University of New Mexico
UNM Digital Repository

Biomedical Sciences ETDs

Electronic Theses and Dissertations

5-1-2012

Functions of autophagy in lipid homeostasis and survival in androgen-dependent prostate cancer cells

Ramesh Kaini

Follow this and additional works at: https://digitalrepository.unm.edu/biom_etds

Part of the Medicine and Health Sciences Commons

Recommended Citation

Kaini, Ramesh. "Functions of autophagy in lipid homeostasis and survival in androgen-dependent prostate cancer cells." (2012). https://digitalrepository.unm.edu/biom_etds/52

This Dissertation is brought to you for free and open access by the Electronic Theses and Dissertations at UNM Digital Repository. It has been accepted for inclusion in Biomedical Sciences ETDs by an authorized administrator of UNM Digital Repository. For more information, please contact disc@unm.edu.

www.manaraa.com

Ramesh Raj Kaini

Candidate

Biomedical Science Graduate Program

Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Dr. Chien-an Andy Hu, Chairperson

Dr. Laurel Sillerud

Dr. Marco Bisoffi

Dr. Vojo Deretic

المنارة للاستشارات

www.manaraa.com

FUNCTIONS OF AUTOPHAGY IN LIPID HOMEOSTASIS AND SURVIVAL IN ANDROGEN-DEPENDENT PROSTATE CANCER CELLS

by

RAMESH RAJ KAINI

M.B.B.S., Institute of Medicine, Tribhuvan University,
Nepal

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Biomedical Sciences

The University of New Mexico
Albuquerque, New Mexico

May, 2012

www.manaraa.com

Acknowledgements

I owe my deepest gratitude to,

My mentor Chien-an Andy Hu, for his constant guidance and support, and for all of his efforts, hard work, persistence and dedication in science.

My co-mentor Laurel Sillerud for his guidance, encouragement, and for his insights on the practice of science.

My dissertation committee: Marco Bisoffi and Vojo Deretic for all of their insightful suggestions.

Department of Defense Prostate Cancer Research Program for funding on my dissertation study.

And, my beloved Indira, for all her confidence & support in my endeavor in the journey of science.

المنارة للاستشارات

www.manaraa.com

FUNCTIONS OF AUTOPHAGY IN LIPID HOMEOSTASIS AND SURVIVAL IN ANDROGEN-DEPENDENT PROSTATE CANCER CELLS

BY

RAMESH RAJ KAINI

M.B.B.S, Institute of Medicine, Tribhuvan University, Nepal

Ph.D., Biomedical Science Graduate Program, University of New Mexico, NM, USA

Abstract

Androgen deprivation therapy, one of the standard treatments for prostate cancer, induces apoptosis as well as autophagy in androgen-responsive PCa cells. As modulation of autophagy is a new paradigm for enhancing the therapeutic efficacy of various cancer therapies, we sought to determine the functions of autophagy during androgen deprivation. In this study, we confirmed that androgen removal or inhibition induces autophagy in two different hormone sensitive prostate cancer cells. Androgen deprivation also caused depletion of lipid droplets which was abrogated on inhibition of autophagy by pharmacological means (3-methyladenine, bafilomycin A1) or using a genetic approach (Atg5 siRNA). In addition, colocalization of lipid droplets and autophagic vesicles was

www.manaraa.com

observed in LNCaP cells, which was further enhanced by blocking the autophagic flux. These findings suggest that autophagy mediates lipid droplet degradation and lipolysis in androgen sensitive prostate cancer cells. Furthermore, inhibition of autophagy by chloroquine during androgen deprivation synergistically killed LNCaP cells in a dose and time dependent manner. We further confirmed that chloroquine caused accumulation of autophagosomes and decreased cytosolic ATP levels. Moreover, chloroquine induced apoptosis in androgen deprived LNCaP cells. These findings suggest that chloroquine may be used as an adjuvant in hormone therapy to improve therapeutic efficacy.

المنارة للاستشارات
www.manaraa.com

Table of contents

List of figures……………………………………………………………………….IX

1. Introduction…..…………………………………………………………………...1

1.1. Hormone ablation therapy is a standard treatment in prostate cancer………………………………………………………………………………… 2

1.2. Androgen deprivation induces autophagy in LNCaP cells…………………….5

1.3. Autophagy plays an important role in cellular bioenergetic balance…………7

1.4. Prostate cancer cells have a unique intermediary metabolism that is regulated by androgens……………………………………………………………13

1.5. Autophagy can be modulated at various stages………………………………19

1.6.Chloroquine is a potent adjunct in various cancer therapies…………………22

1.7.Hypothesis…………………………………………………………………...….24

Chapter 2. Autophagy regulates lipolysis and cell survival through lipid droplet degradation in androgen sensitive prostate cancer cells…………..25

2.1.Abstract……………………………………………….…………….………...26

2.2.Introduction……………………………………………………………………..28

2.3. Materials and methods…………………………………………......…………31

2.4.Results…………………………………………………………………………..36

2.4.1. Androgen ablation induces autophagy in androgen-sensitive PCa cells…..…..36

2.4.2. Androgen deprivation reduces the number of lipid droplets in androgen-

المنارة للاستشارات

www.manaraa.com

sensitive PCa cells……………………………………………………………38

2.4.3. siRNA knockdown of ATG5 reverses LDs degradation in androgen-deprived LNCaP cells………………………………………………………...39

2.4.4. Autophagosomes colocalize with LDs in LNCaP cells during androgen deprivation…………………………………………………………………..40

2.5.Discussion……………………………………………………………………42

2.6.Acknowledgement……………………………………………………………...46

2.7. Figures and figure legends…………………………………………………..47

Chapter 3. Synergistic killing effect of chloroquine and androgen deprivation in LNCaP cells……………………………..………………………..59

3.1.Abstract……………………………………………………………………….60

3.2. Introduction………………………………………………………………….61

3.3. Materials and methods……………………………………………………..64

3.4. Results……………………………………………………………………….67

3.4.1. Chloroquine synergistically kills LNCaP cells during androgen deprivation…………………………………………………………………..67

3.4.2. Chloroquine blocks autophagic degradation and reduces cytosolic ATP in LNCaP cells…………………………………………………………..67

3.4.3. Chloroquine induces apoptosis in LNCaP cells……….……………….68

3.5.Discussion…………………………………………………………………...70

3.6.Acknowledgement…………………………………………….……………74

3.7. Figures and figure legends………………………………..………………...75

المنارة للاستشارات

www.manaraa.com

Chapter 4. Discussion and Future Perspectives……………………………….80

4.1. Autophagy in hormone sensitive PCa cells during androgen deprivation….81

4.2. Autophagy regulated lipolysis in PCa cells……………………………………83

4.3. Effect of chloroquine, an autophagy inhibitor, in survival and bioenergetic

status of LNCaP cells………………………………………………………….85

4.4. Limitations and future implications…………………………………………...88

4.4.1. Autophagy during androgen deprivation………………………………...88

4.4.2. Function of autophagy in lipid metabolism in hormone sensitive PCa

cells……………………………………………………………………………...90

4.4.3. Chloroquine as an adjuvant in hormone therapy………………………91

4.5. Summary………………………………………………………………………92

List of Abbreviations…………………………………………………………...94

References…………………………………………………………………………96

المنارة للاستشارات

www.manaraa.com

List of Figures

Figure 1.1. Progression of PCa and available treatment options……………..4

Figure 1.2. Effects of autophagy in cellular metabolism……………………...12

Figure 1.3. Lipid droplets and regulation of lipolysis…………………………13

Figure 1.4. Metabolism in normal prostate cells……………………………...16

Figure 1.5. Metabolism in prostate cancer……………………………………18

Figure 1.6. Autophagy can be regulated at different stages…………………21

Figure 2.1. Androgen deprivation induces autophagy in LNCaP cells…….47

Figure 2.2. Androgen antagonist CDX induces autophagy flux in LAPC4 cells……………………………………………………………………………..49

Figure 2.3. The crosstalk between androgen, autophagy, and lipid droplets in androgen sensitive PCa cells………………………………………...51

Figure 2.4. Attenuation of autophagy by Si-ATG5 retains LDs in androgen-deprived LNCaP cells………………………………………………………..53

Figure 2.5. Quantitative NMR assay of the effect of androgen and 3-MA on triglycerols in LNCaP cells…………...………………………………….55

Figure 2.6. Autophagosomes colocalize with LDs in LNCaP cells during androgen deprivation………………………………………………………...57

Figure 3.1. Survival effect of chloroquine on LNCaP cells…………………...75

Figure 3.2. Time dependent effect of chloroquine on the proliferation of LNCaP cells…………………………………………………………………….76

Figure 3.3. Effect of chloroquine on autophagic vesicles degradation…….77

Figure 3.4. Chloroquine treatment induces apoptosis on LNCaP cells……78

المنارة للاستشارات

www.manaraa.com

.

Chapter 1

Introduction

المنارة للاستشارات
www.manaraa.com

1.1. Hormone ablation therapy is a standard treatment in prostate cancer.

The prostate is a male specific exocrine gland located around the neck of urinary bladder that encircles the urethra (1). It secretes prostatic fluid that helps in the survival and function of sperm (1). Histologically, the prostate is made of compound tubular structures that are lined by columnar epithelium cells. Cancer of the prostate gland is mainly caused by neoplastic transformation of these epithelial cells (1, 2). The incidence of prostate cancer (PCa) increases with advancing age (3, 4). Autopsy studies showed that approximately 65% of men over 70 years of age have some microscopic evidence of cancer in their prostate gland. However, progression of PCa to the aggressive phenotype is not common, so active surveillance is the mainstay of treatment in many cases (5). The American Cancer Society estimates that that there will be 240,890 newly diagnosed cases of PCa in 2011, which makes it the most common cancer among men in the US (6). Besides watchful waiting, the National Cancer Institute (NCI) recommends three treatment regimens as standard therapies in early stage PCa: surgical resection, radiation therapy and hormone ablation therapy (7). Despite advances in the treatment of PCa, about 33,720 American men will die of it in 2011, making it the second leading cause of cancer death in the US (6).

Androgen is a generic term for a group of natural or synthetic compounds that stimulate male characteristics by binding the androgen receptor (AR). Testosterone is mostly secreted by the testes and is the main androgen in humans. Androgens are synthesized from cholesterol, so they are lipophilic which allows

المنارة للاستشارات
www.manaraa.com

them to easily pass through the cell membrane (8-11). In the cytosol, most testosterone is converted into a more potent form, dihydrotesterone, by an enzyme called 5-α reductase (9). Both testosterone and dihydrotestosterone can bind to the androgen receptor (AR), which is a member of the steroid-thyroid-retinoid nuclear receptor super family. Ligand binding causes AR dimerization and translocation to the nucleus where it acts as a transcription factor. There are many genes which are known to have upstream androgen response elements (ARE). Binding of the androgen-AR complex along with other co-factors leads to transcriptional regulation of hundreds of genes that affect survival, and proliferation of prostate epithelial cells (10-14).

During PCa progression (Figure 1.1), hormonal effects of androgen stimulation are intact during the initial phase of PCa, also called the androgen-dependent phase (15). Charles Brenton Huggins received a Nobel Prize in medicine in 1966 for his discoveries of hormonal treatment of prostate cancer (16). He showed that orchiectomy (surgical removal of the testes), induces regression of PCa. This discovery set the foundation for hormone ablation therapy in PCa treatment. Different drugs are available nowadays that interfere at different steps of androgen synthesis and/or function. Hormone withdrawal or inhibition of AR induces apoptosis in both normal prostate epithelial cells and androgen-dependent prostate cancer cells. However, some of these cancer cells can survive and eventually develop into an androgen-independent phenotype (17, 18). Development of resistance to the hormone is the end stage for the prognosis of PCa for which there are very limited treatment options. During progression

المنارة للاستشارات
www.manaraa.com

alterations in a variety of cellular pathways take place that t help PCa cells survive and proliferate (19, 20). Understanding the cellular mechanisms that permit survival during androgen deprivation may help us to identify novel targets that can be used as an adjuvant in hormone ablation therapy for PCa.

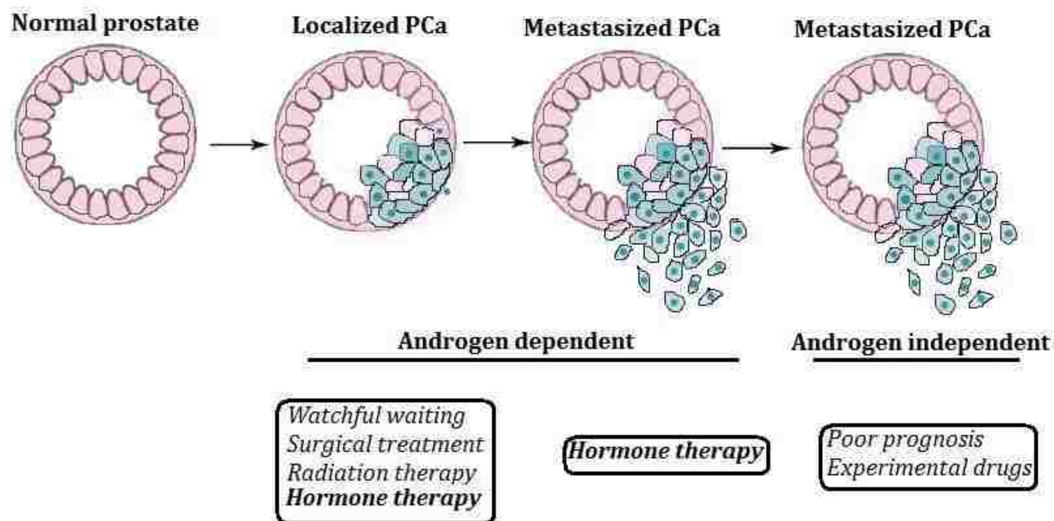


Figure 1.1. Progression of PCa and available treatment options. Most PCa cells originate from the columnar epithelial lining of the peripheral zone of the prostate gland. Cancer cells are initially confined within the basement membrane. During tumor progression, PCa cells acquire the capacity to invade and metastasize to other organs. Initially, they are androgen sensitive and respond well to hormone inhibition or withdrawal. However, some of the cancer cells acquire castration resistance to survive and proliferate in the absence of androgen. Treatment options are limited for castration resistant prostate cancer.

www.manaraa.com

1.2. Androgen deprivation induces autophagy in LNCaP cells.

Historically, autophagy is defined as a highly conserved catabolic process in eukaryotes in which aberrant organelles along with a portion of cytoplasmic constituents are sequestered into double or multilayered membrane vesicles, known as autophagic vesicles (AVs), and then targeted to the lysosomes for degradation (22-24). Autophagy is inducible by nutrient starvation and is pivotal for the maintenance of cellular energy homeostasis (22). In addition, autophagy normally functions in removing damaged organelles, especially mitochondria, and thus reduces the possibility of reactive oxygen species (ROS) leakage from damaged mitochondria into the cytosol (25, 26).

Autophagy has been extensively studied in the last decade. Its function in the pathogenesis of several diseases including its paradoxical function in cancer treatment is a hot topic in research (27-32). Many studies suggest that autophagy plays a prosurvival role in tumor biology. BECLIN1 is a tumor suppressor gene that inhibits tumorigenesis in prostate cancer, breast cancer and ovarian cancer (33). Allelic loss of BECLIN1 occurs at high frequency in prostate and breast cancer. Moreover, BECLIN1+/- mice are tumor prone, suggesting that autophagy is a tumor suppression mechanism. In addition, certain oncogenes, for example, AKT and c-myc, are found to inhibit autophagy (33-35). Likewise, tumor suppressor genes, for example, PTEN and p53, are able to induce autophagy by inhibiting mTOR activity (35-38). Some studies have shown that autophagy has a pro-

المنارة للاستشارات

www.manaraa.com

survival effect in cancer especially in cells at the inner core of the tumor mass which are deprived of nutrients from the circulation (39).

Interestingly, it has been observed that autophagy is induced during different cancer therapies, and may mediate cancer cell death. Many cancer treatments like tamoxifen, rapamycin, and arsenic compounds are found to trigger autophagy (40-43). In PCa, gamma radiation induces autophagy and confers anti-tumor activity by mediating autophagic cell death (44). We previously showed that Apolipoprotein L1, a novel BH3-only protein, induces autophagic cell death in several cancer cells (45, 46). However, the presence of autophagosomes in cancer cells during treatment is not always indicative of a pro-death mechanism. Treatment of two human PCa cell lines, PC-3 and LNCaP, with another promising anti-cancer drug, Sulforaphane, induces autophagy that protects cancer cells from death (47). The effects of autophagy on cell survival and death have intrigued scientists and provoked numerous investigations of whether modulation of autophagy can be a treatment option in cancer.

As stated earlier, hormone ablation therapy is a standard treatment in PCa. Androgens are a steroid hormones that stimulate growth of prostate epithelial cells. Growth factors are key regulators of autophagy in many cell types and do so by modulating the activity of the mTOR signaling pathway (23). It has been reported that in LNCaP cells, androgens stimulate the activity of mTOR, a known inhibitor of autophagy (48). In another study, incubation of LNCaP cells in serum free medium causes loss of phosphorylation of p70 ribosomal S6 kinase, a downstream effector of mTOR. This effect can be reversed by the addition of dihydrotestosterone (DHT)

المنارة للاستشارات
www.manaraa.com

(49). The study further showed that serum deprivation induces autophagy which is markedly suppressed by DHT. These results suggest a negative regulation of autophagy by androgens in LNCaP cells, and that autophagy is induced during androgen-deprivation therapy. However, androgens did not affect autophagy in the androgen-resistant prostate cancer PC3 cell line (49). These results are consistent with an *in vivo* report, where increased numbers of autophagosomes were observed in the prostate epithelial cells of castrated mice (50).

Inhibition of autophagy in LNCaP cells under serum-free conditions causes increased cell death, suggesting that autophagy protects LNCaP cells (49). However, the molecular mechanism underlying the prosurvival effect of autophagy varies in different cells. Elucidating the molecular events that are regulated by autophagy during androgen deprivation is critical to understanding if regulators of autophagy can be used as therapeutic targets in prostate cancer treatment.

1.3. Autophagy plays an important role in cellular bioenergetic balance.

One of the best defined prosurvival functions of autophagy is its impact on cellular bioenergetic balance. Autophagy has been shown to contribute significantly to the degradation of protein aggregates, aberrant organelles, lipid droplets and glycogens (Figure 1.2) (51-54). Thus released amino acids, fatty acids and glucose can serve as precursors for both biosynthesis as well as bioenergetic fuels. This function of autophagy is more pronounced during starvation when there is scarcity

المنارة للاستشارات
www.manaraa.com

of nutrients, but evidence suggests that cells can induce autophagy to maintain energy homeostasis under other conditions as well (55-57).

Autophagy is an evolutionarily conserved process of “self-eating” . The function of autophagy is to target the cargo to the lysosome, which is the cellular digestive organelle. There are three different types of autophagy; macroautophagy, microautophagy, and chaperone mediated autophagy (CMA) (58). Cargo transfer by the double or multilayered membrane vesicles into the lysosome is called macroautophagy, but it is generally denoted by the term “autophagy” (58). Microautophagy takes place in the lysosomal membrane where the membranes are pinched from inside (59). Chaperone-mediated autophagy plays a role in the translocation of cytosolic proteins to the inside of lysosomes (60).

Autophagy can sequester the cargo by both random and selective mechanisms (61-63). Selective autophagic sequestration is named based on the target organelle, for example mitophagy, lipophagy, ribophagy, aggregophagy etc. Different cargos are recognized and targeted to the limiting membranes of the newly formed autophagosomes by adaptor proteins including p62 and NBR1. (62, 63). The initiation of autophagic processes in each of these types of autophagy is a complex process and is regulated by multiple mechanisms. Here, autophagic regulation in response to cellular energy status is described.

Mammalian target of rapamycin complex 1 (mTORC1) is a critical nutrient sensor that regulates autophagy in response to cellular energy status. mTORC1 negatively regulates autophagy (64). The protein kinase activity of mTORC1 phosphorylates ULK1 which then forms a complex with Atg13 and FIP200 (65, 66).

المنارة للاستشارات
www.manaraa.com

An active Atg1-Atg13 complex is required for the initial shuttling of Atg9 to the site of autophagosome formation (66). mTORC1 also negatively regulates autophagy by activating another negative regulator of autophagy, called death associated protein 1 (DAP1) (67). Lower cellular energy is also sensed by another energy sensor, AMP-activated protein kinase (AMPK) (68-71). AMPK positively regulates autophagy by multiple mechanisms. It can inhibit mTORC1 by phosphorylating TSC2 complex and Raptor. It can also affect the induction of autophagy by regulating the activity of p53 (70-72).

Autophagy is a major catabolic process for long-lived proteins (73). In fact, cellular levels of long-lived proteins can be used as a measure of the autophagic flux. Protein aggregates are also sequestered by the autophagosomes and targeted to lysosomes (74). Lysosomal proteases break down the proteins into amino acids, which are then recycled back to the cytosol. Amino acids are used in cells for both protein synthesis and fuel supply depending on the cellular metabolic status. Interestingly, deprivation of essential amino acids also induces autophagy (75).

Another energy depot in cells is glycogen. Usually breakdown of glycogen into glucose occurs in the cytosol; however acid glycosidases present in the lysosomes can contribute to glucose generation during acute nutrient starvation through an autophagolysosomal process (76, 77).

