LPA protects intestinal epithelial cells from apoptosis by inhibiting the mitochondrial pathway

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Deng, Wenlin, De-An Wang, Elvira Gosmanova, Leonard R. Johnson, and Gabor Tigyi. LPA protects intestinal epithelial cells from apoptosis by inhibiting the mitochondrial pathway. Am J Physiol Gastrointest Liver Physiol 284: G821–G829, 2003; 10.1152/ajpgi.00406.2002.—We previously showed (Gastroenterology 123: 206–216, 2002) that lysophosphatidic acid (LPA) protects and rescues rat intestinal epithelial cells (IEC-6) from apoptosis. Here, we provide evidence for the LPA-elicited inhibition of the mitochondrial apoptotic pathway leading to attenuation of caspase-3 activation. Pretreatment of IEC-6 cells with LPA inhibited camptothecin-induced caspase-9 and caspase-3 activation and DNA fragmentation. A caspase-9 inhibitor peptide mimicked the LPA-elicited antia apoptotic activity. LPA elicited ERK1/ERK2 and PKB/Akt phosphorylation. The LPA-elicited antiapoptotic activity and inhibition of caspase-9 activity were abrogated by pertussis toxin, PD 98059, wortmannin, and LY 294002. LPA reduced cytochrome c release from mitochondria and prevented activation of caspase-9. LPA prevented translocation of Bax from cytosol to mitochondria and increased the expression of the antiapoptotic Bcl-2 mRNA and protein. LPA had no effect on Bcl-xL, Bad, and Bak mRNA or protein expression. These data indicate that LPA protects IEC-6 cells from camptothecin-induced apoptosis through G1-coupled inhibition of caspase-3 activation mediated by the attenuation of caspase-9 activation due to diminished cytochrome c release, involving upregulation of Bcl-2 protein expression and prevention of Bax translocation.

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These responses include promoting Schwann cell survival (66), suppressing apoptosis induced during ischemia and reperfusion in cardiomyocytes (64), and by serum withdrawal in fibroblasts (12) and renal tubular cells (37), preventing TNF-α-induced apoptosis in hepatocytes (54), and preventing ceramide-induced apoptosis in T cells (16). Recently, we (11) reported that LPA protected and rescued intestinal epithelial cells from serum withdrawal, TNF-α, and irradiation- and chemotherapy-induced apoptosis. Orally administered, LPA also reduced radiation-induced apoptosis in vivo (11). Despite the well-documented antiapoptotic effects of LPA, the exact mechanisms underlying its protective effect remain unknown.

Under normal conditions, the rates of apoptosis in intestinal epithelia are paired with the rates of mitosis to maintain mucosal integrity (18, 19, 47). Studies have demonstrated that an inappropriately high rate of intestinal epithelial cell apoptosis is the primary cause of iatrogenic enteropathy during cancer chemotherapy and gamma irradiation (25, 28, 48). Most of this pathologically excessive apoptosis occurs in the stem cell region of the intestinal crypt, thus disrupting the balance between epithelial cell production and loss, which maintains villus integrity, and leading to intestinal injury (11, 19, 25, 48). Therefore, preventing excessive intestinal epithelial cell apoptosis is an important potential target for therapeutic intervention. Initiator caspases including caspases-8 and -9 amplify the cascades by activating their downstream effectors caspases-3/CPP32, -6, and -7, which in turn cleave critical cellular protein substrates leading to the systematic dismantling of the cell (46, 51, 59). Caspase-3/CPP32 cleaves the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) (45) and the DNA fragmentation cofactor F-45, resulting in the fragmentation of genomic DNA into nucleosomal fragments, a characteristic feature of apoptosis (39, 40). Selective inhibition of caspase-3/CPP32-mediated PARP cleavage can prevent apoptosis from occurring in vitro and in vivo (45, 58). Caspase-3/CPP32 is also capable of activating the nuclear lamins protease, capase-6, and caspase-7 (13, 56, 59).

