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Autophagy Regulates Lipolysis and Cell Survival Through Lipid Droplet Degradation in Androgen Sensitive Prostate Cancer Cells

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Abstract

BACKGROUND—Androgen deprivation therapy, one of the standard treatments for prostate cancer (PCa) induces apoptosis, as well as autophagy in androgen-responsive PCa cells. As autophagy can promote either cell survival or death, it is important to understand its role in PCa treatment. The objective of our study was to elucidate the function of autophagy in lipid droplet homeostasis and survival in androgen-sensitive PCa cells.

METHODS—To produce androgen deprivation, charcoal filtered serum or the androgen inhibitor casodex were used in LNCaP and LAPC4 cells. Autophagy was monitored by immunofluorescence/confocal microscopy and immunoblot analysis. Levels of intracellular lipid droplets and triacylglycerols after the inhibition of autophagy by 3-methyladenine, bafilomycin A₁ or si-ATG5 were quantified by three independent methods, Oil Red O staining, triacylglycerols lipase assay, and nuclear magnetic resonance.

RESULTS—Androgen deprivation induced autophagy and the depletion of lipid droplets in both of the androgen-sensitive PCa cell lines examined, whereas the blockage of autophagy by pharmacological or genetic means inhibited lipid droplet degradation and therefore lipolysis and cell growth. In addition, under androgen deprivation, increased colocalization of lipid droplets and autophagic vesicles was observed in LNCaP cells, which can be further enhanced by blocking the autophagic flux.

CONCLUSION—Autophagy mediates lipid droplet degradation and lipolysis in androgen-sensitive PCa cells during androgen deprivation which aids the survival of PCa cells during hormone therapy.

Keywords

Prostate cancer; Autophagy; Androgen deprivation; Lipid droplets; Lipolysis; NMR

Introduction

The presence of normal concentrations of androgens is a critical requirement for the survival, proliferation and progression of prostate epithelial cancer (PCa) [1]. These steroids

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act by binding to the androgen receptor (AR) and regulating the expression of hundreds of genes affecting cellular metabolism, survival and growth. One effect of androgen/androgen receptor axis is the stimulation of fatty acid synthase in the human prostate adenocarcinoma cell line LNCaP, resulting in an accumulation of lipid droplets within the cytoplasm [2–4]. The activities of several other enzymes, including Acetyl-CoA carboxylase and ATP-citrate lyase, that are involved in the synthesis of fatty acids, are also increased in PCa [5–8]. Interestingly, fatty acid oxidation is also enhanced in PCa [9–12]. Tumors need unusual amounts of energy and biosynthetic precursors to survive and grow [13], and the alterations in metabolic pathways observed in PCa cells helps them to satisfy this increased bioenergetic demand.

It is well known that androgen ablation induces apoptosis and regression of prostate tumors, and that this forms the basis of hormone ablation therapy in PCa [14, 15]. However, some of the cancer cells can “escape” from the treatment, survive and develop androgen independence by several mechanisms [16, 17]. In order to understand the progression of PCa it is important to investigate the cell survival mechanisms activated during hormone ablation therapy.

Autophagy is a cellular mechanism that is inducible during starvation and stress [18, 19]. The process involves the formation of double or multilayered vesicles, known as autophagosomes, that enwrap cytosolic components and target them to lysosomes for hydrolysis. This mechanism of intracellular bulk digestion and degradation provides precursors for metabolism in cells [18]. A protein known as light chain 3 isoform I (LC3I) of the microtubule associated protein complex 1 is essential for the autophagy pathway: activation and translocation of lipidated LC3 isoform II (LC3II) to autophagosomes is correlated with the induction of autophagy and is one of the markers of autophagy. Like other metabolic pathways, autophagy can be regulated by various inducers and inhibitors. For example, serum or amino acid deprivation induces autophagy, whereas 3-Methyladenine (3-MA), an inhibitor of class III PI3 kinase, blocks the generation of phosphatidylinositol 3-phosphate (PI3P), an essential docking molecule for the formation of phagophores and the early stage of autophagy. In addition, antibiotics, such as bafilomycin A1 (Baf A1) and concanamycin A, are used to investigate the autophagic flux because they inhibit H⁺-V-ATPase activities and acidification of the lysosome, and therefore the final fusion event between the lysosome and autophagic vesicles [20, 21]. In PCa research, it has been shown that when androgen-sensitive LNCaP cells were cultured in serum free medium, autophagy was induced which was diminished by adding dihydrotestosterone, suggesting a negative regulation of autophagy by androgen [22]. In addition, two independent studies have demonstrated that inhibition of autophagy in LNCaP cells under androgen deprivation causes increased cell death, suggesting that autophagy protects PCa cells [22, 23].