Although the contribution of autophagy to protein and glycogen metabolism has been long known, its effect in lipid metabolism was only recently identified. Lipids are stored in the cytosol in single layered organelles called lipid droplets

المنارة للاستشارات
www.manaraa.com

(LDs) (78). These mainly consist of neutral lipids, triglycerides and cholesterol esters. Though the precise mechanism of lipid droplet formation is still elusive, they are presumed to be formed in the ER membrane where the diacylglycerol acyltransferases (DGAT) and acyl CoA: cholesterol acyltransferases (ACAT) enzymes are localized. These enzymes catalyze the final step in the synthesis of triacylglycerols and cholesterol esters, respectively (79).

There are many proteins embedded in the membrane of the lipid droplets, most of which are temporally targeted to the surface (80, 81). Members of the PAT-domain (perilipin, ADRP and TIP47-related proteins) family of proteins are well studied in the function of lipid droplets (78, 81). Among them, perilipin plays an important role in maintaining their homeostasis. Glucagon, catecholamine, and TNFα can promote lipid droplet breakdown (lipolysis) by activation of cyclic-AMP-dependent protein kinase (PKA), which then phosphorylates perilipin. PKA also phosphorylates hormone sensitive lipase (HSL) which is then translocated from the cytosol to the membrane of lipid droplets (79, 82). HSL interacts with perilipin and mediates lipolysis. Phosphorylation of perilipin also recruits another protein, CGI-58, which then promotes the translocation of adipose tissue triglyceride lipase (ATGL). Both HSL and ATGL are cytosolic lipases which break down triglycerides into fatty acids (Figure 1.3). The released fatty acids are further oxidized in the peroxisomes and the mitochondria to generate acetyl-CoA.

An alternative pathway of lipolysis was recently identified and is a significant breakthrough in our understanding of lipid metabolism. Autophagy was observed to mobilize lipid droplets to lysosomes in rat hepatocytes (83). Inhibition of autophagy

المنارة للاستشارات
www.manaraa.com

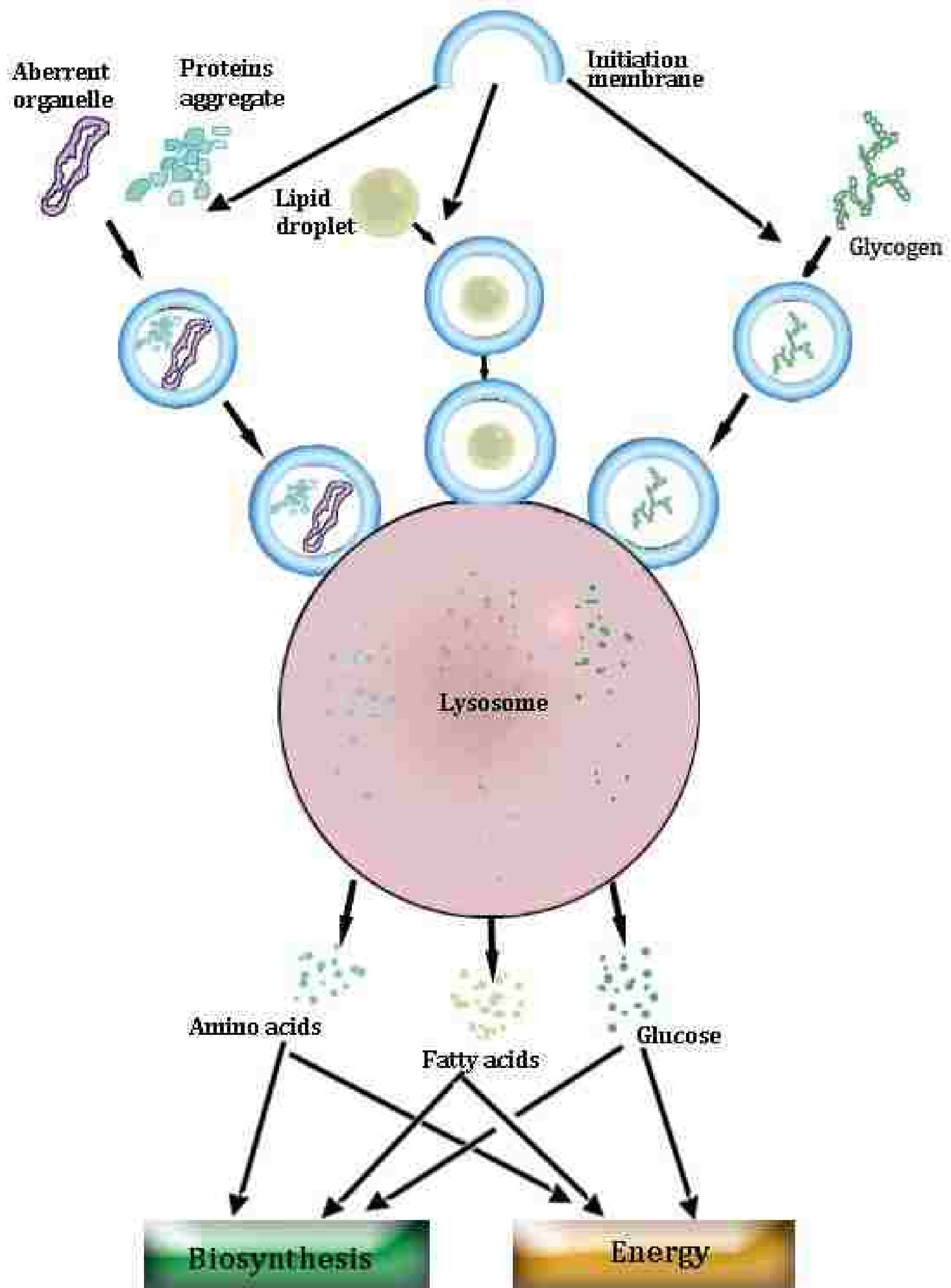
by chemical or genetic means leads to reduced β-oxidation and marked accumulation of lipid droplets. Moreover, autophagosomes and lipid droplets were associated as evidenced by colocalization assay and increased LC3-II in the lipid droplet fraction isolated from rat hepatocytes. Lipid droplets were also observed in the lumen of autophagosomes by electron microscopy. This cellular process of lipid droplet sequestration by autophagosomes is named lipophagy (84-90).

Lysosomes are important cellular organelles for intracellular digestion. They contain different hydrolases like lipases, proteases, glycosidases, and nucleotidases, which help them to degrade macromolecules (85-87). Lipids can also be targeted to lysosomes through the endocytic pathway (91, 92).

Lipophagy could be a critical biochemical pathway in cells that rely on lipid metabolism such as PCa cells.. They are rich in lipid droplets and depend on β-oxidation for their bioenergetic supply (93, 94). Fatty acid synthase (FAS) is highly expressed in LNCaP cells and its inhibition or knockdown significantly inhibits cell survival, suggesting that lipid metabolism is critical for PCa progression (95, 96).

Figure 1.2. Effects of autophagy in cellular metabolism. Autophagy can sequester protein aggregates, aberrant organelles, glycogens and lipid droplets as its cargo and deliver to lysosomes for degradation. The lysosomal acidic hydrolases are known to breakdown these biomolecules into amino acids, fatty acids and glucose, which can further be recycled for biosynthesis or utilized as fuel precursors for generation of energy depending on the cellular nutritional and metabolic status.

المنارة للاستشارات
www.manaraa.com



المنارة للاستشارات
www.manaraa.com

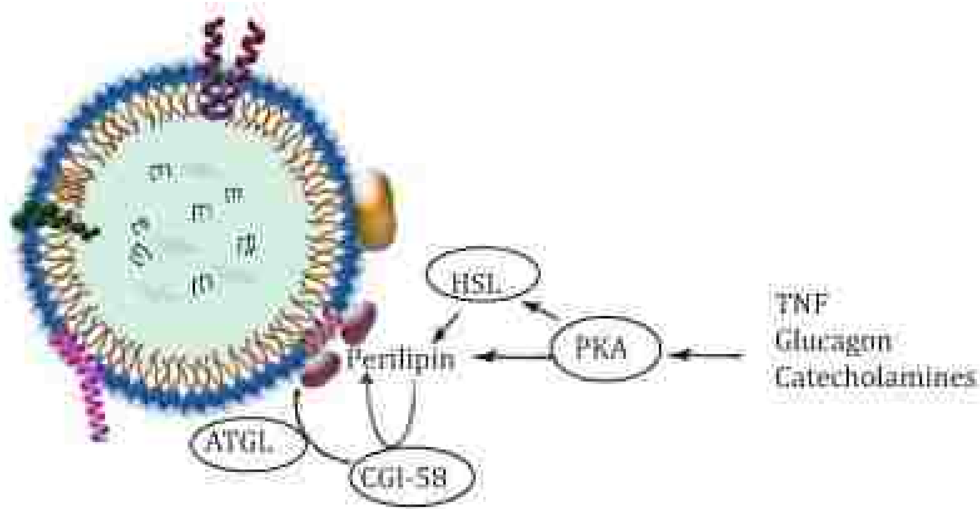


Figure 1.3. Lipid droplets and regulation of lipolysis. Lipid droplets are intracellular vesicles containing triglycerides and cholesteryl esters that are surrounded by a monolayer of lipids. Different PAT-family member proteins (Perilipin, ADRP, TIP-47), anchor protein Rab 18, diacylglycerol transferase (DGAT2) and others are known to reside in the membrane. Targeting of many lipid droplets proteins is temporally regulated. Upstream signals activate PKA which then phosphorylates both perilipin and HSL. Perilipin also recruits another lipase, ATGL, with the help of CGI-58. Translocation of lipases to the membrane results in the breakdown of triglycerides into fatty acids.

1.4. Prostate cancer cells have a unique intermediary metabolism that is regulated by androgens.

المنارة للاستشارات
www.manaraa.com

Cancer cells frequently change their metabolome compared with untransformed cells by reprogramming various metabolic pathways (97, 98). Uncontrolled proliferation of cancer cells requires a higher supply of energy as well as increased amounts of synthetic precursors. Most likely, increased genomic instability in cancer cells, in both nuclear and mitochondrial DNA provides selective pressures to shift to a new metabolome that favors cancer cell survival and proliferation. Over the last century, we have witnessed an enormous surge in our knowledge regarding the biochemical pathways that cells use to maintain energy homeostasis, to sense their bioenergetic status, and to regulate catabolic and anabolic pathways. The observation that cancer cells use an atypical biochemical pathway for their energy supply was made around 70 years ago by Otto H Warburg for which he was awarded the Nobel Prize (99, 100). Typically, cells breakdown single molecule of glucose into two molecules of pyruvate which is then shuttled into mitochondria for use as a substrate in the TCA cycle. During strenuous exercise, muscle cells cannot get oxygen to sustain their TCA cycle at a sufficient rate and use alternate pathways to convert pyruvate to lactate (101). In contradiction to the typical glycolytic pathway, Warburg observed that solid tumor cells use increased lactic fermentation even in the presence of oxygen. This type of atypical glycolysis is called aerobic glycolysis, and is commonly referred to as the "Warburg effect" in his honor (102).

However, the Warburg effect is not an essential event in all types of cancer. Prostate cancer cells have unique intermediary metabolism that is tightly regulated by androgens and their downstream transcription factors (103-105). More

المنارة للاستشارات
www.manaraa.com

interestingly, normal prostate epithelial cells themselves have a distinct metabolic pathway which we need to understand before we look at alterations in malignant cells.

The prostate gland has three anatomic regions called the transition, central and the peripheral zones. About 80% of prostate cancer originates in the peripheral zone, so the metabolism described pertains to the epithelial cells of this region(106). The epithelial cells of the peripheral zone are specialized citrate-producing secretory cells. Citrate is synthesized in mitochondria in the first step of the TCA cycle by condensation of acetyl-CoA with oxaloacetate by the enzyme citrate synthase. Further isomerization of citrate to isocitrate is severely limited in these cells by inactive m-aconitase which causes accumulation of high amounts of citrate in these cells and is subsequently secreted in the prostatic fluid. Normal prostate tissue from peripheral zones has around 13000 nmol/g of citrate (107, 108).

المنارة للاستشارات
www.manaraa.com

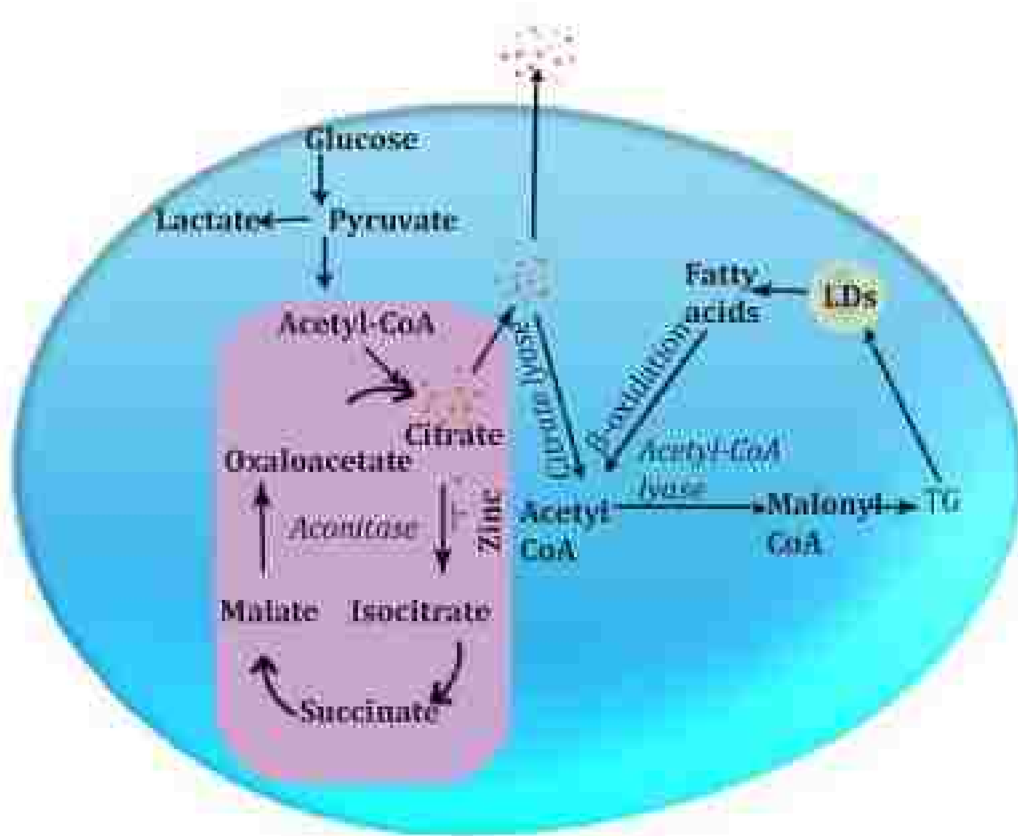


Figure 1.4. Metabolism in normal prostate cells. Prostate epithelial cells have unique metabolism. High intramitochondrial zinc inhibits the enzyme aconitase and shuts down the TCA cycle. The accumulated citrate diffuses to the cytosol. The lipogenic enzymes are also less active, so the majority of citrate is secreted out in the prostatic fluid. This is believed to provide nourishment and other functions for the sperm.

These citrate-producing normal epithelial cells transform metabolically to a citrate-oxidizing phenotype in prostate cancer cells (107, 109). The high level of

للاستشارات المنارة
www.manaraa.com

intramitochondrial zinc is responsible for inhibition of m-aconitase in normal cells. The level of zinc is found to decrease in malignant cells and plays an important role in allowing the further oxidation of citrate in the TCA cycle. However, more importantly, androgens play a key role in regulating the consumption of acetyl-CoA for synthesis of lipids (110-112). The citrate level from prostate cancer tissues falls to 500 nmol/g (107).

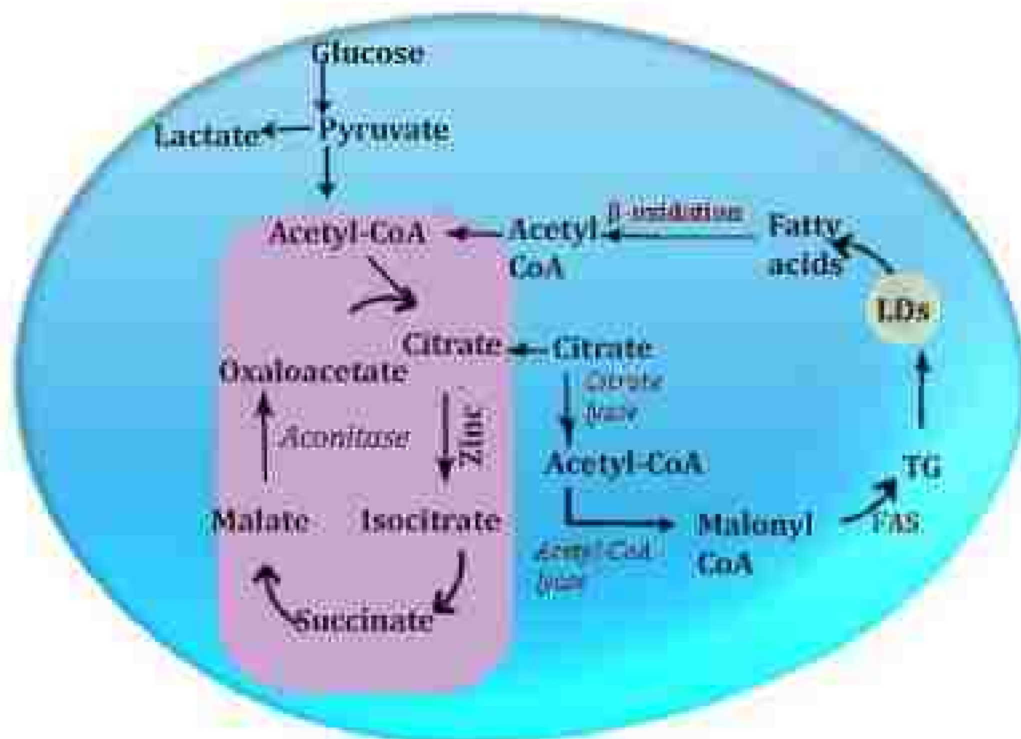
Androgens act by binding to the androgen receptors and regulating the expression of hundreds of genes affecting cellular metabolism, survival, and growth. Androgens affect the transcription of many genes that encode lipogenic enzymes (113). Transcriptional regulation of these genes is a complex process that is regulated by the SREBP (sterol-regulatory element binding protein) family of transcription factors (114). Evidence suggests that androgens affect the activation of SREBPs by a coordinated multistep mechanism (115-117). The most notable enzymes affected by the androgens are fatty acid synthase and ATP-citrate lyase, which are involved in the synthesis of fatty acids. Other lipogenic enzymes that are affected by androgens are acetyl-CoA carboxylase, and acetyl-CoA binding protein (116), and expression of fatty acid synthase is correlated with the tumorigenicity in prostate cancer (95).

Cells use excess fatty acids in the cytosol to produce triacylglycerols which are stored in lipid droplets. Lipid droplets are intracellular depots of neutral lipids (both triacylglycerols and cholesterols) which are enwrapped in a monolayer of phospholipids associated with various proteins (81, 82). Hormone regulated cytosolic lipases are known to break down triacylglycerols in lipid droplets into fatty

المنارة للاستشارات
www.manaraa.com

acids (117). These fatty acids can act as substrates for synthesis of lipids in the endoplasmic reticulum or as energy precursors for β-oxidation in the mitochondria. In prostate cancer cells, androgens also regulate many enzymes involved in fatty acid oxidation, making it the dominant metabolic pathway (94, 109, 118).

This evidence suggests that androgens are key regulators of metabolism in prostate cancer and account for the metabolic transformation from the citrate-secreting phenotype to the citrate-oxidizing phenotype. As metabolic transformation in cancer was recently added to the list of hallmarks of cancer (119), it further emphasizes the importance of studying the metabolic alterations that happen in the PCa cell during androgen deprivation.



المنارة للاستشارات
www.manaraa.com

Figure 1.5. Metabolism in prostate cancer. In comparison to normal prostate epithelial cells, intracellular citrate in PCa cells is markedly reduced. Low amounts of zinc in cancer cells allow m-aconitase to isomerize citrate to isocitrate. This causes increase in flow of the intermediates in the TCA cycle. More importantly, androgens stimulate many enzymes involved in lipogenesis (red colors) and β-oxidation of fatty acids. This metabolic transformation of PCa cells helps them to fulfill their increased bioenergetic demand.

1.5. Autophagy can be modulated at various stages.

Autophagy is generally divided into three phases: initiation, elongation, and maturation (22). The phosphatidylinositol phosphates (PIPs) play an important role in providing docking sites for various membrane trafficking events (22-24). During the initiation phase of autophagosome formation, class III PI3K vacuolar protein sorting 34 (Vps34) forms a complex with BECLIN1 and p150; and phosphorylates the PI3P to provide docking sites for recruitment of other molecules (120). Bcl-2, JNK1, AMBRA1, Atg14 and Bif-1 are known to differentially regulate the activity of this complex (121-125). Vps34 can be inhibited by 3-methyladenine or BECLIN1 knockdown (74). Furthermore, a ubiquitin-like reaction mediated by Atg7 (E1-like enzyme) and Atg10 (E2-like enzyme) conjugates Atg5 and Atg12; which later interacts with Atg16L proteins to make a complex of Atg12-Atg5-Atg16L (23). This complex is important for the formation of autophagosomes. Knockdown of Atg 5 and Atg 7 genes revealed that they are essential in the formation of

المنارة للاستشارات
www.manaraa.com

autophagosomes. Therefore these genes are commonly targeted for inhibiting autophagy in experimental studies.

