Differential Regulation of Autophagy by Therapeutic and Physiologic Glucose Restriction under Normoxia and Hypoxia

Haibin Xi

University of Miami, xhbkirby@hotmail.com

Follow this and additional works at: https://scholarlyrepository.miami.edu/oa_dissertations

Recommended Citation
Xi, Haibin, "Differential Regulation of Autophagy by Therapeutic and Physiologic Glucose Restriction under Normoxia and Hypoxia" (2012). Open Access Dissertations. 870.
https://scholarlyrepository.miami.edu/oa_dissertations/870
UNIVERSITY OF MIAMI

DIFFERENTIAL REGULATION OF AUTOPHAGY BY THERAPEUTIC AND PHYSIOLOGIC GLUCOSE RESTRICTION UNDER NORMOXIA AND HYPOXIA

By

Haibin Xi

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

December 2012
DIFFERENTIAL REGULATION OF AUTOPHAGY BY THERAPEUTIC AND PHYSIOLOGIC GLUCOSE RESTRICTION UNDER NORMOXIA AND HYPOXIA

Haibin Xi

Approved:

Theodore J. Lampidis, Ph.D.  M. Brian Blake, Ph.D.
Professor of Cell Biology  Dean of the Graduate School

Niramol Savaraj, M.D.  Carlos T. Moraes, Ph.D.
Professor of Medicine  Professor of Neurology and Cell Biology

Mansoor M. Ahmed, Ph.D.  Julio C. Barredo, M.D.
Program Director  Professor of Pediatrics
Radiotherapy Development Branch
Radiation Research Program
Division of Cancer Treatment and Diagnosis
National Cancer Institute
Advances in our understanding of tumor biology achieved in the past decade have convincingly revealed the coupling of oncogenic transformation with increased glucose utilization, also known as aerobic glycolysis or the “Warburg effect”, that was observed more than 80 years ago. Although the altered glucose metabolism renders tumor cells selective growth and survival advantages, it also creates a therapeutic window to target cancer using extrinsic sugar analogs such as 2-deoxyglucose (2-DG). On the other hand, certain malignant cell populations inevitably face glucose starvation (GS) intrinsically arising as a solid tumor grows and the nutrient demand exceeds supply.

In order to overcome the deleterious effects of both therapeutic (2-DG) and physiologic (GS) glucose restriction, tumor cells mount an evolutionarily conserved intracellular bulk degradation process called autophagy, among others, for adaptation and survival under these metabolic stresses. Therefore, delineating the precise role and regulation of autophagy in response to therapeutic as well as physiologic glucose restriction is critical not only for a better understanding of the biology of cancer but improving the treatment outcomes.
Acting as a glucose analog, 2-DG blocks glycolysis and thereby reduces cellular ATP levels. Due to its unique structure, 2-DG also mimics mannose and thus interferes with N-linked glycosylation leading to the induction of endoplasmic reticulum (ER) stress. Thus, in Chapter 1 of this dissertation we set out to determine which pathway 2-DG interferes with is responsible for activating autophagy. We show that 2-DG activates autophagy predominantly through the induction of ER stress rather than ATP reduction. Furthermore, we find that 2-DG-induced ER stress stimulates autophagy through $\text{Ca}^{2+}$-CaMKK$\beta$ signaling-dependent AMPK activation. In contrast, we show that although GS leads to ER stress which contributes to autophagy activation, it does so by a different mechanism. In addition to ER stress, GS also stimulates autophagy through lowering ATP and activating the canonical LKB1-AMPK energy sensing pathway as well as through increasing reactive oxygen species (ROS) resulting in the activation of ERK.

While under normoxic conditions 2-DG only results in moderate ATP reduction, under hypoxic conditions this sugar analog elicits severe ATP depletion due to the lack of ATP production from the mitochondria. Accordingly, in Chapter 2 we investigate how 2-DG regulates autophagy under hypoxia. In a model of chemical hypoxia where mitochondrial ATP synthase is inhibited, 2-DG suppresses rather than induces autophagy. Similar observations are observed in a genetic model of hypoxia where cells are devoid of mitochondrial DNA. Moreover, in a more physiologic environmental model of hypoxia, both 2-DG and GS reduce autophagy in cells cultured under low $O_2$ tensions. This blockage is accompanied by the disruption of the PI3K III-Beclin1 complex for autophagy initiation, conjugation of Atg12 to Atg5 for autophagosome expansion, as well as inhibition of the functional lysosomal compartment for autophagic degradation.
It has been reported that autophagy responds to various cellular stresses to play either a pro- or anti-death role. Therefore, in Chapter 3 we study the functional role of autophagy activation in modulating 2-DG’s cytotoxic effects. Our results demonstrate that autophagy protects cancer cells against 2-DG-elicited cell death and apoptosis, apparently through relieving 2-DG-induced ER stress.

Mechanistically, our data support a model where therapeutic and physiologic glucose restriction differentially activate autophagy under normoxia, while similarly inhibit this process under hypoxia. Functionally, our work shows that 2-DG-induced autophagy ameliorates ER stress and counteracts 2-DG cytotoxicity. Overall, this study delineates the molecular mechanisms and functional roles of autophagy regulation by glucose restriction under different environmental conditions, and therefore may provide useful information for improving 2-DG’s anti-tumor efficacy as well as for a better understanding of the influence of GS on tumor pathophysiology.
DEDICATION

This dissertation is dedicated to my mother and father, Juelin Li and Tianfu Xi. Their continuous love and support inspire me to keep pursuing my goals. It is also dedicated to my wife, Ying Zhang. Her love and sacrifice has helped me progress through the most challenging times.

