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Lysophosphatidic acid prevents apoptosis of Caco-2 colon cancer cells via activation of mitogen-activated protein kinase and phosphorylation of Bad

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Abstract

Lysophosphatidic acids (LPA) exert growth factor-like effects through specific G protein-coupled receptors. The presence of different LPA receptors often determines the specific signaling mechanisms and the physiological consequences of LPA in different environments. Among the four members of the LPA receptor family, LPA₂ has been shown to be overexpressed in colon cancer suggesting that the signaling by LPA₂ may potentiate growth and survival of tumor cells. In this study, we examined the effect of LPA on survival of colon cancer cells using Caco-2 cells as a cell model system. LPA rescued Caco-2 cells from apoptosis elicited by the chemotherapeutic drug, etoposide. This protection was accompanied by abrogation of etoposide-induced stimulation of caspase activity via a mechanism dependent on Erk and PI3K. In contrast, perturbation of cellular signaling mediated by the LPA₂ receptor by knockdown of a scaffold protein NHERF2 abrogated the protective effect of LPA. Etoposide decreased the expression of Bcl-2, which was reversed by LPA. Etoposide decreased the phosphorylation level of the proapoptotic protein Bad in an Erk-dependent manner, without changing Bad expression. We further show that LPA treatment resulted in delayed activation of Erk. These results indicate that LPA protects Caco-2 cells from apoptotic insult by a mechanism involving Erk, Bad, and Bcl-2.

Keywords

lysophosphatidic acids; MAPK; receptor; apoptosis

1. INTRODUCTION

In normal adult colon, enterocytes generated from stem cells located at the base of the crypt migrate towards the luminal surface. During this process, enterocytes located at the tip of small intestine villi or at the luminal surface of the colon are thought to undergo apoptosis. Apoptosis is counterbalanced by the proliferation and differentiation of progenitor cells derived from stem cells. Deregulation of apoptosis and cell division can result in hyperplasia and tumorigenesis of the intestine and colon. For example, expression of Bcl-2, an anti-apoptotic protein, is elevated the majority of dysplastic, adenomatous lesions [1]. Overexpression of Bcl-2 in human

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melanoma cells promotes a migratory and invasive phenotype, contributing to tumor progression [2].

Lysophosphatidic acid (LPA) is a bioactive glycerophospholipid that is generated and released by platelets, macrophages, epithelial cells, and some tumor cells [3-5]. LPA binds to specific G protein-coupled receptors (GPCRs) on the cell surface to exert diverse growth factor-like effects, such as proliferation, apoptosis, contraction, and migration [6-8]. Thus far, four distinct membrane receptors that bind LPA have been identified, LPA₁, LPA₂, LPA₃, and LPA₄/GPR23 [9-12]. All the LPA receptors can couple to three families of heterotrimeric GTP binding proteins, including G_{q/11}, G_{i/o}, and G_{12/13} [6-8]. G proteins, in turn, activate a number of signaling cascades, including the activation of phospholipase C (PLC) with subsequent phosphatidylinositol-(4,5)-biphosphate hydrolysis, the activation of the phosphoinositide-3-kinase (PI3K)-Akt and the mitogen-activated protein kinase (MAPK) pathways, and the small GTPase RhoA that mediates the remodeling of the actin cytoskeleton [6-8].

The effect of LPA in cellular proliferation and survival has been studied extensively, and is partly attributed to the capacity of LPA to regulate apoptosis. However, the effect of LPA on cell survival and apoptosis varies among different cell types. LPA mediates survival of ovarian cancer cells, macrophages, fibroblasts, and neonatal cardiac myocytes, while promoting apoptosis in hippocampal neurons and PC12 cells [7,13]. The varied effects of LPA on apoptosis are dependent on the presence of specific LPA receptors, but also on the cellular context. For example, activation of LPA₁ prevented apoptosis in primary lymphocytic leukemia cells, whereas the same LPA₁ induced apoptosis and anoikis in ovarian cancer cells [14,15]. In untransformed rat intestinal epithelial IEC-6 cells, both LPA₁ and LPA₂ are thought to be responsible for the anti-apoptotic effect of LPA [16], but the effect in colon cancer cells is not known.