Thus far, two caspase-activating cascades upstream of caspase-3/CPP32 are well characterized: the first is...
initiated from cell surface death receptors, whereas the second is triggered by changes in mitochondrial integrity (5, 65). The loss of mitochondrial integrity leads to cytochrome c release from the intermembrane space into the cytosol (31, 67). Once in the cytosol, cytochrome c interacts with Apaf-1 to activate caspase-9, which in turn directly activates caspase-3/CPP32 (38). The mitochondrion-mediated caspase activation is highly regulated by a group of proteins designated as the Bcl-2 family that consists of both anti- and proapoptotic members. Among the antiapoptotic proteins are Bcl-2, Bcl-xl, and Bcl-w. The major proapoptotic proteins include Bax, Bad, Bid, and Bak. These proteins may interact with each other or work alone to regulate cytochrome c release. Although the exact process of cytochrome c release is still not elucidated, previous studies have suggested that Bcl-2 family proteins regulate cytochrome c redistribution by affecting the mitochondrial permeability transition pore (34, 41, 42).

Camptothecin is an inhibitor of DNA topoisomerase I that is in FDA Phase III clinical trial (9, 10). We recently characterized the induction of apoptosis by camptothecin in intestinal epithelial cells (IEC)-6 cells, a nontransformed crypt cell line derived from rat small intestine with an intact p53 gene (11, 49, 70) and found that LPA inhibited camptothecin-induced caspase-3/CPP32 activation (11). In the present study, we further probed the antiapoptotic signaling events that mediate the LPA-elicted inhibition of caspase-3/CPP32 activation. First, we showed that LPA dose dependently attenuates caspase-9 activity in IEC-6 cells. Second, we showed that LPA stimulated phosphorylation of ERK1/ERK2 and PKB/Akt. Consistent with a G protein-coupled receptor (GPCR)-elicited activity, the LPA-elicited inhibition of caspase-3/CPP32 and caspase-9 activity was blocked by pertussis toxin (PTX), PD 98059, LY 294002, and wortmannin. Third, LPA inhibited both camptothecin-induced cytochrome c release and caspase-9 activity. Inhibition of caspase-9 by z-Leu-Glu-(OCH3)-His-Asp(OCH3)-fluoromethyl ketone (z-LEHD-fmk) prevented camptothecin-induced apoptosis. Fourth, LPA prevented camptothecin-induced Bax translocation from cytosol to mitochondria and upregulated the expression of Bcl-2 mRNA and protein. These data demonstrate that one of the mechanisms activated by LPA involves the attenuation of the mitochondrial apoptotic pathway mediated by G protein-coupled signals, which in turn results in decreased activation of caspase-3/CPP32.

MATERIALS AND METHODS

Reagents. LPA (oleoyl) was purchased from Avanti Polar Lipids (Alabaster, AL). LPA was applied to cells complexed with fatty acid-free BSA (Sigma, St. Louis, MO), as previously described (62). Camptothecin was purchased from Sigma. PTX, PD 98059, LY 294002, wortmannin, N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pna) and N-acetyl-Leu-Glu-His-Asp-p-nitroanilide (Ac-LEHD-pna), colorimetric caspase substrates, were purchased from BioMol Laboratories (Plymouth Meeting, PA). Caspase-9 inhibitor z-LEHD-fmk; rabbit anti-caspase-3/CPP32; anti-cytochrome c; anti-Bax, anti-Bcl-xl, anti-Bad, and anti-Bak polyclonal antibodies; mouse anti-Bcl-2; and antiactin monoclonal antibodies were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA). Rabbit anti-caspase-9, anti-p44/42 MAPK, anti-Akt polyclonal, mouse antiphospho-44/42, and antiphospho-Akt monoclonal antibodies were purchased from Cell Signaling (Beverly, MA). Horseradish peroxidase-conjugated secondary antibody was purchased from Sigma.

Cell culture. IEC-6 cells were obtained from the American Type Culture Collection (Manassas, VA) at passage 13 and grown in DMEM supplemented with 5% fetal bovine serum, insulin (10 μg/ml), and gentamycin sulfate (50 μg/ml) at 37°C in a humidified 90% air-10% CO2 atmosphere. The medium was changed every other day. Cells between passages 15 and 21 were used for the experiments. Subconfluent cells were washed twice and replaced by DMEM without serum the night before experiments unless indicated otherwise.