And, in loving memory of grandmother and grandfather, Qi Yang and Shaofu Li.
ACKNOWLEDGMENTS

Firstly, I would like to thank my research mentor Dr. Theodore Lampidis. His dedication to students covers not only scientific techniques and methods, but all the other elements required for being a qualified scientist. Especially, his patient guidance greatly helped me effectively present my work and communicate ideas both privately and publicly. More than a mentor for research, he is also a great teacher who aided my personal growth during my graduate student life.

I would also like to express my sincere gratitude to my physician mentor, Dr. Niramol Savaraj. Her scientific knowledge and insights have been invaluable. Despite her incredibly busy schedule as a physician scientist, she is always willing to help. Her kind support made my study as well as life much easier as a foreigner.

I am grateful to the other members of my dissertation committee, Drs. Carlos Moraes, Julio Barredo and Mansoor Ahmed. They devoted their precious time and continuously provided me with great suggestions to help develop my project.

I would like to acknowledge the members of Dr. Lampidis’ lab, Dr. Huaping Liu, Dr. Metin Kurtoglu, Dr. Howard Leung, Katherine Philips and Elizabeth Sullivan, as well as those of Dr. Savaraj’s, Dr. Medhi Wangpaichitr, Dr. Min You and Dr. Chunjing Wu, for their kind help and discussion. Being the “mother” of the lab, Huaping, with her incredible personality, made the lab not just a pleasant place to work but more like a family. She also offered help concerning my personal life on countless occasions. As a senior student and more experienced scientist, Metin was a great help to me during my rookie year in the lab and in formulating my dissertation proposal.
My appreciation extends to the Cancer Biology program director, Dr. Kerry Burnstein, for her tremendous efforts in moving forward our program and ensuring that every student obtains continuously better education and training. Thanks to our wonderful coordinators, Ms. Kathy Salce, Diane Dames and Sadiz Torres for making a painless stay in the program. Especially, Kathy was always on top of everything and made it possible for me to focus as much as I could on my research.

Last but certainly not least, I would like to thank my good friends Zhe Ma, Ge Tao and Medhi Wangpaichitr. They made my graduate student career an inspiring and enjoyable experience. I apologize to many of my colleagues and friends whose names cannot be all listed here. I want you to know that I would never have made it this far without your help and encouragement.
CHAPTER 1

Under Normoxia 2-Deoxyglucose and Glucose Starvation Differentially Activate Autophagy

Activate Autophagy ........................................................................................................ 1

1.1 Overview .................................................................................................................... 1

1.2 Results ...................................................................................................................... 11

2-DG-induced ER stress and ATP reduction correlate with activation of autophagy ........................................................................................................ 11

Mannose reverses 2-DG-induced autophagy and ER stress but not ATP reduction ............................................................................................................... 12

2-DG displays similar kinetics as tunicamycin in activating autophagy ................. 14

The glycolytic inhibitor oxamate does not activate autophagy .............................. 16

2-DG-induced ER stress activates AMPK via the Ca$^{2+}$-CaMKKβ pathway leading to autophagy ........................................................................................................ 18

The UPR transducers are not required for 2-DG-induced autophagy................. 22

Both ATP reduction and ER stress induced by GS contribute to autophagy activation ................................................................................................................. 23

GS-induced ER stress leads to autophagy independently of CaMKKβ or the
CHAPTER 2

Under Hypoxia 2-Deoxyglucose and Glucose Starvation Similarly Inhibits Autophagy

2.1 Overview ................................................................. 40

2.2 Results ................................................................. 45

2-DG suppresses autophagy in the chemical model of hypoxia ............... 45

2-DG reduces autophagy in the genetic model of hypoxia ....................... 47

2-DG and GS inhibit autophagy in the environmental model of hypoxia .... 48

2-DG and GS block autophagy under severe hypoxia at multiple levels .... 51

2.3 Discussion ........................................................................ 55

CHAPTER 3

Autophagy Protects Tumor Cells against 2-Deoxyglucose Cytotoxicity via

Relieving Endoplasmic Reticulum Stress ............................................. 59

3.1 Overview ........................................................................ 59

3.2 Results ........................................................................... 62

Inhibition of autophagy exacerbates whereas enhancement of autophagy...
ameliorates 2-DG cytotoxicity................................................................. 62
Blockage of autophagy increases 2-DG-induced ER stress and apoptosis........ 62
3.3 Discussion.......................................................................................... 65

MATERIALS AND METHODS ..................................................................... 68

CONCLUDING REMARKS ........................................................................ 77

REFERENCES............................................................................................. 81
LIST OF FIGURES

Figure 1.1. AMPK regulates various aspects of cellular metabolism and energy
balance for cellular adaptation under metabolic stresses ............................................. 3

Figure 1.2. Chemical structures of glucose, mannose and 2-DG .................................. 4

Figure 1.3. 2-DG interferes with mannose metabolism and hence N-linked
glycosylation ........................................................................................................... 5

Figure 1.4. Schematic of UPR signaling ...................................................................... 6

Figure 1.5. The process of autophagy ......................................................................... 8

Figure 1.6. 2-DG induces ATP reduction, ER stress and autophagy .......................... 12

Figure 1.7. Mannose reverses 2-DG-induced ER stress and autophagy without
affecting ATP reduction .......................................................................................... 13

Figure 1.8. 2-DG induces autophagy in a similar kinetics as the classical ER
stressor TM ............................................................................................................ 15

Figure 1.9. OX induces ATP reduction but not autophagy .......................................... 17

Figure 1.10. CaMKKβ mediates 2-DG-induced AMPK activation and autophagy
upregulation ............................................................................................................. 19

Figure 1.11. 2-DG results in [Ca^{2+}]_{ER} leakage and [Ca^{2+}]_{c} increase leading to
activation of AMPK and autophagy ........................................................................ 20