Studies have shown the presence of high levels of LPA in the ascitic fluid of patients with ovarian cancer as well as increased expression of LPA₂ in several types of cancers, including ovarian, colorectal, and thyroid cancer. [17-21]. These studies, in conjunction with multiple effects mediated by LPA, suggest that over-expression of LPA₂ may enhance the development of cancer. Recent studies have identified scaffold proteins that play pivotal roles in signaling elicited by LPA₂ through their interaction with the carboxyl terminus of LPA₂. These scaffold proteins include TRIP6 and at least three PDZ (PSD-95/DlgA/ZO-1) -containing proteins, Na⁺/H⁺ exchanger regulatory factor 2 (NHERF2), PDZ-RhoGEF, and leukemia-associated RhoGEF [21-25]. We have shown previously that knockdown of NHERF2 in Caco-2 human colon cancer cells significantly attenuated the ability of LPA₂ to induce activation of extracellular signal-regulated kinase-1/2 (Erk), Akt, and interleukin-8 (IL-8) [21]. The consequence of the NHERF2 knockdown was similar to knockdown of LPA₂ as LPA₂ is the major LPA receptor expressed in human colon cancer cells such as Caco-2 cells.

In the present study, we show that LPA protects the human colon cancer Caco-2 cells from apoptosis induced by the chemotherapeutic drug etoposide. LPA promotes survival of Caco-2 cells via a NHERF2-dependent mechanism that results in activation of Erk and inactivation of pro-apoptotic Bad and caspase 3.

2. MATERIALS AND METHODS

2.1. Cell culture and treatment

Caco-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml streptomycin, and 100 U/ml penicillin at 37° C in 95% air/ 5% CO₂ atmosphere. Stable knockdown of NHERF2 in Caco-2 cells using small hairpin RNA (shRNA) targeted against NHERF2 has previously been described [21]. Caco-2

cells with stable knockdown of NHERF2 (Caco-2/CL4) and control transfected cells (Caco-2/pSiL) were grown in the above media supplemented with 300 unit/ml hygromycin. 1-Oleoyl-2-hydroxy-sn-glycero-3-phosphatidic acid was obtained from Avanti Polar Lipids and prepared in PBS containing 0.1% fatty acid-free BSA. Cells were serum-starved for 24 h prior to treatment with LPA. In some cases, cells were pretreated for 15-30 min with 20 μ M U0126, 50 μ M LY294002, 5 μ M U73122, 25 μ M Zvad.fmk before exposure to LPA. As controls, cells were treated with the same volume of vehicle (DMSO) or PBS with 0.1% BSA. Etoposide (Sigma) was prepared in Me₂SO. All the inhibitors were obtained from Calbiochem.

2.2. Annexin V staining

Cells seeded at a density of 1×10^5 per 60 mm culture plate were serum-starved for 24 h and treated with 200 μ M etoposide, etoposide together with LPA, or carrier for 24 h. Cells resuspended in binding buffer (10mM HEPES/NaOH, pH.7.4, 140 mM NaCl, 2.5 mM CaCl₂) were stained with 5 μ l of annexin-FITC and 5 μ g/ml propidium iodide (PI). Viable, unstained cells in untreated samples were used as negative controls. Cells were then analyzed by flow cytometry using a FACSCalibur cytometer (BD Biosciences), and CellQuest (BD Biosciences) was used to calculate the amount of apoptotic cells.

2.3. Caspase-3/7 activity

Caspase-3 and caspase-7 activities were determined using Caspase-Glo 3/7 Assay kit (Promega). Briefly, cells in 96-well plates (5,000 cells/well) were serum-starved for 24 h and treated with etoposide with or without LPA for another 24 h. Caspase-Glo reagent (100 μ l) was added to an equal amount of media in each well and incubated at room temperature for 30 min. The luminescence of each sample was measured using a Luminoskan Ascent (Thermo Labsystems).

2.4. Western blot

Cells were lysed in lysis buffer (10 mM Tris-Cl, pH 7.4, 100 mM NaCl, 1mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 10% glycerol, 2 mM Na orthovanadate, 10 mM Na fluoride, 20 mM Na pyrophosphate, 25 mM β -glycerophosphate, 1% Triton X-100, and protease inhibitors). The cell debris was removed by centrifugation at 14,000 g at 4°C for 10 min and protein concentration was determined by the Bicinchoninic Acid assay (Sigma). Equal amounts of lysate were resolved by SDS-PAGE, and Western blot was performed as previously described [21]. Anti-Bad, anti-phospho-Bad, anti-Erk, and anti-phospho-Erk antibodies were purchased from Cell Signaling.

2.5. Statistical analysis

Densitometric analysis was performed on the Typhoon phosphoimager (Amersham) using the Image Quant program. Statistical significance was assessed by one-way ANOVA using Origin software. Data are presented as the means $\pm$ standard error (SE).