Lipid droplets (LDs) are intracellular depots of neutral lipids (both triacylglycerols and cholesterol) which are enveloped by a monolayer of phospholipids and associated proteins [24]. LDs can be metabolized by at least two different pathways. First, hormone-regulated cytosolic lipases break down the triacylglycerols into fatty acids which are then utilized for β -oxidation [24, 25]. Second, lipolysis can be mediated by autophagy. A recent study showed that autophagosomes sequester lipid droplets and target them to the lysosome for lipolysis in rat hepatocytes [26]. Autophagic vesicles (AVs; autophagosomes, amphisomes and autolysosomes), and LDs have been found to be colocalized in starved rat hepatocytes. This alternate pathway of lipolysis by autophagy is significant for intracellular lipid metabolism [27, 28], and could be critical for PCa cell survival under androgen deprivation conditions.

As lipolysis appears to provide critical support for the progression of prostate cancer, we propose that autophagy regulates lipolysis in androgen-sensitive PCa cells during androgen ablation. In this study, we sought to determine how androgen-sensitive PCa cells could ramp up an alternate pathway of lipid catabolism during androgen deprivation. We find that in two different androgen-sensitive PCa cell lines, LNCaP and LAPC4, AVs sequester and target lipid droplets to lysosomes during androgen ablation.

Materials and Methods

Chemical and reagents

We purchased charcoal-filtered fetal bovine serum (CFM) from Hyclone (Thermo Fisher, MA, USA), RPMI 1640, low glucose DMEM medium and regular FBS from Invitrogen (Eugene, OR, USA), Methyltrienolone (R1881) from PerkinElmer (Waltham, MA, USA), 3-Methyladenine (3-MA), Oil Red O, deuterium oxide (D₂O), deuteriochloroform (CDCl₃), tetramethylsilane (TMS), d₆-2,2-Dimethyl-2-silapentane-5-sulfonic acid (DSS) and casodex (CDX) from Sigma-Aldrich (St Luis, MO, USA), HCSLipidTOX Red, Lipofectamine, and G418 from Invitrogen, and bafilomycin A₁ (Baf A1) from A.G. Scientific, Inc (San Diego, CA, USA).

Cell culture and transfection with pEGFP-LC3

Human prostate cancer cells LNCaP (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 medium with 10% (v/v) fetal bovine serum (FBS) at 37°C and 5% CO₂ as previously described [29]. LAPC4 cells, an androgen-sensitive, AR-wildtype PCa cell line, a gift from Dr. Todd Thompson (University of New Mexico Health Science Center) [7, 30, 31], were grown in low glucose DMEM medium with 5 % (v/v) FBS. Media containing regular FBS were designated as CM. To treat cells, both cell lines were grown in medium with charcoal-filtered FBS, designated as CFM, in the presence or absence of androgen analogue (R1881), antiandrogen (casodex/CDX), or autophagy inhibitors (3-MA or Baf A1), as indicated. The pEGFP-LC3 plasmid containing a fusion gene of the two full-length cDNA sequences encoding the enhanced green fluorescent protein (EGFP) and microtubule-associated protein 1 light chain 3 (LC3), was a gift from Dr. Noburu Mizushima (National Institute for Basic Biology, Okazaki, Japan). pEGFP-LC3 was linearized with StuI and transfected into LNCaP cells using Lipofectamine. Stably-transfected clones were selected by continuous culture in G418 (400 µg/ml).

Cell viability assay

LNCaP cells were seeded in 96-well plates (10,000 cells/well) and cultured in CM, CFM or CFM+3-MA for 4 days. The cell viability was then examined by 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay following the manufacturer's protocol (Vybrant MTT assay, Invitrogen).

Si RNA knockdown of ATG5

Si-Control and si-ATG5 duplexes (27-mers; #SR306286), containing 3 target sequences, were purchased from Origene (Rockville, MD, USA). LNCaP cells were seeded in 6-well plates (200,000 cells/well) in complete medium (CM) and the transfection was performed the next day by adding siRNA to the respective wells to a final dose of 5 or 20 nM following the manufacturer's protocol. The efficiency of target silencing was assessed by immunoblotting analysis with anti-Atg5 antibody (Cell Signaling, #2630). In addition, si-ATG5 treated cells were assayed for the content of LDs by Oil Red O staining (see below).