Figure 1.12. AMPK stimulated by 2-DG-induced ER stress positively regulates
autophagy activation by 2-DG .................................................................................. 21
Figure 1.13. 2-DG does not require the UPR transducers to activate autophagy

Figure 1.14. ATP reduction contributes to GS-induced autophagy through the LKB1-AMPK axis

Figure 1.15. ER stress is involved in GS-induced autophagy

Figure 1.16. Neither CaMKKβ nor the UPR transducers are required for GS-induced autophagy

Figure 1.17. ERK activation plays an important role in GS- but not 2-DG-induced autophagy

Figure 1.18. ERK activation elicited by GS is not subsequent to ER stress induction

Figure 1.19. GS induces ROS-ERK signaling leading to autophagy in a MEK activation-independent manner

Figure 1.20. TSC-mediated suppression of mTOR is required for 2-DG-induced autophagy and partially contributes to GS-induced autophagy

Figure 1.21. Model of differential autophagy activation by 2-DG vs. GS

Figure 2.1. Mechanisms of hypoxia-induced advancement of tumor progression and resistance to therapies

Figure 2.2. 2-DG selectively kills hypoxic tumor cells

Figure 2.3. 2-DG decreases autophagy activity in the chemical model of hypoxia

Figure 2.4. 2-DG suppresses autophagy activity in the genetic model of hypoxia

Figure 2.5. 2-DG inhibits autophagy activity in the environmental model of hypoxia

Figure 2.6. GS inhibits autophagy activity under severe hypoxia
Figure 2.7. Both 2-DG and GS suppress autophagy activity under a model of chronic hypoxia ................................................................. 51

Figure 2.8. 2-DG decreases the expression of the core autophagy machinery components under severe hypoxia ............................... 52

Figure 2.9. 2-DG and GS inhibit autophagy at multiple stages ......................... 54

Figure 2.10. Model of similar autophagy inhibition by 2-DG and GS under hypoxia..................................................................................... 57

Figure 3.1. Mode of autophagy determination of life or death............................... 61

Figure 3.2. 3-MA increases and rapamycin decreases 2-DG cytotoxicity........... 63

Figure 3.3. Atg 7 knockdown enhances 2-DG-induced ER stress, apoptosis and cell death........................................................................... 64
CHAPTER 1

Under Normoxia 2-Deoxyglucose and Glucose Starvation Differentially Activate Autophagy

1.1 Overview

The rewiring of cellular metabolism is an integral part of oncogenic transformation, and has been recognized as one of the “new generation” hallmarks of cancer (Cairns et al., 2011; Hanahan and Weinberg, 2011). Perhaps the most striking cancer-associated metabolic alteration is the increased utilization of glucose and its conversion to lactate even under normal O₂ concentrations (normoxia), an observation originally made by Otto Warburg and co-workers in the 1920s (Koppenol et al., 2011). Although a variety of mechanisms have been proposed to explain this phenomenon of aerobic glycolysis or “Warburg effect”, it still remains one of the biggest puzzles in cancer biology. Regardless of how it evolves, increased glucose metabolism has clearly been demonstrated to provide a selective advantage for tumor cell growth and survival (Jones and Thompson, 2009; Kroemer and Pouyssegur, 2008). Nevertheless, it is this same cancer hallmark that opens up a therapeutic window to treat cancer through targeting its reprogrammed glucose metabolism (Tennant et al., 2010; Vander Heiden, 2011).

Among the various approaches to targeting cancer’s sweet spot, the sugar analog 2-deoxyglucose (2-DG) is among the most widely studied, and is being investigated in both clinical trials and pre-clinical models (Dwarakanath et al., 2009; Dwarakanath, 2009; Farooque et al., 2009; Gupta et al., 2009; Kurtoglu et al., 2007b; Merchan et al., 2010;
Raez et al., 2012; Stein et al., 2010). Similar to its natural counterpart glucose, 2-DG is taken up by the cell surface glucose transporters (GLUTs) and phosphorylated and thereby trapped inside the cells by hexokinase (HK), the first enzyme in the glycolytic pathway. However, unlike the HK-phosphorylated glucose molecule, glucose-6-phosphate (G-6-P), 2-DG-6-phosphate (2-DG-6-P) cannot be recognized and utilized by the second glycolytic enzyme phosphoglucone isomerase (PGI). Therefore, 2-DG-6-P progressively accumulates inside the cells and competitively inhibits PGI resulting in depletion of fructose-6-phosphate (F-6-P) and blockage of glycolysis (Wick et al., 1957). In addition to this primary location of glycolytic inhibition, accumulation of 2-DG-6-P to high enough concentrations also leads to allosteric inhibition of HK, thereby further blocking glycolysis and interfering with glucose metabolism (Chen and Gueron, 1992; Lampidis et al., 2006).