3. RESULTS

3.1. LPA protects Caco-2 cells from etoposide-induced apoptosis

Etoposide is a widely used clinical agent for cancer chemotherapy that targets topoisomerase II [26]. We initially tested different concentrations of etoposide in Caco-2 cells to determine an optimum concentration to induce apoptosis. Although a range of 75 to 500 μ M etoposide resulted in an increase in the apoptotic population, 150-200 μ M etoposide was needed for a consistent increase in apoptotic population within 24 h (data not shown) and all the studies reported in this work used 200 μ M etoposide. Because previous studies have shown that cell

survival and anoikis of intestinal enterocytes vary depending on the state of differentiation [27,28], all our studies were performed with differentiated Caco-2 cells.

To determine whether LPA can protect colon cancer cells from apoptosis, serum-starved Caco-2 cells were treated with etoposide (200 μ M) for 24 h in the presence or absence of LPA (20 μ M). As controls, cells were treated with either an equal amount of Me₂SO, solvent used to prepare etoposide, or LPA alone. Apoptosis was determined by Annexin V and propidium iodide (PI) staining, and apoptotic cells were defined as Annexin-positive but PI-negative (Figure 1). Following 24 h serum-starvation, 16.5 ± 5.5 % of cells were apoptotic. LPA alone did not have a significant effect (16.2 ± 4.2 %). Exposure to etoposide increased the apoptotic population to 56.3 ± 6.9 %, more than a three-fold increase compared to the control. Etoposide-induced apoptosis was blocked by addition of LPA (etoposide/LPA) in which the apoptotic cell level was decreased to 21.2 ± 4.9 %. These results suggested that LPA effectively protected Caco-2 cells from etoposide-induced apoptotic death.

3.2. Protection of Caco-2 from etoposide-induced apoptosis is dependent on the presence of NHERF2

We have shown previously that Caco-2 cells predominantly express LPA₂ with the expression level of LPA₁ at a significantly lower level and no expression of LPA₃, making LPA₂ only a significant LPA receptor in these cells [21]. LPA₂ has carboxyl-terminal motifs that allow interaction with a PDZ (PSD-95/DlgA/ZO-1) domain of the Na⁺/H⁺ exchanger regulatory factor 2 (NHERF2), which functions as a scaffold to link signaling proteins, such as PKA and PKC α , to GPCRs and transporters at the cell surface [21,23,29-31]. NHERF2 links LPA₂ to PLC- β 3 and hence affects the activation of downstream targets, such as Erk and cyclooxygenase-2 [23]. We have previously shown that knockdown of NHERF2 expression in Caco-2 cells by RNA interference drastically down-regulates the LPA₂-G α _i-Akt pathway and induction of IL-8 [21]. Moreover, over-expression of NHERF2 reconstitutes the signaling mediated by LPA₂ [23]. Collectively these data demonstrate that displacement of the LPA₂-NHERF2 interaction severely perturbs the cellular signaling mediated by LPA₂. Therefore, to determine whether the perturbation of LPA₂-mediated signaling compromises the LPA-induced anti-apoptotic responses, we examined the effects of etoposide and LPA in Caco-2 cells with stable knockdown of NHERF2 (Caco-2/CL4) and control transfected Caco-2 cells (Caco-2/pSiL) [21]. These cells were treated with etoposide, carrier, or LPA for 24 h and apoptosis was determined by Annexin V labeling. In both Caco-2/pSiL and Caco-2/CL4 cells, etoposide doubled the number of cells undergoing apoptosis compared with untreated serum-starved control cells (Figure 2). However, in contrast to Caco-2/pSiL (Figure 2A), LPA did not protect Caco-2/CL4 from etoposide-induced apoptotic death (Figure 2B). These results demonstrated that the LPA-induced survival effect on Caco-2 cells was dependent on the presence of NHERF2 and intact LPA₂ signaling.

3.3. LPA inactivates caspase-3/7

We estimated that a minimum of 12 h is needed to induce apoptotic death of Caco-2 cells by etoposide. This is based on histogram analysis to determine the subG1 cell population (apoptotic) following exposure to etoposide for varying times [32]. An increase in the subG1 population was observed after 12 h of treatment (data not shown).

Caspases are downstream effectors common to many paradigms of apoptosis [33]. To determine the effect of LPA on caspase activation, Caco-2/pSiL cells were treated with etoposide for 24 h in the presence or absence of 20 μ M LPA. Etoposide induced a three-fold increase in caspase-3/7 activity (Figure 3A). Co-incubation with LPA reduced etoposide-induced caspase-3/7 activation by approximately 50%, demonstrating a direct effect on caspases by LPA treatment. In contrast, LPA alone did not significantly alter caspase activity.