Another ubiquitin-like conjugation process involving lipidation of LC3 is important for elongation of the autophagic membrane (126-127). LC3 is first cleaved by Atg4 to expose its C-terminal glycine. Then Atg7 (E1-like enzyme) and Atg3 (E2-like enzyme) mediate conjugation of the C-terminal glycine of LC3 with the amino head of phosphotidylethanolamine (PE). LC3 is a cytosolic protein, but after lipidation it translocates to the newly forming autophagic membranes. This translocation process is widely used to monitor autophagic activity in cell systems. Importantly, the lipidated form runs a little faster in polyacrylamide gel electrophoresis and forms a distinct band in immunoblots (128).

During the process of autophagosome formation, cargo can be targeted selectively into the lumen of autophagosomes by various adaptor proteins like p62, Nix, NBR1 and ALFY (62, 63, 129, 130). However, the mechanism of selectivity in targeting the lipid droplets is still unknown. In some experiments, autophagosomes were observed to form from the membranes of lipid droplets, supporting the new concept that autophagosomes may start forming at the target site (83).

Once the autophagosomes are fully formed, they fuse with late endosomes or lysosomes, and the cargo is delivered to the lumen of lysosomes. Different acidic hydrolases are known that degrade those biomolecules to their basic building blocks (85-87). Acidity is important in the activity of these hydrolases, thus drugs that increase the pH of lysosomes interfere in the degradation of autophagosomes. Bafilomycin A1 is a H^+K^+ ATPase inhibitor, which diminishes the

المنارة للاستشارات
www.manaraa.com

acidification of lysosomes (131). Another drug, chloroquine, sequesters protons and increases the pH (132). Alteration in pH also affects the fusion process of autophagosomes with lysosomes, causing accumulation of autophagosomes.

Recently it was found the BECLIN1/Vps 34 complex also plays a role in the maturation of autophagosomes. Interestingly, UVRAG positively regulates the activity of BECLIN1 in the maturation process while Rubicon does the opposite (133, 134).

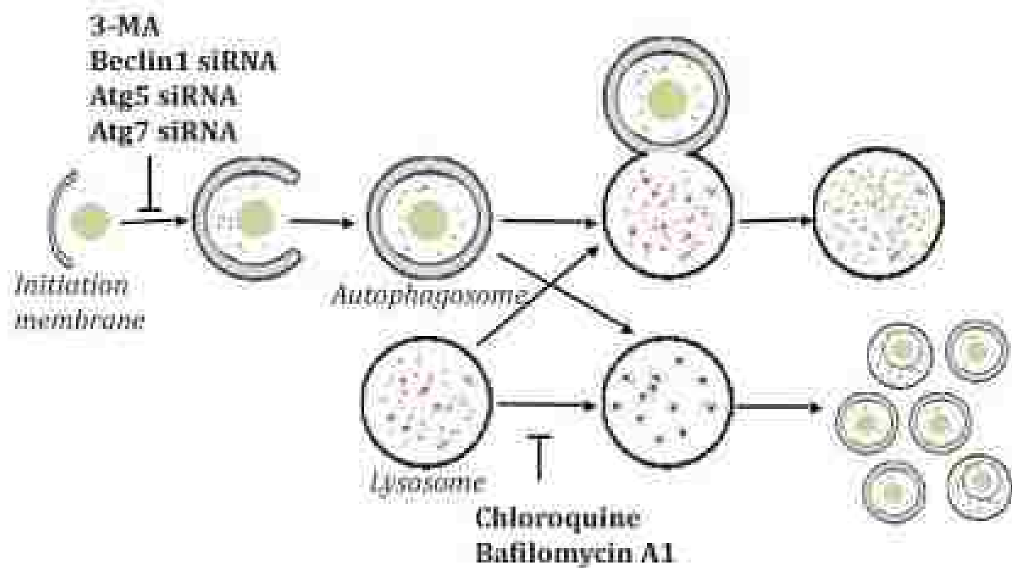


Figure 1.6. Autophagy can be regulated at different stages. Autophagosome formation can be divided into 3 different phases: initiation, membrane elongation and maturation. Beclin1 siRNA, Atg5 siRNA, Atg7 siRNA and 3-MA are widely used to inhibit the autophagy initiation phase, so there will be no autophagosome formation at all. Bafilomycin A1 and chloroquine are used to inhibit the maturation

المنارة للاستشارات
www.manaraa.com

process of autophagosomes. They cause accumulation of autophagosomes and autophagolysosomes.

1.6. Chloroquine is a potent adjunct in various cancer therapies.

Chloroquine (N'-(7-chloroquinolin-4-yl0-N,N-diethyl-pentane-1,4-diamine) is a drug that has been used widely since the second world war for chemotherapy against malaria (135). It is also used as mild immunosuppressant in the treatment of rheumatoid arthritis and Lupus (136, 137). Recently, it has been observed that chloroquine can be used as an adjunct in various cancer therapies.

Chloroquine is a weak base (pka1=8.1, pka2=10.2) which can exist in both protonated and unprotonated forms. Its charge determines its pharmacodynamics and biodistribution (135). Because the unprotonated form is freely diffusible through the plasma membrane, chloroquine has a large volume of distribution. But once it gets into the lysosome, it gets protonated and cannot diffuse through the membrane. This trapping of chloroquine in the lysosomal compartment causes an increase in pH and subsequent inactivation of acidic hydrolases. This effect of chloroquine also inhibits the maturation of autophagosomes (138).

Inhibition of the maturation process of autophagy by chloroquine is potentially important in cancer therapies where cells induce autophagy as a protective mechanism. Interestingly, chloroquine may also affect cancer growth in other ways; chloroquine can act as a weak DNA intercalating agent and halts the DNA repair process (139). This function has been found to be useful in potentiating the killing effect of radiation in some cancer cells (135). However,

المنارة للاستشارات
www.manaraa.com

effects of chloroquine on arresting the maturation of autophagosomes have been observed in many cell types. In human retina ARPE-19 cells, chloroquine caused arrest of autophagy, lysosomal dilatation and subsequent cell death (140). Similarly in fibroblasts, leukocytes and myocytes, chloroquine caused accumulation of autophagic vacuoles and affected cellular metabolism (141-144).Recently, it was shown to enhance the therapeutic efficacy of topotecan in lung cancer cells by inhibiting autophagy (145).

Similarly, in HT-29 colon cancer cells, chloroquine was observed to potentiate the anti-cancer effects of 5-Fluorouracil (5-FU) (146). However, on investigation it was found that 5-FU induces autophagy and use of chloroquine inhibits autophagosomal degradation. In another study, chloroquine induced dose dependent cell death in 5 different glioblastoma cell lines that were of different p53 status (147). It suggests that chloroquine also affects the cell death beyond its effects on DNA repair processes. Furthermore, they found that chloroquine induces accumulation of autophagic vacuoles in all cell lines and affects the levels and subcellular distribution of Cathepsin D. These findings suggest that altered lysosomal function may play a role in chloroquine induced cell death. Inhibition of autophagy by chloroquine also sensitizes PCa cells to Src family kinase inhibitors like saracatinib and PP2 (148). Src kinase inhibitors induce autophagy in both LNCaP and PC-3 cells. Autophagy blockade by chloroquine or 3-MA or Atg7 siRNA during Src kinase inhibitor treatment caused more cell death *in vivo* and *in vitro*.

المنارة للاستشارات
www.manaraa.com

1.7 Hypothesis

Androgen deprivation induces autophagy in hormone sensitive prostate adenocarcinoma LNCaP cells. Elucidating the molecular effects of autophagy during androgen deprivation is important in understanding whether modulators of autophagy can be used as therapeutic agents in prostate cancer treatment. Recently, autophagy has been shown to regulate lipolysis in rat hepatocytes. Prostate cancer cells are also rich in lipid droplets and rely predominantly on lipid oxidation. This novel function of autophagy may be crucial in survival of prostate cancer cells during androgen deprivation. **Therefore, I hypothesize that autophagy mediates lipophagy in androgen-dependent prostate cancer cells and thus helps them to survive in the absence of androgen.**

للاستشارات
www.manaraa.com

Chapter 2

Autophagy regulates lipolysis and cell survival through lipid droplet degradation in androgen sensitive prostate cancer cells

Ramesh R. Kaini, Laurel O. Sillerud, Siqin Zhaorigetu, Chien-An A. Hu*

Department of Biochemistry and Molecular Biology , University of New Mexico Health Sciences Center, Albuquerque, NM, USA

*Corresponding author

1 University of New Mexico, MSC08 4670, Albuquerque, NM, 87131-001, USA

Tel: 505-272-8816, Fax: 505-272-6587, Email: AHu@salud.unm.edu

Prostate. 2012 Jan 31. doi: 10.1002/pros.22489. [Epub ahead of print]

Received 9 July 2011; accepted 2 January 2012

المنارة للاستشارات

www.manaraa.com

2.1. 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 triacyglycerols after the inhibition of autophagy by 3-methyladenine, bafilomycin A_1 or si-ATG5 were quantified by three independent methods, Oil Red O staining, triacyglycerols 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.

للاستشارات المنارة

www.manaraa.com

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 ablation therapy.

Keywords: Prostate cancer, Autophagy, Androgen deprivation, Lipid droplets, Lipolysis, NMR

المنارة للاستشارات

www.manaraa.com

2.2 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 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 (96, 149, 150). 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 (14, 93, 151, 152). Interestingly, fatty acid oxidation is also enhanced in PCa (94, 103, 107, 108). Tumors need unusual amounts of energy and biosynthetic precursors to survive and grow (97) 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 (153, 154). However, some of the cancer cells can “escape” from the treatment, survive and develop androgen independence by several mechanisms (19, 20). In order to understand the progression of PCa it is important to investigate the cell survival mechanisms activated during hormone ablation therapy.

المنارة للاستشارات
www.manaraa.com

Autophagy is a cellular mechanism that is inducible during starvation and stress (22, 45). 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 (22). 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 (155, 156). 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 (49). In addition, two independent studies have demonstrated that inhibition of

المنارة للاستشارات
www.manaraa.com

autophagy in LNCaP cells under androgen deprivation causes increased cell death, suggesting that autophagy protects PCa cells (49, 157).

Lipid droplets (LDs) are intracellular depots of neutral lipids (both triacylglycerols and cholesterols) which are enveloped by a monolayer of phospholipids and associated proteins (158). 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 (81, 158). 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 (83). 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 (85, 88), 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.

المنارة للاستشارات
www.manaraa.com

2.3. 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 (D_2O), deuterochloroform ($CDCl_3$), tetramethylsilane (TMS), d_6-2,2-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 A_1 (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% CO_2 as previously described (159). LAPC4 cells, an androgen-sensitive, AR- wildtype PCa cell line, a gift from Dr. Todd Thompson (University of New Mexico Health Science Center) (160, 161), 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

المنارة للاستشارات
www.manaraa.com

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 g/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 knowdown 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

المنارة للاستشارات
www.manaraa.com

antibodies, anti-LC3 (MBL, PM036), anti-GFP (Santa Cruz, sc-8334), or anti-□-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

المنارة للاستشارات
www.manaraa.com

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 D_2O instead of H_2O 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.* (162), dried under a stream of nitrogen, dissolved in 0.5 ml $CDCl_3$ 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 $(-CH_2-)_n$ signal at □ = 1.29 ppm was integrated with respect to the DSS signal at □ = 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 = 14) side chains as previously described (151).

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.

المنارة للاستشارات
www.manaraa.com

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

المنارة للاستشارات
www.manaraa.com

2.4. Results

2.4.1. 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. 2.1A, 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. 2.1A, panel b) and increased the number of AVs observed per cell by seven-fold (Fig. 2.1B, 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. 2.1A panel c, and Fig. 2.1B, 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. 2.1A, 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. 2.1C), suggesting that autophagy induced by androgen deprivation is pro-survival. This observation is consistent with results previously reported (165,

المنارة للاستشارات
www.manaraa.com

166). 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. There was a greater increase of LC3II in cells of CFM+Baf A1 (Fig. 2.1D, lane 5) than for cells in CM, CFM, or CM+Baf A1 (Fig. 2.1D, 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 (165, 166). 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. 2.1, 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. 2.1E), 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. 2.2A, panel b), compared to cells in CM alone (Fig. 2.2A, 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

المنارة للاستشارات
www.manaraa.com

and LC3I (Fig. 2.2B). Moreover, cells cultured in either CFM or CM+CDX for 24 hrs showed a loss of cytosolic p62 (Fig. 2.2C). 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 lumenal 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 (163, 164). These data confirm that androgen attenuation induces autophagy and therefore p62 degradation.

2.4.2. 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. 2.3A, panel a), as previously reported (93, 150). The cellular triacylglycerol content was found to be ~14.5 pM/µg protein (Fig. 2.3C, 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. 2.3A, panel b) and in the amount of triacylglycerols (Fig. 2.3C, column b) by day 5, an effect that was reversed by addition of the androgen analogue, R1881 (Fig 2.3A, panel c; and Fig. 2.3C, column c), or the autophagy inhibitor 3-MA (Fig 2.3A, panel d; and Fig. 2.3C, column d). Our observation of a decrease in lipid droplet formation when androgens are withdrawn is consistent with data

المنارة للاستشارات
www.manaraa.com

reported by Swinnen et al. (150). 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 (165, 166). LAPC4 cells were cultured in the absence (Fig. 2.3B, panel a) and presence (Fig. 2.3B, 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. 2.3B, panels b and c; Fig. 2.3D columns #2 and #3) compared to the control cells grown in CM (Fig. 2.3B, panel a; and Fig. 2.3D, column #1). In addition, biochemical assays for triacylglycerols also showed a greater than 70% reduction in intracellular triacylglycerols in the treated group (Fig. 2.3D, 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.

2.4.3. 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 2.4A, 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

المنارة للاستشارات
www.manaraa.com

CFM+si-ATG5 (Figure 2.4B), 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 (^{1}H-NMR). Integrals of the methylene $(-CH_2-)_n$ signals at 1.29 ppm (as shown in Fig. 2.5, 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. Consistent with the optical (microscopy) and biochemical (TG enzymatic) assays, ^{1}H-NMR also showed the reduction of intracellular TGs in cells deprived of androgen (Fig. 2.5, panel b; and Fig. 2.5E, column b). This effect can be reversed by treatment of the cells with the androgen analogue R1881 (Fig. 2.5, panel c; and Fig. 5E, column c) or the autophagy inhibitor 3-MA (Fig. 2.5, panel d; and Fig. 5E, column d).

2.4.4. 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 2.6, EGFP-LC3 (green) was mainly cytosolic and there were abundant LDs (red) in

المنارة للاستشارات
www.manaraa.com

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 l; panel m, column 3; panel n, column 3). In addition, blocking the autophagic flux by means of Baf A_1 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 (131), increased the number of colocalized AV/LD dots, indicating that Baf A1 restricted intracellular LDs degradation in LNCaP cells.

المنارة للاستشارات
www.manaraa.com

2.5.Discussion

Hormone ablation 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 (19, 167) and then this treatment is no longer useful. Because metabolic deregulation is commonly observed during tumoriogenesis, 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 A_1 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 (35, 40, 44, 47), its role in the survival or

المنارة للاستشارات

www.manaraa.com

death of prostate cancer cells has not been extensively examined. What is known is that radiation therapy of PCa cells induced cytoprotective autophagy (44) and that incubation of LNCaP cells, but not PC3 cells (an androgen-insensitive PCa line) in serum free medium induced autophagy (49, 157). 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 (49, 157). 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 (168). 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 2.1). 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 (165, 166), 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 (94, 107). Otto Warburg found that most cancer cells rely primarily on aerobic glycolysis to generate ATP to meet their energy demand (97, 99). 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 (94,

المنارة للاستشارات
www.manaraa.com

169). Importantly, androgens stimulate both the lipogenic and fatty acid β-oxidation pathways in androgen-dependent PCa cells to fulfill their increased bioenergetic needs (14, 93, 94, 107, 118, 151, 152). Recently, Singh and colleagues reported that autophagosomes sequestered LDs and mediated lysosomal lipolysis in rat hepatocytes (83). 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 2.3A, 2.3C, 2.4 and 2.5e), which is consistent with results previously reported (150). The same result was observed in LAPC4 cells when androgens were depleted using CFM or the AR was inhibited with CDX (Figure 2.3B and 2.3D). 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 2.1, 2.3 and 2.4). 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 2.3 and 2.4). 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 A_1 (Baf A1) has been reported to inhibit lysosomal acidification/hydrolysis, to block autophagic degradation, and to

المنارة للاستشارات
www.manaraa.com

cause the accumulation of autophagosomes (131). 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. 2.6, 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 A_1 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 2.3D, 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 (85, 88), 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.

المنارة للاستشارات
www.manaraa.com

2.6. Acknowledgements

We thank colleagues of the fluorescence microscopy shared facility, which is supported by University of New Mexico Health Science Center and the University of New Mexico Cancer Center. The NMR studies were carried out at the UNM Department of Chemistry High-Field NMR facility.

المنارة للاستشارات
www.manaraa.com

2.7. Figures and figure legends:

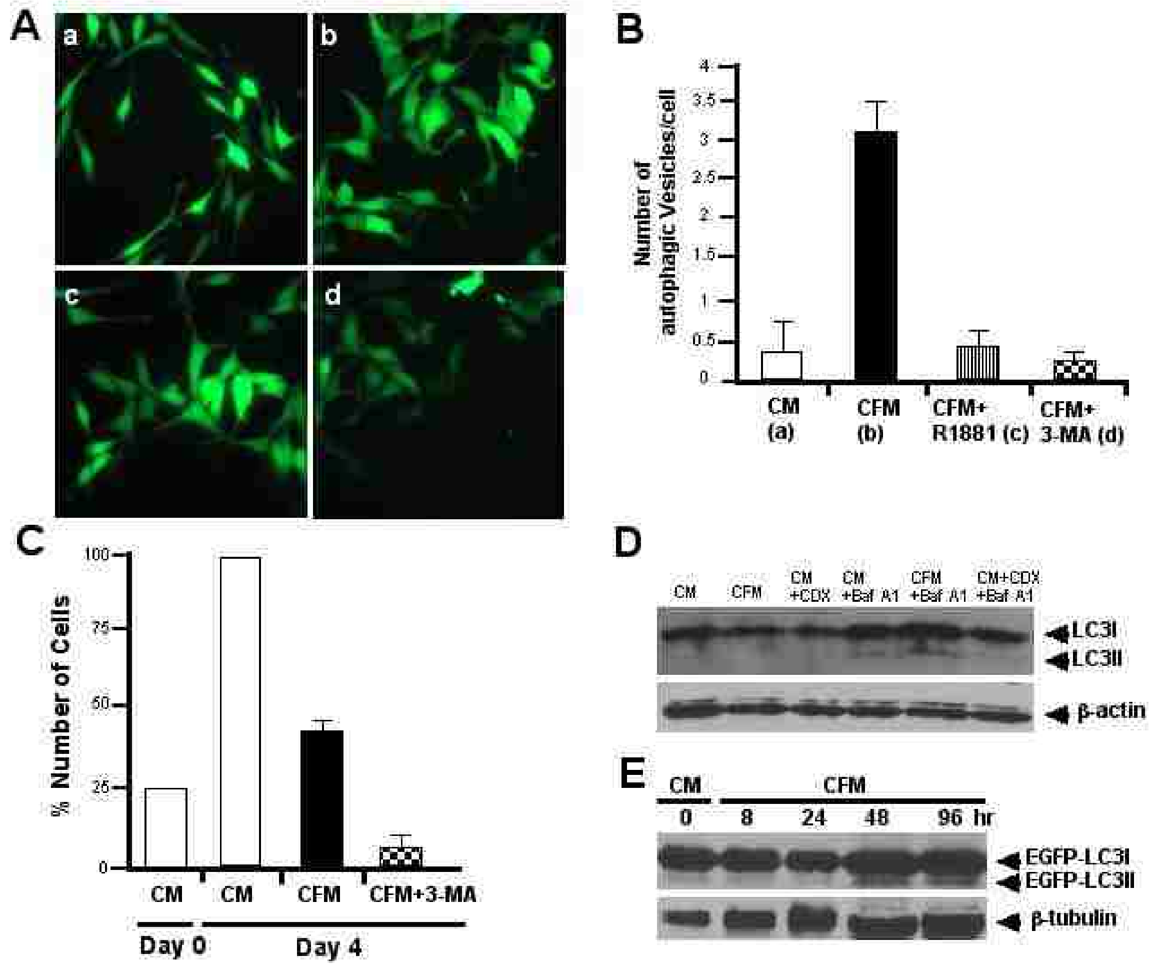


Figure 2.1. Androgen deprivation induces autophagy in LNCaP cells, which can be reversed by autophagy inhibitors, 3-MA and Bafilomycin A1 (Baf A1). A. LNCaP.EGFP-LC3 cells (LNCaP cells stably transfected with pEGFP.LC3) were incubated for 2 days in indicated medium, (a) CM, complete medium; (b) CFM, charcoal-filtered fetal bovine serum medium; (c) CFM+R1881; and (d) CFM+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

المنارة للاستشارات
www.manaraa.com

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.