DNA fragmentation ELISA. The DNA fragmentation ELISA method followed a modified procedure provided with the cell death detection ELISA kit from Boehringer (Indianapolis, IN). Briefly, cells were harvested and lysed in DNA lysis buffer for 30 min at 4°C to pellet debris. An aliquot of the supernatant was incubated with immunoreagents (antihistone-biotin plus anti-DNA peroxidase conjugated antibody) in 96-well streptavidin-coated plates on a shaker for 2 h. After washing with the incubation buffer, 100 μl substrate buffer was added to each well and incubated for an additional 5–10 min. DNA absorbance was read at 405 nm in a microplate reader. Duplicates of the samples were used to quantify protein by using the bichinonic acid (BCA) kit from Pierce (Rockford, IL). DNA fragmentation was expressed as absorbance units per microgram protein per minute.

Caspase activity assay. Cells were harvested and washed with cold Dulbecco’s PBS buffer. The cell pellet was taken up in ice-cold 50 mM HEPES (pH 7.4) buffer containing 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM DTT, 0.1 mM EDTA, and 0.1% Triton-X100. After 30-min incubation on ice, the lysate was centrifuged at 10,000 g at 4°C for 10 min. The supernatant was used for the measurement of caspase-3/CPP32 activity by using the Ac-DEVD-pNA chromogenic substrate and for measurement of caspase-9 activity by using the Ac-LEHD-pNA colorimetric caspase substrate. Each reaction (150 μl) contained 100 μg of cytosolic proteins, assay buffer (in mM: 50 HEPES, pH 7.4, 100 NaCl, 10 DTT, and 1 EDTA and 0.1% CHAPS), and 10 μl of 2 mM Ac-DEVD-pNA or Ac-LEHD-pNA dissolved in assay buffer. The enzymatic reaction was carried out in 96-well plates at 37°C. Absorbance was read at 405-nm in a microplate reader. Protein concentration was measured by using the Bradford protein assay kit (Bio-Rad, Hercules, CA), and caspase activity was expressed as picomole pNA cleaved per minute per microgram protein.

Preparation of cellular lysates. For preparation of mitochondrial and cytosolic fractions, a previously described (67) procedure of differential centrifugation was used. Briefly, IEC-6 cells were harvested and washed with PBS once, then resuspended with five volumes of ice-cold lysis buffer (in mM: 20 HEPES-KOH, pH 7.5, 10 KCl, 1.5 MgCl2, 1 DTT, 1 EDTA, 1 EGTA, and 0.1% FMSF) containing 250 mM sucrose. Cells were homogenized with a Dounce homogenizer and centrifuged at 10,000 g for 15 min at 4°C to pellet the mitochondrial fraction, whereas the resulting supernatants were further centrifuged at 100,000 g for 1 h at 4°C to produce a supernatant corresponding to the cytosolic fraction designated S-100. Protein concentration was measured by...
using the Bradford protein assay kit. For preparation of total cellular protein lysates, IEC-6 cells were washed with PBS and lysed in ice-cold M-PER mammalian protein extraction buffer. The protein concentration was quantified by using the BCA kit (Pierce, Rockford, IL).

**Western blot analysis.** Caspase-3/CPP32 was measured in the cytosol prepared for the caspase-3 activity assay as described above. Cytochrome c and Bax were detected in the mitochondrial and cytosolic S-100 fractions. Caspase-9 activation, Bcl-2, Bcl-xl, Bad, and Bak were evaluated in total cellular protein lysates. Twenty micrograms of protein was mixed with an equal amount of 2 × SDS-PAGE sample buffer, denatured by boiling for 5 min, followed by separation in 15% gels, and transfer to nitrocellulose membranes. Membranes were blocked with 5% (vol/vol) bovine serum in PBST (10 mM phosphate buffer, pH 7.4, 0.05% Tween-20) for 30 min at room temperature, and probed with the primary antibody for 1 h. After three washes with PBST, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody diluted in PBST for 1 h, followed by four washes and developed with the enhanced chemiluminescence Western blotting detection system (New England Nuclear Life Science Products, Boston, MA).