As shown in Figure 3B, etoposide induced apoptosis of Caco-2/CL4 cells, but co-treatment with LPA did not attenuate caspase activity in these cells, affirming the importance of LPA₂ and NHERF2 in protection of the cells from the apoptotic insult. In Figure 3C, Caco-2 cells were treated with the general caspase inhibitor, Zvad.fmk [34]. As expected, there was no change in the caspase activity was incurred by etoposide or LPA under this condition. These data collectively confirm that the induction of caspase-3/7 activity is an essential step in etoposide-induced apoptosis and that LPA rescues cells by inhibition of caspases.

3.4. LPA-induced signaling leads to Bad phosphorylation

Caspase-3 and -7 can be activated by either activation of cell surface receptors or changes in mitochondrial integrity [33]. The mitochondrial-mediated caspase activation is highly regulated by members of the Bcl-2 family. The Bcl-2 family consists of pro-survival proteins (Bcl-2, Bcl-x_L, and Bcl-w) and pro-apoptotic proteins (Bax, Bad, and Bim) [35]. To determine whether etoposide and LPA affect the Bcl-2 family of proteins, we examined expression levels of Bcl-2 and Bcl-x_L. Etoposide treatment for 13 h resulted in a small decrease in the expression level of Bcl-2 (Figure 4A), whereas no change in Bcl-x_L was observed (Figure 4B). Similarly, we observed no change in Bax expression (data not shown). The change in Bcl-2 protein expression by etoposide was partially blocked in the presence of the MEK inhibitor, U0126, but not in the presence of the PI3K inhibitor, LY294002 (Figure 4A). However, the basal expression levels of Bcl-2 in the presence of either inhibitor was slightly lower compared with in the absence of the inhibitors. Bad, a very distant Bcl-2 family member, promotes apoptosis by binding to either Bcl-2 or Bcl-x_L and displacing them from apoptotic Bax [36]. Phosphorylation of Bad on either Ser-112 or Ser-136 is thought to promote cell survival [37-40]. Phosphorylation at either of these residues promotes interaction with the 14-3-3 protein instead of Bcl-2 or Bcl-x_L, resulting in the liberation of the anti-apoptotic protein and consequent promotion of cell survival [37,40]. Akt phosphorylates Bad at Ser-112, whereas Ser-136 is reported to be phosphorylated by MAPKs [38,39,41]. To determine whether phosphorylation of Bad is involved in LPA-mediated cell survival, we treated cells with etoposide, etoposide/LPA or carrier for 4h, but there was no significant changes in either phosphorylation or expression levels of Bad (Figure 5A). However, as shown in Figure 5B, a change in Bad phosphorylation was observed at 13 h in the presence of etoposide, at which time there was a significant increase in the subG1 population. Etoposide decreased phospho-Bad at Ser-112, as determined by Western blot using an antibody specific for detecting phosphorylation at Ser-112 of Bad. Co-incubation with LPA restored phosphorylation at Ser-112 to a level similar to the control. In comparison, the effect of etoposide or etoposide/LPA on phosphorylation at Ser-136 of Bad was not significant. To confirm the role of MAPK pathway in phosphorylation of Bad, phosphorylation on Ser-112 of Bad was determined in the presence of the mitogen-activated kinase kinase 1 (MEK1) inhibitor U0126 (20 μM), the PI3K inhibitor LY294002 (50 μM), or pertussis toxin (50 ng/ml), which uncouples Gα_i. As shown in Figure 5C, U0126 abrogated the increase in phosphorylation at Ser-112 by etoposide + LPA compared with etoposide alone. On the other hand, LY290042 or PTX showed no effect on phosphorylation at Ser-112. The lack of an effect on phosphorylation at Ser-112 by PTX is consistent with a previous report that LPA-mediated activation of Erk in Caco-2 cells is not PTX-sensitive [21]. In contrast to the changes in the phosphorylation at Ser-112, the phosphorylation level at Ser-136 was not changed even after 24h treatment in the absence or presence of inhibitors (Figure 5D).

