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Lysophosphatidic acid represses autophagy in prostate carcinoma cells

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Running title: Lysophosphatidic acid is a potential repressor of autophagy in PC3, LNCaP and Du145 cells.

Abstract

Lysophosphatidic acid (LPA) is a small signaling phospholipid that mediates diverse functions including cell proliferation, migration and survival by engaging LPA-agonized G-protein coupled receptors. Autophagy is a survival mechanism in response to nutrient depletion or organellar damage that encloses idle or damaged organelles within autophagosomes that are then delivered to lysosomes for degradation. However, the relationship between LPA and autophagy is largely unknown. The purpose of this study is to elucidate whether LPA affects autophagy through the ERK1/2 and/or the Akt/mTOR signaling pathways. In this study, we investigated the effect of LPA on autophagy-regulating pathways in various prostate-derived cancer cells including PC3, LNCaP and Du145 cells grown in complete medium and exposed to serum-free medium. Using Western blotting and ELISA, we determined that LPA stimulates the ERK and mTOR pathways in complete and serum-free medium. The mTOR pathway led to phosphorylation of S6K and ULK, which respectively stimulates protein synthesis and arrests autophagy. Consistent with this, LPA exposure suppressed autophagy as measured by LC3 maturation and formation of GFP-LC3 puncta. Altogether, these results suggest that LPA suffices to activate mTORC1 and suppress autophagy in prostate cancer cells.

Keywords: Autophagy, lysophosphatidic acid, prostate cancer

1. Introduction

Prostate cancer occurs when growth, mitogenic and survival pathways become dysregulated and usurped by cancerous cells in prostate tissues. These pathways include signals downstream of growth factors and mitogens and regulate homeostatic processes like protein synthesis rates and autophagy. In particular, lysophosphatidic acid (LPA) has been linked to prostate cancer growth and invasion (Bektas, Payne, Liu et al. 2005; Hwang, Lee, Zhang et al. 2016; Qi, Park, Gibbs et al. 1998). LPA mediates a wide range of biological actions such as cell proliferation, cell survival, and tumor cell invasion by engaging one of at least six G protein-coupled receptors (GPCR) that are known as LPAR1-6 (Aikawa, Hashimoto, Kano et al. 2015; Daaka 2002; Umezue-Goto, Tanyi, Lahad et al. 2004). Depending on the isoform, LPA receptors signal through different heterotrimeric G α subunit families such as G $\alpha_{12/13}$, G $\alpha_{q/11}$, G $\alpha_{i/o}$ and G α_s . One of the best known functions of LPA is to drive G $\alpha_{i/o}$ -mediated activation of the Raf–MEK–ERK–MAPK pathway, a powerful mitogenic pathway with a multitude of targets including transcription factors (Gibbs, Rubio, Zhang et al. 2009; McCain 2013; Riaz, Huang and Johansson 2016; Winter, Fox, Kester et al. 2010). For example, activated LPA receptors stimulate ERK1/2 mediated proliferation and survival of androgen-insensitive prostate cancer cells (Guo, Kasbohm, Arora et al. 2006; Spiegel and Milstien 2005; Zeng, Kakehi, Nouh et al. 2009). In addition, LPA is also reported to stimulate the phosphatidylinositol 3-kinase [PI-3K]-Akt signaling pathway, leading to stimulatory phosphorylation of Thr308 and Ser473 of Akt (Hwang, Lee, Zhang et al. 2016; Riaz, Huang and Johansson 2016). At least in prostate carcinoma PC3 cells, LPA-dependent activation of Akt is necessary for cell survival (Raj, Sekula, Guo et al. 2004).

Akt is a kinase with myriad targets including the pathway that modulates the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) (Lapante and Sabatini 2009). More specifically, Akt phosphorylates and represses the Tuberous Sclerosis Complex 1 and 2 which is a GTPase-

activating protein (GAP) complex (Chiang and Abraham 2005; Gerstein, Lu, Van Nostrand et al.). Suppression of TSC1/2 promotes GTP-loading of the Rheb GTPase, which then stimulates mTORC1 (Inoki, Li, Xu et al. 2003). Alternatively, the presence of essential amino acids like leucine, engages the Ragulator complex to modulate the Rag GTPase complex, which in turn stimulates mTORC1 (Efeyan, Zoncu and Sabatini 2012; Jewell, Kim, Russell et al. 2015). mTORC1 phosphorylates ribosomal protein S6 kinases (S6Ks), which stimulates translation, and the eukaryotic translation initiation factor 4E-binding proteins (4E-BPs), repressing these proteins to release their inhibitory effect on translation initiation (Ma and Blenis 2009). In addition, mTORC1 phosphorylates and represses ULK1, an autophagy-promoting kinase – hence mTORC1 represses autophagy. By contrast, the absence of growth factors and/or of amino acids inactivates mTORC1, slowing mRNA translation rates and promoting autophagy. Overall, the broad roles of mTOR/S6K signaling pathway implicates this hub kinase in many important diseases including cancer, diabetes, and obesity (Tavares, Pavan, Amaral et al. 2015). While LPA was reported to induce PI3K/Akt/mTOR/p70S6K pathways in prostate cancer cells, its effect on downstream mTORC1 pathways like autophagy has not been examined (Lee, Park, Lee et al. 2006; Lin 2015; New, Wu, Kwok et al. 2007).