While most differentiated normal cells produce energy (ATP) through O$_2$-dependent mitochondrial oxidative phosphorylation (OXSPHOS), cancer cells derive a considerable amount of ATP from glycolysis due to their upregulated glycolytic activity and reprogrammed energy metabolism (Cairns et al., 2011; Seyfried and Mukherjee, 2005). Therefore, glycolytic inhibition conferred by 2-DG results in ATP reduction even when cells are grown in the presence of normal O$_2$ tensions. Cells respond to lowering of ATP (increase of AMP/ATP ratios) via activating the evolutionarily-conserved energy sensor AMP-activated protein kinase (AMPK), which subsequently serves as a master regulator of cellular metabolism (Fig. 1.1) (Mihaylova and Shaw, 2011; Shackelford and Shaw, 2009). The activated AMPK elicits a plethora of downstream effects, which include increased GLUTs expression and cell surface translocation, upregulated
Figure 1.1. AMPK regulates various aspects of cellular metabolism and energy balance for cellular adaptation under metabolic stresses. The activated AMPK increases glucose metabolism by upregulating GLUTs gene expression and their cell surface translocation for increased glucose uptake, as well as by stimulating PFK2 thus enhancing glycolysis. AMPK regulates lipid metabolism via CPT1 stimulation and FAS inhibition, resulting in upregulation of fatty acid oxidation and downregulation of fatty acid synthesis, respectively. With regard to protein metabolism, AMPK blocks protein synthesis through suppressing mTOR and thus translation initiation, as well as via activating eEF2K which subsequently inhibits translation elongation. Overall, AMPK enhances energy-producing catabolic pathways, i.e. glycolysis and fatty acid oxidation, while suppressing energy-consuming anabolic processes, i.e. fatty acid and protein synthesis. Moreover, through p53 activation and cyclin inhibition, AMPK also slows down cell cycle and proliferation thereby allowing cells sufficient time to adapt to and recover from metabolic stresses. AMPK, AMP-activated protein kinase; CPT1, carnitine palmitoyltransferase 1; eEF2K, eukaryotic elongation factor 2 kinase; FAS, fatty acid synthase; GLUT, glucose transporter; mTOR, mammalian target of rapamycin; PFK2, phosphofructokinase 2.

glycolytic activity, enhanced mitochondrial biogenesis and function, accelerated fatty acid (FA) β-oxidation and decelerated FA synthesis, decreased mRNA translation, and
halted cell proliferation. Collectively, AMPK promotes ATP-generating catabolic activities while blocks ATP-consuming anabolic processes thereby helping cells adapt to bioenergetic stress.

Figure 1.2. Chemical structures of glucose, mannose and 2-DG. Note that the only difference between glucose and mannose is the conformation of the hydroxyl group at C2. Since 2-DG does not have a hydroxyl group at this position, it mimics both glucose and mannose.

Due to its unique chemical structure, 2-DG not only mimics glucose but also mannose (Fig. 1.2). Mannose plays an indispensible role in protein N-linked glycosylation taking place inside the endoplasmic reticulum (ER). A series of publications in the late 1970s demonstrated that during the conversion of 2-DG to GDP-2-DG, this sugar analog depletes the intracellular pool of GDP and thereby suppresses the production of GDP-mannose, which is normally added onto the N-linked glycosylation precursors lipid-linked oligosaccharides (LLOs). Moreover, GDP-2-DG can also be fraudulently incorporated onto the growing LLO chains. However, once a 2-DG molecule sits on a growing LLO, no mannose molecules can be further incorporated. Thus, 2-DG competes with mannose and interferes with N-linked glycosylation by reducing substrate
availability as well as prematurely terminating precursor assembly (Fig. 1.3) (Datema and Schwarz, 1978; Datema and Schwarz, 1979).

Proper N-linked glycosylation is necessary to ensure correct folding of most of the cell surface and secretory proteins synthesized inside the ER (Schroder and Kaufman, 2005). Therefore, by competing with mannose and disrupting of N-linked glycosylation, 2-DG causes accumulation of unfolded and misfolded proteins in the ER lumen. In response to this protein folding abnormality, cells mount a series of signaling events collectively called the unfolded protein response (UPR) (Fig. 1.4) (Schroder and
Kaufman, 2005; Szegezdi et al., 2006). In higher eukaryotes, there are three ER-resident transmembrane proteins that are mainly responsible for transmitting the UPR signals.

**Figure 1.4. Schematic of UPR signaling.** When unfolded/misfolded proteins are accumulated inside the ER, the molecular chaperone Grp78 will bind and help protein folding, thus relieving the inhibition on the three UPR transducers. Once PERK is activated, it phosphorylates and inhibits eIF2. While this event blocks translation initiation of the majority of mRNAs thereby reducing protein synthesis and the folding burden of the ER, it selectively upregulates a subset of mRNAs which transduce the UPR signals into the nucleus, including the transcription factor ATF4. As for ATF6, it translocates to the Golgi where it is cleaved to generate an active transcription factor to regulate gene expression. Through its atypical endoribonuclease activity, IRE1 splices the XBP1 mRNA, which allows it to be translated into a functional transcription factor. Collectively, the transcription factors produced downstream of the UPR modulate a variety of gene expression programs, including upregulating chaperones and ER protein degradation to aid in cellular adaptation to ER stress. ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; CHOP, C/EBP homologous protein; eIF2, eukaryotic initiation factor 2; Grp78, glucose-regulated protein, 78 kDa; IRE1, inositol-requiring enzyme 1; PERK, PKR-like ER kinase; sXBP1, spliced XBP1; XBP1, X-box binding protein 1. Taken from Szegezdi et al., 2006.

These so-called UPR transducers include PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6). In a coordinated fashion,
the UPR transducers facilitate cellular adaptation to ER stress through relieving ER folding burden, increasing ER protein degradation, as well as enhancing ER folding capacity.

It has long been thought that due to the dual activity of 2-DG in inhibiting glycolysis hence reducing ATP as well as interfering with glycosylation thus inducing ER stress, this sugar analog mimics the naturally occurring microenvironment of glucose starvation (GS) that most solid tumors undergo as they evolve. While therapeutic glucose restriction (2-DG) holds great promise in selectively targeting cancer cells, pathophysiologic glucose restriction (GS) has been shown to play a pivotal role in the progression of tumors (Buchakjian and Kornbluth, 2010; Yun et al., 2009). Therefore, studying how cells respond to therapeutic as well as physiologic glucose restriction may provide useful information not only for improving the efficacy of anti-tumor treatment but a better understanding of tumorigenesis and cancer biology.