**RT-PCR.** Isolation of total RNA and synthesis of cDNA were performed as described previously (61). The real-time PCR primers of mouse Bcl2, Bax, and Bad were designed by using the Primer Express software (Applied Biosystems, Foster City, CA). The primers used in the present study were Bcl2 forward 5′-TGTGTTGAGAGCGTCACAACA-3′, reverse 5′-TGC CGGTTC AGGTACTCAGTC-3′; Bax forward 5′-GGGCTCTCTCTTCTACGG-3′, reverse 5′-TGGGACCTACGCAAAAGA-3′; Bak-1 forward 5′-GGGCGGGAA TGCTA AGACT-3′, reverse 5′-CCC CAGCTGAGTCCTACCTCT-3′; and β-actin forward 5′-TGTTGCTTGTGGATCCGTGGCT-3′, reverse 5′-CTGG GATCCACTAGT-3′. PCR reactions were performed by using an ABI Prism 7700 sequence detection system (Applied Biosystems). The SYBR Green PCR Core Reagent kit (Applied Biosystems) was used for the PCR reaction. The reaction contained 25 μl of SYBR reagent, 2 μl of diluted cDNA, and 30 nM primers in 50-μl volume. The thermal cycling conditions involved an initial denaturation step at 95°C for 10 s followed by an extension step at 65°C for 1 min. Quantitative values were obtained from the threshold cycle value (Ct), which is the point where a significant increase of fluorescence is first detected. The transcript number of mouse β-actin was quantified as an endogenous RNA control, and each sample was normalized on the basis of its β-actin content. Relative levels of Bcl2, Bax, and Bad mRNA were also normalized to the nontreated control group (calibrator). Calculation of mRNA copy number was done by using the formulas available at http://dorak.int.uh.edu/genetics/real.time.html (20). Final results, expressed as n-fold difference in mouse Bcl2, Bax, and Bad expression relative to β-actin and the nontreated control, termed N, were calculated as N = 2 (ΔCt sample -ΔCt calibrator), where ΔCt values of the sample and calibrator are determined by subtracting the average Ct value of a mouse Bcl2, Bax, and Bad genes from the average Ct value of the β-actin gene.

**Statistical analysis.** All data are expressed as means ± SE. Each experiment was repeated at least three times. Student’s t-test was used for comparison between the control and treatment groups. A P value of <0.05 was considered significant.

**RESULTS**

LPA prevents camptothecin-induced apoptosis in IEC-6 cells. Genomic DNA fragmentation is one of the most distinctive features of apoptosis, and activation of the effector caspase-3/CPP32 is an essential step in this pathway (40, 45). Treatment of IEC-6 cells with camptothecin at a concentration of 20 μM for 4 h resulted in a sixfold increase in DNA fragmentation and a fourfold increase in caspase-3/CPP32 activity compared with controls. Pretreatment with 10 μM LPA significantly attenuated the camptothecin-induced DNA fragmentation by 48% (P < 0.001, Fig. 1A). Correspondingly, LPA pretreatment significantly inhibited the increase of caspase-3/CPP32 activity by 56% after 4 h of camptothecin exposure (P < 0.001, Fig. 1B). Analysis of caspase-3/CPP32 activation by Western blot confirmed that the LPA-elicited inhibition of caspase-3/CPP32 activity correlated with the reduction of its activation, as indicated by the reduction in camptothecin-induced conversion of the 32-kDa procaspase-3/CPP32 into its 20-kDa active unit form (Fig. 1C).

Fig. 1. Lysophosphatidic acid (LPA) protects intestinal epithelial cell (IEC-6) cells against camptothecin (CAM)-induced apoptosis by inhibiting caspase-3/CPP32. IEC-6 cells were washed twice and starved in serum-free DMEM overnight. LPA at a concentration of 20 μM equal to that found in serum or vehicle was applied 15 min before 20 μM CAM treatment. DNA fragmentation (A) and caspase-3/CPP32 activity (B) were measured 4 h after CAM exposure. Pro-caspase-3/CPP32 and its active units (C) were evaluated by Western blot. A total of 20 μg of cytosolic protein was loaded in each lane.