Autophagy is a cellular catabolic process employed to remove damaged organelles and unused cytoplasmic components to regenerate amino acids and other monomeric subunits during nutrient depletion (Galluzzi, Pietrocola, Levine et al. 2014). During autophagy, target organelles are engulfed by a double-membrane vacuole, known as an autophagosome, which can be recognized by staining for microtubule-associated protein 1 light chain 3 (LC3) (Murugan and Amaravadi 2016). After engulfment, autophagosomes are then targeted to and fuse with lysosomes to form autophagolysosomes, wherein the cytoplasmic cargos are degraded. In this process, LC3-I is converted to form a LC3-II. Thus, LC3-I/II ratio can be used to track the level of

autophagy and autophagic flux (Murugan and Amaravadi 2016). The released monomeric nutrients like amino acids are then exported into the cytosol to support general cell metabolism (Glick, Barth and Macleod 2010; Liu, Pei, Yang et al. 2017; Pattingre, Espert, Biard-Piechaczyk et al. 2008). There is a growing appreciation that autophagy has a dual role in cancer. Active and continuous autophagy can cause autophagic cell death (Yang, Chee, Huang et al. 2011). On the other hand, autophagy can protect cancer cells during nutrient stress because of their continuous consumption of cellular resources (Liu, Pei, Yang et al. 2017; Xu, Wu, Gu et al. 2013).

Overall, LPA is highly effective in driving growth and anabolic pathways and likely uses multiple LPA receptors to cross-talk and coordinate these growth and survival pathways (Winter, Jefferson and Kimball 2011). Since LPA promotes prostate cancer cell survival and modulates ERK and PI3K-Akt, we decided to examine the effect of LPA on mTORC1 pathway and autophagy in prostate carcinoma cells. We observed that LPA addition to specific prostate cancer cells can suppress autophagy, even in serum-starved cells.

2. Material and methods

2.1. Reagents and antibodies

The LPA used in our studies was 1-oleoyl-2-hydroxy-sn-glycerol-3-phosphate (Avanti Polar Lipids, 857130, Alabama, USA) since this is the predominant LPA found in biological samples and LPA receptor agonist (Ma, Nagai, Chun et al. 2013; Xie, Gibbs, Mukhin et al. 2002). Stock solutions of LPA were maintained by dissolving in phosphate buffered saline (PBS, pH 7.2) in the presence of 0.1% (w/v) bovine serum albumin (BSA) (BioShop, 9048-46-8, Canada Inc.) and were then diluted with or without serum containing medium to a final concentration of 10 μ M. mTOR was inhibited with 100 nM torin 1 (Tocris, 4247, Bristol, United Kingdom), MEK1/2 was blocked with 10 μ M U0126 (Cell Signaling, 9903, Danvers, MA) and Akt was arrested with 10 μ M Akt1/2 kinase

inhibitor (Sigma, 6730, USA). Primary antibodies used for Western blotting were rabbit anti-S6K (p70S6 Kinase, 49D7) monoclonal antibody (mAb; 2708S), rabbit anti-phospho-p70S6 Kinase (Thr389) mAb (108D2; 9234), rabbit anti-ULK1 mAb (D8H5; 8054), rabbit anti-phospho-ULK1 polyclonal antibody (Ser757; 6888), and rabbit anti-LC3B polyclonal antibody (2775) were all purchased from Cell Signaling Technologies (Danvers, MA) and all were used at 1:1000 dilution. Secondary antibodies were horseradish peroxidase (HRP)–linked goat anti-rabbit (Cell Signaling, catalog no: 7074, Danvers, MA) used at dilution 1:1000.

2.2. Cell culture

The prostate carcinoma PC3, LNCaP and Du145 cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and were grown in RPMI 1640 (Gibco, catalog no: 21870076, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (Wisent, catalog no: 080450, Canada), L-glutamine (Wisent, catalog no: 609-065-EL, Canada) and penicillin/streptomycin antibiotics (Wisent, catalog no: 450-201-EL, Canada) at 37°C with 5% CO₂. For serum starvation, cells were grown in complete RPMI-1640 medium until 70% confluent, then cultures were rinsed with PBS and exposed in serum-free RPMI-1640.

2.3. DNA synthesis assay

Cells were seeded in 96-well plates at a density of 5x10³ cells/well, grown for 24 hours, then incubated with 1, 5, 10 and 15 µM LPA for 2 hours. The LPA-containing medium was then removed and fresh medium containing 100 µM BrdU was added and incubated for 8 hours. DNA synthesis was measured with the Cell Proliferation ELISA BrdU colorimetric assay kit (Roche Diagnostics GmbH, catalog no: 1164722901, Mannheim, Germany) as instructed by the manufacturer. The reaction product was quantified by measuring the absorbance at 370 nm using ELISA reader (model: Cambridge Scientific, Thermo Labsystems, Multiskan Spectrum,

Microplate Reader, Finland).