Immunoblotting

Cells were lysed and sonicated in RIPA buffer containing protease inhibitors. Twenty microgram of proteins were separated in each lane using either 12 or 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% milk in 1X TBST, incubated with primary antibodies, anti-LC3 (MBL, PM036), anti-GFP (Santa Cruz, sc-8334), or anti- β -actin (Sigma, A1978), and then probed with corresponding goat anti-rabbit or anti-mouse HP-labeled secondary antibodies (Bio-Rad, Hercules, CA, USA).

LC3-II Translocation assay

LNCaP cells stably transfected with pEGFP-LC3 (LNCaP.EGFP-LC3) were grown on cover slips, washed in 1X PBS and then incubated in CFM in the presence or absence of R1881 or 3-MA for various time points, and then observed with a Zeiss immunofluorescence microscope. In addition, LAPC4 cells were fixed in 4% paraformaldehyde after CDX treatment, probed with anti-LC3 antibody and then Texas Red labeled goat anti-rabbit secondary antibody, and observed with a confocal microscope (Zeiss LSM 510). Distinct puncta and cells were counted from ten random visual fields for each slide. The number of puncta per cell was used as a measure of autophagic activity.

Oil Red O staining

Cells were grown on coverslips in indicated medium, fixed in 10% paraformaldehyde, stained with Oil Red O for 15 minutes, and then rinsed in water. Images were acquired using a brightfield microscope (Zeiss Axioscope). The numbers of LDs per cell were measured.

Triacylglycerol lipase assay

The amount of intracellular triacylglycerols were determined using a triacylglycerol assay kit (BioVision, Mountain view, CA, USA). In brief, cells were harvested, centrifuged and resuspended in 10 ml corresponding medium. Equal amounts of cells were aliquoted for either triacylglycerol or DNA/protein quantification. The amount of total triacylglycerols was reported per microgram of DNA or protein from an equal number of cells.

Nuclear Magnetic Resonance Spectroscopy

Three million cells were washed 3 times in PBS prepared with D₂O instead of H₂O to replace most of the solvent hydrogen with deuterium. The cell pellets after the final wash were resuspended in 0.5 ml of DPBS, to which 1 mM DSS was added, and placed into 5 mm NMR tubes (Wilmad Glass, NJ). Triacylglycerols were extracted from cell pellets by the method of Folch *et al.* [32], dried under a stream of nitrogen, dissolved in 0.5 ml CDCl₃ containing 0.1 % TMS and placed into 5 mm NMR tubes. Proton NMR spectra were taken at 37 °C (maintained with a VT1000 temperature controller) with the aid of a Bruker Avance 500 MHz spectrometer running TopSpin. A 6000 Hz sweep following a 90° pulse was collected into 16K data points in 1.32 sec with a relaxation delay of 10 sec to ensure that the spectra resulting from 64 scans were fully-relaxed. The lipid methylene (-CH₂-)_n signal at δ = 1.29 ppm was integrated with respect to the DSS signal at δ = 0.00 ppm in order to measure the intracellular triacylglycerols. The lipid methylene integral was corrected for the fact that it represented only 84 out of the 104 total protons from a triacylglycerol molecule containing three assumed oleic acid (n = 17) side chains as previously described [5].

Co-localization assay

LNCaP.EGFP-LC3 cells were fixed in 4% paraformaldehyde and stained with HCSLipidTOX Red. Cells were imaged using a Zeiss LSM 510 confocal microscope with a 63X objective. Images were analyzed for colocalization of LipidTOX and GFP fluorescence.

Statistical analysis

Each experiment was performed in triplicate and the data are presented as the mean $\pm$ standard deviation (SD). Data were analyzed by Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