Macroautophagy (hereafter simply referred to as autophagy) is an ancient bulk degradation process which has been increasingly recognized as an important cellular adaptive mechanism in response to a wide variety of stresses including metabolic stress. The term autophagy (auto, self; phagy, eating) was coined by Christian de Duve in his seminal description of this phenomenon back in 1963. However, the breakthrough of its molecular characterization arrived only until the 1990’s when a series of yeast genetic screens identified a growing list of the autophagy-related genes (Atg) as critical players involved in this process (Klionsky, 2007). These yeast analyses, along with the following studies in mammalian and other systems have largely shaped our current view of autophagy as a complex process comprised of six distinct stages (Fig. 1.5) (He and
The induction of autophagy is stimulated by the activation of upstream positive regulators such as the Atg1 kinase.
complex as well as suppression of negative modulators like mammalian target of rapamycin (mTOR). Upon receiving the induction signals, an autophagy nucleation complex containing Beclin1 (Atg6), class III phosphatidylinositol 3’ kinase (PI3K III, also called Vps34) and other proteins is assembled on a piece of isolation membrane to form the phagophore assembly site (PAS), which serves as a platform to recruit additional proteins for later steps. Next, the PAS directs the de novo biogenesis of a double-membrane vesicle called autophagosome through elongation and expansion of the original isolation membrane. Tow ubiquitin-like conjugation systems are indispensable for this step -- the conjugation of Atg12 to Atg5 and microtubule-associated proteins light chain 3 (LC3, also called Atg8) to phosphoethanolamine (PE), mediated by the shared E1-like enzyme Atg7 and their respective E2-like enzyme Atg10 and Atg3. After autophagosome maturation, all the Atg proteins except the PE-lipidated LC3 (LC3-II) molecules associated with the inner autophagosomal membrane are retrieved by an Atg9- and Atg18-dependent mechanism for another round of autophagosome biogenesis. The matured autophagosomes then fuse with the lysosomes to form so-called autolysosomes. Finally, the cytoplasmic contents including various organelles engulfed by the autophagosomes are degraded and recycled inside the autolysosomes.

Autophagy is critical in maintaining normal cellular homeostasis and ensuring proper organismal development (Levine and Klionsky, 2004). It has also been shown to play essential roles in various human diseases including infection, muscular dystrophy, heart failure, neurodegenerative diseases and cancer (Levine and Kroemer, 2008; Rubinsztein et al., 2007). During tumorigenesis, autophagy has been proposed to act as a double-edged sword (Amaravadi et al., 2011; Chen and Karantza, 2011; Eng and
Abraham, 2011; Maiuri et al., 2009; Mathew et al., 2007; Maycotte and Thorburn, 2011). At early stages of tumor progression, physiologic autophagy activity prevents malignant transformation by reducing chronic inflammation and maintaining genomic stability. However, at later stages, autophagy protects tumor cells from pathophysiologic stresses arising in the tumor microenvironment as well as therapeutic stresses induced by a wide variety of treatment modalities.

It has been shown that both energetic and ER stress activate autophagy (Hoyer-Hansen and Jaattela, 2007b; Rabinowitz and White, 2010; Singh and Cuervo, 2011; Verfaillie et al., 2010)(Hoyer-Hansen and Jaattela, 2007b; Rabinowitz and White, 2010; Singh and Cuervo, 2011; Stein et al., 2010). In cells depleted with ATP, increased AMP/ATP ratios activate AMPK, which in turn inhibits mTOR leading to autophagy induction (Hoyer-Hansen and Jaattela, 2007a). Additionally, AMPK has also been reported to stimulate autophagy by phosphorylating and activating unc-51-like kinase 1 (ULK1, the mammalian homolog of yeast Atg1) (Egan et al., 2011; Kim et al., 2011; Lee et al., 2010). In ER stressed cells, there have been varying reports on the pathways involved in signaling autophagy. In some studies, the UPR transducer PERK is shown to play a role (Kouroku et al., 2007; Lepine et al., 2011; Rouschop et al., 2010) while in others it does not (Ogata et al., 2006; Py et al., 2009). Similarly, varying results for the other two UPR transducers, IRE1 (Ogata et al., 2006; Rouschop et al., 2010; Sakaki et al., 2008) and ATF6 (Lepine et al., 2011; Rouschop et al., 2010; Sakaki et al., 2008) have been reported for their roles in activating autophagy. The discrepancies in the results from these reports may be explained by the different agents used to induce ER stress as well as time points and cell types assayed.
Although 2-DG has been recently reported to activate autophagy (DiPaola et al., 2008; Wu et al., 2009), the mechanism(s) by which this occurs remains unknown. Similarly, the relative roles of ATP reduction and ER stress in GS-induced autophagy are also not completely understood. Therefore, in this chapter of the dissertation, we determine whether ER stress and/or ATP reduction play a predominant role in both 2-DG- and GS-induced autophagy, and examine the detailed signaling pathways involved therein.