3.5. The MAPK pathway mediates LPA protection against apoptosis

LPA stimulates both Erk and Akt in Caco-2 cells [21] and as shown above the phosphorylation of Bad indicates that the MAPK pathway plays a dominant role in the protection of Caco-2 cells. To confirm that activation of Erk is involved in the prosurvival signaling elicited by LPA, we determined the caspase activity in Caco-2/pSiL cells incubated with carrier, etoposide, or

etoposide/LPA for 24 h in the presence of U0126 or LY294002 (Figure 6). The basal caspase activity was elevated following the pretreatment with either inhibitor compared to carrier-treated cells (Figure 3A), suggesting that the basal activities of these kinases are important for the viability of the cells. Because of the elevated basal caspase activity, the inclusion of the inhibitors diminished the magnitude of changes in caspase activity elicited by etoposide exposure. Despite the smaller effect on the caspase activity, LPA failed to prevent an increase in caspase activity in the presence of U0126 (Figure 5A). Our previous study has shown that LPA-induced Erk activation is dependent on phospholipase C β (PLC β) since inhibition of PLC β decreased activation of Erk [21]. Similarly to U0126, the PLC β blocker, U73122, also abolished the effect of LPA (Figure 5B). Although we could not detect phosphorylation at Ser-136 of Bad, LY294002 almost completely abrogated the protective effect of LPA (Figure 5C). Interestingly, in cells treated with U0126 etoposide/LPA exposure resulted in an increase in the caspase activity compared with etoposide alone. U73122 also resulted in a small, although statistically insignificant, increase in the caspase activity with LPA/etoposide compared with etoposide alone. Figure 5D and E show that U0126 and LY294002, respectively, had no effect on the caspase activity in Caco-2/CL4 cells when compared with those in the absence of the inhibitors (Figure 3B).

3.6. LPA dependent pro-survival signals are mediated through delayed activation of Erk in Caco-2 cells

The phosphorylation of Bad induced by LPA suggests that the anti-apoptotic effect of LPA in Caco-2 cells is mediated by the activation of Erk and to a lesser extent Akt. To further examine the involvement of the MAPK and PI3K-Akt pathways, we next examined Erk and Akt activation in response to LPA by determining the phosphorylation levels by Western blot analysis.

Figure 6A shows that LPA increased phosphorylation level of Erk in Caco-2 cells at 30 min, which decreased to the basal level at 4 h. Etoposide alone resulted in an increase in Erk phosphorylation level at 30 min. In addition, we occasionally observed a small increase in Erk phosphorylation at 12 h. In contrast, co-treatment of the cells with etoposide and LPA restored the acute phosphorylation of Erk. In addition, there was sustained phosphorylation of Erk, which reached the maximum level at 12 h. In contrast to the control cells, LPA treatment did not elicit phosphorylation of Erk in Caco-2/CL4 cells. Moreover, etoposide/LPA treatment of Caco-2/CL4 failed to induce delayed activation of Erk.

The kinetic of phosphorylation of Akt by LPA was relatively slower, exhibiting a small increase at 30 min that was sustained for the next 12 h (Figure 6B). Etoposide alone showed no effect on Akt and co-incubation with LPA restored the levels of Akt phosphorylation almost to the levels observed by LPA alone. LPA treatment also stimulated the phosphorylation levels of Akt in Caco-2/CL4 cells and the levels of phosphorylation were lower compared to control cells as previously shown [21]. The effects of LPA/etoposide in Caco-2/CL4 cells were again similar to those in the control cells.

4. DISCUSSION

Apoptosis is a normal physiological process critical for the development and function of multicellular organisms. Apoptosis seems to be one of the major safeguards against uncontrolled proliferation. Proliferation and apoptosis are intimately related. Many cancer cells are often hypersensitive to apoptosis induction, but can avoid apoptosis because they have lost or can bypass check points that control cell cycle progression [33,42]. Over-expression of LPA₂ has been associated with different types of cancers, including ovarian and colon cancers, suggesting that LPA may act to promote survival of cancerous cells even in the presence of chemotherapy [18-21,43]. Herein, we show that LPA provides survival signals to Caco-2 cells,

protecting cells from etoposide-induced apoptosis via a NHERF2-dependent mechanism. It has previously been shown that NHERF2 interacts with LPA₂, but not other LPA receptors, and plays a pivotal role in regulation of cellular signaling elicited by LPA₂ [21,23]. Knockdown of NHERF2 in Caco-2 and HeLa cells drastically compromised LPA₂-mediated signaling, such as activation of Akt, Erk, and PLC β . Consistent with the earlier reports, we found that Caco-2/CL4 cells, with NHERF2 knocked down, were not protected by LPA from etoposide-induced apoptosis. Although we did not show that LPA₂ is directly responsible for the anti-apoptotic effect, there is compelling evidence indicating that this effect is mediated by LPA₂ [21,23]. First, other known LPA receptors are expressed at much lower levels or absent in Caco-2 and other colon cancer cells including Caco-2, making LPA₂ the only LPA receptor with a significant cellular function. Second, knockdown of NHERF2 or LPA₂ similarly attenuated LPA-mediated induction of IL-8 in colon cancer cells. Third, LPA₂ is the only LPA receptor that interacts with NHERF2.