Results

Androgen ablation induces autophagy in androgen-sensitive PCa cells

To study the effects of androgen deprivation on autophagy and on lipid droplet degradation in androgen responsive PCa cells, we generated 6 independent clones of LNCaP cells, which we denoted LNCaP.EGFP-LC3, stably-transfected with pEGFP.LC3. When these clones were grown in complete medium (CM), the intensities of the EGFP fluorescence in the cytosol were comparable among the independent LNCaP.EGFP-LC3 clones (Fig. 1A, panel a). Incubation of LNCaP.EGFP.LC3 clones in charcoal-filtered FBS medium (CFM) induced translocation of EGFP.LC3 from the cytosol to punctate autophagic vesicles (AVs) (Fig. 1A, panel b) and increased the number of AVs observed per cell by seven-fold (Fig. 1B, column b). This effect was due to the lack of androgens in CFM, because it could be reversed by the addition of the androgen analogue, R1881 to the CFM (Fig. 1A panel c, and Fig. 1B, column c) where the number of AVs returned to that observed (~ 0.6 per cell) for cells cultured in CM. Inhibition of autophagy by 3-MA further reduced the formation of AVs (to ~ 0.2 per cell) indicating that these are *bona fide* autophagosomes that are induced in LNCaP cells during androgen deprivation (Fig. 1A, panel d, and Fig. 1B, column d). In addition, using MTT assay, we showed that blocking of autophagy by 3-MA induced cell death in LNCaP cells cultured in CFM (Fig. 1C), suggesting that autophagy induced by androgen deprivation is pro-survival. This observation is consistent with results previously reported [22, 23]. To confirm that androgen deprivation indeed induced autophagy in LNCaP cells, we treated cells cultured in CFM with Baf A1, an inhibitor of autolysosome formation in the final stage of autophagy [20, 21]. There was a greater increase of LC3II in cells of CFM+Baf A1 (Fig. 1D, lane 5) than for cells in CM, CFM, or CM+Baf A1 (Fig. 1D, lanes 1, 2 and 4, respectively), indicating that androgen deprivation induced autophagy. Interestingly, CDX, an antiandrogen and AR inhibitor, had no effect on autophagy in LNCaP cells cultured in CM (lane 3). The basis for this observation is likely due to the mutant AR, which is insensitive to CDX, in LNCaP cells [35, 36]. Consistently, when LNCaP cells were co-treated with CDX and Baf A1 in CFM, we observed a moderate increase of LC3II, possibly due to the blocking of endogenous autophagy by Baf A1 in LNCaP cells (Fig. 1, lane 6). Moreover, using immunoblot analysis, we found that androgen deprivation resulting from culture of LNCaP.EGFP-LC3 cells in CFM caused an increase of EGFP-LC3II (Fig. 1E), indicating that autophagy was induced when hormones were removed.

To confirm that the induction of autophagy during androgen deprivation was not specific to LNCaP cells, we examined another androgen-sensitive PCa cell line, LAPC4. Immunofluorescence microscopy analysis using an antibody against LC3 showed increased AV formation when LAPC4 cells were treated with CDX for 24 hrs in CM (Fig. 2A, panel b), compared to cells in CM alone (Fig. 2A, panel a) or in CFM alone (data not shown). In addition, LAPC4 cells cultured in CM in the presence of CDX and/or Baf A1 for 24 hrs showed elevated levels of endogenous, lipidated LC3II and an increase in the ratio between LC3II and LC3I (Fig. 2B). Moreover, cells cultured in either CFM or CM+CDX for 24 hrs showed a loss of cytosolic p62 (Fig. 2C). P62 (SQSTM1/sequestome 1) is an adaptor protein whose binding to aggregated proteins and organelles tags them for autophagic degradation. Through binding with the lipidated LC3II molecules that are localized in the luminal side of the inner autophagosomal membrane, p62 sequestomes are selectively and specifically

incorporated into autophagosomes and subsequently degraded by autophagy. Thus, the total level of p62 inversely correlates with LC3II-dependent autophagic activity, a marker for autophagic efficiency [33, 34]. These data confirm that androgen attenuation induces autophagy and therefore p62 degradation.

Androgen deprivation reduces the number of lipid droplets in androgen-sensitive PCa cells