2.4. Western blotting analysis

Cells were seeded in 6-well plates and grown for 24 hours. Then, cells were incubated in either serum-supplemented medium or serum-free medium with or without 10 μ M LPA and with or without inhibitors for 2 hours for S6K and ULK1, and 6 hours for LC3 detection. To inhibit ERK, Akt and mTOR, we used 10 μ M U0126, 10 μ M Akt1/2 kinase inhibitor or 100 nM torin1, respectively. Total cell lysates were prepared by resuspending and homogenizing cells in RIPA buffer containing protease inhibitor (Complete, mini-tablet EDTA-free) and phosphatase (PhosSTOP) inhibitor tablets from Roche (Indianapolis, IN) dissolved in PBS. Cell lysates were cleared by centrifugation for 10 min at 13,000 $\times g$. The supernatant was then mixed with 2 $\times$ Laemmli sample buffer (Bio-Rad, Hercules, CA) and boiled for 5 min at 95°C. Lysates were then loaded and separated on a 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). The membranes were incubated with primary antibodies, followed by HRP-linked secondary antibodies in tris-buffered saline plus 0.1% tween-20 containing 5% nonfat milk or bovine serum albumin (BSA; BioShop Canada, Burlington, ON), followed by enhanced chemiluminescence detection using the Amersham ECL western blotting detection Reagent (BioRad, USA). The quantification of Western blots was done by densitometry using Image Lab software (Bio-Rad, Version 6.0.0, USA).

2.5. Enzyme-Linked Immunosorbent Assay

We used ELISA kits to detect phospho-ERK1(T202/Y204)/phospho-Erk2(T185/Y187) and pan-ERK1/2 (catalog no: PEL-Erk-T202-T, RayBiotech, RayBio, Norcross, GA, USA). The concentration of phospho-mTOR and total mTOR were determined using ELISA kits to detect human phospho-mTOR (S2448) and total mTOR (catalog no: PEL-mTOR-S2448-T, RayBio, Norcross, GA, USA). In

both cases, we followed the manufacturer's instructions and measured the absorbance at 450 nm wavelength using a multifunction microplate reader (Multiskan Ascent 354 Microplate Reader, Thermo Labsystems, Finland).

2.6. Fluorescence Microscopy And Quantification

For autophagy detection, PC3 cells were transfected with LC3-GFP plasmid for overnight expression using FuGENE HD (Promega Corp.) according to the manufacturer's instructions. Following overnight over-expression, cells were serum starved or incubated with inhibitors, previously described, for 2 hours. Cells were washed 3x with PBS and fixed with 4% paraformaldehyde for 15 mins. Afterwards, LC3-GFP was imaged with a Quorum Discovery spinning disc confocal microscope system equipped with a Leica DMI8 microscope connected to an iXon 897 EMCCD camera, and controlled by Quorum WAVE FX powered by MetaMorph software (Quorum Technologies, Guelph, ON). For quantification of LC3 punctate structures, particle detection tools in ImageJ (v. 1.47 bundled with 64-bit Java) were used, where background subtraction and thresholding was done to eliminate noise-derived particles. Autophagy index was determined by normalizing the average number of LC3-GFP puncta in each condition to LC3-GFP puncta in complete medium

2.7. Statistical analysis

Data were analyzed using GraphPad Prism software (Version 7.0). All data presented in our study were obtained from at least three independent experiments and expressed as means $\pm$ standard deviation (S.D.). Statistical significance was determined by using ANOVA and a Tukey's post-hoc test. Differences between groups were considered significant if $p \leq 0.05$.

3. Results

LPA is reported to stimulate proliferation of various cancer cell lines including prostate cancer-derived PC3 cells (Hao, Tan, Xu et al. 2007). To expand on these results, we assessed the effective concentration of LPA needed to stimulate proliferation of various prostate carcinoma cells. As a proxy, we measured DNA replication (S phase) by using BrDU incorporation. We exposed PC3, LNCaP, and Du145 cells to 1, 5, 10 and 15 μ M of LPA for 2 hours and measured the extent of proliferation. Indeed, addition of 10-15 μ M LPA stimulated DNA replication rates in all three carcinoma cells relative to lower LPA concentrations (Fig. 1). Since 10 μ M LPA was as effective as 15 μ M, we employed the former LPA concentration for the remaining studies. Therefore, we used 2 hours incubation for further experiments.

3.1. LPA increases ERK1/2 phosphorylation in prostate carcinoma cells

LPA activates MEK/ERK signaling in a number of cancer cell lines (Du, Sun, Hu et al. 2010; Kue, Taub, Harrington et al. 2002). In this study, we examined the ability of LPA to potentiate ERK phosphorylation in prostate cancer cells grown in complete medium (Fig. 2A) or rescue ERK activation in cells exposed to serum-free medium (Fig. 2B). To achieve this, phospho- and total ERK1/2 levels were measured by ELISA and the results were given as a ratio of phospho-ERK to total ERK. When PC3, LNCaP, and Du145 cells were maintained in complete medium, LPA exposure for 2 h significantly increased the amount of phospho-ERK1/2 relative to resting cells or those treated with BSA-alone, which controls for BSA as an LPA carrier (Fig. 2A).