The MAPK (JNK, p38 and Erk) pathways have been previously associated with apoptosis. The JNK and p38 kinase pathways are the classical pathways involved in apoptosis, whereas Erk is considered a pro-survival kinase in most cases [27,28,44-46]. However, recent studies have challenged this unilateral role of Erk in cell survival that activation of Erk can play an active role in inducing apoptosis and functions upstream of mitochondria signaling [44,47,48]. In addition, it has been shown recently that conversion of sustained Erk activation to transient abrogates the pro-apoptotic effect of estrogen [49]. On the other hand, the sustained and delayed Erk activation provides survival signals to human keratinocytes, suggesting a dual role of Erk on cell survival [50]. Treatment of Caco-2 cells with etoposide elevated caspase activity resulting in cell death, which was prevented by co-incubation with LPA (Figure 3A). As expected, the survival of Caco-2 cells was dependent on activation of Erk as the pharmacological inhibition of MEK1 completely abrogated the protective effect of LPA against etoposide-induced apoptosis (Figure 6A). On the other hand, etoposide alone resulted in acute phosphorylation of Erk at 30 min and occasionally weak and delayed Erk phosphorylation at 12-24 h (Figure 7A). Previous studies have shown that etoposide-induced apoptosis of fibroblasts and keratinocytes is associated with an increase in Erk phosphorylation [51,52]. In fibroblasts, etoposide induced caspase activity, which was completely abrogated by inhibition of Erk activity [51,52]. In keeping with these reports, apoptosis of hepatocytes by *Clostridium difficile* toxin is accompanied by acute stimulation of Erk [53]. We found that U0126 attenuated etoposide-induced caspase activity (Figure 6A) but still resulted in a significant increase compared with control-treated cells (Figure 3A). Taken together, activation of Erk by etoposide has a limited role in apoptosis of Caco-2 cells and does not appear to be the main mechanism underlying etoposide-induced apoptosis of Caco-2 cells. Subsequently, we show that co-incubation of Caco-2 cells with LPA restored acute phosphorylation of Erk and potentiated delayed phosphorylation of Erk. The specificity of these responses to LPA is supported by the lack of similar changes in Caco-2/CL4 cells in which LPA-induced signaling is perturbed by knockdown of NHERF2 [21]. Although the delayed phosphorylation of Erk by etoposide/LPA appears to mimic the effect by etoposide alone, the levels of Erk phosphorylation were substantially greater than those in the presence of etoposide alone. A previous study has shown that a gradual and sustained increase in phosphorylation of Erk by LPA rescued hepatocytes from *C. difficile* toxin-induced apoptosis [53]. In this report, in addition to the sustained phosphorylation of Erk, LPA resulted in phosphorylation of p90RSK, which lies upstream of Bad [53]. Erk represents an important converging point of diverse cellular processes that are regulated by precise spatio-temporal control mechanisms [27,28,44,54]. Since there are a large number of Erk substrates and diverse biological processes that Erk regulates, the eventual outcome of Erk activation is determined by the dynamic compartmentalization and the spatial arrangement of Erk [54]. Hence, it is conceivable that despite the resemblance of Erk phosphorylation by etoposide and etoposide/LPA, the different levels of Erk phosphorylation

may result in activation of different sets of target genes leading to cell death by etoposide, whereas etoposide/LPA promotes cell survival.