To investigate the effect of androgen deprivation on lipid droplet (LD) homeostasis in androgen-sensitive prostate cancer cells, we first showed that LDs indeed accumulated in LNCaP cells grown in CM (Fig. 3A, panel a), as previously reported [4, 8]. The cellular triacylglycerol content was found to be ~14.5 pM/mg protein (Fig. 3C, column a). In contrast to this, cells grown in androgen-depleted CFM showed at-least a four-fold reduction in the number of LDs (Fig. 3A, panel b) and in the amount of triacylglycerols (Fig. 3C, column b) by day 5, an effect that was reversed by addition of the androgen analogue, R1881 (Fig 3A, panel c; and Fig. 3C, column c), or the autophagy inhibitor 3-MA (Fig 3A, panel d; and Fig. 3C, column d). Our observation of a decrease in lipid droplet formation when androgens are withdrawn is consistent with data reported by Swinnen et al.[4]. To confirm the effect of androgens on the LD content, we employed androgen receptor inhibitor, CDX, to block the androgen/AR axis. The LAPC4 cell line was used for this experiment because it possesses a wild-type AR. In contrast, it is well documented that the AR gene in LNCaP is mutated [35, 36]. LAPC4 cells were cultured in the absence (Fig. 3B, panel a) and presence (Fig. 3B, panel b) of CDX for 5 days and LD formation was measured. Oil Red O staining showed a marked reduction in the number of LDs in the treated cells (Fig. 3B, panels b and c; Fig. 3D columns #2 and #3) compared to the control cells grown in CM (Fig. 3B, panel a; and Fig. 3D, column #1). In addition, biochemical assays for triacylglycerols also showed a greater than 70% reduction in intracellular triacylglycerols in the treated group (Fig. 3D, 2nd and 3rd columns). Taken together, these results show that androgen deprivation depletes LDs in androgen-sensitive PCa cells, and autophagy inhibitors reverse the phenotype.

siRNA knockdown of ATG5 reverses LD degradation in androgen-deprived LNCaP cells

To determine if autophagy is the causal factor of LD degradation in LNCaP cells grown in androgen deprivation medium, we treated cells cultured in CFM with si-ATG5. As shown in Figure 4A, immunoblot analysis demonstrated that 20 nM siATG5 greatly reduced the level of Atg5 expression in cells, as compared with lipofectamine only or si-Control. Importantly, Oil Red O staining showed that siATG5 significantly blocked the LD degradation in cells cultured in CFM+si-ATG5 (Figure 4B), demonstrating that Atg5-dependent autophagy is essential for androgen deprivation-induced LD degradation.

To confirm that the lipolysis seen in LNCaP cells is not due to artifacts from the cell manipulation or disruption associated with either the optical or the biochemical assays, a quantitative NMR method was used to assess the effect of androgen and 3-MA on triacylglycerol (TG) catabolism in intact LNCaP cells. Total cellular TGs in treated cells, such as those in Figure 3A and C, were quantified using proton nuclear magnetic resonance (¹H-NMR). Integrals of the methylene (-CH₂-)_n signals at 1.29 ppm (as shown in Fig. 5, panel a), were used to calculate the total amount of TGs in each sample, standardized to the amount of protein in an equal volume of sample [5]. Consistent with the optical (microscopy) and biochemical (TG enzymatic) assays, ¹H-NMR also showed the reduction of intracellular TGs in cells deprived of androgen (Fig. 5, panel b; and Fig. 5E, column b). This effect can be reversed by treatment of the cells with the androgen analogue R1881 (Fig. 5, panel c; and Fig. 5E, column c) or the autophagy inhibitor 3-MA (Fig. 5, panel d; and Fig. 5E, column d).

Autophagosomes colocalize with LDs in LNCaP cells during androgen deprivation

To further investigate the role of autophagy in lipid metabolism, LNCaP.EGFP-LC3 cells were incubated in indicated medium, fixed, stained with LipidTOX, and observed with a confocal microscope. As shown in Figure 6, EGFP-LC3 (green) was mainly cytosolic and there were abundant LDs (red) in cells grown in CM (panels a and b). However, there was almost no colocalization of EGFP.LC3 and LDs in these cells (panel c; panel n, column 1). However, when LNCaP cells were cultured in CFM, which lacked androgen, the EGFP.LC3 molecules translocated to green punctate structures, presumed AVs (panel d; panel m, column 2), which partially colocalized with the red LDs (panels f; panel n, column 2). The effect of androgen deprivation could be reversed by adding back the androgen analogue R1881 (panels g to i; panel m, column 3; panel n, column 3). In addition, blocking the autophagic flux by means of Baf A₁ resulted in a greater accumulation of AVs (panel j; panel m, column 4) and LDs (panel k) and the number of colocalized AVs and LDs (panel l; panel n, column 4), indicating that Baf A₁ restricted intracellular LD degradation in LNCaP cells. Panel o showed a representative image of a cell filled with AVs partially colocalized with LDs, and a magnified image of an LD enwrapped by AVs. Together, these results show that autophagosomes sequester and target LDs for lysosomal hydrolysis during androgen ablation in androgen-sensitive PCa cells. Baf A₁, an inhibitor of autophagic flux [37], increased the number of colocalized AV/LD dots, indicating that Baf A₁ restricted intracellular LDs degradation in LNCaP cells.