We then assayed the ability of LPA to support ERK activation during serum starvation. First, and as expected, serum depletion led to a significant decrease in phospho-ERK relative to total ERK in PC3, LNCaP and Du145 cells (Fig. 2B). Second, supplementing free medium with BSA alone failed to potentiate the levels of phospho-ERK in any of the serum-starved cell lines (Fig. 2B). In

comparison, all prostate cancer cells displayed significantly higher phosphorylation of ERK1/2 when exposed to serum-free medium supplemented with LPA than cells exposed to serum-free medium or BSA-only medium (Fig. 2B). Collectively, these data indicate that LPA potentiates phosphorylation of ERK1/2 in the presence of serum and is sufficient to rescue ERK activation in serum-deprived conditions.

3.2. LPA increases mTOR phosphorylation in prostate carcinoma cells

In addition to the ERK1/2 MAPK pathway, the PI3K/Akt signaling pathway is a key driver in prostate cancer progression (Kinkade, Castillo-Martin, Puzio-Kuter et al. 2008). LPA is known to potentiate the Akt pathway (Baudhuin, Cristina, Lu et al. 2002). Since Akt is a driver of the mTORC1 pathway, we evaluated whether LPA activates mTOR by assessing the levels of phospho-mTOR relative to total mTOR levels in PC3, LNCaP, and Du145 cells grown in complete medium or depleted for serum. First, cells grown in complete medium were kept as is or exposed to BSA-alone or 10 μ M LPA for 2 hours prior to cell lysis and quantification of phospho- and total mTOR using an ELISA kit. LPA treatment significantly increased the phosphorylation of mTOR in PC3, LNCaP, and Du145 cells compared with control conditions (Fig. 3A). Torin-1 is a selective inhibitor of mTOR both in mTORC1 and mTORC2. Since mTORC2 is important to activate Akt, we expect this to reduce phosphorylation of mTOR itself (Dan, Antonia and Baldwin 2016). Indeed, torin1 was able to block phosphorylation of mTOR even in the presence of LPA. Following, we rechallenged cells with LPA after they were serum starved. First, as expected, serum-deprivation reduced phospho-mTOR levels relative to serum-exposed cells and this was not rescued by the addition BSA-only treatment (Fig. 3B). Strikingly, LPA supplementation to serum-free medium was sufficient to increase phospho-mTOR levels in a torin-1-dependent manner (Fig. 3B). Overall, this work shows that LPA signaling can promote the mTOR pathway in prostate cancer cells.

3.3. LPA increases the phosphorylation of S6K in PC3, LNCaP and Du145 cells

Since LPA activates mTOR in prostate cancer cells, we next assessed by Western blotting the phosphorylation status of S6K, a key mTORC1 substrate that controls protein translation activity in serum-replete and depleted conditions. For these experiments, we measured the signal ratio of phospho-S6K to total S6K obtained by Western blotting. First, torin1 pre-treatment nearly eliminated phospho-S6K completely in all three prostate cancer lines showing that this is dependent on mTOR activity (Fig. 4A). Second, we showed that LPA treatment for 2 hours led to a significant increase in phosphorylation of S6K in serum-repleted prostate cancer cells (Fig. 4B). Given that LPA activates ERK and mTOR and that there is evidence for cross-talk between the ERK and the PI3K-Akt-mTOR pathways, we then examined if LPA-stimulation of phospho-S6K is dependent on these pathways in non-starved cells by pre-treating PC3, LNCaP and Du145 cells with the MEK inhibitor U0126 and the Akt inhibitor Akti. Importantly, we found that both MEK and Akt inhibitors reduced the level of LPA-induced phospho-S6K levels, where Du145 cells were the most dependent on the Erk and Akt pathway (Fig. 4A). Taken together, these results suggest that the phosphorylation of S6K is dependent on both ERK and Akt signaling.

We then investigated phospho-S6K in all three prostate cancer cell lines under serum-starved conditions to better understand how these cells respond to serum starvation stress. First, we observed a significant reduction in S6K phosphorylation in all three cell lines growing in serum-free medium compared to serum-replete conditions (Fig. 4B). We then assessed whether LPA exposure could stimulate phosphorylation of S6K in serum-starved PC3, LNCaP and Du145 cells. Interestingly, we observed cell-specific differences in this response. First, relative to cells exposed to serum-free medium alone, DU145 and PC3 cells treated with LPA in serum-free medium exhibited significantly higher phosphorylation of S6K (Fig. 4B). In comparison, LPA did

not boost the levels of phospho-S6K in serum-starved LNCaP cells relative to serum-starved LNCaP cells (Fig. 4B).