1.2 Results

2-DG-induced ER stress and ATP reduction correlate with activation of autophagy

To determine whether perturbation of energy and/or ER homeostasis by 2-DG leads to autophagy activation, ATP levels and ER stress markers were assayed in two different human cancer cell lines, i.e. pancreatic cancer cells 1420 (Fig. 1.6A) and melanoma cells MDA-MB-435 (Christgen and Lehmann, 2007) (Fig. 1.6B). In both cell lines, 2-DG decreased ATP levels while increased the ER stress markers glucose-regulated protein 78 kDa (Grp78) and C/EBP homologous protein (CHOP). Concomitantly, activation of autophagy was also observed as assayed by the autophagy marker LC3B-II (Fig. 1.6A and B). Overall, these data show that 2-DG stimulates autophagy which correlates with ER stress induction and ATP reduction, suggesting that either or both of these activities of 2-DG are activating autophagy.
Figure 1.6. 2-DG induces ATP reduction, ER stress and autophagy. (A) 1420 cells were treated with 4 mM of 2-DG for 5 h followed by measurement of intracellular ATP levels (top panel) or 16 h followed by Western blot analysis of Grp78, CHOP and LC3B-II levels. (B) MDA-MB-435 cells were similarly treated and analyzed as in (A), except that 10 mM of 2-DG was used. LC3B-I*, the appearance of this band depends on the lot and type of the primary antibodies used, the type of cells tested, as well as the blot exposure time applied. Error bars represent standard deviation (SD) of triplicate samples. Results are representative of at least three independent experiments. **, P<0.01 and ***, P<0.001 compared to control 1420 (A) and MDA-MB-435 (B) cells, respectively.

Mannose reverses 2-DG-induced autophagy and ER stress but not ATP reduction

Recently, our lab showed that exogenous mannose reverses 2-DG-induced ER stress (Kurtoglu et al., 2007a). Therefore, mannose was added to 2-DG treated cells to study autophagy activation when ER stress was abolished. Consistent with our previous work, in 1420 cells 1 mM of mannose reversed 2-DG (4 mM)-induced upregulation of Grp78 and CHOP (Fig. 1.7A). Most noteworthy, under these conditions LC3B-II levels almost reverted back to those found in untreated control cells, signifying blockage of
Figure 1.7. Mannose reverses 2-DG-induced ER stress and autophagy without affecting ATP reduction. (A) 1420 cells were treated with 4 mM of 2-DG with or without 1 mM of mannose (Man) for 16 h, and Grp78, CHOP and LC3B-II levels were examined by Western blot. (B) 1420 cells were exposed to 2-DG alone or in combination with Man for 16 h, and the endogenous LC3B (green) distribution was assessed by fluorescent microscopy. Nuclei were counterstained by DAPI (blue). (C) Cellular ATP levels were measured in 1420 cells treated as in (A) except for 5 h. Western blot (D), fluorescent microscopy (E) and ATP measurement (F) were applied in MDA-MB-435 cells as indicated in (A), (B) and (C), respectively, except that 2-DG was used at 10 mM. Error bars represent SD of triplicate samples. Results are representative of at least three independent experiments. NS, statistically not significant compared to 2-DG only-treated 1420 (A) and MDA-MB-435 (B) cells.

Autophagy stimulation. The ability of mannose to reverse 2-DG-induced autophagy was further confirmed by another widely used method to detect autophagy, namely the appearance of LC3B puncta (Klionsky et al., 2012) (Fig. 1.7B). In contrast to the reversal of ER stress and autophagy activation, mannose failed to restore 2-DG-induced decreased
ATP to untreated levels (Fig. 1.7C). These data suggest that 2-DG activates autophagy through induction of ER stress and not by depleting ATP.

To study whether activation of autophagy by 2-DG through ER stress is a general phenomenon, mannose reversal experiments were extended to three other human cancer cell lines. In MDA-MB-435 cells, mannose was found to completely reverse the increased expression of LC3B-II as well as Grp78 and CHOP assayed by Western blot (Fig. 1.7D); and to abolish the punctate pattern of LC3B assessed by immunofluorescence (Fig. 1.7E). In agreement with the results obtained in 1420 cells, mannose did not affect 2-DG-induced ATP reduction in this cell line (Fig. 1.7F). Similar findings were observed in the breast cancer cells SKBR3 and glioblastoma multiforme cells T98G (data not shown). Taken together, data from these experiments demonstrate that induction of ER stress is the predominant mechanism by which 2-DG stimulates autophagy in multiple human cancer cell lines.

2-DG displays similar kinetics as tunicamycin in activating autophagy

Since tunicamycin (TM) induces ER stress similarly to 2-DG in that both of them interfere with N-linked glycosylation (Duksin and Mahoney, 1982), the kinetics of this agent to activate autophagy was investigated and compared to that of 2-DG in 1420 cells. Both drugs were found to induce ER stress in a similar manner during a 24 h period of time, as assessed by the expression of Grp78 and CHOP (Fig. 1.8A). Treatment with either drug increased peak levels of LC3B-II at 16 h with decreasing levels observed at 24 h after drug exposure (Fig. 1.8A). To compare more carefully the autophagic flux induced by 2-DG and TM, we conducted similar experiments in the presence of EST
1520 cells were treated with 4 mM of 2-DG or 1 μg/ml of TM in the absence (A) or presence (B) of 4 μg/ml of EST. Grp78, CHOP and LC3B-II levels were examined by Western blot at the indicated time points. Blots are from one of at least two independent experiments.

(E64d), a lysosomal protease inhibitor that can block the degradation of LC3B-II after the autophagosomes fuse with the lysosomes. Thus, under these conditions activation of autophagy is less likely to be underestimated (Klionsky et al., 2012). As shown in Fig. 1.8B, similar results of increased levels of LC3B-II were obtained when EST was applied. Interestingly, a sustained upregulation of LC3B-II after 24 h (and 48 h, data not shown) exposure to both drugs was observed in the presence of EST as compared to the decrease found when cells were treated in the absence of EST, suggesting that accelerated autophagic degradation is occurring at this time point of drug treatment. Overall, these data illustrate that 2-DG and TM display similar kinetics in stimulating autophagy

Figure 1.8. 2-DG induces autophagy in a similar kinetics as the classical ER stressor TM.
activity, further supporting the conclusion that 2-DG-activated autophagy results as a consequence of its induction of ER stress.