†P < 0.001 compared with CAM alone; †P < 0.001 compared with control. Data shown are means ± SE of 3 experiments.

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LPA inhibits camptothecin-induced cytochrome c release and caspase-9 activation. Release of cytochrome c into the cytosol and subsequent activation of caspase-9 are essential events of the mitochondrion-mediated release and caspase-9 activation. Western blot analysis of cytochrome c in the mitochondrial and corresponding cytosolic S-100 fractions showed that treatment of IEC-6 cells with 20 μM camptothecin for 4 h induced a large amount of cytochrome c release and that this increase in cytosolic cytochrome c correlated with its decrease in the mitochondrial fraction (Fig. 2A). As early as 2 h after camptothecin treatment, caspase activity increased twofold and continued to increase by reaching a 3.5-fold stimulation at 4 h (Fig. 2B). Treatment with 10 μM LPA not only prevented most of the camptothecin-induced cytochrome c release from the mitochondria, but also significantly decreased caspase-9 activity in response to camptothecin at 2 and 4 h after treatment (P < 0.001, Fig. 2B). Further analysis of caspase-9 activation by Western blot demonstrated that the LPA-elicited inhibition of caspase-9 activity was due to the reduction of its activation, as shown by the fact that the camptothecin-induced conversion of the 54-kDa procaspase-9 into its 40- and 38-kDa active forms was markedly reduced by LPA pretreatment (Fig. 2C). LPA reaches a serum concentration of 10 μmol/l but its plasma concentration is 100-fold less (2). A dose-response relationship between LPA concentration and camptothecin-induced caspase-9 activity showed that as little as 1 μM LPA significantly inhibited the activity of the enzyme (Fig. 2D), suggesting that a physiological concentration of the lipid is sufficient to exert an inhibitory effect on caspase-9.

LPA elicits antiapoptotic effects through a G_i-coupled pathway. LPA activates G_i-coupled receptors to promote survival (8, 11, 14). Western blot analysis showed that LPA induced a significant increase of ERK1/ERK2 and Akt phosphorylation, which was sustained for up to 120 min (Fig. 3A). ERK1/2 phosphorylation was blocked by 25 ng/ml PTX or 50 μM PD 98059, but not by 50 nM wortmannin or 50 μM LY 294002. PKB/Akt phosphorylation was blocked by PTX, wortmannin, or LY 294002, but not by the MEK-1 inhibitor PD 98059 (Fig. 3B). Pretreatment of IEC-6 cells with 25 ng/ml PTX for 12 h, 50 μM PD 98059 for 1 h, and 50 nM wortmannin or 50 μM LY 294002 incubation for 30 min decreased LPA-elicited antiapoptotic activity by >80% as evaluated by DNA fragmentation ELISA and caspase-3/CPP32 activity (Fig. 3, C and D). Further experiments revealed that PTX or PD 98059 abolished >90% of the LPA-elicited inhibition of caspase-9 activity, whereas wortmannin or LY 294002 almost completely abrogated the inhibition of caspase-9 activity by LPA (Fig. 3E). Caspase-9 inhibitor z-LEHD-fmk mimicked the LPA-elicited antiapoptotic activity and decreased camptothecin-induced DNA fragmentation by 56% and caspase-3 activity by 70% (P < 0.001, Fig. 3, C and D). The antiapoptotic activity of z-LEHD-fmk was not significantly affected by the upstream inhibitors of ERK1/ERK2 or PKB/Akt phosphorylation (Fig. 3, C and D). These observations taken together suggest that camptothecin-induced apoptosis can be effectively attenuated by the inhibition of caspase-9 either by z-LEHD-fmk or by LPA.