We then examined the role of ERK and Akt-mTOR pathways in stimulating S6K in serum-starved prostate cancer cells. PC3, LNCaP, and Du145 cells were incubated in serum-free medium containing both ERK inhibitor and LPA. Interestingly, LPA-promoted S6K phosphorylation in PC3 and LNCaP cells independently of ERK pathway (Fig. 4B), bringing phospho-S6K to levels similar or just above serum-replete levels. Strikingly, phospho-S6K levels were blunted in Du145 cells exposed to LPA and ERK inhibition, suggesting that these cells are highly dependent on ERK activity for stimulating the mTOR pathways, unlike PC3 and LNCaP cells (Fig. 4B).

We next investigated whether the PI3K/Akt pathway participated in the stimulation of S6K in serum-depleted prostate cancer cells. Therefore, we treated PC3, LNCaP and Du145 cells with both LPA and Akt inhibitor (Fig. 4B). We found that Akt inhibitor strongly hindered phospho-S6K levels during treatment with LPA across all cells with Du145 cells being the most sensitive again (Fig. 4B). Overall, these results suggest that the synergy between the PI3K/AKT pathway and the Ras/Raf/MEK/ERK pathway plays an important role in the LPA-induced mTOR activation and S6K phosphorylation but that the extent of cross-talk and interaction depends on cell type.

3.4. LPA treatment increases phosphorylation of ULK1 in prostate carcinoma cells

Uncordinated-51-like kinase 1 (ULK1) initiates the early steps of autophagy. In conditions of nutrient abundance, the ULK1 complex is inhibited through mTOR-mediated phosphorylation on ULK1. By contrast, mTOR dissociates from the ULK1 complex during starvation, causing dephosphorylation of the ULK1 complex and initiating autophagy (Nazio and Cecconi 2017). According to our results above, LPA activates mTOR in prostate cancer cells dependent on PI3K-Akt and/or ERK pathways with the extent of interaction dependent on cell background. Thus, we investigated whether the LPA signaling had an effect on ULK1 in PC3, LNCaP, and Du145 cells by

blotting for phospho-ULK1 using serum-grown and serum-deprived conditions. We found that LPA treatment of serum-replete cells caused a minor increase on phospho-ULK1 levels across the three cell lines with the most significant increase in Du145 cells (Fig. 5A). Nevertheless, inhibition of ERK and Akt pathways both led to a significant drop in phospho-ULK suggesting that these pathways regulate autophagy in prostate cells, particularly LNCaP and Du145 cells.

Since LPA activates mTOR in serum-starved cells (Fig. 3), we asked whether LPA could up-regulate phospho-ULK levels in serum-deprived cells and regulate autophagy (Shang, Chen, Du et al. 2011). As observed in Fig. 5B, ULK1 phosphorylation was much lower in the absence of serum than the control group for all cell types. Importantly, when we added LPA to serum-free medium, we found that cells treated with LPA had higher levels of ULK1 phosphorylation compared to the serum-free group in PC3 and Du145 cells, but not in LNCaP cells (Fig. 5B). This is consistent with LPA stimulation of phospho-mTOR in serum-driven PC3 and Du145 (Fig. 3).

We next inhibited ERK to examine whether LPA mediated ERK / mTOR signaling pathway plays a role in ULK phosphorylation. When the ERK inhibitor and LPA were added to LNCaP cells growing in serum-free medium, ULK1 phosphorylation was lower than in cells exposed to serum-free medium containing LPA (Fig. 5B). To then used the Akt inhibitor to investigate the role of the Akt/mTOR signaling pathway on LPA-mediated regulation of ULK1 in serum-free medium (Fig. 5B). We found that Akt reduced ULK1 phosphorylation in PC3 and LNCaP cells exposed to serum-free medium containing LPA, the Akt inhibitor was more effective at inducing ULK dephosphorylation than the ERK inhibitor in PC3 and LNCaP cells.

3.5. Lysophosphatidic acid suppresses autophagy in serum-starved prostate cancer cells

As LPA activates mTOR and phosphorylates ULK1, we asked whether LPA suppresses autophagy by probing for LC3 maturation and formation of LC3-labelled puncta. LC3 is a canonical autophagosomal marker that exist as two isoforms, LC3-I and LC3-II. LC3-I is the immature and

cytosolic isoform that predominates in low autophagic conditions. In comparison, autophagy-promoting signals causes phosphatidylethanolamine conjugation to LC3-I to form LC3-II, which is recruited to autophagosomal membranes (Tanida, Ueno and Kominami 2008). Thus, we can estimate the levels of autophagy by quantifying the LC3-II:LC3-I ratio using Western blotting and by scoring the number of LC3-labelled autophagosomes.