المنارة للاستشارات
www.manaraa.com

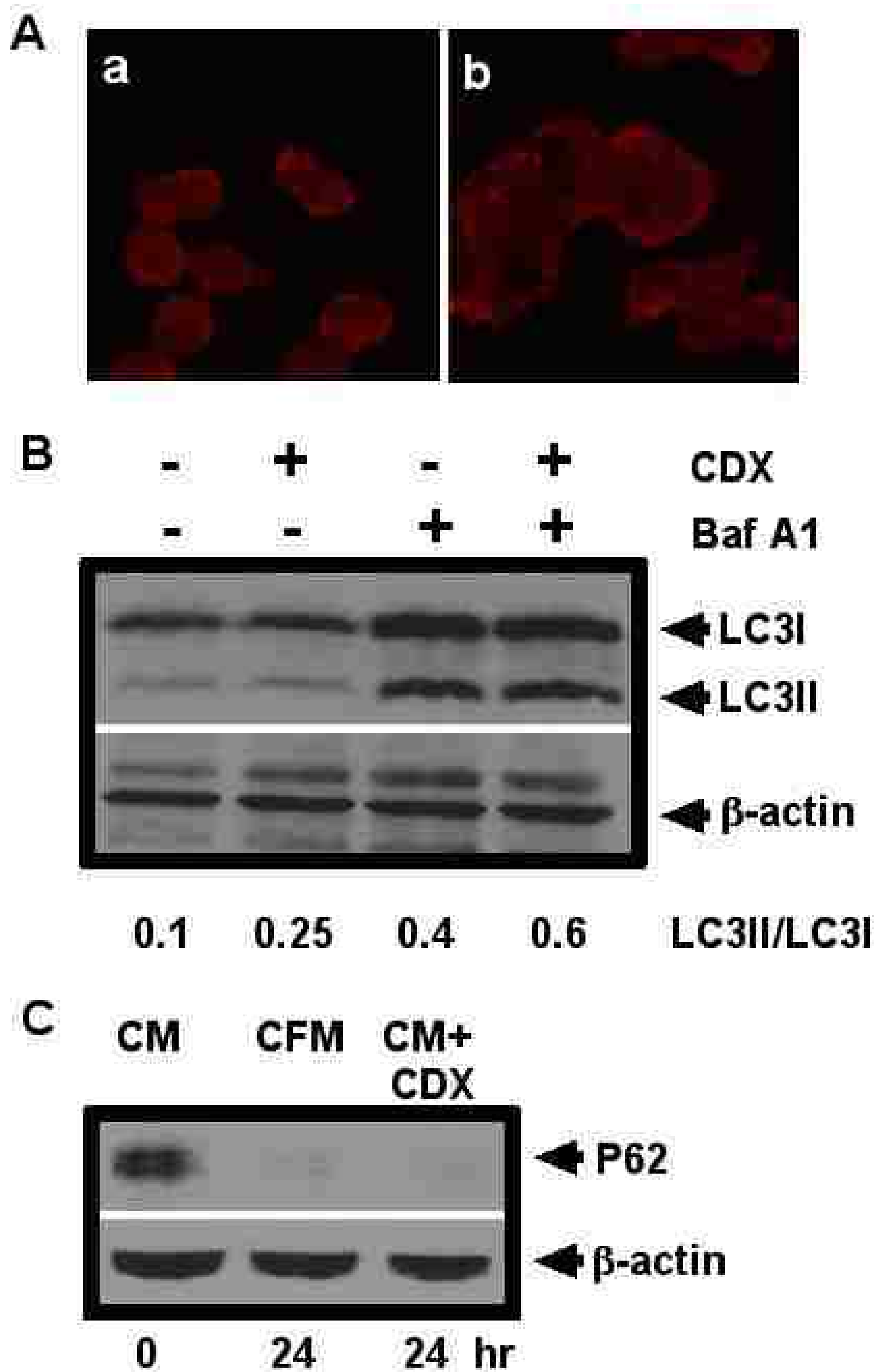


Figure 2.2. Androgen antagonist CDX induces autophagy in LAPC4 cells. A. LAPC4 cells were incubated for 24 hrs in (a) CM or (b) CM + 5 μM casodex (CDX). Cells were mounted in coverslips, probed with anti-

المنارة للاستشارات
www.manaraa.com

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 μM CDX; (lane 3) CM+ 100 nM 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.

المنارة للاستشارات

www.manaraa.com

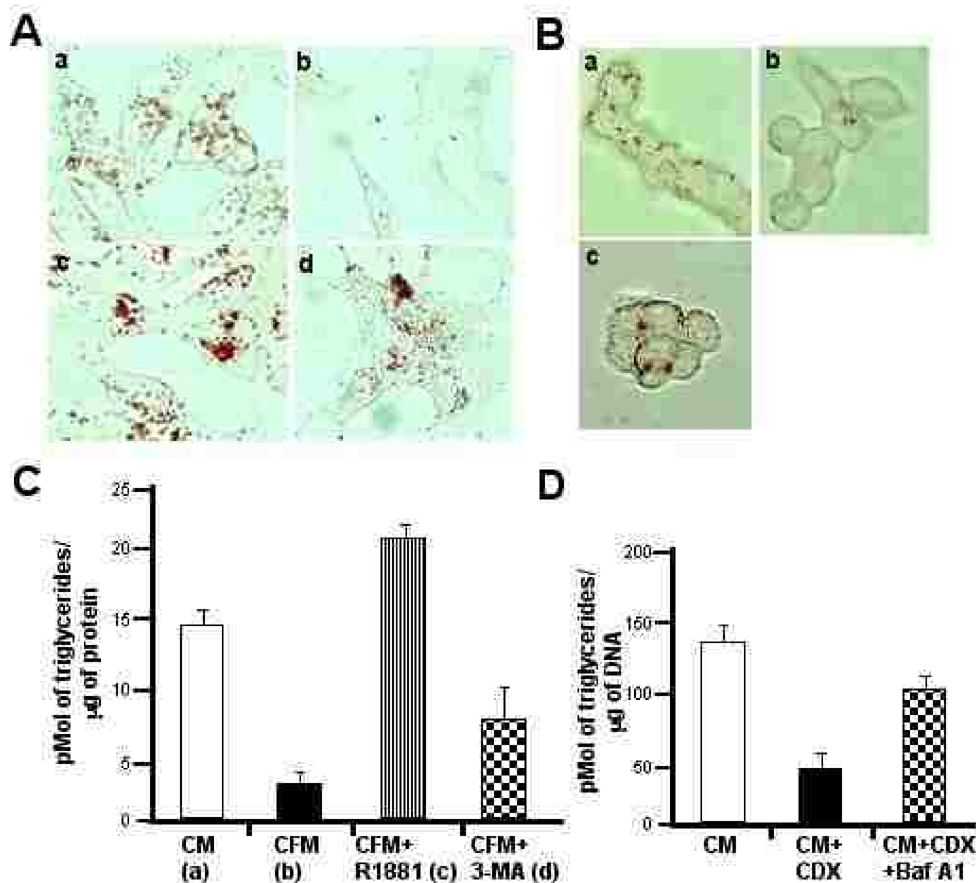


Figure 2.3. The crosstalk between androgen, autophagy and lipid droplets in androgen sensitive PCa cells. A. LNCaP cells were incubated for 5 days in indicated medium, (a) CM, complete medium; (b) CFM (c) CFM+R1881, and (d) CFM+3MA. Cells were stained with Oil Red O, mounted in coverslips, and observed 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

المنارة للاستشارات
www.manaraa.com

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.

المنارة للاستشارات
www.manaraa.com

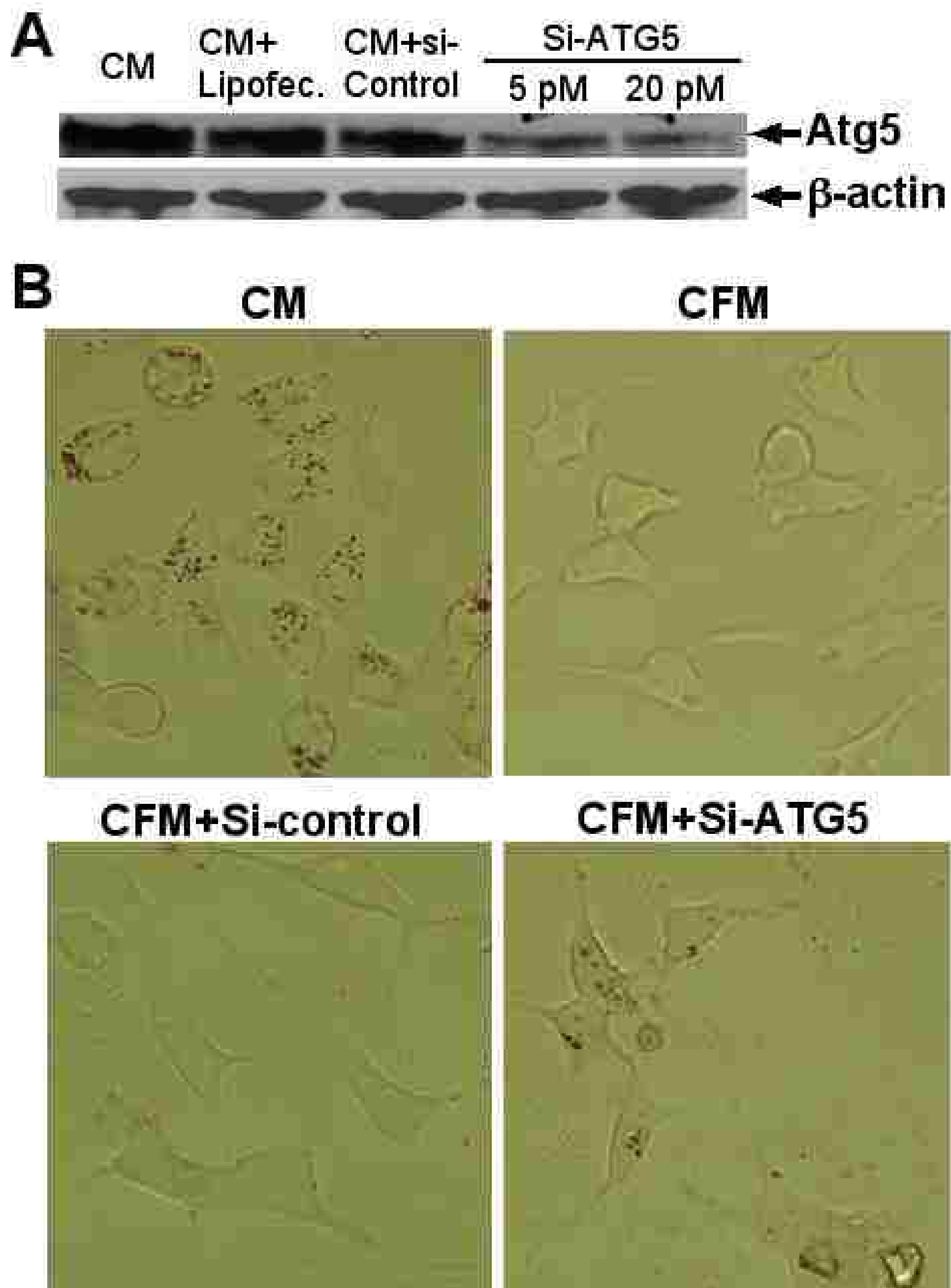


Figure 2.4. Attenuation of autophagy by Si-ATG5 retains LDs in androgen-deprived LNCaP cells. A. Si-ATG5 knockdown of Atg5 was confirmed by immunoblot analysis. Twenty µg of total soluble protein extracts were used from

المنارة للاستشارات
www.manaraa.com

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 pM) 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.

المنارة للاستشارات
www.manaraa.com

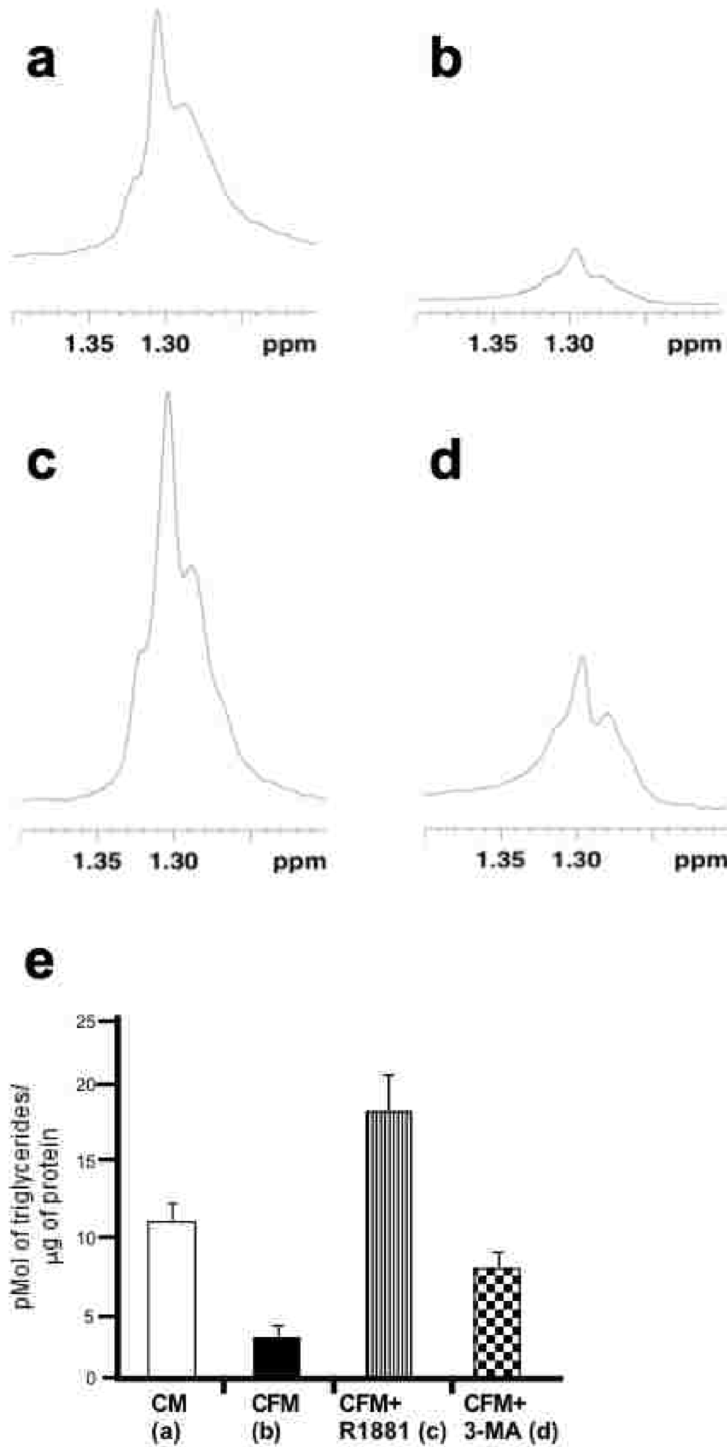


Figure 2.5. Quantitative NMR assay of the effect of androgen and 3-MA on triacylglycerols in LNCaP cells. Total cellular triglycerides in treated cells were quantified using proton nuclear magnetic resonance (^{1}H-NMR). All cells were

www.manaraa.com

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 $(-CH_2-)_n$ 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.

المنارة للاستشارات
www.manaraa.com

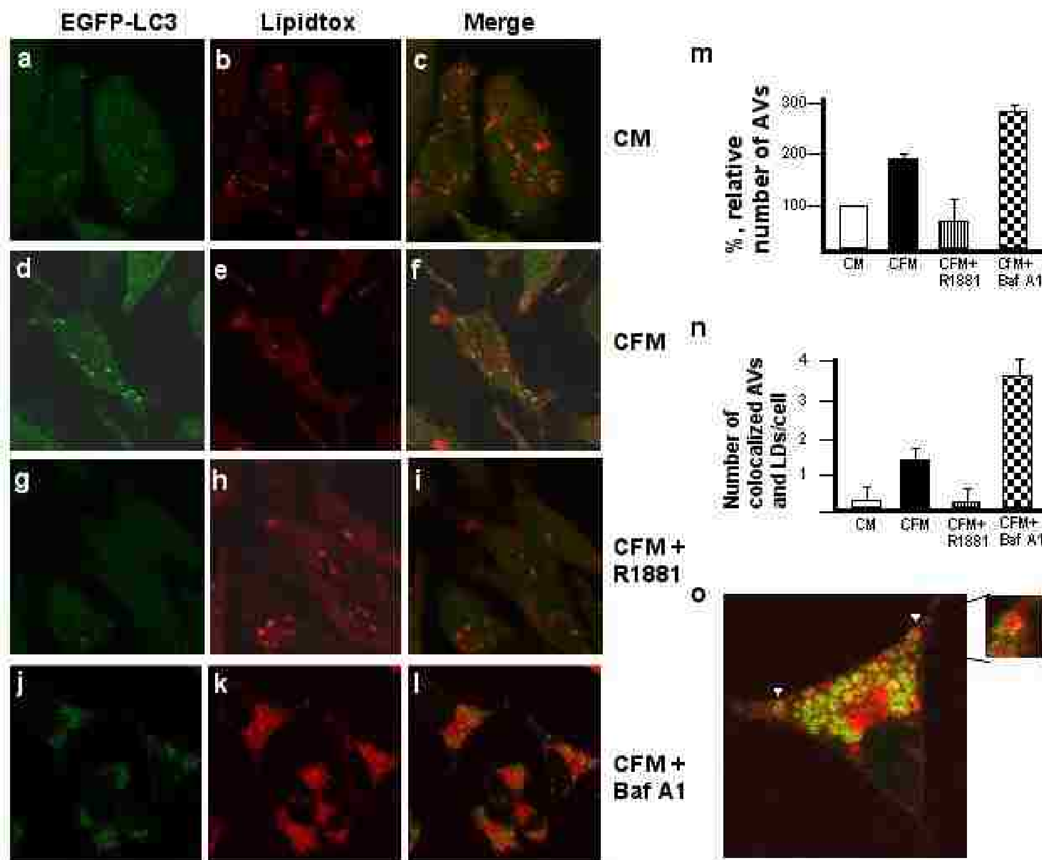


Figure 2.6. Autophagosomes colocalize with LDs in LNCaP cells during androgen deprivation. LNCaP.EGFP-LC3 cells were incubated for 3 days in indicated medium, (a) CM, (d) CFM; (g) CFM+R1881, and (j) CFM plus BAF A1 for 12 hours. Cells were fixed, stained with HCSLipidTOX Red, and observed with a Zeiss 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

المنارة للاستشارات
www.manaraa.com

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 A_1 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).

المنارة للاستشارات
www.manaraa.com

Chapter 3

Synergistic killing effect of chloroquine and androgen deprivation in LNCaP cells

Ramesh Raj Kaini, Chien-An Andy Hu*

Department of Biochemistry and Molecular Biology , University of New Mexico Health Sciences Center, Albuquerque, NM, USA

*Corresponding author

1 University of New Mexico, MSC08 4670, Albuquerque, NM, 87131-001, USA

Tel: 505-272-8816, Fax: 505-272-6587, Email: AHu@salud.unm.edu

المنارة للاستشارات

www.manaraa.com

3.1. Abstract

Modulation of autophagy is a new paradigm in enhancing the therapeutic efficacy of various cancer treatments. Recently, a novel function of chloroquine in inhibiting degradation of autophagic vesicles has led to studies on cancer cells whether chloroquine can be used as an adjuvant in targeting autophagic prosurvival mechanism. We previously observed that autophagy plays a protective role during hormone ablation therapy in prostate cancer cells by consuming lipid droplets. Here, to further investigate the importance of autophagy in PCa cell survival and dissect the role of CLQ in PCa cell death, we treated hormone responsive LNCaP cells with chloroquine during androgen deprivation. We observed that chloroquine synergistically kills LNCaP cells during androgen deprivation in a dose and time dependent manner. We further confirmed that chloroquine inhibits the maturation of autophagic vesicles and decreases the cytosolic ATP. Moreover, chloroquine induces apoptosis in androgen deprived LNCaP cells. Our finding suggests that chloroquine may be useful as an adjuvant in hormone ablation therapy to improve the therapeutic efficacy.

Keywords: Prostate cancer, Chloroquine, Androgen deprivation, Autophagy

المنارة للاستشارات

www.manaraa.com

3.2. Introduction

Prostate cancer (PCa) is the most common cancer among men of all races in the United States (1, 3, 4). About 1 in 6 men will be diagnosed of PCa in his lifetime. There are three standard types of treatment in prostate cancer: surgical resection, radiation therapy and hormone ablation therapy. In general, surgical resection and radiotherapy are conducted when the tumor is localized (3, 5). However, once metastasized, prostate tumors are hard to cure and bear poor prognosis (3, 5, 6). It is well known that androgens are essential for survival, proliferation, and progression of PCa cells (8, 21). Androgen ablation induces apoptosis and regression of hormone responsive PCa cells (153, 154). Thus, hormone ablation therapy is a treatment option when PCa cells metastasize (5). However, unfortunately, some PCa cells can escape and survive the treatment and subsequently develop hormone/castration resistant phenotype (19, 20). Developing adjuvant therapy that would enhance the therapeutic effectiveness of hormone ablation therapy is crucial for PCa treatment.