Discussion

Anti-hormone therapy is a standard treatment for initially androgen-sensitive prostate cancers. Unfortunately, these tumors generally progress in time to an androgen independent phenotype by activating various cellular pathways for survival in an androgen-depleted environment [16, 38] and then this treatment is no longer useful. Because metabolic deregulation is commonly observed during tumorigenesis, we sought to determine if alternate metabolic pathways were activated during the adaptation of prostate cancer cells to androgen deprivation. We confirmed that either culturing cells in an androgen deprived medium, or inhibiting the wild-type AR, induced autophagy and cell death in androgen-sensitive PCa cell lines. Moreover, we found, using histochemical, biophysical and biochemical techniques, that the inhibition of autophagy resulted in the retention of cytoplasmic lipid droplets in these cells. We further found that autophagosomes colocalized with LDs. Inhibiting the autophagic lysosomal degradation of the lipid droplets by using Bafilomycin A₁ or si-ATG5 also caused retention of intracellular triacylglycerols. These findings show that, in the absence of androgens, autophagy was activated in androgen-sensitive PCa cells with the consequence that LDs were sequestered and targeted to the lysosomes for hydrolysis. Activation of this alternate pathway of lipolysis during androgen ablation could be a critical event supporting the survival and progression of prostate tumors into an androgen-independent phenotype.

Even though autophagy has been found to be activated during a variety of treatments for different cancer types [39–42], its role in the survival or death of prostate cancer cells has not been extensively examined. What is known is that radiation therapy of PCa cells induced cytoprotective autophagy [40] and that incubation of LNCaP cells, but not PC3 cells (an androgen-insensitive PCa line) in serum free medium induced autophagy [22, 23]. Li and colleagues also reported that the inhibition of autophagy led to increased apoptosis of LNCaP cells in serum free medium compared to medium containing either dihydrotestosterone or serum, suggesting that autophagy protects LNCaP cells during androgen deprivation [22, 23]. Studies using other tumor cell types have shown that growth factors in serum are potential regulators of autophagy through activation of the mTOR pathway, which is an inhibitor of autophagy [43]. We induced autophagy in LNCaP and

LAPC4 cells through androgen deprivation by using charcoal-filtered serum medium, and in LAPC4 cells by growing them in the presence of the antiandrogen, casodex (CDX) (Figure 1). Interestingly, however, CDX did not affect autophagy in LNCaP cells, in which the AR gene is mutated. It has been reported that CDX stimulated the mutant AR [35, 36], which may be the reason that LNCaP cells were insensitive to CDX treatment.

Prostate cancer cells have a unique intermediary metabolism that markedly differs from that found in other cancer cell types [9, 11]. Otto Warburg found that most cancer cells rely primarily on aerobic glycolysis to generate ATP to meet their energy demand [13, 44]. However, glucose does not play a major metabolic role in androgen-dependent PCa cells because LNCaP cells can grow with a medium glucose concentration as low as 0.28 mM [9, 45]. Importantly, androgens stimulate both the lipogenic and fatty acid β -oxidation pathways in androgen-dependent PCa cells to fulfill their increased bioenergetic needs [5–11]. Recently, Singh and colleagues reported that autophagosomes sequestered LDs and mediated lysosomal lipolysis in rat hepatocytes [26]. This increased delivery of LDs to the lysosome by autophagosomes was observed under conditions of starvation or lipid overload. We found that this novel function of autophagy also serves as an alternate pathway for lipolysis in androgen-sensitive PCa cells during androgen deprivation: Incubation of LNCaP cells in CFM significantly reduced the amount of LDs by day 6 (Figures 3A, 3C, 4 and 5e), which is consistent with results previously reported [4]. The same result was observed in LAPC4 cells when androgens were depleted using CFM or the AR was inhibited with CDX (Figure 3B and 3D). In addition, 3-MA, a phosphatidylinositol-3-kinase inhibitor used extensively to block autophagosome formation at the initiation phase, blocked autophagosome formation and inhibited autophagy and therefore LD degradation in LNCaP cells (Figures 1, 3 and 4). All three independent methods to measure LDs or their major constituent triacylglycerols showed that inhibition of autophagy caused retention of LDs indicating that autophagosomes play a role in the metabolism of LDs (Figures 3 and 4). We further showed that autophagosomes colocalize with LDs by using fluorescent microscopy. On average, 1.5 LDs were observed to colocalize with autophagosomes per cell. Treatment of LNCaP cells with Bafilomycin A₁ (Baf A₁) has been reported to inhibit lysosomal acidification/hydrolysis, to block autophagic degradation, and to cause the accumulation of autophagosomes [37]. In our study, the treatment of LNCaP cells with Baf A₁ indeed led to the accumulation of autophagic vesicles and increased the amount of colocalization of them with LDs (Fig. 5, panels j, l and o; column 4 of panels m and n). We also measured intracellular triacylglycerols after the inhibition of autophagic degradation in both LNCaP and LAPC4 cells. For example, LAPC4 cells treated with Baf A₁ for 24 hours under conditions of androgen deprivation led to an increase in the amount of TGs compared to cells grown only under androgen deprivation (Figure 3D, column 3). These data indicated that autophagosomes sequestered and targeted LDs to the lysosome in androgen-sensitive PCa cells when androgens were absent. Lysosomal lipases are known to hydrolyze triacylglycerols into glycerols and fatty acids [27, 28], and these fatty acids can either be translocated to peroxisomes and mitochondria for β -oxidation for energy production or used for new membrane synthesis for proliferation. The existence of an alternate pathway of lipolysis in PCa cells may help to explain how androgen responsive PCa cells adapt to survive in the absence of the key metabolic regulator, androgens.