The fate of cell's death and survival is often determined by the Bcl-2 family proteins of anti- and proapoptotic regulators [35]. Bcl-2 and its close relatives, Bcl-X_L and Bcl-w, protect cells from a wide range of cytotoxic agents. Other Bcl-2 relatives, such as Bax, Bak, and Bad, bind to Bcl-2 to promote rather than antagonize apoptosis. LPA rescued normal rat intestinal IEC-6 cells from camptothecin-induced apoptosis by inactivation of caspase 9 and 3 and upregulation of Bcl-2 expression [55]. In confluent Caco-2 cell cultures, inhibition of the MEK/Erk pathway induced apoptosis with a significant decrease in the expression of Bcl-2 and Bcl-X_L, whereas a decrease in Bcl-2 but not Bcl-X_L was observed in subconfluent Caco-2 or human intestinal crypt cells [28,56]. In human T lymphoblastoma cells, Bax expression was suppressed in response to LPA with no effect on Bcl-2 or Bad [57]. We observed a decrease in Bcl-2 in etoposide-treated Caco-2 cells, consistent with earlier studies [28,55,56]. This decrease in the expression of Bcl-2 was blocked by inhibition of the MEK/Erk pathway. However, we did not observe a significant change in the expression level of Bcl-X_L or Bax, although the Caco-2 cultures were about 5-7 days post-confluence. On the other hand, LPA treatment resulted in Bad phosphorylation. Phosphorylation of Bad as a pro-survival mechanism has been shown in a number of studies. Estradiol inactivates Bad in breast cancer cells via pathways dependent on both Erk and Akt [58]. Studies have identified Ser-112 and Ser-136 as the predominant phosphorylation sites of Bad [38,39,59]. Akt phosphorylates Bad at Ser-136 both *in vitro* and *in vivo* [38,39], whereas p90 ribosomal S6 kinase (p90RSK), which is activated by Erk, phosphorylates Bad at Ser-112 [41,60]. LPA induces Bad phosphorylation in HeLa cells via an Akt-dependent pathway [61]. In Caco-2 cells, we found that LPA increased Bad phosphorylation at Ser-112, but no change at Ser-136 was incurred. In fact, the phosphorylation level of Ser-136 was not changed by the inhibition of the PI3K or MEK/Erk pathways. The phosphorylation at Ser-112 correlates with the on-set of delayed activation of Erk at 13 h post-LPA treatment (Figure 5 and 7A). In addition, LPA-induced phosphorylation at Ser112 of Bad was prevented by U0126, but not by LY294002 or PTX. These data collectively suggest that the MAPK pathway is the major component responsible for the cell survival effect of LPA in Caco-2 cells. In addition to Ser-112 and Ser-136, Bad is phosphorylated at Ser-155 by protein kinase A (PKA) [62]. LPA₂, which is the major LPA receptor in Caco-2 cells and only LPA receptor interacting with NHERF2, is known to inhibit forskolin -induced cAMP production and hence phosphorylation at Ser-155 was not studied in the current work [63].

The importance of Akt in LPA-mediated survival has been previously suggested. Activation of Akt protects primary lymphocytic leukemia cells and ovarian cancer cells from apoptosis, whereas G_i-mediated activation of MAPK is necessary for the survival of fibroblasts [13,14, 64]. In these fibroblasts, the PI3K-Akt pathway showed a limited contribution to the survival activity of LPA [13]. In addition, studies have shown that the PI3K-Akt pathway plays a critical role in the survival of intestinal epithelial cells [27,28,56]. In the present study, the inhibition of PI3K enhanced apoptotic cell death and completely abrogated the protective effect of LPA (Figure 6D). This effect is not caused by non-specific inhibition of Erk by LY294002 since the inhibition of PI3K by LY290042 does not affect LPA-induced activation of Erk in these cells as we have previously demonstrated [21]. Despite the significant effect by LY290042 on the caspase activity (Figure 6), we could not see a significant difference in the time-course of phosphorylation of Akt between control and NHERF2-knockdown cells treated with LPA alone or etoposide/LPA (Figure 7). However, the expression level of Bcl-2 was down-regulated in the presence of LY2940042, which is consistent with the increase in the basal caspase activity in the presence of the inhibitor (Figure 6). Although the change in Bcl-2 expression level by the inhibition of PI3K is a plausible mechanism, alternative mechanisms are noteworthy. There is a close relationship between caspase activity and viability, and the role of Akt has been often attributed to the viability and growth of cells [65,66]. Our data showed

that the activation of Erk was almost completely abrogated in Caco-2/CL4 cells despite the robust activation of Akt. Therefore, these data suggest that the activation alone is not sufficient to protect cells but is necessary for the protection. Taken together, it seems plausible that the PI3K-Akt pathway may sensitize the cells to etoposide-induced apoptosis by compromising the viability of Caco-2 cells. In support of this speculation, the basal caspase activity was elevated three-fold in the presence of LY294002 in contrast to a relatively smaller effect by U0126 or U73122 that blocked the MAPK pathway. Alternatively, LPA may protect cells from apoptosis in a PI3K-dependent but Akt-independent pathway. For example, microinjection of either wild-type or constitutively active Akt constructs in neurons does not protect against the neurons from extracellular amyloid β peptide-induced toxicity [67]. In addition, other downstream effectors of PI3K, such as serum and glucocorticoid-inducible kinase 1, SGK1, which is activated by the phosphoinositide-dependent kinase has been involved in protecting cells from cell death [68,69].