We previously reported that during hormone ablation therapy, autophagy is induced in two different hormone sensitive PCa cell lines, LNCaP and LAPC4 cells (chapter 2). Autophagy is a cytosolic mechanism in which a portion of the cytosol constituents including aberrant organelles is enwrapped by newly formed vesicles, known as autophagic vesicles and targeted to lysosomes for hydrolysis (22, 45). These autophagic vesicles (AVs) can be induced during androgen deprivation and can sequester lipid droplets, a mechanism known as lipophagy (85-88). As PCa cells are rich in lipid droplets and rely predominantly on β-

المنارة للاستشارات

www.manaraa.com

oxidation for their bioenergetic needs, this alternate pathway of lipolysis is crucial for their survival during hormone ablation therapy (94, 118). Inhibition of autophagy by 3-Methyladenine (3-MA) significantly killed more cells growing in the absence of androgen than cells growing in regular medium, suggesting that autophagy protects LNCaP cells during androgen deprivation (chapter 2). Similar results were reported in two other studies where genetic knockdown of autophagy caused more cell death in the absence of androgen (49, 157).

Modulation of autophagy in cancer treatment is a new and exciting field. It has been shown that inhibition of autophagy by either 3-MA or Chloroquine (CLQ) sensitizes LNCaP cells to Src kinase inhibitors such as saracatinib and PP2 (148). 3-MA is a PI3 kinase inhibitor which blocks the early stage of autophagosome formation, whereas CLQ is a lysosomotropic drug which inhibits the autolysosomal degradation (146). CLQ causes accumulation of AVs but prevents its major function, i.e. breaking down the cytosolic and vesicular components for recycling of biomolecules for energy generation. CLQ has been used widely as an effective antimalarial and anti-rheumatoid drug (135). Recent studies have been conducted to seek CLQ as an adjuvant in different cancer therapies. CLQ has been shown to potentiate the cytotoxic effects of 5-fluorouracil in colon cancer cells (146). Combining CLQ with Akt inhibitors also significantly enhanced the cell death effect in breast cancer cells (170). CLQ also potentiated the efficacy of carmustine on glioma cells *in vivo* and *in vitro* (171). Taken together, these reports suggest that CLQ is a potential candidate to enhance the anti-cancer effect of hormone ablation therapy in PCa. As

المنارة للاستشارات
www.manaraa.com

autophagy induced during androgen ablation helps to protect LNCaP cells, we hypothesized that CLQ augments the apoptotic effect of androgen deprivation in LNCaP cells. We treated LNCaP cells with CLQ in the presence or absence of androgen and characterized the cell death effects and pathways that were activated.

المنارة للاستشارات
www.manaraa.com

3.3. Materials and Methods

Chemicals and reagents. Charcoal-filtered fetal bovine serum (CFM) was purchased from Hyclone (Thermo Fisher, MA, USA), RPMI 1640, and regular FBS was from Gibco/Invitrogen (Eugene, OR, USA). Chloroquine diphosphate salt was purchased from Sigma Aldrich (St Louis, MO, USA) and dissolved in dH_2O. Trihydrochloride trihydrate was purchased from Molecular Probe (Eugene, OR, 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 ^{0}C and 5% CO_2. pEGFP-LC3 stably transfected LNCaP cell lines were generated as described in chapter 1. Cells were grown in CM or CFM in the presence or absence of CLQ at the indicated concentrations.

Cell viability assay. LNCaP cells were seeded in equal number (10,000 cells/ well) in 96 well plates and cultured in CM or CFM overnight. CLQ was added at 200 uM and incubated for 24 hours or other time points where specified. The cell viability was then examined by using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to manufacturer's instructions (Vybrant MTT assay, Invitrogen, Eugene, OR, 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 CM or CFM in the presence or absence of CLQ for 24 hours, and

المنارة للاستشارات
www.manaraa.com

then observed under the LSM 510 confocal microscope. Number of cells and distinct puncta were counted from ten random visual fields for each slide. The number of puncta per cell was used as a measure of autophagic activity.

ATP measurement. Equal number of LNCaP cells (10000 cells/ well) were seeded in 96 well plates and incubated in CM or CFM. Cells were treated with CLQ (200 uM) for 24 hours. Intracellular ATP levels were measured using a colorimetric assay kit (Abcam, San Francisco, CA, USA) followed the manufacturer's instruction.

Annexin-V/PI assay. Cells were treated with CLQ for 36 hours in CM or CFM. Both, the floating cells and the attached cells were harvested. Cell pellets were washed twice with 1X DPBS and then resuspended in 1X Binding buffer at a concentration of 1 x 10^6 cells/ ml. One hundred µl of the suspension (1x10^5 cells) was used to label with Annexin V- FITC and Propodium Iodide (BD Biosciences, San Diego, CA, USA) and analyzed by flow cytometry.

DNA fragmentation assay. LNCaP cells were seeded in equal number and cultured in CM or CFM and then treated with CLQ for another 36 hours. The floating cells were collected, washed with ice-cold DPBS, lysed with lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA, and 1% Triton X-100] for 20 min on ice, and centrifuged at 11000 x g for 20 min. The supernatant was collected and treated with RNase A and Proteinase K for 1 hour at 37^0 C. DNA was extracted using phenol-chloroform method, resuspended in equal volume of 1 x TE, and then

المنارة للاستشارات
www.manaraa.com

electrophoresed in 0.8% agarose gel. DNA was visualized using Ethidium Bromide staining.

Hoechst nuclear staining. LNCaP cells were inoculated on coverslips in 6 well plates, cultured, and treated with CLQ for 36 hrs as in DNA fragmentation assay. Attached cells were washed and stained with Hoechst 33342 and observed under a Zeiss Axioskop microscope.

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

المنارة للاستشارات
www.manaraa.com

3.4. Results

3.4.1. Chloroquine synergistically kills LNCaP cells during androgen deprivation.

To study the effect of CLQ on the survival of LNCaP cells, ten thousands cells were seeded in 96 well plates in regular medium (CM) or androgen deprived medium (CFM). Both groups were treated with increasing concentrations of CLQ (0 -600 μM) for 24 hours. Viable cells were quantified using MTT assay. The cytotoxicity of CLQ was observed that was correlated with concentrations of CLQ in both conditions (Figure 3.1). When cells were treated with 200 μM of CLQ for 24 hours, there was little effect on cells grown in CM, whereas there was 15% reduction in cell grown in CFM. At higher concentrations, CLQ has greater cytotoxicity in cells treated with androgen deprivation.

Because CLQ has a long half life ($t^1/_2$= 2- 6 days) (135, 138), we looked at the time dependent effect of CLQ during androgen deprivation. Treatment of LNCaP cells with CLQ at 200 μM for 2 days in CFM reduced the number of viable cells by 30%, whereas treatment for 3 days reduced the number by more than 50% compared to cells growing in the CFM only (Figure 3.2 A & B).

3.4.2. Chloroquine blocks autophagic degradation and reduces cytosolic ATP in LNCaP cells.

To investigate if CLQ affected the maturation of autophagic vesicles, we incubated LNCaP.EGFP-LC3 cells with CLQ (200 μM) in the presence or absence of androgen. We previously showed that incubation of LNCaP cells in

www.manaraa.com

CFM induces translocation of cytosolic EGFP-LC3 to puncta like autophagic vesicles. As shown in Figure 3.3, CLQ also induced increase of puncta in cells cultured in CM; however the accumulation of puncta was even greater in cells cultured in CFM, suggesting that CLQ blocks degradation of autophagic vesicles (Figure 3.3 A, panel c & d).

To study the effect of CLQ on bioenergetics, the cytosolic ATP was measured in LNCaP cells treated with CLQ in CM or CFM. Interestingly, without CLQ, there was no change in the amount of ATP in cells cultured in CM and CFM (Figure 3.3 B, column c and a). However, CLQ alone decreased the cytosolic ATP by 20% in cells growing in CM (Figure 3.3 B, column b), whereas combinational treatment of CLQ and CFM decreased the cytosolic ATP level by 38% (Figure 3.3 B, column d), suggesting that autophagy dependent metabolism is more prominent in androgen deprived LNCaP cells.

3.4.3. Chloroquine induces apoptosis in LNCaP cells.

To characterize the cell death pathway during CLQ treatment, cells were incubated in CM or CFM in the presence or absence of CLQ. Cells were harvested at 36-hr time point and labeled using Annexin V-FITC and propodium iodide co-staining, and analyzed by flow cytometry. We observed that androgen deprivation or CLQ treatment alone has a little effect on apoptosis (Figure 3.4 A, panel b & d), whereas CLQ synergistically increased apoptotic cells in cells cultured in CFM (Figure 3.4 A, panel c).

المنارة للاستشارات
www.manaraa.com

To further confirm that CLQ induced apoptosis during androgen deprivation, we investigated DNA laddering and chromatin condensation, two other hallmarks of apoptosis. DNA was fragmented in laddered pattern from cells treated with CLQ and CFM (Figure 3.4 B, third lane), but not from cells treated with CLQ or androgen deprivation alone. Further, combined treatment of LNCaP cells with CLQ and androgen ablation caused significant alterations in nuclear morphology, known as nuclear condensation/fragmentation (Figure 3.4 C, panel C). The nuclei were in crescent shape and looked fragmented.

المنارة للاستشارات
www.manaraa.com

3.5. Discussion

In this study, we showed that combinational treatment of chloroquine and androgen deprivation has better efficacy in killing LNCaP cells: CLQ synergistically kills in a dose and time dependent manner with androgen deprivation in LNCaP cells. We also observed that CLQ blocked the degradation of autophagic vesicles. Furthermore, we confirmed that autophagy is important for the generation of bioenergetic precursors as CLQ treatment lowered the cytosolic ATP. Moreover, CLQ treatment enhanced the apoptosis in androgen deprived LNCaP cells, as evidenced by Annexin V-FITC/ PI analysis, DNA laddering and nuclear staining.

CLQ has been used to treat different diseases (for e.g. malaria, rheumatoid arthritis and lupus) for more than 60 years (135, 136, 137). The application of CLQ as an adjuvant in cancer therapy has emerged because it can work as a weak intercalating agent and halt the DNA repair process (139). This function has been found useful in potentiating the killing effect of radiated cancer cells (135). CLQ is a weak base that would be widely distributed in human body if administrated. In its unprotonated form, CLQ can diffuse through the plasma membrane. However, when CLQ is in the acidic organelles like late endosomes, and lysosomes, it is protonated and trapped inside the lumen of the organelles (135, 138, 140). This causes increase in the pH which inactivates the lysosomal hydrolases, and thus lysosomal function. CLQ caused inhibition of maturation of autophagic vesicles and accumulation of autolysosomes in several types of human cells (138, 140-144).

www.manaraa.com

Effects of chloroquine on maturation of autophagic vesicles are potentially important in cancer therapies where cells induce autophagy as a pro-survival mechanism. In HT-29 colorectal cancer cells, treatment with 5-fluorouracil (5-FU) induces autophagy, however, in the presence of CLQ, autophagosomal degradation is inhibited and therefore potentiates the anti-cancer effect of 5-FU (146). In another study, CLQ induces dose dependent cell death in 5 different glioblastoma cell lines independent of p53 status suggesting that CLQ also affects cell death beyond its effect in DNA repair process. Furthermore, they found that CLQ induces accumulation of autophagic vesicles in the cell lines they studied and affects the levels and subcellular distribution of Cathepsin D (147). These findings suggest that altered lysosomal function may play a role in CLQ-induced cell death. Inhibition of autophagy by CLQ also sensitizes prostate cancer cells to Src family kinase inhibitors like saracatinib and PP2 (148). It has been shown that Src kinase inhibitors induce autophagy in both LNCaP and PC3 cells. Inhibition of autophagy by CLQ, 3-MA or si-Atg7 during Src kinase inhibitors treatment, more cell death was observed.

We and others demonstrated that autophagy is induced in hormone sensitive prostate cancer cells either by incubation in androgen deficient medium or treatment of an androgen inhibitor, Casodex. Chemical inhibition of autophagy by 3-MA or genetic ablation of autophagy machinery (si-Beclin 1, si-Atg 5) causes more cell death, suggesting that autophagy protects PCa cells during androgen deprivation. We also observed that one survival mechanism of autophagy is to sequester lipid droplets and target to lysosomes for the generation of precursors

المنارة للاستشارات
www.manaraa.com

for cellular bioenergetic needs. Lysosomes are cellular digestive organelles where acidic hydrolases degrade complex biological molecules and help in recycling of biomolecules (85-87). Autophagy is well known to mediate recycling of biomolecules inside the cells during starvation. It starts with sequestration of a portion of the cytosol with organelles in a double or multilayered vesicle which then fuses with lysosomes. As CLQ alters the function of lysosomes, it inhibits autophagosomal degradation and causes accumulation of autophagic vesicles (146). Here, we showed that CLQ causes accumulation of autophagic vesicles in androgen deprived LNCaP cells. Accumulation of autophagic vesicles in LNCaP cells was also observed when cells were treated with H^+ V^+ ATPase inhibitor, Bafilomycin A1 (chapter 2). CLQ also caused accumulation of AVs in the presence of androgen, but less in the amount than in the cells grown in androgen deprived medium. This is consistent with our earlier finding that basal autophagy is active in LNCaP cells (chapter 2). Moreover, CLQ causes reduction in cytosolic ATP level, suggesting that autophagy is important for regenerating bioenergetic fuels during androgen deprivation. Interestingly, inhibition of autophagy by CLQ induces apoptosis in LNCaP cells. During apoptosis in CLQ treated LNCaP cells, there was a change in nuclear morphology and DNA fragmentation. These results are consistent with other reports that autophagy inhibition by CLQ induces cell death in a variety of cancer cell types.

Hormone ablation therapy is a major treatment modality in PCa (5). However, it relapses as hormone/castration resistant phenotype in many patients by some poorly defined mechanisms (17-21). We speculate that the effect of

المنارة للاستشارات
www.manaraa.com

hormone ablation therapy may be potentiated and synergized by inhibiting one of the cancer survival mechanisms. Chloroquine is a drug already in clinical usage, repurposing it as an adjuvant in hormone ablation therapy is practical and cost effective. Further study in pre-clinical trial is warranted in animal models of hormone sensitive PCa that is required to test the efficacy and efficiency of CLQ *in vivo*.

المنارة للاستشارات

www.manaraa.com

3.6. Acknowledgements

We thank colleagues of the fluorescence microscopy shared facility, which is supported by University of New Mexico Health Science Center and the University of New Mexico Cancer Center.

This work was supported by DOD PCRP Predoctoral Award W81XWH-08-1-0183 to RRK, and NIH/NCI Grants 5R01 CA106644 to CAH.

المنارة للاستشارات
www.manaraa.com

3.7.Figures and figure legends

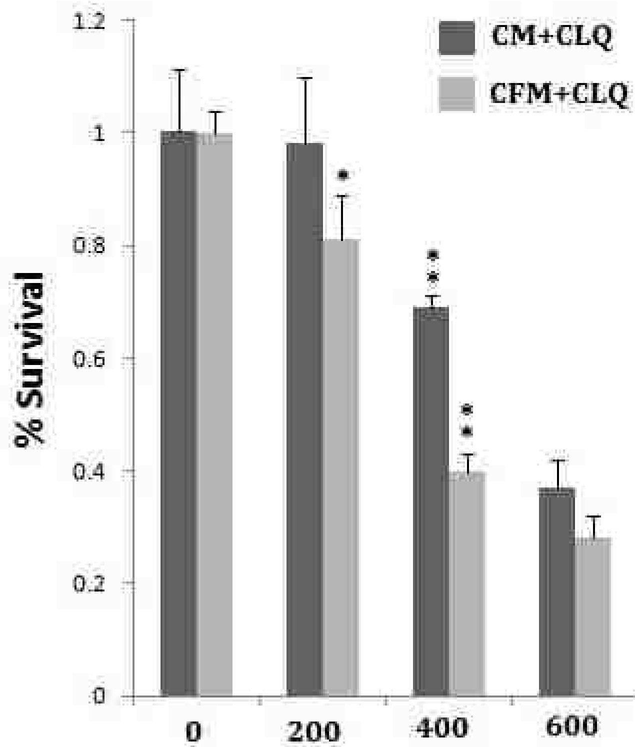


Figure 3.1: Survival effect of chloroquine (CLQ) on LNCaP cells. Cells were incubated in the presence (CM) or absence of androgen (CFM). CLQ was added at increasing concentration (0-600 uM) and treated for 24 hours. Viable cells were quantified by MTT assay.

المنارة للاستشارات
www.manaraa.com

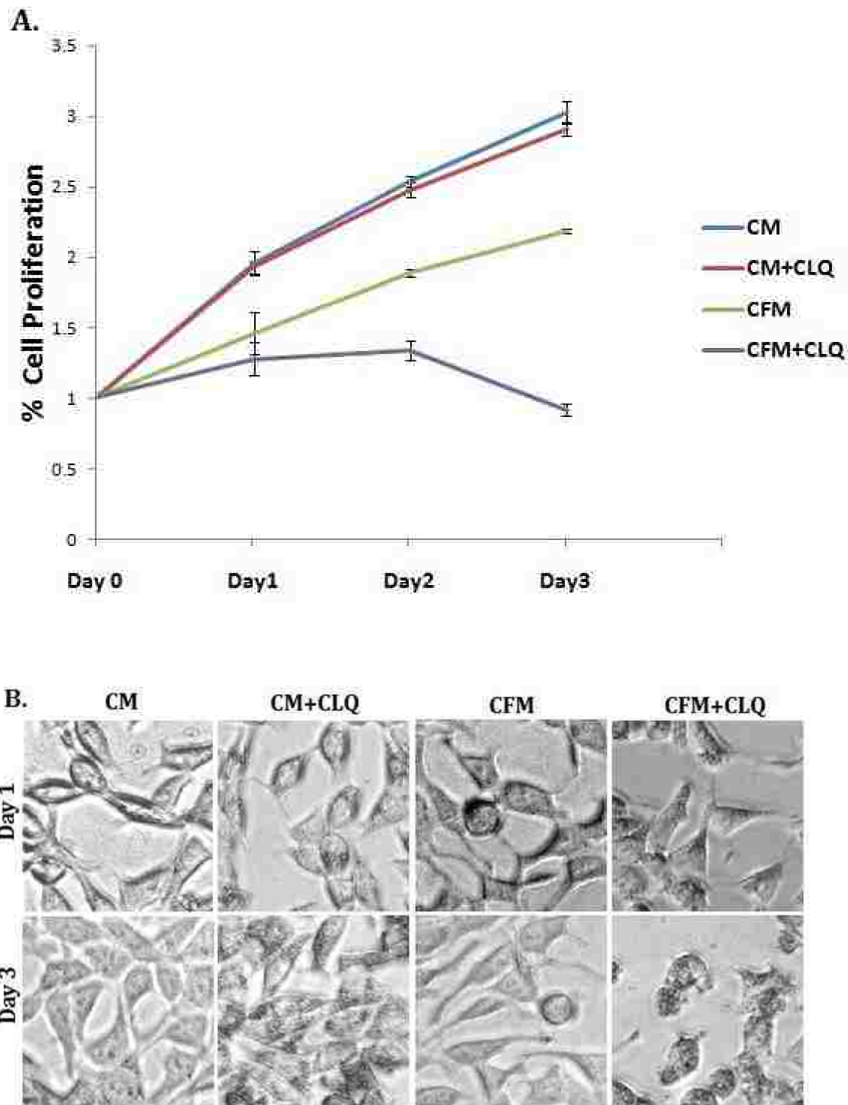


Figure 3.2: Time dependent effect of CLQ on the proliferation of LNCaP cells. A. Equal number of cells were seeded and incubated in CM or CFM. CLQ (200 μM) was added and treated for 1 day, 2 days and 3 days. Viable cells were quantified by using MTT assay at each time point. B. Microscopic view of cells from day 1 and day 3 from the same experiment.