The glycolytic inhibitor oxamate does not activate autophagy

Recent reports indicate that various environmental stresses or drug treatments that can lead to energy reduction induce autophagy (Kuma et al., 2004; Lum et al., 2005; Meley et al., 2006; Papandreou et al., 2008; Wei et al., 2010; Xu et al., 2007). This raises the possibility that the nature of a certain stress condition may determine the involvement of ATP reduction in signaling autophagy activation. To better understand the role of ATP reduction in autophagy stimulation, we monitored autophagy activity as described above when cells were treated with oxamate (OX), a glycolytic inhibitor that decreases ATP levels but does not induce ER stress. In MDA-MB-435 cells three different doses of OX (2, 5 and 10 mg/ml) were studied so that the range of ATP reduction achieved by these doses was similar to that reached by 4 mM of 2-DG (Fig. 1.9A, top panel). As shown in Fig. 1.9A, bottom panel, at doses of OX where ATP levels were reduced similarly or greater than that by 2-DG there was no increase in LC3B-II, while 2-DG (as a positive control) induced an obvious increase in this autophagy marker. Similar experiments were performed in MDA-MB-435 cells, and the same results were obtained (Fig. 1.9B). In addition, following the kinetics of autophagy flux showed that at 5 mg/ml of OX, where ATP levels were reduced to ~50%, LC3B-II levels within 24 h were not significantly affected regardless of the presence or absence of EST (Fig. 1.9C). Consistent with the Western blot results of LC3B processing, OX at 5 mg/ml did not increase LC3B puncta in either cell lines (data not shown), further confirming that there was no increase of
autophagy when cells were reduced of ATP by OX. Taken together, these data show that ATP reduction induced by OX at levels equivalent to that of 2-DG does not stimulate autophagy in the cell lines used in this study, supporting our conclusion that 2-DG, when lowering ATP moderately in our experimental settings, activates autophagy mainly via ER stress.

Figure 1.9. OX induces ATP reduction but not autophagy. (A) ATP levels (top, 5 h) and Grp78, CHOP and LC3B-II levels (bottom, 16 h) were measured in 1420 cells treated by 2-DG or OX. (B) MDA-MB-435 cells were similarly treated and analyzed as in (A), except for the use of 2-DG at 10 mM. (C) 1420 cells were treated with 5 mg/ml of OX in the presence or absence of 4 wg/ml of EST. Western blot was performed to detect LC3B-II expression at the indicated time points. Error bars represent SD of triplicate samples. ATP and Western blot results are representative of three and two independent experiments, respectively. **, P<0.01 and ***, P<0.001 compared to control 1420 (A) and MDA-MB-435 (B) cells.
2-DG-induced ER stress activates AMPK via the Ca$^{2+}$-CaMKKβ pathway leading to autophagy

It is known that ER stress can disrupt Ca$^{2+}$ homeostasis inside the ER, which in turn leads to Ca$^{2+}$ leakage into other cellular compartments (Deniaud et al., 2008; Zong et al., 2003). It has also been reported that massive increases in cytoplasmic Ca$^{2+}$ concentrations ([Ca$^{2+}$]$_{i}$) stimulate autophagy through Ca$^{2+}$/calmodulin-dependent kinase kinase β (CaMKKβ) and the subsequent activation of AMPK (Hoyer-Hansen et al., 2007). These observations led us to investigate whether the activity of 2-DG to induce ER stress leads to AMPK activation (which up till the present has been thought to occur mainly as a response to ATP depletion) via Ca$^{2+}$-CaMKKβ and in turn stimulates autophagy. As shown in Fig. 1.10A, in 1420 cells 2-DG increased the expression of LC3B-II and the phosphorylation of AMPKα at Thr172 (pAMPKα). Importantly, the CaMKKβ inhibitor STO-609 (STO) reduced both LC3B-II and pAMPKα levels upregulated by 2-DG. Similarly, knockdown of CaMKKβ also attenuated 2-DG-induced LC3B-II as well as phosphorylation of acetyl-CoA carboxylase at Ser79 (pACC), an indicator of AMPK activity (Fig. 1.10B). Moreover, the classical ER stressor TM which did not reduce cellular ATP levels (data not shown), also induced increases in AMPK activity and LC3B-II levels, both of which were diminished by STO or CaMKKβ knockdown (Fig. 1.10A and 1.10B). In Fig. 1.10C, quantification of the dot formation of the enhanced green fluorescent protein (EGFP)-LC3B is presented, which served as another marker of autophagy (Klionsky et al., 2012), further confirming that when CaMKKβ was knocked down 2-DG-induced autophagy was reduced.
Figure 1.10. CaMKKβ mediates 2-DG-induced AMPK activation and autophagy upregulation. (A) 1420 cells were treated with 4 mM of 2-DG or 1 μg/ml of TM with or without 40 μM of STO for 16 h. Expression of pAMPKα, AMPKα and LC3B-II were examined by Western blot. The numbers below the bands represent the ratios of the blot intensity of pAMPKα to total AMPKα or LC3B-II to β-actin. (B) 1420 cells were transfected with either siRNAs against luciferase (siLuc, as a control) or CaMKKβ (siCaMKKβ), treated by 2-DG or TM for 16 h and analyzed by Western blot for pACC and LC3B-II levels. The numbers below the bands represent the ratios of the blot intensity of pACC or LC3B-II to β-actin. Right panel shows the knockdown efficiency assessed by qPCR. Western blot results represent one out of at least three individual experiments. (C) 1420 cells stably expressing EGFP-LC3B fusion proteins were transfected with either siLuc or siCaMKKβ, followed by 16 h of 2-DG exposure. The distribution of EGFP-LC3B (green) was monitored under a confocal microscope (left, representative of four independent experiments) and the number of green dots per cell was quantified by ImageJ with error bars representing SD (right). ##, P<0.01 compared to 2-DG-treated cells transfected with siLuc.