In summary, our results demonstrate that LPA protects Caco-2 cells from apoptosis and this protection involves activation of both MAPK and PI3K. LPA treatment is accompanied by upregulation of Bcl-2 expression, an increase in the phosphorylation level of Bad, and delayed and prolonged activation of Erk. The perturbation of LPA₂-elicited signaling by knock down of NHERF2 significantly abrogates the ability of LPA to protect cells from apoptotic death. The pharmacological inhibition of PI3K significantly compromised the survival of Caco-2 cells. Although the precise role of PI3K needs further studies, there was a decrease in the expression level of Bcl-2.

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Figure 1. LPA inhibits etoposide-induced apoptosis in Caco-2 cells

Caco-2 cells were serum-starved overnight and treated for 24 h with vehicle (Control), 200 μ M etoposide (Etop), 200 μ M etoposide + 20 μ M LPA, or 20 μ M LPA. Cells were harvested, stained with Annexin V and PI, and analyzed by FACS analysis. The Annexin V-positive and PI-negative population was defined as cells undergoing apoptosis. Representative results from three separate sets of experiments are shown.

Figure 2. LPA does not suppress apoptosis in Caco-2/CL4 cells

(A) Caco-2/pSiL and (B) Caco-2/CL4 cells were treated for 24 h with etoposide or etoposide + LPA. Cells were stained with Annexin V only and analyzed by FACS. n=3. *, P<0.01 compared with control treated cells, ** P<0.01 compared with etoposide treated cells. ns, not significant.

Figure 3. LPA inhibits etoposide-induced caspase activity

(A) Caco-2/pSiL and (B) Caco-2/CL4 were treated with etoposide or etoposide + LPA. Caspase-3/7 activity was determined by using the Caspase Glo 3/7 assay as described in Materials and Methods. $n=3$. *, $P<0.01$ compared with control cells, **, $P<0.01$ compared with etoposide treated cells. ns, not significant. (C) Caco-2 cells were preincubated with Zvad.fmk before being treated as above. Caspase-3/7 activity was determined as described above. $n=3$.

Figure 4. LPA modulates the expression level of Bcl-2

(A) Caco-2 cells were etoposide or etoposide + LPA for 13h in the absence or presence of U0126 or LY294002. The expression level of Bcl-2 was determined using an anti-Bcl-2 antibody. n=3. (B) The expression levels of Bcl-X_L are shown. n=3.

Figure 5. LPA inactivates pro-apoptotic Bad

Caco-2 cells were treated with etoposide or etoposide + LPA for 4 h (A) or 13 h (B). Phosphorylation of Bad was determined using anti-phospho-Bad antibodies that specifically recognize Bad phosphorylation at either Ser-112 or Ser-136. The amounts of total Bad were determined using an anti-Bad antibody. n=3. (C) Phosphorylation of Bad at Ser-112 was determined in the presence of U0126, LY294002, and PTX. Phosphorylation at Ser-112 of Bad was determined. n=3. (D) Phosphorylation of Bad at Ser-136 were determined in Caco-2 cells treated with etoposide or etoposide + LPA for 24 h. n=3.

Figure 6. LPA-mediated caspase 3/7 inactivation is dependent on Erk and Akt signaling in Caco-2/pSiL cells

Caco-2/pSiL cells (A-C) and Caco-2/CL4 (D, E) were treated with etoposide or etoposide + LPA in the presence of 20 μM UO126 (A, D), 5 μM U73122 (B), or 50 μM LY294002 (C, E). Caspase-3/7 activity was determined as described earlier. n=3. *, P<0.05 compared with control cells. **, P<0.05 compared with etoposide treated cells. ns, not significant.

Figure 7. LPA mediated pro-survival effects are associated with prolonged Erk activation
Caco-2/pSiL (A, B) and Caco-2/CL4 (C, D) cells were treated with LPA, etoposide, or both for different times. Treated cells were lysed and equal amounts of lysates were subjected to SDS-PAGE. The amounts of phosphorylated-Erk (A, C) or phosphorylated-Akt (B, D) were determined using anti-phospho-Erk or Akt antibodies, respectively. Membranes were stripped and reprobed with anti-Erk or anti-Akt antibodies to determine total Erk or Akt protein expression. Relative changes in phosphorylation normalized to the total protein are indicated. Representative results of three experiments are shown.