المنارة للاستشارات
www.manaraa.com

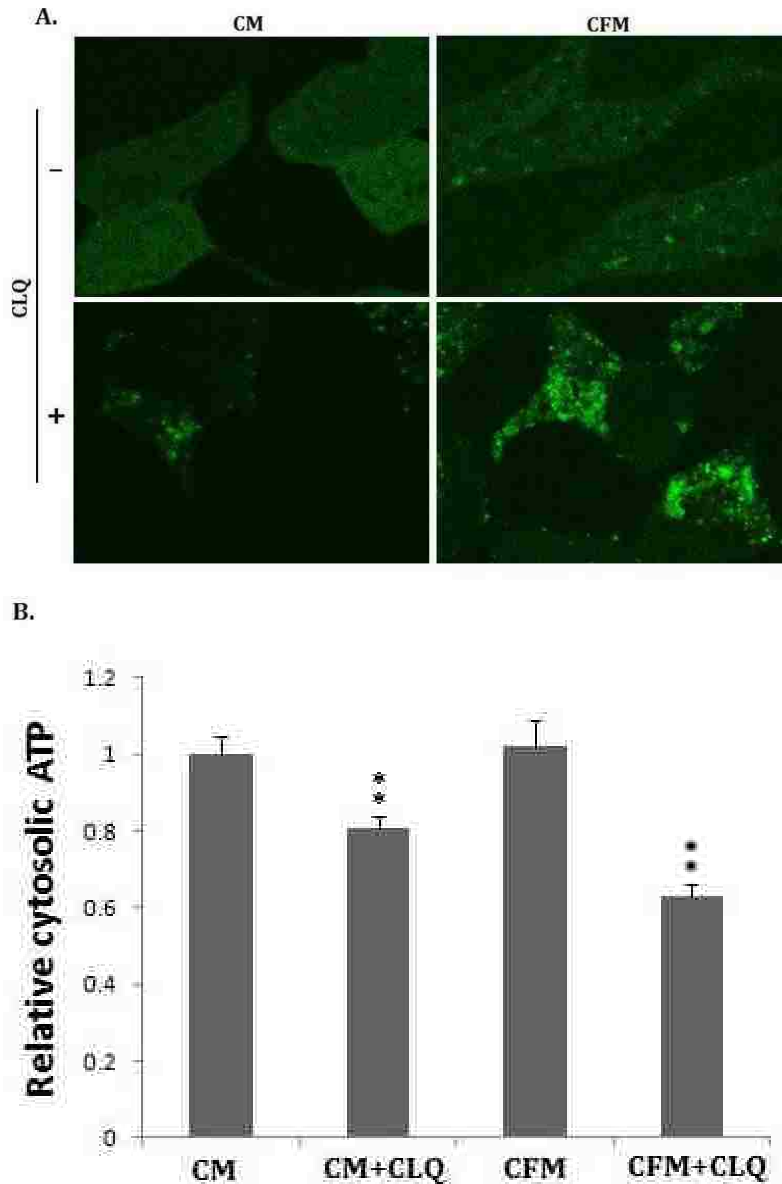


Figure 3.3: Effect of CLQ on autophagic vesicles. A. LNCaP.EGFP-LC3 cells were incubated in CM or CFM as indicated. CLQ (200 μM) was added in the medium and incubated for 24 hours. Cells were fixed and observed under confocal microscope. B. Intracellular ATP level was measured in LNCaP cells treated as above.

المنارة للاستشارات
www.manaraa.com

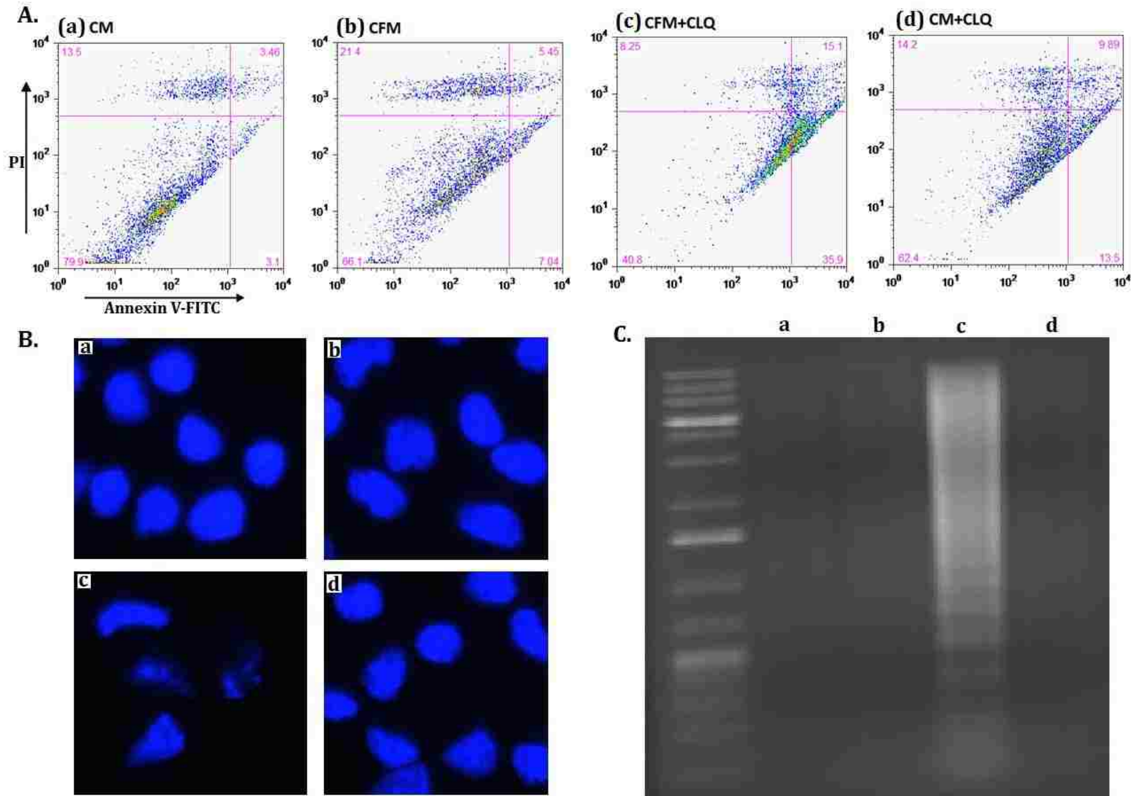


Figure 3.4: CLQ treatment induces apoptosis in LNCaP cells during androgen deprivation. A. Cells were incubated in CM or CFM and treated with chloroquine (200 µM) for 48 hours. Cells were harvested and labeled with Annexin-V-FITC and PI and analyzed by flow cytometer. The lower right quadrant of each group represents the percentage of cells undergoing apoptosis. **B.** Cells grown on coverslips were treated with CLQ (200 uM) in CM or CFM for 36 hours. The attached cells were stained with Hoechst nuclear stain and observed under microscope. **B.** DNA laddering assay of cells treated with chloroquine. Equal number of cells were seeded and treated as indicated for 36

www.manaraa.com

hours. DNA was extracted from cells floating in the medium (dead cells) and separated in 0.8% agarose gel.

المنارة للاستشارات
www.manaraa.com

Chapter 4

Discussion and Future Perspectives

المنارة للاستشارات
www.manaraa.com

4.1 Autophagy in hormone sensitive Prostate cancer cells during androgen deprivation

The function of autophagy in the pathogenesis of cancer is an exciting yet under-studied field. Autophagy can be activated during a variety of treatments in different cancer types. However, its role in the tumorigenesis and effects in various Prostate cancer (PCa) therapies are less studied.

As hormone ablation therapy is common in PCa treatment, we seek to determine the fate of autophagy in hormone sensitive PCa cells. Previously, it was shown that incubation of LNCaP (androgen sensitive), but not PC-3 cells (androgen insensitive), in serum free medium induced autophagy (49). Androgens may inhibit autophagy which is supported by the observation that addition of the androgen analogue DHT inhibited autophagy in LNCaP cells. That result was consistent with earlier reports that increased autophagic vesicles were observed in prostate epithelial cells from castrated mice (50). 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 a negative regulator of autophagy (48). Thus, we sought to further investigate the autophagy induction during androgen deprivation by using a charcoal-filtered serum containing medium (CFM). Charcoal filtration removes the steroid hormones out from the serum, but not other growth factors. We confirmed that incubation of LNCaP cells in CFM induces autophagy, which was abrogated by

المنارة للاستشارات
www.manaraa.com

the addition of androgen analogue, R1881. Increased translocation of cytosolic EGFP.LC3 to puncta-like structures was observed by confocal microscopy. Immunoblot analysis also showed increased autophagic flux during androgen deprivation. However, we did not observe autophagy induction in LNCaP cells in the presence of androgen antagonist, bicalutamide (CDX) in normal medium. It has been documented that the LNCaP cell line carries mutations in the androgen receptor (AR) gene (165, 166). Instead, CDX has been shown to stimulate the activity of mutant AR, which may be the reason that LNCaP cells were insensitive to CDX treatment.

To confirm that the induction of autophagy during androgen deprivation is not specific to LNCaP cells, we examined another hormone sensitive PCa cell line, LAPC4, which is known to have wild type AR. Immunofluorescence microscopy using an antibody against LC3 showed increased formation of autophagic puncta structures when LAPC4 cells were treated with CDX. Immunoblot analysis of LC3 expressions (ILC3II/LC3I) also showed increased autophagic flux. To further confirm this observation, we carried out Western blot analysis of p62 in LAPC4 cells. p62 is a cytosolic protein that is degraded by autophagy and is commonly used to measure the autophagic flux. Treatment of LAPC4 cells with CDX resulted in the loss of cytosolic p62. These results confirmed that autophagy is induced in two different hormone sensitive PCa cells during androgen deprivation.

Li et al have shown that inhibition of autophagy during incubation of LNCaP cells in serum free medium caused increased cell death (49)). In these

المنارة للاستشارات
www.manaraa.com

experiments, autophagy was inhibited by both pharmacological and genetic means. Addition of an androgen analog rescued the effect on cell death effect seen on incubation in serum-free medium, suggesting the protective role of autophagy during this treatment. When we treated the LNCaP cells grown in CFM with 3-MA, a significant reduction in cell number was observed compared to the non-treated group, which further confirmed the protective role of autophagy during androgen deprivation.

4.2. Autophagy regulated lipolysis in Prostate Cancer cells

One of the main goals of the present study was to elucidate the effects of autophagy on cell survival that was observed during androgen deprivation. In Chapter 2 we showed that autophagy regulates lipolysis through degradation of lipid droplets in androgen sensitive PCa cells. We first confirmed that LNCaP and LAPC4 are enriched in lipid droplets. Incubation of LNCaP cells in androgen-deprived medium significantly lowered the number of intracellular lipid droplets and triglycerides by 80%, which was reversed by the addition of the androgen analogue R1881. However, androgen inhibitor CDX had no effect on lipid droplet content in LNCaP cells. Therefore, we chose to use LAPC4 cells which carry the wild type AR, to further explore the co-relationship between autophagy and lipolysis. We observed 70% reduction in intracellular triglycerides by day 5 on treatment of LAPC4 with CDX.

To evaluate the effect of autophagy on lipolysis, we inhibited autophagy with 3-MA and quantified the intracellular levels of lipid droplets or triglycerides

www.manaraa.com

using three different methods (biochemical, biophysical and histochemical) in LNCaP cells. All three methods showed that autophagy inhibition during androgen deprivation caused partial retention of lipid droplets. Consistent with this, retention of lipid droplets was also observed when autophagy was inhibited by siRNA. To further confirm these observations, we inhibited autophagy in LAPC4 cells using 3-MA. Oil Red O staining showed that depletion of lipid droplets by CDX treatment was reversed on the addition of 3-MA. Taken together, these results suggest that autophagy plays a role in lipolysis in hormone sensitive PCa cells.

To further investigate the role of autophagy in lipid metabolism, we stained LNCaP cells stably expressing pEGFP-LC3 with the neutral lipid droplet-specific fluorescent dye, HCS LipidTOX™ Red. Confocal microscopy images showed that the green puncta from the EGFP-LC3 molecules colocalized with the red puncta from LipidTOX™ when androgen was withdrawn. The degree of colocalization was enhanced when Bafilomycin A1 was added to the medium, suggesting that autophagosomes sequester and target lipid droplets for lysosomal hydrolysis during androgen ablation in hormone sensitive PCa cells. Furthermore, quantification of triglycerides in Bafilomycin A1 treated LNCaP and LAPC4 cells showed retention of lipid droplets in both cell lines.

PCa cells have a unique intermediary metabolism (2). Contrary to many other types of solid tumors, they use lipid metabolism for their increased bioenergetic needs. Androgens play a key role in the metabolism of PCa that influences several lipogenic and lipolytic enzymes. PCa metabolism in the

المنارة للاستشارات
www.manaraa.com

absence of androgen is less studied and here we observed that PCa cells ramp up an alternate pathway of lipolysis in the absence of androgens, which could provide a survival strategy during hormone ablation therapy.

Autophagy has long been known to contribute significantly to cellular energetic balance, especially during periods of starvation, but its role in mediating lipolysis has only recently been established. Singh and colleagues reported that autophagosomes sequestered lipid droplets and target them to lysosomes for lipolysis in rat hepatocytes (83). The increased delivery of lipid droplets was observed under conditions of starvation or lipid overload. Autophagy-mediated lipolysis was also reported in adipocytes and other cell types, but not in PCa cells.

Lysosomes are vesicles that contain acidic lipases. Lipid droplets targeted to lysosomes through the autophagic machinery cause bulk lipolysis and cleave triglycerides into fatty acids. The released fatty acids can provide precursors for energy during androgen deprivation.

4.3. Effect of Chloroquine, an autophagy inhibitor, in survival and bioenergetic status of LNCaP cells.

With our increased understanding of the effects of autophagy in survival of cancer cells, modulation of autophagy as an adjunct to enhance the killing effect of existing therapeutic agents has become a topic of intense interest. We and others have shown that autophagy plays a protective role during androgen deprivation in PCa cells. We further identified that autophagy mediates lipolysis

المنارة للاستشارات
www.manaraa.com

by targeting lipid droplets to lysosomes for hydrolysis. Recently, chloroquine (CLQ) and other members of this family of drugs have been used to block autophagy by preventing autophagolysosomal degradation (135). Thus, we used CLQ for combination treatment with hormone ablation therapy in LNCaP cells and characterized the effects on cell death and bioenergetic status.

In this study, we observed that CLQ synergistically killed LNCaP cells during androgen deprivation in a dose- and time-dependent fashion. The higher the concentration, the better was the killing effect. Interestingly, treatment with CLQ for longer durations also significantly enhanced the killing effect. This is particularly important in the context that CLQ has long half life in the body (2–6 days). At 200 μM, a reduction of more than 50% reduction in cell number was observed on day 3 of androgen deprivation whereas there was no effect of CLQ in cells growing in the presence of androgen.

Confocal microscopy of CLQ treated EGFP-LC3 expressing LNCaP cells, revealed increased accumulation of puncta, which were presumably the autophagic vacuoles. Furthermore, CLQ treatment reduced the intracellular ATP level, supporting the role of autophagy in supplying energy precursors during androgen deprivation.

To characterize the cell death pathway activated during CLQ treatment, cells were labeled with Annexin V-FITC and propidium iodide and analyzed by flow cytometry. This showed that an increased percentage of cells died through apoptosis in the group co-treated with CLQ and androgen deprivation. Enhanced

المنارة للاستشارات
www.manaraa.com

apoptosis in the co-treated group was further confirmed by using a DNA ladder assay and direct observation of crescent shaped and fragmented nuclei.

CLQ has been used as a drug in clinical practice for more than 60 years and is currently widely used for prophylaxis and treatment in malaria, rheumatoid arthritis, and lupus (135-137). Recently, its application as an adjunct in cancer therapy has emerged because of its known function in cellular physiology. CLQ is a weak base and has a wide distribution in the human body. It has a tendency to accumulate in acidic vesicles such as lysosomes and neutralize the pH, which inactivates lysosomal enzymes (138, 140). This effect of CLQ is potentially important in cancer therapies where cells induce autophagy as a protective mechanism. Recently, it was observed that 5-FU induces autophagy in HT-29 colon cancer cells, and co-treatment with CLQ inhibits autophagosomal degradation and potentiates the anti-cancer effect of 5-FU (146). In another study, CLQ induced accumulation of autophagic vacuoles in 5 different glioblastoma cell lines and affected the survival of these cells (147). These findings suggest that altered lysosomal function may play a role in CLQ induced cell death. Inhibition of autophagy by CLQ also sensitizes PCa cells to Src family kinase inhibitors like saracatinib and PP2 in both LNCaP and PC3 cells (149). Moreover, combination treatments of CLQ or its analogs with many established chemotherapeutic agents are already in different phases of clinical trials. Notably, co-treatment with hydroxychloroquine and docetaxel in PCa showed a synergistic killing effect on PCa cells and is already in phase II clinical trials (135).

المنارة للاستشارات
www.manaraa.com

The effect of CLQ on autophagic degradation was similar to the effect seen with Bafilomycin A1 treatment which also increases the pH of lysosome by inhibiting the $H^+ K^+$ ATPase. In chapter 2, we showed that Bafilomycin A1 treatment of LNCaP cells caused the accumulation of autophagic vesicles and inhibited lipophagy.

4.4 Limitations and future implications

The experimental findings presented in this work imply several limitations that need to be addressed further. In this section these limitations will be explained as they pertain to each of the important findings and possible future implications will be highlighted.

4.4.1 Autophagy during androgen deprivation

Literature reviews show that the first evidence of autophagy inhibition by androgens date back to 1999 when Kwong et al. reported increased autophagic vesicles in the prostate of castrated mice (50). This observation was from the normal prostate gland. Later, Xu et al. showed that androgens activate the mTOR pathway and promote proliferation of LNCaP cells (48). As the mTOR pathway is a key regulator of autophagy, it was speculated that autophagy may be induced during androgen deprivation. In 2008, Li et al. showed that incubation of LNCaP cells in a serum-free medium induces autophagy, which is abrogated on addition of an androgen analog (49). This study further showed that androgen deprivation was also associated with low mTOR activity as evidenced by loss of phosphorylation of p70S6K (Thr421/Ser424). Consistent with these reports,

المنارة للاستشارات
www.manaraa.com

another group recently showed that incubation of LNCaP cells in a serum-free medium under hypoxic conditions activates the AMPK pathway and increases autophagy (172).

As most animal serum samples used in tissue culture medium contain several growth factors including androgens, we tried to mimic androgen-deprivation conditions by using medium containing charcoal-filtered serum. Charcoal filters out most of the androgens along with other steroid factors. Thus, a charcoal-filtered serum medium mimics the hormone ablation therapy better than the serum free medium, but still these conditions may vary from what occurs in human tissues *in vivo*. We further confirmed the inhibition of autophagy by androgens in another androgen sensitive PCa cell line, LAPC4. However, all these are *in vitro* experiments. Thus, further evaluation of autophagic activity are required either using PCa tissue samples from patients who have undergone hormone ablation therapy, or tissue samples from experimental animal models of hormone sensitive PCa cells.

Although autophagy inhibition significantly enhanced the killing of androgen-deprived PCa cells, it cannot be guaranteed that it will alter the course of PCa progression in patients. When LNCaP cells were continuously cultured in androgen-deprived medium, almost all cells were dead by the end of the third week (our observations). In human PCa patients, hormone ablation therapy causes regression of tumors. However, after an indefinite period (mostly in years), most patients show remission of PCa with an androgen/castration-independent phenotype. Studies have identified multiple mechanisms of how

المنارة للاستشارات
www.manaraa.com

PCa cells can survive in the absence of androgens, but the molecular mechanisms of progression to that stage are still elusive. One possibility is that during hormone ablation therapy, some of these cancer cells go into a dormant phase and later recommence proliferation when the environment becomes favorable. In this study, we did not directly address the effect of autophagy on progression to the androgen-independent phenotype. However, it is possible that killing the hormone sensitive PCa cells in a more effective way may lower the chance of evasion of cell death and progression to an androgen-independent phenotype.

4.4.2 Function of autophagy in lipid metabolism in hormone sensitive Prostate cancer cells

Regulation of lipolysis by autophagy, also named lipophagy, is a novel function of autophagy that was first reported in 2009 (83). Soon after, a number of cancer cell types were also reported to use the autophagic machinery for regulating lipid metabolism (84-90). In chapter 2, we characterized this novel function of autophagy in hormone-sensitive PCa cells.

As previously discussed, it is possible that our observations in cellular systems may not accurately represent cell responses *in vivo*. Lipophagy during androgen deprivation at least should be tested in autophagy-deficient prostate glands in an animal model. In addition, further studies are needed to elucidate the mechanism of lipid droplet sequestration by autophagosomes. Is it a selective process? Are there specific adaptor proteins used to target the lipid droplets to

المنارة للاستشارات
www.manaraa.com

autophagosomes? We do not have the answers, yet several lines of evidence suggest that autophagosomes may start to form on lipid droplets. Because nutrient starvation also induced lipophagy in our cell systems, it is possible that lipophagy is a common cellular stress response to altered metabolic status.

4.4.3 Chloroquine as an adjuvant in hormone ablation therapy.

In chapter 3, I showed that CLQ synergistically killed androgen-deprived LNCaP cells through apoptosis. There are several limitations in those experiments that need to be addressed before CLQ can be repurposed as an adjuvant in hormone-sensitive PCa.

In our experiments, we used 200 µM of CLQ to treat the cells. Alhough, only 15% cell death was observed after 24 hours, it was more pronounced on day 2 and day 3. As CLQ has a long half life *in vivo*, we treated the cells with CLQ for 36 hours to 48 hours to characterize the pathways activated during the treatment. There is limited research on the bioavailability of CLQ in prostate gland. Because response to CLQ is dose dependent, bioavailability in PCa cells *in vivo* is an important factor. Thus, in the future a key study will be to test the efficacy of the co-treatment of CLQ and androgen deprivation in animal models of PCa.

There are other available analogs of the CLQ family, such as hydroxychloroquine, mefloquine, quinine, quinacrine, NSC305819, NSC10010 and NSC86371. These compounds may have variable therapeutic efficacy in different cancer cell systems, and therefore they should be tested and then

المنارة للاستشارات
www.manaraa.com

compared with CLQ to identify the best adjuvant to synergize with hormone ablation therapy.