LPA prevents camptothecin-induced Bax translocation from cytosol to mitochondria. Bax is a proapoptotic protein from the Bcl-2 family that mediates apoptosis by affecting the mitochondrial permeability transition pore (34, 42) and subsequently, the release of cytochrome c from mitochondria. Western blot analysis of Bax in the mitochondrial and corresponding cytosolic fractions revealed that, in control IEC-6 cells, Bax was...
located predominantly in the cytosol with only a small portion in the mitochondrial fraction (Fig. 4A). Treatment of IEC-6 cells with 20 μM camptothecin for 4 h resulted in the redistribution of Bax with a large amount of the 23 kDa Bax translocated to the mitochondria compared with the control. The increase in Bax in the mitochondrial fraction accounted for its decrease in the cytosol (Fig. 4A). Pretreatment with LPA significantly suppressed camptothecin-induced Bax translocation (Fig. 4A). Neither LPA nor camptothecin had any effect on the total cellular Bax protein level (Fig. 4B). Consistent with the unchanged Bax protein levels, RT-PCR analysis of cellular Bax mRNA expression revealed no differences (Fig. 4C).

LPA upregulates Bcl-2 but not Bcl-xl, Bad, and Bak. Bcl-2 prevents the release of cytochrome c from the mitochondria and thus prevents caspase activation (31). With the use of Western blot and RT-PCR techniques, we examined the effect of LPA on Bcl-2 protein level and mRNA expression (Fig. 5). Treatment of IEC-6 cells with 20 μM camptothecin had little effect on either Bcl-2 protein (Fig. 5, A and B) or mRNA levels (Fig. 5C). In contrast, LPA pretreatment within 2 h induced a significant increase in Bcl-2 protein level (Fig. 5, A and B), and a significant increase at 4 h after camptothecin exposure (Fig. 5, A and B). Interestingly, LPA pretreatment elicited a significant increase of

Fig. 3. LPA protection and inhibition of caspase-9 requires Gα, MEK, and phosphatidylinositol 3-kinase (PI3-kinase). A: time course of LPA-elicited ERK1/2 and Akt activation in IEC-6 cells. Note that LPA elicited a long-lasting biphasic activation of ERK1/2 and Akt. B: serum-starved IEC-6 cells were treated with pertussis toxin (PTX) (25 ng/ml) for 12 h, PD-98059 (50 μM) for 1 h, and LY 294002 (50 μM) or wortmannin (50 nM) for 30 min, followed by the addition of LPA (10 μM), caspase-9 inhibitor z-LEHD-fmk (LEHD), or vehicle 15 min before 20-μM CAM treatment. Activation of ERK1/ERK2 and PKB/Akt were examined by Western blot at 0 or 5 min after LPA incubation. DNA fragmentation (C), caspase-3/CPP32 activity (D), and caspase-9 activity (E) were evaluated 4 h after CAM exposure. * P < 0.001 compared with CAM alone; † P < 0.001 compared with CAM plus LPA. Results are representative of 3 independent experiments.

Fig. 4. LPA prevents CAM-induced Bax translocation from cytosol to mitochondria. IEC-6 cells were washed twice and starved in serum-free DMEM overnight. LPA (10 μM) or vehicle was applied 15 min before 20 μM CAM. Western blot analysis of Bax protein levels in mitochondrial and corresponding cytosolic S-100 fractions (A) and Western blot analysis of total cellular Bax protein level 4 h after CAM exposure (B). A total of 20 μg of cytosolic protein was loaded in each lane. C: real-time RT-PCR evaluation of cellular Bax mRNA expression. Data represent the means ± SE of 3 experiments.
Bcl-2 mRNA expression at the 2-h time point ($P < 0.001$, Fig. 5C), whereas no effect was observed at the 4-h time point compared with camptothecin exposure alone. Cells treated with LPA alone showed a threefold increase in Bcl-2 mRNA expression at the 2-h time point, which continued to increase to the 4-h time point compared with control ($P < 0.001$, Fig. 5C). We also evaluated other members of the Bcl-2 family including Bcl-xl, Bad, and Bak. Pretreatment with 25 µM LPA had no effect on either protein levels or mRNA expression of these Bcl-2 family members (data not shown).