Using Western blots to quantitate the LC3-II:LC3-I ratio, we observed that LPA had a minor effect on LC3-II:LC3-I ratio in PC3 and LNCaP cells grown in serum-containing media, consistent with low basal levels of autophagy in serum-grown cells (Fig. 6A,B). By comparison, whereas serum-depleted PC3 and LNCaP cells displayed higher LC3-II:LC3-I ratio, indicating autophagy promotion, LPA-treated serum-starved cells exhibited significantly lower LC3-II:LC3-I ratio (Fig. 6A, B). Collectively, these data indicate that LPA can repress autophagic signals during serum-starvation. To complement this autophagy measure, we also transfected PC3 cells with GFP-LC3, which can be used to quantify formation of autophagosomes. First, we showed that LPA does not alter the number of LC3 puncta in serum-replete conditions (Fig. 6C, D), consistent with the observed LC3-II:LC3-I ratio in Fig. 6A. In addition, within 2 h of LPA with and without ERK and Akt inhibition, cells exhibited similar level of autophagy (Fig. 6C, D). In comparison, whereas there was a striking increase in LC3-labelled structures in serum-starved PC3 cells, LPA addition to serum-starved PC3 cells repressed formation of these LC3-positive puncta (Fig 6C, E). Surprisingly, LPA could reduce autophagy even in cells inhibited for ERK or Akt, suggesting that LPA can modulate autophagy independently of ERK and Akt activation (Fig. 6). Collectively, these data show that LPA is a potent antagonist of autophagy upon growth factor depletion.

4. Discussion

LPA signaling via the LPA receptors plays a role in the pathogenesis of prostate cancer

through effects on cell proliferation, migration, and survival (Aikawa, Hashimoto, Kano et al. 2015). In part, LPA contributes to proliferation by engaging multiple cross-talking signalling pathways such as the MEK/ERK and PI3K-Akt pathways, which are central to the progression of cancer (Kinkade, Castillo-Martin, Puzio-Kuter et al. 2008). Indeed, inhibition of the PI3K/Akt pathway hindered prostate cancer growth (Butler, Marlein, Walker et al. 2017). A major target of the PI3K/Akt pathway is mTORC1, a key hub kinase that modulates anabolism and represses catabolic processes like autophagy. Yet, to the best of our knowledge there are no reports examining whether LPA modulates mTOR signaling and the autophagic response in prostate cancer cells. This is particularly important question since studies on autophagy in cancer cells have lead to opposing conclusions; some studies indicate that autophagy suppresses carcinogenesis, while others suggest that autophagy promotes carcinogenesis (White 2015). In this study, we tried to characterize downstream LPA signals by examining the state of the ERK and Akt-mTOR signaling pathways in various prostate carcinoma cells and the impact of LPA on autophagy in these cells. We found that LPA caused higher ERK and mTOR phosphorylation in PC3, LNCaP, and Du145 prostate carcinomas in both serum-containing and serum-free media. The fact that LPA can stimulate both ERK and mTOR in cells depleted of serum shows that LPA is a powerful growth factor, a conclusion further corroborated by higher incorporation of BrDU into cells exposed to LPA.

Despite that LPA activates ERK and mTOR pathways in PC3, LNCaP and Du145 prostate cancer cells in the presence and absence of serum, the observed effects on downstream targets of mTOR was complex and context-dependent. First, LPA boosted the levels of phospho-S6K in all three cell lines grown in serum in a manner dependent on ERK and Akt. This is consistent with observations made by Winter et al. that showed an increase of phosphorylated S6K after adding LPA to Rat2 fibroblasts grown in 0.5% FBS containing medium or serum-free medium (Winter,

Fox, Kester et al. 2010). However, only PC3 cells displayed a large increase in phospho-S6K upon addition of LPA in the absence of serum, this despite higher levels of active mTOR in all cell types. Similarly, while all cells depended on Akt to stimulate S6K upon LPA exposure in serum-starved cells, only starving Du145 cells required ERK to activate S6K. It is not clear from our experiments how ERK may modulate S6K, but there are studies suggesting that ERK phosphorylates TSC2 to block the TSC1-TSC2 complex, thus stimulating Rheb and mTORC1 activity (Ballif, Roux, Gerber et al. 2005; Sun, Ren, Zhu et al. 2009). Taken together, LPA likely drives anabolic pathways such as protein synthesis by stimulating the mTORC1-S6K axis. However, the sensitivity and the signals that feed into mTOR-mediated metabolism may depend on cell type and the presence of additional signals.

In comparison to S6K, serum-exposed cells did not generally display significantly higher phospho-ULK1 in LPA and serum-treated cells relative to serum-only conditions, suggesting that basal levels of mTORC1 suffice to repress ULK1. This is consistent with low levels of autophagy observed in serum-fed cells with and without LPA exposure. In comparison, all prostate cancer cells that were serum-starved exhibited a dramatic decrease in phospho-ULK and a strong increase in autophagosome formation. Strikingly, addition of LPA to serum-starved cells significantly boosted phospho-ULK in PC3 and Du145 cells but not in LNCaP, despite higher mTOR activity, suggesting that mTOR substrates may be subject to additional regulation. For example, protein kinase A activated by LPA is a negative regulator of autophagy and may result in the inactivation of ULK1 leading to the suppression of autophagy (Björn Stork 2012; He and Klionsky 2009; Lagadari, Truta-Feles, Lehmann et al. 2009). Importantly, LPA inhibited starvation-induced autophagy in PC3 cells consistent with a significant increase in phospho-ULK in serum-starved and LPA-treated PC3 cells. Perplexingly, LPA-mediated inhibition of autophagy remained unhindered when cells were also exposed to ERK and Akt inhibitors, indicating that LPA can