As discussed earlier, co-treatment of CLQ and androgen deprivation should be tested for its impact on progression to an androgen-independent phenotype, both *in vivo* and *in vitro*. Previously, LNCaP cells have been cultured continuously in androgen-deprived medium for several passages, and shown the formation of clones that were androgen independent. One can test how CLQ affects the generation of androgen independent clones *in vitro*, but the real impact *in vivo* can only be assessed in longitudinal clinical studies.

4.5 Summary

The present work probed the function of autophagy on lipid metabolism and survival in hormone sensitive PCa cells. Two different androgen sensitive PCa cells underwent autophagy during androgen deprivation, which is one of the standard treatments for early-stage hormone sensitive PCa. The autophagosomes generated in response to androgen removal mediated an alternative lipolytic pathway. Pharmacologic inhibition of autophagy caused retention of lipid droplets and triglycerides. Similar results were observed after genetic siRNA-based inhibition of autophagy. Furthermore, autophagosomes were observed to colocalize with lipid droplets during androgen deprivation. These findings demonstrate that, in the absence of androgens, autophagy was activated in hormone sensitive PCa cells with the consequence that lipid droplets were sequestered and targeted to the lysosomes for hydrolysis. Consistent with

المنارة للاستشارات
www.manaraa.com

this model, inhibition of autophagolysosomal degradation caused retention of intracellular triglycerides.

Autophagy induced during androgen ablation aided in survival of these cancer cells. Inhibition of autophagy caused a synergistic cell death effect during androgen deprivation in a dose- and time-dependent manner. It also caused a decrease in intracellular ATP levels. Moreover, cells died through apoptosis when autophagy was inhibited during androgen deprivation.

PCa cells are rich in lipid droplets and rely on lipid metabolism for their bioenergetic needs. The identification of lipophagy in PCa cells shows how cancer cells ramp up alternate metabolic pathways for their survival. Characterization of the effects on cell death of the autophagy inhibitor CLQ during androgen deprivation opens the possibility of repurposing CLQ as an adjunct in hormone ablation therapy in PCa.

المنارة للاستشارات
www.manaraa.com

List of abbreviations

3-MA	3-methyladenine
ACAT	Acetyl-Coenzyme A acetyltransferase
ADRP	Adipose Differentiation Related Protein
AMPK	Adenosine Monophosphate-Activated Protein Kinase
AR	Androgen receptor
ARE	Androgen response element
ATGL	Adipose Triglyceride Lipase
AVs	Autophagic vacuoles
Baf A1	Bafilomycin A1
CDX	Casodex (Bicalutamide)
CFM	Charcoal filtered serum containing medium
CLQ	Chloroquine
CM	Complete medium
CMA	Chaperone mediated autophagy
DAP1	Death associated protein 1
DGAT	Diglyceride acyltransferase
DHT	Dihydrotesteterone
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FIP 200	Focal adhesion kinase [FAK] family interacting protein
HSL	Hormone sensitive lipase
MAP-LC3	Microtubule associated protein - light chain 3

المنارة للاستشارات
www.manaraa.com

mTOR	Mammalian target of Rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI	National cancer institute
NMR	Nuclear magnetic resonance
PBS	Phosphate buffer solution
P53	Tumor protein 53
P70S6K	p70 ribosomal protein S6 kinase
PCa	Prostate cancer
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
ROS	Reactive oxygen species
SREBP	Sterol Regulatory Element-Binding Proteins
TG	Triglycerides
TIP47	Tail-interacting protein of 47 kDa
TNF-α	Tumor necrosis factor-α
TSC2	Tuberous sclerosis protein 2
Vps34	Vacuolar protein sorting protein 34

المنارة للاستشارات
www.manaraa.com

References

1. Kumar V, Abbas AK, Fausto N. Robbins & Cotran Pathologic basis of disease, 7th edition. Philadelphia: Elsevier Inc, 2005. Print

2. Costello LC, Franklin RB. The intermediary metabolism of the prostate: a key to understanding the pathogenesis and progression of prostate malignancy. Oncology. 2000 Nov;59(4):269-82

3. “Basic information about prostate cancer.” Center for disease control and prevention. n.d. Web. 12 Aug 2011. http://www.cdc.gov/uscs.

4. Patel AR, Klein EA. Risk factors for prostate cancer. Nat Clin Pract Urol. 2009 Feb;6(2):87-95.

5. Jaret P. “Your prostate cancer treatment options.” WebMD. n.d. Web. 3 Oct 2011.
http://www.webmd.com/prostate-cancer/guide/prostate-cancer-treatments.

6. “Prostate Cancer.” American cancer society. n.d. Web. 3 Oct 2011.
http://www.cancer.org/Cancer/ProstateCancer/DetailedGuide/prostate-cancer-key-statistics

7.Chodak GW, Warren KS.Watchful waiting for prostate cancer: a review article. Prostate Cancer Prostatic Dis. 2006;9(1):25-9.

8.Dehm SM, Tindall DJ. Molecular regulation of androgen action in prostate cancer. J Cell Biochem 2006;99:333-344.

9. Taplin ME, Ho SM. Clinical review 134: The endocrinology of prostate cancer. J Clin Endocrinol Metab. 2001 Aug;86(8):3467-77.

المنارة للاستشارات
www.manaraa.com

10. Brinkmann, A. O. et al. Mechanisms of androgen receptor activation and function. J. Steroid Biochem. Mol. Biol.1999. 69: 307–313.

11. Foradori CD, Weiser MJ, Handa RJ. Non-genomic actions of androgens. Front Neuroendocrinol. 2008 May;29(2):169-81.

12. Nelson PS, Clegg N, Arnold H, Ferguson C, Bonham M, White J, Hood L, Lin B. The program of androgen-responsive genes in neoplastic prostate epithelium. Proc Natl Acad Sci U S A 2002;99:11890-11895.

13. Lamont KR, Tindall DJ. Androgen regulation of gene expression. Adv Cancer Res. 2010;107:137-62.

14. DePrimo SE, Diehn M, Nelson JB, Reiter RE, Matese J, Fero M, Tibshirani R, Brown PO, Brooks JD. Transcriptional programs activated by exposure of human prostate cancer cells to androgen. Genome Biol 2002;3:RESEARCH0032.

15.Debes JD, Tindall DJ. Mechanisms of androgen-refractory prostate cancer. N Engl J Med. 2004 Oct 7;351(15):1488-90.

16. Huggins C. Endocrine-induced regression of cancers. Science. 1967 May 26;156(3778):1050-4.

17. Pienta KJ, Bradley D. Mechanisms underlying the development of androgen-independent prostate cancer. Clin Cancer Res. 2006 Mar 15; 12(6):1665-71. PubMed PMID: 16551847.

18 Cohen MB, Rokhlin OW. Mechanisms of prostate cancer cell survival after inhibition of AR expression. J Cell Biochem. 2009 Feb 15;106(3):363-71.

19. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nat Rev Cancer. 2001 Oct, 1(1):34-45.

المنارة للاستشارات

www.manaraa.com

20. Dutt SS, Gao AC. Molecular mechanisms of castration-resistant prostate cancer progression. Future Oncol. 2009 Nov; 5(9):1403-13. PubMed PMID: 19903068.

21. Bisoffi M, Klima I, Gresko E, Durfee PN, Hines WC, Griffith JK, Studer UE, Thalmann GN. Expression profiles of androgen independent bone metastatic prostate cancer cells indicate up-regulation of the putative serine-threonine kinase GS3955. J Urol. 2004 Sep;172(3):1145-50.

22. Mizushima N, Ohsumi Y, Yoshimori T. Autophagosome formation in mammalian cells. Cell Struct Funct. 2002 Dec; 27(6):421-9.

23. Yang Z, Klionsky DJ. An overview of the molecular mechanism of autophagy. Curr Top Microbiol Immunol. 2009;335:1-32.

24. Burman C, Ktistakis NT. Autophagosome formation in mammalian cells. Semin Immunopathol. 2010 Dec;32(4):397-413.

25. Shintani T, Klionsky DJ. Autophagy in health and disease: a double-edged sword. Science. 2004 Nov 5; 306(5698):990-5.

26. Monastyrska I, Klionsky DJ. Autophagy in organelle homeostasis: peroxisome turnover. Mol Aspects Med. 2006 Oct-Dec;27(5-6):483-94.

27. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008 Jan 11; 132(1):27-42.

28. Deretic V. Autophagy in infection. Curr Opin Cell Biol. 2010 Apr; 22(2):252-62.

المنارة للاستشارات

www.manaraa.com

29. Bishop NA, Lu T, Yankner BA. Neural mechanisms of ageing and cognitive decline. Nature. 2010 Mar 25; 464(7288):529-35.

30 Cao DJ, Gillette TG, Hill JA. Cardiomyocyte autophagy: remodeling, repairing, and reconstructing the heart. Curr Hypertens Rep. 2009 Dec; 11(6):406-11.

31.Shea L, Raben N. Autophagy in skeletal muscle: implications for Pompe disease. Int J Clin Pharmacol Ther. 2009; 47 Suppl 1:S42-7.

32. Levine B, Deretic V. Unveiling the roles of autophagy in innate and adaptive immunity. Nat Rev Immunol. 2007 Oct; 7(10):767-77.

33. Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci. 2003 Dec 9;100(25):15077-82.

34. Marx J. Autophagy: is it cancer's friend or foe? Science. 2006 May 26; 312(5777):1160-1.

35. Levine B. Unraveling the role of autophagy in cancer. Autophagy. 2006 Apr-Jun; 2(2):65-6.

36. Chen N, Karantza-Wadsworth V. Role and regulation of autophagy in cancer. Biochim Biophys Acta. 2009 Sep; 1793(9):1516-23.

37. Levine B. Cell biology: autophagy and cancer. Nature. 2007 Apr 12; 446(7137):745-7

38. Gozuacik D, Kimchi A. Autophagy as a cell death and tumor suppressor mechanism. Oncogene. 2004 Apr 12; 23(16):2891-906.

المنارة للاستشارات

www.manaraa.com

39. Chen HY, White E. Role of autophagy in cancer prevention. Cancer Prev Res. 2011 Jul;4(7):973-83.

40. Botti J, Djavaheri-Mergny M, Pilatte Y, Codogno P. Autophagy signaling and the cogwheels of cancer. Autophagy. 2006 Apr-Jun; 2(2):67-73.

41. Bursch W, Ellinger A, Kienzl H, Török L, Pandey S, Sikorska M, Walker R, Hermann RS. Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy. Carcinogenesis. 1996 Aug;17(8):1595-607.

42.Ciuffreda L, Di Sanza C, Incani UC, Milella M. The mTOR pathway: a new target in cancer therapy. Curr Cancer Drug Targets. 2010 Aug;10(5):484-95.

43.Azad MB, Chen Y, Gibson SB. Regulation of autophagy by reactive oxygen species (ROS): implications for cancer progression and treatment. Antioxid Redox Signal. 2009 Apr;11(4):777-90.

44. . Cao C, Subhawong T, Albert JM, Kim KW, Geng L, Sekhar KR, Gi YJ, Lu B. Inhibition of mammalian target of rapamycin or apoptotic pathway induces autophagy and radiosensitizes PTEN null prostate cancer cells. Cancer Res. 2006 Oct 15; 66(20):10040-7.

45.Wan G, Zhaorigetu S, Liu Z, Kaini R, Jiang Z, Hu CA. Apolipoprotein L1, a novel Bcl-2 homology domain 3-only lipid-binding protein, induces autophagic cell death. J Biol Chem. 2008 Aug 1;283(31):21540-9.

المنارة للاستشارات

www.manaraa.com

46.Zhaorigetu S, Wan G, Kaini R, Jiang Z, Hu CA. ApoL1, a BH3-only lipid-binding protein, induces autophagic cell death. Autophagy. 2008 Nov;4(8):1079-82.

47. Herman-Antosiewicz A, Johnson DE, Singh SV. Sulforaphane causes autophagy to inhibit release of cytochrome C and apoptosis in human prostate cancer cells. Cancer Res. 2006 Jun 1; 66(11):5828-35.

48. Xu Y, Chen SY, Ross KN, Balk SP. Androgens induce prostate cancer cell proliferation through mammalian target of rapamycin activation and post-transcriptional increases in cyclin D proteins. Cancer Res. 2006 Aug 1; 66(15):7783-92.

49.Li M, Jiang X, Liu D, Na Y, Gao GF, Xi Z. Autophagy protects LNCaP cells under androgen deprivation conditions. Autophagy. 2008 Jan 1; 4(1):54-60.

50. Kwong J, Choi HL, Huang Y, Chan FL. Ultrastructural and biochemical observations on the early changes in apoptotic epithelial cells of the rat prostate induced by castration. Cell Tissue Res. 1999 Oct; 298(1):123-36.

51.Rubinsztein DC. The roles of intracellular protein-degradation pathways in neurodegeneration. Nature. 2006 Oct 19;443(7113):780-6.

52. Yorimitsu T, Klionsky DJ. Autophagy: molecular machinery for self-eating. Cell Death Differ. 2005 Nov;12 Suppl 2:1542-52.

53. Dong H, Czaja MJ. Regulation of lipid droplets by autophagy. Trends Endocrinol Metab. 2011 Jun;22(6):234-40.

المنارة للاستشارات
www.manaraa.com

54. Schworer CM, Cox JR, Mortimore GE.Alteration of lysosomal density by sequestered glycogen during deprivation-induced autophagy in rat liver. Biochem Biophys Res Commun. 1979 Mar 15;87(1):163-70.

55.Rabinowitz JD, White E. Autophagy and metabolism. Science. 2010 Dec 3;330(6009):1344-8.

56.Chen N, Karantza V. Autophagy as a therapeutic target in cancer. Cancer Biol Ther. 2011 Jan 15;11(2):157-68.

57.Czaja MJ. Functions of autophagy in hepatic and pancreatic physiology and disease. Gastroenterology. 2011 Jun;140(7):1895-908.

58.Klionsky DJ, Cuervo AM, Seglen PO. Methods for monitoring autophagy from yeast to human. Autophagy. 2007 May-Jun;3(3):181-206.

59.Mijaljica D, Prescott M, Devenish RJ. Microautophagy in mammalian cells: revisiting a 40-year-old conundrum. Autophagy. 2011 Jul;7(7):673-82.

60. Li W, Yang Q, Mao Z.Chaperone-mediated autophagy: machinery, regulation and biological consequences. Cell Mol Life Sci. 2011 Mar;68(5):749-63.

61. Weidberg H, Shvets E, Elazar Z. Biogenesis and cargo selectivity of autophagosomes. Annu Rev Biochem. 2011 Jun 7;80:125-56.

62.Bjørkøy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H, Johansen T. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J Cell Biol. 2005 Nov 21; 171(4):603-14.

المنارة للاستشارات

www.manaraa.com

63.Kirkin V, Lamark T, Sou YS, Bjørkøy G, Nunn JL, Bruun JA, Shvets E, McEwan DG, Clausen TH, Wild P, Bilusic I, Theurillat JP, Øvervatn A, Ishii T, Elazar Z, Komatsu M, Dikic I,Johansen T. A role for NBR1 in autophagosomal degradation of ubiquitinated substrates. Mol Cell. 2009 Feb 27;33(4):505-16.

64. Meijer AJ, Codogno P. Regulation and role of autophagy in mammalian cells. Int J Biochem Cell Biol. 2004 Dec;36(12):2445-62.

65. Hosokawa N, Hara T, Kaizuka T, Kishi C, Takamura A, Miura Y, Iemura S, Natsume T, Takehana K, Yamada N, Guan JL, Oshiro N, Mizushima N. Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. Mol Biol Cell. 2009 Apr;20(7):1981-91.

66. Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, Kundu M, Kim DH. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol Biol Cell. 2009 Apr;20(7):1992-2003.

67.Koren I, Reem E, Kimchi A. DAP1, a novel substrate of mTOR, negatively regulates autophagy. Curr Biol. 2010 Jun 22;20(12):1093-8

68.Hardie DG. AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. Genes Dev. 2011 Sep 15;25(18):1895-908.

69.Carling D, Mayer FV, Sanders MJ, Gamblin SJ. AMP-activated protein kinase: nature's energy sensor. Nat Chem Biol. 2011 Jul 18;7(8):512-8.

70.Samari HR, Seglen PO. Inhibition of hepatocytic autophagy by adenosine, aminoimidazole-4-carboxamide riboside, and N6-mercaptopurine riboside.

المنارة للاستشارات

www.manaraa.com

Evidence for involvement of amp-activated protein kinase. J Biol Chem. 1998 Sep 11;273(37):23758-63.

71. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell. 2003 Nov 26;115(5):577-90.

72.Vakana E, Altman JK, Platanias LC. Targeting AMPK in the treatment of malignancies. J Cell Biochem. 2011 Sep 16. doi: 10.1002/jcb.23369. [Epub ahead of print]

73.Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell. 2004 Apr;6(4):463-77.

74.Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, Baba M, Baehrecke EH, Bahr BA, Ballabio A, Bamber BA, Bassham DC, Bergamini E, Bi X, Biard-Piechaczyk M, Blum JS, Bredesen DE, Brodsky JL, Brumell JH, Brunk UT, Bursch W, Camougrand N, Cebollero E, Cecconi F, Chen Y, Chin LS, Choi A, Chu CT, Chung J,Clarke PG, Clark RS, Clarke SG, Clavé C, Cleveland JL, Codogno P, Colombo MI, Coto-Montes A, Cregg JM, Cuervo AM, Debnath J, Demarchi F, Dennis PB, Dennis PA,Deretic V, Devenish RJ, Di Sano F, Dice JF, Difiglia M, Dinesh-Kumar S, Distelhorst CW, Djavaheri-Mergny M, Dorsey FC, Dröge W, Dron M, Dunn WA Jr, Duszenko M, Eissa NT, Elazar Z, Esclatine A, Eskelinen EL, Fésüs L, Finley KD, Fuentes JM, Fueyo J, Fujisaki K, Galliot B, Gao FB, Gewirtz DA, Gibson SB, Gohla A, Goldberg AL, Gonzalez R,González-Estévez C, Gorski S, Gottlieb RA, Häussinger D, He YW, Heidenreich K, Hill JA, Høyer-Hansen M, Hu X, Huang WP, Iwasaki A, Jäättelä M, Jackson WT, Jiang X, Jin S, Johansen T, Jung JU, Kadowaki M, Kang C, Kelekar A, Kessel DH, Kiel

المنارة للاستشارات

www.manaraa.com

JA, Kim HP, Kimchi A, Kinsella TJ, Kiselyov K, Kitamoto K, Knecht E, Komatsu M, Kominami E,Kondo S, Kovács AL, Kroemer G, Kuan CY, Kumar R, Kundu M, Landry J, Laporte M, Le W, Lei HY, Lenardo MJ, Levine B, Lieberman A, Lim KL, Lin FC, Liou W, Liu LF, Lopez-Berestein G, López-Otín C, Lu B, Macleod KF, Malorni W, Martinet W, Matsuoka K, Mautner J, Meijer AJ, Meléndez A, Michels P, Miotto G, Mistiaen WP, Mizushima N, Mograbi B,Monastyrska I, Moore MN, Moreira PI, Moriyasu Y, Motyl T, Münz C, Murphy LO, Naqvi NI, Neufeld TP, Nishino I, Nixon RA, Noda T, Nürnberg B, Ogawa M, Oleinick NL, Olsen LJ, Ozpolat B, Paglin S, Palmer GE, Papassideri I, Parkes M, Perlmutter DH, Perry G, Piacentini M, Pinkas-Kramarski R, Prescott M, Proikas-Cezanne T, Raben N, Rami A,Reggiori F, Rohrer B, Rubinsztein DC, Ryan KM, Sadoshima J, Sakagami H, Sakai Y, Sandri M, Sasakawa C, Sass M, Schneider C, Seglen PO, Seleverstov O, Settleman J,Shacka JJ, Shapiro IM, Sibirny A, Silva-Zacarin EC, Simon HU, Simone C, Simonsen A, Smith MA, Spanel-Borowski K, Srinivas V, Steeves M, Stenmark H, Stromhaug PE,Subauste CS, Sugimoto S, Sulzer D, Suzuki T, Swanson MS, Tabas I, Takeshita F, Talbot NJ, Tallóczy Z, Tanaka K, Tanaka K, Tanida I, Taylor GS, Taylor JP, Terman A,Tettamanti G, Thompson CB, Thumm M, Tolkovsky AM, Tooze SA, Truant R, Tumanovska LV, Uchiyama Y, Ueno T, Uzcátegui NL, van der Klei I, Vaquero EC, Vellai T, Vogel MW, Wang HG, Webster P, Wiley JW, Xi Z, Xiao G, Yahalom J, Yang JM, Yap G, Yin XM, Yoshimori T, Yu L, Yue Z, Yuzaki M, Zabirnyk O, Zheng X, Zhu X, Deter RL. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy. 2008 Feb;4(2):151-75.

المنارة للاستشارات
www.manaraa.com

75.Grinde B, Seglen PO. Effects of amino acids and amino acid analogues on lysosomal protein degradation in isolated rat hepatocytes. Acta Biol Med Ger. 1981;40(10-11):1603-12.