**DISCUSSION**

Despite the fact that LPA has been found to be a strong antiapoptotic agent in a number of cell lines, the mechanism of the protective effect has not been elucidated (11, 12, 16, 37, 54, 64, 66). We previously reported that LPA prevented IEC-6 cells from apoptosis induced by four different mechanisms including the activation of the intrinsic apoptotic pathway by camptothecin exposure, gamma irradiation, or the activation of the extrinsic apoptotic pathway by TNF-α treatment and serum withdrawal. Similar to IEC-6 cells, LPA reduced the number of apoptotic cells in the crypts of mice exposed to gamma irradiation (11). These findings identify LPA as a potential therapeutic agent in the enteropathies resulting from chemo and irradiation therapy (11). In the present study, we used camptothecin-induced apoptosis of IEC-6 cells as the model to elucidate the mechanisms underlying LPA-elicited antiapoptotic activity. Camptothecin stabilizes the covalent topoisomerase I-DNA complexes through the cell cycle leading to frank DNA strand breaks (23, 57), which initiate a series of DNA damage-related signals, cell-cycle arrest, and an orderly engagement of the enzymatic machinery that results in apoptosis (27, 43). It remains unclear how DNA strand breaks lead to cell death, but sufficient evidence indicates that mitochondria are the main checkpoint upstream of effector caspase activation. The biochemical events involved in the process of camptothecin-initiated apoptosis have been described by several investigators (35, 52, 70) and include the activation of the intrinsic/mitochondrial apoptotic pathway. These include cytochrome c release from mitochondria, activation of caspase-9 and caspase-3/CPP32, and internucleosomal DNA fragmentation (Figs. 1 and 2).

We demonstrate that LPA inhibits camptothecin-induced caspase-3/CPP32 activation and subsequent DNA fragmentation (Fig. 1). Three different polypeptides (Apaf-1, cytochrome c, caspase-9) have been identified as being necessary and sufficient for triggering
caspase-3/CPP32 activation (38, 71). Apaf-1 serves as a docking protein that complexes with both cytochrome c and caspase-9. So far, the precise cytochrome c binding site on Apaf-1 has not been clarified, but the caspase recruitment domains (CARDs) on Apaf-1 mediate the recruitment of caspases that have similar CARDs at their NH2 terminus (22, 71). Normally, the CARDs on Apaf-1 are not exposed. Cytochrome c must complex with Apaf-1 to expose the CARDs to recruit caspase-9 (6, 38). Subsequently, the docking of caspase-9 to Apaf-1 leads to cleavage and conversion of pro-caspase-9 into its active form, which in turn activates caspase-3/CPP32 (38, 46, 71). We have demonstrated that the camptothecin-induced release of cytochrome c and increase in caspase-9 activity were both inhibited by LPA pretreatment by using concentrations in the physiological 1–10 μM range (Fig. 2). These data, with the definite cause-effect relationship between cytochrome c and caspase-9, clearly indicate that the antiapoptotic activity of LPA is mediated, at least in part, through inhibition of camptothecin-elicited cytochrome c release.

It is well established that Gi-protein-mediated activation of ERK1/ERK2 contributes to the LPA-elicited survival signals (12, 54, 66), whereas LPA also stimulates PTX-sensitive activation of phosphatidylinositol 3-kinase (PI3-kinase)/Akt antiapoptotic pathway (12). In agreement with these reports, we found significant increase of both ERK1/ERK2 and PKB/Akt phosphorylation in IEC-6 cells, which lasted for at least 2 h (Fig. 3A). The significance of the sustained activation of these two enzymes is presently unclear. Blocking of these Gαi-coupled pathways cancelled most of the LPA-elicited antiapoptotic activity (Fig. 3, C and D), indicating Gαi-mediated activation of ERK1/ERK2 and PI3-kinase/Akt both are required but alone not sufficient for the antiapoptotic activity of LPA. Interestingly, activation of ERK1/ERK2 was insensitive to inhibition of PI3-kinase, and inhibition of MEK-1 by PD 98059 didn’t inhibit PI3-kinase activation. Further experiments showed that LPA-elicited inhibition of caspase-9 activity was sensitive to PTX, PD 98059, LY 294002, and wortmannin (Fig. 3D), which bridged the gap between the upstream activation of ERK1/ERK2 and PI3-kinase/Akt and the downstream activation of caspase-3/CPP32, suggesting that the LPA-elicited activation of ERK1/ERK2 and PI3-kinase/Akt signaled through the mitochondrial pathway. Based on the present data, it appears that activation of both ERK1/ERK2 and PI3-kinase/Akt is required for the protective effect of LPA. Further experiments are needed to delineate the molecular contribution and cascades of the antiapoptotic pathways.

