RNeasy Mini kit (Qiagen, Hilden, Germany). One μg of total RNA was used for cDNA synthesis using a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer’s instruction. Quantitative PCR was performed with iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) on a Mastercycler Realplex (Eppendorf, Hamburg, Germany). Expression levels determined in triplicate per sample were normalized to β-actin. PCR primer sequences are listed in Table 1.

**Rac1 GTPase Activity Assay**

GTP-bound Rac1 was determined using a G-LISA Activation Assay Kit according to the manufacturer’s instructions (Cytoskeleton, Denver, CO).

**Confocal IF**

After being flushed with cold PBS, the medial small intestine and colon segments were incubated overnight in 30% sucrose in PBS for cryoprotection. Six-micron cryostat sections were prepared and stored at −80°C until needed. The frozen sections were fixed with ice-cold 100% ethanol and acetone at the ratio of 1:1 for 10 minutes at −20°C. The cell monolayers on Transwell filters were washed 3 times with ice-cold PBS, followed by fixation with 4% paraformaldehyde in PBS for 10 minutes at room temperature. For claudin-4 staining, cells were fixed with...
100% ice cold ethanol for 20 minutes at −20°C. After fixation, tissues and cells were permeabilized with 0.2% Triton X-100 for 10 minutes, blocked in PBS containing 5% normal goat serum for 30 minutes, and incubated for 1 hour with a specific primary antibody at room temperature. The monolayers were rinsed 5 times with PBS for 5 minutes and incubated with fluorescence tag-labeled secondary antibodies for 30 minutes at room temperature. After five 5-minute washes with PBS, specimens were mounted with ProLong Glass Antifade Reagent (Invitrogen) and observed under a Nikon A1R HD confocal microscope (Nikon Instruments Inc, New York, NY) coupled to a Plan Apo λ 60x Oil lens.

**Statistical Analysis**

Statistics were performed using independent samples, two-tailed Student t test or analysis of variance, followed by Tukey post hoc test using Prism 6 software (GraphPad Software, La Jolla, CA). Results are presented as mean ± standard deviation (SD). A value of \( P < 0.05 \) was considered significant.

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