modulate autophagy through additional pathways. For example, it was reported that LPA decreases the levels of p53 which promotes autophagy by inhibiting mTOR pathways via the activation of AMPK (Itahana and Itahana 2018; Murph, Hurst-Kennedy, Newton et al. 2007). Thus, LPA likely drives mitogenic signals, anabolic reactions like protein synthesis, and suppresses autophagy in at least a subset of prostate cancer cells. The cell specific effects may reflect differences of whether cells are androgen sensitive or not. For example, LNCaP cells are androgen-sensitive, while Du145 and PC3 cells are independent and more sensitive to ERK-inhibition. Generally, LNCaP cells are assumed to represent early stages of cancer, while PC3 cells are representative of late prostate cancer, with both cell lines exhibiting differences in the genes they express (Dozmorov, Hurst, Culkin et al. 2009; Marchiani, Tamburrino, Nesi et al. 2010). Strikingly, we show that LPA stimulation activates ERK activity in LNCaP cells. In fact, these three cell lines may show differences in terms of response to LPA because mRNA levels of autotaxin, the enzyme responsible for LPA production, was lower in LNCaP cells than PC3 and Du145 cells (Nouh, Wu, Okazoe et al. 2009). In some studies, LPA response was not detected in LNCaP cells (Gibbs, Rubio, Zhang et al. 2009; Sivashanmugam, Tang and Daaka 2004). Gibbs and colleagues (Gibbs, Rubio, Zhang et al. 2009) reported that ERK activity did not change in LNCaP cells after a 15 minute incubation of LPA, in contrast, we see ERK activation after 2 hours of incubation. This discrepancy could likely be explained by the difference in experimental conditions and kinetics in those studies. Interestingly, it has been reported that LNCaP cells overexpress LPAR3 receptor mRNA compared to the other prostate cancer cell lines (Zeng, Kakehi, Nouh et al. 2009) and LPA stimulation through LPAR3 activates ERK in C9 cells. (Alcantara-Hernandez, Hernandez-Mendez, Campos-Martinez et al. 2015; Ishii, Contos, Fukushima et al. 2000). Therefore it may be reasonable to find that ERK phosphorylation is increased after treatment with LPA in LNCaP cells.

Autophagy has recently received intense interest as a target for a variety of diseases,

especially cancer. The most extensively studied condition in inducing autophagy is starvation (Cheng, Ren, Hait et al. 2013). Starvation-induced autophagy is for generating energy by degradation processes to maintain cell survival (Toepfer, Childress, Parikh et al. 2011). On the other hand, overt and continuous autophagy is known as a type 2 cell death mechanism and can be seen during starvation (Liu and Levine 2015). Thus, the balance of autophagy may support survival or trigger death (Choi 2012). For example, prostate cancer tumorigenesis is increased by reducing ULK expression to suppress autophagy (Puto, Brognard and Hunter 2015). On the other hand, activation of autophagy promotes survival of cancer cells because autophagy may promote resistance of the cell to starvation-stress (White 2015). Given that LPA hindered autophagy in PC3 cells, this suggests that LPA may carry out signal transduction to prevent autophagic cell death in order to protect the cancer cells. We speculate that cancer cells that are distressed and undergoing nutrient deprivation, LPA may be generated to block autophagic cell death.

In summary, the results from this study indicate that LPA activates mTOR through MEK-ERK and PI3K-Akt pathways resulting in S6K and ULK1 phosphorylation, concomitant with the suppression of autophagy. We speculate that LPA is a critical molecule that protects prostate cancer cells from autophagic cell death. If so, blocking LPA receptor-Akt-ERK/mTOR pathway may be beneficial in patients with prostate cancer by increasing autophagic cell death.

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Conflict of Interest

There is no conflict of interest.

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Figure Legends

Fig. 1. The proliferative effect of LPA on prostate cancer PC3, LNCaP, and Du145 cells. Cells were incubated in the presence of 1, 5, 10 or 15 μ M LPA for 2 hours. As a proxy for proliferation, we measured the level of DNA synthesis by using the BrdU incorporation assay. Data are expressed as mean $\pm$ SD from ten independent experiments. *:p<0.05: compared to 1 μ M LPA, **: p<0.05: compared to 5 μ M LPA.

Fig. 2. LPA-mediated ERK activation in PC3, LNCaP and Du145 cells. ERK activation is represented as the ratio of phospho-ERK to total ERK protein using ELISA. (A) Cells were treated with medium (control), medium + BSA, or lysophosphatidic acid (LPA, 10 μ M) for 2 hours. (B) Cells were treated with medium (control), serum-free medium (SF), serum-free medium + BSA, serum-free medium + LPA for 2 hours. The medium containing the BSA in which LPA was dissolved was also the control group. All data are expressed as mean $\pm$ SD from four independent experiments. *: p<0.05 compared to control, +: p<0.05 compared to SF group.