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Figure 1. Androgen deprivation induces autophagy in LNCaP cells, which can be reversed by autophagy inhibitors, 3-MA and Bafilomycin A1 (Baf A1)

A. LNCaP.EGFP-LC3 cells (LNCaP cells stably transfected with pEGFP.LC3) were incubated for 2 days in indicated medium, (a) CM, complete medium; (b) CFM, charcoal-filtered fetal bovine serum medium; (c) CFM+R1881, CFM plus R1881; and (d) CFM+3MA, CFM plus 3-methyladenine (3-MA). Cells were mounted in coverslips and observed with a Zeiss LSM 510 confocal microscope. **B.** Numbers of cells and puncta were counted from 10 random visual fields for each group. Average numbers of puncta per cell were plotted in the graph. **C.** Blocking autophagy by 3-MA, an inhibitor of the initial stage of autophagy, induced cell death in LNCaP cells cultured in CFM by day 4, as analyzed by MTT assay. **D.** Induction of autophagy was confirmed in LNCaP cells treated with CFM. Immunoblot analysis showed that treatment of Baf A1, a blocker of the autolysosome formation in the final stage of autophagy, in cells cultured in CFM resulted in greater increase of LC3II (lane 5) compared to cells cultured in CM (lane 4), indicating that androgen deprivation induced autophagy. CDX, an AR inhibitor, showed no effect on LC3 II level and thus autophagy in LNCaP cells cultured in CM (lane 3), whereas combinational treatment of CDX and Baf A1 showed moderate increase of LC3-II, suggesting that the endogenous autophagy is inhibited by Baf A1. **E.** Time- dependent activation of autophagy in LNCaP.EGFP-LC3 cells cultured in medium CFM. Immunoblot analysis showed that the level of EGFP-LC3II (45 kDa) was increased in a time-dependent manner in cells grown in CFM.

Figure 2. Androgen antagonist CDX induces autophagy in LAPC4 cells

A. LAPC4 cells were incubated for 24 hrs in (a) CM or (b) CM plus (+) 5 μ M casodex (CDX). Cells were mounted in coverslips, probed with anti-LC3 antibody and then Texas Red labeled goat anti-rabbit secondary antibody, and observed under a Zeiss immunofluorescence microscope. There was a marked increase in LC3II-containing punctate structures, presumed to be autophagic vesicles (AVs; Fig. 2A, panel b). **B.** LAPC4 cells were incubated for 24 hrs in (lane 1) CM; (lane 2) CM+5 μ M CDX; (lane 3) CM+5 μ M bafilomycin A1 (Baf A1), an inhibitor of autophagic flux; or (lane 4) CM+CDX+Baf A1. Immunoblot analysis showed that levels of LC3II were increased in cells treated with CDX or Baf A1 (lanes 2 and 3), and an additive effect on LC3II activation when cells were treated with CDX and Baf A1 (lane 4). The ratios of LC3II/LC3I are 0.1, 0.25, 0.4 and 0.6, corresponding to lane 1, 2, 3, 4, respectively. **C.** Immunoblot analysis showed that p62, a marker of effective autophagy, was markedly downregulated in LAPC4 cells treated with androgen deprivation (CFM; lane 2) or androgen antagonist CDX (lane 3), suggesting that androgen attenuation induces autophagy and therefore p62 degradation.