76.Kotoulas OB, Kalamidas SA, Kondomerkos DJ. Glycogen autophagy in glucose homeostasis. Pathol Res Pract. 2006;202(9):631-8

77. Shea L, Raben N. Autophagy in skeletal muscle: implications for Pompe disease. Int J Clin Pharmacol Ther. 2009;47 Suppl 1:S42-7.

78.Beller M, Thiel K, Thul PJ, Jäckle H. Lipid droplets: a dynamic organelle moves into focus. FEBS Lett. 2010 Jun 3;584(11):2176-82.

79.Krahmer N, Guo Y, Farese RV Jr, Walther TC. SnapShot: Lipid Droplets. Cell. 2009 Nov 25;139(5):1024-1024.e1.

80. Ducharme NA, Bickel PE. Lipid droplets in lipogenesis and lipolysis. Endocrinology. 2008 Mar;149(3):942-9.

81.Brasaemle DL. Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipiddroplets and control of lipolysis. J Lipid Res. 2007 Dec;48(12):2547-59.

82. Slavin BG, Ong JM, Kern PA. Hormonal regulation of hormone-sensitive lipase activity and mRNA levels in isolated rat adipocytes. J Lipid Res. 1994 Sep;35(9):1535-41.

83.Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, Tanaka K, Cuervo AM, Czaja MJ. Autophagy regulates lipid metabolism. Nature. 2009 Apr 30; 458(7242):1131-5.

المنارة للاستشارات
www.manaraa.com

84. Weidberg H, Shvets E, Elazar Z. Lipophagy: selective catabolism designed for lipids. Dev Cell. 2009 May; 16(5):628-30.

85. Czaja MJ. Autophagy in health and disease: 2. Regulation of lipid metabolism and storage by autophagy: pathophysiological implications. Am J Physiol Cell Physiol. 2010 May;298(5):C973-8.

86.Dong H, Czaja MJ. Regulation of lipid droplets by autophagy. Trends Endocrinol Metab. 2011 Jun;22(6):234-40.

87.Singh R, Cuervo AM. Autophagy in the cellular energetic balance. Cell Metab. 2011 May 4;13(5):495-504.

88.Rodriguez-Navarro JA, Cuervo AM. Autophagy and lipids: tightening the knot. Semin Immunopathol. 2010 Dec;32(4):343-53.

89.Kovsan J, Bashan N, Greenberg AS, Rudich A. Potential role of autophagy in modulation of lipid metabolism. Am J Physiol Endocrinol Metab. 2010 Jan;298(1):E1-7.

90.Ding WX, Manley S, Ni HM. The emerging role of autophagy in alcoholic liver disease. Exp Biol Med (Maywood). 2011 May 1;236(5):546-56.

91. Heeren J, Beisiegel U. Intracellular metabolism of triglyceride-rich lipoproteins. Curr Opin Lipidol. 2001 Jun;12(3):255-60.

92. Czaja MJ, Cuervo AM. Lipases in lysosomes, what for? Autophagy. 2009 Aug; 5(6):866-7.

المنارة للاستشارات

www.manaraa.com

93.Swinnen JV, Heemers H, van de Sande T, de SE, Brusselmans K, Heyns W, Verhoeven G. Androgens, lipogenesis and prostate cancer. J Steroid Biochem Mol Biol 2004;92:273-279.

94.Liu Y. Fatty acid oxidation is a dominant bioenergetic pathway in prostate cancer. Prostate Cancer Prostatic Dis 2006;9:230-234.

95. Migita T, Ruiz S, Fornari A, Fiorentino M, Priolo C, Zadra G, Inazuka F, Grisanzio C, Palescandolo E, Shin E, Fiore C, Xie W, Kung AL, Febbo PG, Subramanian A, Mucci L, Ma J,Signoretti S, Stampfer M, Hahn WC, Finn S, Loda M. Fatty acid synthase: a metabolic enzyme and candidate oncogene in prostate cancer. J Natl Cancer Inst. 2009 Apr 1;101(7):519-32.

96. Baron A, Migita T, Tang D, Loda M. Fatty acid synthase: a metabolic oncogene in prostate cancer? J Cell Biochem. 2004 Jan 1;91(1):47-53.

97. Garber K. Energy deregulation: licensing tumors to grow. Science 2006;312:1158-1159.

98.Malhi H, Gores GJ. Cancer therapy: back to metabolism. Cancer Biol Ther. 2006 Aug;5(8):986-7.

99.Warburg O. On the origin of cancer cells. Science 1956;123:309-314.

100.Koppenol WH, Bounds PL, Dang CV. Otto Warburg's contributions to current concepts of cancer metabolism. Nat Rev Cancer. 2011 May;11(5):325-37.

101.DeBerardinis RJ. Is cancer a disease of abnormal cellular metabolism? New angles on an old idea. Genet Med. 2008 Nov;10(11):767-77.

102.Kim JW, Dang CV. Cancer's molecular sweet tooth and the Warburg effect. Cancer Res. 2006 Sep 15;66(18):8927-30.

المنارة للاستشارات

www.manaraa.com

103. Swinnen JV, Verhoeven G. Androgens and the control of lipid metabolism in human prostate cancer cells. J Steroid Biochem Mol Biol 1998;65:191-198.

104. Corona G, Baldi E, Maggi M. Androgen regulation of prostate cancer: where are we now? J Endocrinol Invest. 2011 Mar;34(3):232-43.

105.Heemers H, Maes B, Foufelle F, Heyns W, Verhoeven G, Swinnen JV. Androgens stimulate lipogenic gene expression in prostate cancer cells by activation of the sterol regulatory element-binding protein cleavage activating protein/sterol regulatory element-binding protein pathway. Mol Endocrinol. 2001 Oct;15(10):1817-28.

106. Luo J, Duggan DJ, Chen Y, Sauvageot J, Ewing CM, Bittner ML, Trent JM, Isaacs WB. Human prostate cancer and benign prostatic hyperplasia: molecular dissection by gene expression profiling. Cancer Res. 2001 Jun 15;61(12):4683-8.

107. Costello LC, Franklin RB. The intermediary metabolism of the prostate: a key to understanding the pathogenesis and progression of prostate malignancy. Oncology 2000;59:269-282.

108. Costello LC, Franklin RB. The clinical relevance of the metabolism of prostate cancer; zinc and tumor suppression: connecting the dots. Mol Cancer. 2006 May 15;5:17.

109. Costello LC, Franklin RB. Bioenergetic theory of prostate malignancy. Prostate. 1994 Sep;25(3):162-6.

المنارة للاستشارات

www.manaraa.com

110.Costello LC, Franklin RB, Feng P. Mitochondrial function, zinc, and intermediary metabolism relationships in normal prostate and prostate cancer. Mitochondrion. 2005 Jun;5(3):143-53.

111. Singh KK, Desouki MM, Franklin RB, Costello LC. Mitochondrial aconitase and citrate metabolism in malignant and nonmalignant human prostate tissues. Mol Cancer. 2006 Apr 4;5:14.

112. Costello LC, Franklin R, Stacey R. Mitochondrial isocitrate dehydrogenase and isocitrate oxidation of rat ventral prostate. Enzyme. 1976;21(6):495-506.

113. Huang H, Tindall DJ. The role of the androgen receptor in prostate cancer. Crit Rev Eukaryot Gene Expr. 2002;12(3):193-207.

114. Swinnen JV, Ulrix W, Heyns W, Verhoeven G. Coordinate regulation of lipogenic gene expression by androgens: evidence for a cascade mechanism involving sterol regulatory element binding proteins. Proc Natl Acad Sci U S A. 1997 Nov 25;94(24):12975-80.

115.Shimano H, Yahagi N, Amemiya-Kudo M, Hasty AH, Osuga J, Tamura Y, Shionoiri F, Iizuka Y, Ohashi K, Harada K, Gotoda T, Ishibashi S, Yamada N. Sterol regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes. J Biol Chem. 1999 Dec 10;274(50):35832-9.

116. Heemers H, Vanderhoydonc F, Roskams T, Shechter I, Heyns W, Verhoeven G, Swinnen JV. Androgens stimulate coordinated lipogenic gene expression in normal target tissues in vivo. Mol Cell Endocrinol. 2003 Jul 31;205(1-2):21-31.

المنارة للاستشارات

www.manaraa.com

117. Jocken JW, Blaak EE. Catecholamine-induced lipolysis in adipose tissue and skeletal muscle in obesity. Physiol Behav. 2008 May 23;94(2):219-30.

118. Zha S, Ferdinandusse S, Hicks JL, Denis S, Dunn TA, Wanders RJ, Luo J, De Marzo AM, Isaacs WB. Peroxisomal branched chain fatty acid beta-oxidation pathway is upregulated in prostate cancer. Prostate. 2005 Jun 1;63(4):316-23.

119. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646-74.

120. Tanida I. Autophagosome formation and molecular mechanism of autophagy. Antioxid Redox Signal. 2011 Jun;14(11):2201-14.

121.Liang C, E X, Jung JU. Downregulation of autophagy by herpesvirus Bcl-2 homologs. Autophagy. 2008 Apr;4(3):268-72

122. Wei Y, Pattingre S, Sinha S, Bassik M, Levine B. JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. Mol Cell. 2008 Jun 20;30(6):678-88.

123. Fimia GM, Stoykova A, Romagnoli A, Giunta L, Di Bartolomeo S, Nardacci R, Corazzari M, Fuoco C, Ucar A, Schwartz P, Gruss P, Piacentini M, Chowdhury K, Cecconi F. Ambra1 regulates autophagy and development of the nervous system. Nature. 2007 Jun 28;447(7148):1121-5.

124. Takahashi Y, Coppola D, Matsushita N, Cualing HD, Sun M, Sato Y, Liang C, Jung JU, Cheng JQ, Mulé JJ, Pledger WJ, Wang HG. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. Nat Cell Biol. 2007 Oct;9(10):1142-51.

المنارة للاستشارات
www.manaraa.com

125. Itakura E, Mizushima N. Atg14 and UVRAG: mutually exclusive subunits of mammalian Beclin 1-PI3K complexes. Autophagy. 2009 May;5(4):534-6.

126. Tanida I, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol. 2004 Dec;36(12):2503-18.

127. Tanida I, Sou YS, Ezaki J, Minematsu-Ikeguchi N, Ueno T, Kominami E. HsAtg4B/HsApg4B/autophagin-1 cleaves the carboxyl termini of three human Atg8 homologues and delipidates microtubule-associated protein light chain 3- and GABAA receptor-associated protein-phospholipid conjugates. J Biol Chem. 2004 Aug 27;279(35):36268-76

128. Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. Autophagy. 2007 Nov-Dec;3(6):542-5.

129. Schweers RL, Zhang J, Randall MS, Loyd MR, Li W, Dorsey FC, Kundu M, Opferman JT, Cleveland JL, Miller JL, Ney PA. NIX is required for programmed mitochondrial clearance during reticulocyte maturation. Proc Natl Acad Sci U S A. 2007 Dec 4;104(49):19500-5.

130. Clausen TH, Lamark T, Isakson P, Finley K, Larsen KB, Brech A, Øvervatn A, Stenmark H, Bjørkøy G, Simonsen A, Johansen T. p62/SQSTM1 and ALFY interact to facilitate the formation of p62 bodies/ALIS and their degradation by autophagy. Autophagy. 2010 Apr;6(3):330-44.

131. Yamamoto A, Tagawa Y, Yoshimori T, Moriyama Y, Masaki R, Tashiro Y. Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. Cell Struct Funct. 1998 Feb;23(1):33-42.

المنارة للاستشارات
www.manaraa.com

132. Livesey KM, Tang D, Zeh HJ, Lotze MT. Autophagy inhibition in combination cancer treatment. Curr Opin Investig Drugs. 2009 Dec;10(12):1269-79.

133. Zhong Y, Wang QJ, Li X, Yan Y, Backer JM, Chait BT, Heintz N, Yue Z. Distinct regulation of autophagic activity by Atg14L and Rubicon associated with Beclin 1-phosphatidylinositol-3-kinasecomplex. Nat Cell Biol. 2009 Apr;11(4):468-76.

134. Matsunaga K, Saitoh T, Tabata K, Omori H, Satoh T, Kurotori N, Maejima I, Shirahama-Noda K, Ichimura T, Isobe T, Akira S, Noda T, Yoshimori T. Two Beclin 1binding proteins, Atg14L and Rubicon, reciprocally regulate autophagy at different stages. Nat Cell Biol. 2009 Apr;11(4):385-96.

135.Solomon VR, Lee H. Chloroquine and its analogs: a new promise of an old drug for effective and safe cancer therapies. Eur J Pharmacol. 2009 Dec 25;625(1-3):220-33.

136. Meinão IM, Sato EI, Andrade LE, Ferraz MB, Atra E. Controlled trial with chloroquine diphosphate in systemic lupus erythematosus. Lupus. 1996 Jun;5(3):237-41.

137. Haydu GG. Rheumatoid arthritis therapy; a rationale and the use of chloroquine diphosphate. Am J Med Sci. 1953 Jan;225(1):71-5.

138. Chen PM, Gombart ZJ, Chen JW. Chloroquine treatment of ARPE-19 cells leads to lysosome dilation and intracellular lipid accumulation: possible implications of lysosomal dysfunction in macular degeneration. Cell Biosci. 2011 Mar 8;1(1):10.

المنارة للاستشارات

www.manaraa.com

139. Meshnick SR. Chloroquine as intercalator: a hypothesis revived. Parasitol Today. 1990 Mar;6(3):77-9.

140. Yoon YH, Cho KS, Hwang JJ, Lee SJ, Choi JA, Koh JY. Induction of lysosomal dilatation, arrested autophagy, and cell death by chloroquine in cultured ARPE-19 cells. Invest Ophthalmol Vis Sci. 2010 Nov;51(11):6030-7.

141. Ramser B, Kokot A, Metze D, Weiss N, Luger TA, Böhm M. Hydroxychloroquine modulates metabolic activity and proliferation and induces autophagic cell death of human dermal fibroblasts. J Invest Dermatol. 2009 Oct;129(10):2419-26.

142. Jones CJ, Jayson MI. Chloroquine: its effect on leucocyte auto- and heterophagocytosis. Ann Rheum Dis. 1984 Apr;43(2):205-12.

143. Trout JJ, Stauber WT, Schottelius BA. Increased autophagy in chloroquine-treated tonic and phasic muscles: an alternative view. Tissue Cell. 1981;13(2):393-401.

144. Oikarinen A. Hydroxychloroquine induces autophagic cell death of human dermal fibroblasts: implications for treating fibrotic skin diseases.J Invest Dermatol. 2009 Oct;129(10):2333-5.

145.Wang Y, Peng RQ, Li DD, Ding Y, Wu XQ, Zeng YX, Zhu XF, Zhang XS. Chloroquine enhances the cytotoxicity of topotecan by inhibiting autophagy in lung cancer cells. Chin J Cancer. 2011 Oct;30(10):690-700.

146. Sasaki K, Tsuno NH, Sunami E, Tsurita G, Kawai K, Okaji Y, Nishikawa T, Shuno Y, Hongo K, Hiyoshi M, Kaneko M, Kitayama J, Takahashi K, Nagawa H.

المنارة للاستشارات

www.manaraa.com

Chloroquine potentiates the anti-cancer effect of 5-fluorouracil on colon cancer cells. BMC Cancer. 2010 Jul 15;10:370.

147. Geng Y, Kohli L, Klocke BJ, Roth KA. Chloroquine-induced autophagic vacuole accumulation and cell death in glioma cells is p53 independent. Neuro Oncol. 2010 May;12(5):473-81.

148. Wu Z, Chang PC, Yang JC, Chu CY, Wang LY, Chen NT, Ma AH, Desai SJ, Lo SH, Evans CP, Lam KS, Kung HJ. Autophagy Blockade Sensitizes Prostate Cancer Cells towards Src Family Kinase Inhibitors. Genes Cancer. 2010 Jan;1(1):40-9.

149. Swinnen JV, Esquenet M, Goossens K, Heyns W, Verhoeven G. Androgens stimulate fatty acid synthase in the human prostate cancer cell line LNCaP. Cancer Res 1997;57:1086-1090.

150. Swinnen JV, Van Veldhoven PP, Esquenet M, Heyns W, Verhoeven G. Androgens markedly stimulate the accumulation of neutral lipids in the human prostatic adenocarcinoma cell line LNCaP. Endocrinology 1996;137:4468-4474.

151. Averna TA, Kline EE, Smith AY, Sillerud LO. A decrease in 1H nuclear magnetic resonance spectroscopically determined citrate in human seminal fluid accompanies the development of prostate adenocarcinoma. J Urol 2005;173:433-438.

152. Nelson PS, Clegg N, Arnold H, Ferguson C, Bonham M, White J, Hood L, Lin B. The program of androgen-responsive genes in neoplastic prostate epithelium. Proc Natl Acad Sci U S A 2002;99:11890-11895.

المنارة للاستشارات

www.manaraa.com

153. Szende B, Romics I, Vass L. Apoptosis in prostate cancer after hormonal treatment. Lancet 1993;342:1422.

154. Denmeade SR, Lin XS, Isaacs JT. Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. Prostate 1996;28:251-265.

155. Levine B, Mizushima N & Virgin HW (2011) Autophagy in immunity and inflammation. Nature 469, 323-335

156. Ravikumar, B., Sarkar, S., Davies, J. E., Futter, M., Garcia-Arencibia, M., Green-Thompson, Z. W., Jimenez-Sanchez, M., Korolchuk, V. I., Lichtenberg, M., Luo, S., Massey, D. C., Menzies, F. M., Moreau, K., Narayanan, U., Renna, M., Siddiqi, F. H., Underwood, B. R., Winslow, A. R., and Rubinsztein, D. C. Regulation of mammalian autophagy in physiology and pathophysiology. (2010) Physiol Rev 90, 1383-1435

157. Bennett HL, Fleming JT, O'Prey J, Ryan KM, Leung HY. Androgens modulate autophagy and cell death via regulation of the endoplasmic reticulum chaperone glucose-regulated protein 78/BiP in prostate cancer cells. Cell Death Dis 2010;1:e72.

158. Farese RV, Jr., Walther TC. Lipid droplets finally get a little R-E-S-P-E-C-T. Cell 2009;139:855-860.

159. Liu Z, Lu H, Shi H, Du Y, Yu J, Gu S, Chen X, Liu KJ, Hu CA. PUMA overexpression induces reactive oxygen species generation and proteasome-mediated stathmin degradation in colorectal cancer cells. Cancer Res 2005;65:1647-1654.

المنارة للاستشارات
www.manaraa.com

160. Thompson TA, Wilding G. Androgen antagonist activity by the antioxidant moiety of vitamin E, 2,2,5,7,8-pentamethyl-6-chromanol in human prostate carcinoma cells. Mol Cancer Ther 2003;2:797-803.

161. Sobel RE, Sadar MD. Cell lines used in prostate cancer research: a compendium of old and new lines--part 2. J Urol 2005;173:360-372.

162. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497-509.

163. Kim PK, Hailey DW, Mullen RT, Lippincott-Schwartz J. Ubiquitin signals autophagic degradation of cytosolic proteins and peroxisomes. Proc Natl Acad Sci U S A 2008;105:20567-20574.

164. Ichimura Y, Komatsu M. Selective degradation of p62 by autophagy. Semin Immunopathol 2010;32:431-436.

165. Brinkmann AO, Kuiper GG, Ris-Stalpers C, van Rooij HC, Romalo G, Trifiro M, Mulder E, Pinsky L, Schweikert HU, Trapman J. Androgen receptor abnormalities. J Steroid Biochem Mol Biol 1991;40:349-352.

166. Hara T, Miyazaki J, Araki H, Yamaoka M, Kanzaki N, Kusaka M, Miyamoto M. Novel mutations of androgen receptor: a possible mechanism of bicalutamide withdrawal syndrome. Cancer Res 2003;63:149-153.

167. Devlin HL, Mudryj M. Progression of prostate cancer: multiple pathways to androgen independence. Cancer Lett 2009;274:177-186.

168. Mehrpour M, Esclatine A, Beau I, Codogno P. Overview of macroautophagy regulation in mammalian cells. Cell Res 2010;20:748-762.

المنارة للاستشارات
www.manaraa.com

169. Singh G, Lakkis CL, Laucirica R, Epner DE. Regulation of prostate cancer cell division by glucose. J Cell Physiol 1999;180:431-438.

170. Espina V, Mariani BD, Gallagher RI, Tran K, Banks S, Wiedemann J, Huryk H, Mueller C, Adamo L, Deng J, Petricoin EF, Pastore L, Zaman S, Menezes G, Mize J, Johal J, Edmiston K, Liotta LA. Malignant precursor cells pre-exist in human breast DCIS and require autophagy for survival. PLoS One. 2010 Apr 20;5(4):e10240.

171.Sotelo J, Briceño E, López-González MA. Adding chloroquine to conventional treatment for glioblastoma multiforme: a randomized, double-blind, placebo-controlled trial. Ann Intern Med. 2006 Mar 7;144(5):337-43.

172.Chhipa RR, Wu Y, Ip C. AMPK-mediated autophagy is a survival mechanism in androgen-dependent prostate cancer cells subjected to androgen deprivation and hypoxia. Cell Signal. 2011 Sep;23(9):1466-72.

المنارة للاستشارات

www.manaraa.com