Fig. 3. LPA-mediated mTOR activation in PC3, LNCaP and Du145 cells. mTOR activation is represented as the ratio of phospho-mTOR to total mTOR using ELISA. (A) Cells were treated with medium (control), medium + BSA, 10 μ M LPA, or LPA plus 100 nM torin 1 for 2 hours. (B) Cells were treated with medium (control), serum-free medium, serum-free medium + BSA, serum-free medium + LPA for 2 hours. The medium containing the BSA in which LPA was dissolved was also the control group. All data are expressed as mean $\pm$ SD from four independent experiments. *: $p < 0.05$ compared to control, **: $p < 0.05$ compared to LPA group, +: $p < 0.05$ compared to SF group.

Fig. 4. LPA-mediated activation of S6K in prostate cancer cells. Western blot analysis was used to quantify phospho-S6K and total S6K in PC3, LNCaP and Du145 cells. S6K-specific bands were quantified by Image Lab software. (A) Cells were treated with medium (control), torin 1 (100 nM), lysophosphatidic acid (LPA, 10 μ M), LPA + ERK inhibitor (10 μ M) and LPA + Akt inhibitor (10 μ M) for 2 hours. (B) Cells were treated with medium (control), serum free medium (SF), serum free medium + LPA (10 μ M) (SF + LPA), serum free medium + LPA (10 μ M) + ERK inhibitor (10 μ M) (SF + LPA + ERKi), serum free medium + LPA (10 μ M) + Akt inhibitor (10 μ M) (SF + LPA + Akti) for 2 hours. All data are expressed as mean $\pm$ SD from three independent experiments. *: $p < 0.05$ compared to control group, **: $p < 0.05$ compared to LPA group, ϕ : $p < 0.05$ compared to LPA+ERK inhibitor group. +: $p < 0.05$ compared to SF group, ϕ : $p < 0.05$ compared to SF + LPA group, δ : $p < 0.05$ compared to SF + LPA + ERKi group.

Fig. 5. LPA-mediated regulation of ULK1 in prostate cancer cells. Western blot analysis was used to quantify phospho-ULK1 and total ULK in PC3, LNCaP, and Du145 cells. ULK-1-specific bands were quantified by ImageJ software and the ratio of phospho-ULK1 to total ULK1 was quantified. (A) Cells were treated with medium (control), lysophosphatidic acid (LPA, 10 μ M), LPA + ERK inhibitor (10 μ M) and LPA + Akt inhibitor (10 μ M) for 2 hours. (B) Cells were treated with medium

(control), serum free medium (SF), serum free medium + LPA (10 μ M) (SF + LPA), serum free medium + LPA (10 μ M) + ERK inhibitor (10 μ M) (SF + LPA + ERKi), serum free medium + LPA (10 μ M) + Akt inhibitor (10 μ M) (SF + LPA + Akti) for 2 hours. All data are expressed as mean $\pm$ SD from three independent experiments. *: $p < 0.05$ compared to control, **: $p < 0.05$ compared to LPA group, δ : $p < 0.05$ compared to LPA+ERK inhibitor group, +: $p < 0.05$ compared to SF group, ϕ : $p < 0.05$ compared to SF+LPA group.

Fig. 6. The effect of LPA on autophagy in prostate cancer cells. A and B: LC3-I induction and conversion of LC3-I to LC3-II were determined by western blot analysis. PC3 cells (A) were treated with medium (control), lysophosphatidic acid (LPA, 10 μ M), serum free medium (SF), serum free medium + LPA (10 μ M) (SF + LPA) for 6 hours. LNCaP cells (B) were treated with medium (control), lysophosphatidic acid (LPA, 10 μ M), serum free medium (SF), serum free medium + LPA (10 μ M) (SF + LPA) for 6 hours. C: PC3 cells transiently transfected with GFP-LC3 were incubated in complete medium (control) or serum-free medium (SF) with and without 10 μ M LPA and with and without 10 μ M ERK inhibitors (U0126) or 10 μ M Akt inhibitor for 2 hours. After treatments, cells were assessed by spinning disc confocal microscopy to quantify the number of GFP-LC3 puncta as an indicator of autophagy. Scale bar equals 10 μ m. D and E: in serum-replete (D) and serum-depleted (E) PC3 cells autophagic index was determined by normalizing the average number of LC3-GFP puncta in each condition to LC3-GFP puncta in complete medium. All data are expressed as mean $\pm$ SD from three independent experiments. *: $p < 0.05$ compared to control, ϕ : $p < 0.05$ compared to LPA group, +: $p < 0.05$ compared to SF group, δ : $p < 0.05$ compared to SF+ERK inhibitor group, ϕ : $p < 0.05$ compared to SF+Akt inhibitor group.

DNA synthesis
Absorbance (370 nm)

A

B

A

B

A **B**

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C

Complete medium

SF medium

D **E**