Figure 3. The crosstalk between androgen, autophagy and lipid droplets in androgen sensitive PCa cells

A. LNCaP cells were incubated for 2 days in indicated medium, (a) CM, complete medium; (b) CFM (c) CFM+R1881, CFM plus R1881; and (d) CFM+3MA. Cells were stained with Oil Red O, mounted in coverslips, and observed with a Zeiss Axioscope brightfield microscope. Androgen deprivation in CFM (b) caused depletion of lipid droplets in LNCaP cells, which could be reversed by adding back androgen (R1881; c) or blocking autophagy with 3-MA (d). **B.** LAPC4 cells were incubated for 5 days in indicated medium, (a) CM, (b) CM+CDX, and (c) CM+CDX+3-MA. Cells were stained with Oil Red O, mounted in coverslips, and observed with a Zeiss Axioscope brightfield microscope. Inhibition of the AR by CDX induced depletion of lipid droplets (panel b), whereas 3-MA, an inhibitor of PI3K and therefore autophagy, reversed the CDX effect on lipid droplets (panel c). **C.** the quantitative assay of intracellular triacylglycerols in LNCaP cells described in **A.** **D.** the quantitative assay of intracellular triacylglycerols in LAPC4 cells cultured in CM, CM+CDX and CM+CDX+Baf A1.

Figure 4. Attenuation of autophagy by Si-ATG5 retains LDs in androgen-deprived LNCaP cells
A. Si-ATG5 knockdown of Atg5 was confirmed by immunoblot analysis. Twenty μ g of total soluble protein extracts were used from cells cultured in CM in the presence of lipofectamine (lipofect.), si-Control, and 5 and 20 nM si-ATG5. **B.** Treatment of Si-ATG5 (20 nM) blunted CFM-induced LD degradation. Oil Red O staining indicated that siRNA knockdown of ATG5 restored LD content in cells cultured in CFM (panel 4). All the stained images were taken with the same microscope (Zeiss A1) with a CCD camera (Optronics), and the same parameters.

Figure 5. Quantitative NMR assay of the effect of androgen and 3-MA on triacylglycerols in LNCaP cells

Total cellular triglycerides in treated cells were quantified using proton nuclear magnetic resonance (¹H-NMR). All cells were cultured in (a) CM; (b) CFM; (c) CFM+R1881, CFM for 3 days and then treated with R1881 for another 3 days; and (d) CFM+3-MA, CFM for 3 days and then treated 3-MA for another 3 days. On day 6, cells were washed, trypsinized & harvested. Integrals of the methylene ($-\text{CH}_2-$)_n signals at 1.29 ppm are typical examples of which were used to calculate the total amount of triglycerides in each sample and standardized to the amount of protein in an equal volume of sample used for NMR spectroscopy. The bar graph (e) shows the averages for $n = 3$ measurements for each treatment.

Figure 6. Autophagosomes colocalize with LDs in LNCaP cells during androgen deprivation

LNCaP.EGFP-LC3 cells were incubated for 3 days in indicated medium, (a) CM, (d) CFM; (g) CFM+R1881, and (j) CFM+Baf A1, CFM plus BAF A1 for 12 hours. Cells were fixed, stained with HCSLipidTOX Red, and observed with a Zeiss LSM 510 confocal microscope. Panels of EGFP.LC3 (green), of LipidTOX (red) and merged are as indicated.

Colocalization of autophagic vesicles (AVs; green) and lipid droplets (red) were yellow dots shown in the merged panels. In CM, EGFP-LC3 was mainly cytosolic and there were abundant LDs in cells (panels a and b). There was almost no colocalization of EGFP.LC3 and LDs in these cells (panel c; column 1 in panel n). However, when cells were cultured in CFM, which lacked androgen, the EGFP.LC3 molecules translocated to green punctate structures, presumed AVs (panel d; column 2 in panel m), which partially colocalized with the red LDs (panels f; column 2 in panel n). The effect of androgen deprivation could be reversed by adding back R1881 (panels g to i; column 3 in panels m and n). In addition, blocking the autophagic flux by Baf A₁ resulted in a greater accumulation of AVs (panel j; column 4 in panel m) and LDs (panel k) and the number of colocalized AVs and LDs (panel l; column 4 in panel n), indicating that Baf A₁ restricted intracellular LD degradation in LNCaP cells. Panel o showed a representative image of a cell filled with AVs partially colocalized with LDs, and a magnified image of a LD enwrapped by AVs (inset).