Mitochondria play a critical role in the control of apoptosis (33). Cytochrome c resides primarily in the intermembrane space of the mitochondria and is released into the cytosol in response to various apoptotic agents (30, 33). To elucidate the signaling events upstream of the LPA-elicited inhibition of cytochrome c release, we investigated the Bcl-2 family members. Bax is a proapoptotic protein from the Bcl-2 family that induces cytochrome c release (5, 41, 42). Under normal conditions, Bax is localized predominantly in the cytosol in monomeric form (1, 24). In response to apoptotic stimuli, it is activated and translocates to the mitochondria where it stimulates cytochrome c release (33, 41). Previous studies revealed that camptothecin-induced apoptosis was associated with the translocation of Bax from the cytosol and accumulation in the mitochondrial intermembrane (3, 15). In agreement with these reports, we found significant Bax translocation after camptothecin exposure (Fig. 3). LPA inhibited camptothecin-induced Bax translocation, leading to the inhibition of cytochrome c release (Figs. 2 and 3). Although it is unclear how Bax regulates the process of cytochrome c release from mitochondria, proposed mechanisms include the regulation of the mitochondrial permeability transition pore (34, 41, 42) or formation of ion channels in the mitochondrial outer membrane (1). Most importantly, Bax directly interacts with the antiapoptotic proteins Bcl-2 and Bcl-xl (29). In the present study, we found no LPA-elicited change in Bax mRNA expression, suggesting that Bax is not transcriptionally regulated by the lipid growth factor.

Bcl-2 regulates apoptosis by inhibiting the release of cytochrome c from mitochondria (31). Overexpression of Bcl-2 prevents cytochrome c release (67). We have demonstrated that LPA induces a significant increase in Bcl-2 (Fig. 4) at both the mRNA and protein levels, indicating a prominent role for Bcl-2 in LPA-elicited antiapoptotic activity. Bcl-2 family proteins contain up to four highly conserved sequence regions designated as Bcl-2 homology domains, which enable them to form homo- or heterodimers (21, 29). The antiapoptotic activity of Bcl-2 has been reported to correlate with its ability to heterodimerize with proapoptotic proteins Bax and Bad, inhibiting their biological activities (21, 55, 68, 69). Recently, direct interactions between Bcl-2 and Bax in the mitochondrion have been reported (41). It appears that the relative ratio between anti- and proapoptotic proteins may be a primary factor in determining cytochrome c release (4, 7, 21, 29, 68). Therefore, the LPA-induced increase in Bcl-2 (Fig. 4) favorably shifts the ratio between Bcl-2 and proapoptotic proteins, which leads to a direct inhibition of Bax and Bad activities, and subsequent inhibition of cytochrome c release. Additional evidence suggests that Bcl-2 is also capable of regulating apoptosis independently, which potentially widens the downstream targets affected by increased levels of Bcl-2 in LPA-treated cells (32, 50).

Taken together, these data indicate that LPA protects IEC-6 cells against camptothecin-induced apoptosis through the activation of the mitochondrial pathway at multiple steps. LPA-elicited transcriptionsal up-regulation of Bcl-2 and subsequent inhibition of Bax translocation from the cytosol to the mitochondria contribute to the prevention of cytochrome c release. The decrease in cytochrome c and the resulting decrease in caspase-9 activity prevent caspase-3/CPP32 activation.
and subsequent DNA fragmentation. Elucidation of the pathway that regulates Bcl-2 expression linked to LPA receptors is subject to ongoing studies. A report by Lassus et al. (36), which appeared after the completion of the present study, places caspase-2 upstream of caspase-9 in the activation of the apoptotic cascade. The possible effect of LPA on the regulation of caspase-2 is subject of future investigation.

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