Since CaMKKβ is known to be activated by Ca^{2+}, using the cell-permeable ratiometric [Ca^{2+}]_c indicator Indo-1-AM (Indo-1), we found that both 2-DG and TM upregulated [Ca^{2+}]_c (Fig. 1.11A and 1.11B, top panel). Additionally, thapsigargin (TG) was used to deplete [Ca^{2+}]_ER and therefore increase [Ca^{2+}]_c in cells left untreated or
Figure 1.11. 2-DG results in \([Ca^{2+}]_{\text{ER}}\) leakage and \([Ca^{2+}]_c\) increase leading to activation of AMPK and autophagy. (A) 1420 cells were treated with 4 mM of 2-DG or 1 \(\mu\)g/ml of TM for 14 h, followed by loading with Indo-1. The ratios of the dye fluorescence emitted at 405 nm to 485 nm were measured both before and after the addition of TG. Representative traces of the kinetics of the ratios of different samples (triplicates for each treatment condition) are shown. (B) Quantification of the average baseline ratios measured for 10 min (upper) and the average TG-increased ratios (post TG addition ratios minus baseline ratios) measured for the first 10 min after TG exposure (bottom). Error bars represent SD of four independent experiments. *, \(P<0.05\) and **, \(P<0.01\) compared to vehicle-treated controls. (C) 1420 cells were treated with 2-DG or TM with or without 20 \(\mu\)M of BAPTA for 16 h. Expression of pAMPK\(\alpha\), AMPK\(\alpha\) and LC3B-II were examined by Western blot. The numbers below the bands represent the ratios of the blot intensity of pAMPK\(\alpha\) to total AMPK\(\alpha\) or LC3B-II to \(\beta\)-actin. One representative experiment from at least three individual ones is shown.

pretreated with 2-DG or TM. Pretreatment with either drugs resulted in smaller increases in \([Ca^{2+}]_c\) (the difference between \([Ca^{2+}]_c\) measured before and after TG addition) (Fig.
1.11A and 1.11B, bottom panel), indicating that ER Ca\(^{2+}\) storage was partially depleted by both pretreatments. These results support our hypothesis that 2-DG and TM induce ER Ca\(^{2+}\) leakage thereby increasing [Ca\(^{2+}\)]\(_{c}\). Notably, as observed with blockage of CaMKK\(\beta\), reducing [Ca\(^{2+}\)]\(_{c}\) by the cell-permeable Ca\(^{2+}\) chelator BAPTA-AM (BAPTA) also attenuated 2-DG- or TM-increased LC3B-II and pAMPK\(\alpha\) (Fig. 1.11C), which supports the results above indicating the involvement of CaMKK\(\beta\) in ER stress activation of AMPK and autophagy.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-DG</td>
<td>siLuc</td>
</tr>
<tr>
<td>- Man</td>
<td>Ctrl</td>
</tr>
<tr>
<td>+ Man</td>
<td>pACC</td>
</tr>
<tr>
<td>AMPK(\alpha)</td>
<td>AMPK(\alpha)</td>
</tr>
<tr>
<td>1.0</td>
<td>11.8</td>
</tr>
<tr>
<td>pAMPK(\alpha)</td>
<td>(\beta)-Actin</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>4.5</td>
<td>11.8</td>
</tr>
<tr>
<td>0.9</td>
<td>16.6</td>
</tr>
<tr>
<td>3.3</td>
<td>1.4</td>
</tr>
</tbody>
</table>

**Figure 1.12. AMPK stimulated by 2-DG-induced ER stress positively regulates autophagy activation by 2-DG.** (A) 1420 cells were treated with 4 mM of 2-DG for 16 h in the presence or absence of 1 mM of mannose (Man), followed by Western blot analysis of the pAMPK\(\alpha\) and AMPK\(\alpha\) expression. The numbers represent the ratios of the blot intensity of pAMPK\(\alpha\) to total AMPK\(\alpha\). (B) 1420 cells were transfected with siLuc or siAMPK\(\alpha\)1, treated by 4 mM of 2-DG or 1 \(\mu\)g/ml of TM for 16 h and analyzed by Western blot for pACC, AMPK\(\alpha\) and LC3B-II levels. The numbers represent the ratios of the blot intensity of pACC or LC3B-II to \(\beta\)-actin. Results represent one out of four individual experiments.

In order to further demonstrate that 2-DG can activate AMPK through ER stress induction that is independent of its ATP reducing activity, we added exogenous mannose, which we have shown above reverses 2-DG-induced ER stress without affecting ATP
reduction. As shown in Fig. 1.12A, the addition of mannose partially reversed pAMPKα upregulation induced by 16 h of 2-DG treatment. To determine whether ER stress-induced activation of AMPK plays a role in 2-DG- or TM-induced autophagy, AMPKα1 was knocked down. Results shown in Fig. 1.12B demonstrate that knockdown of AMPKα1 attenuated LC3B-II expression induced by both drugs. Although 2-DG is well-known to activate AMPK through lowering of ATP, our results presented here collectively indicate that 2-DG as well as TM also activates AMPK in response to ER stress through the Ca^{2+}-CaMKKβ pathway leading to autophagy induction.

The UPR transducers are not required for 2-DG-induced autophagy

Since the UPR transducers have been implicated in ER stress-induced autophagy (Hoyer-Hansen and Jaattela, 2007b; Verfaillie et al., 2010), we investigated whether they played a role in 2-DG-induced autophagy. Using PERK wild type (PERK^{+/+}) and knockout (PERK^{-/-}) mouse embryonic fibroblasts (MEFs), we found that 2-DG increased LC3B-II to comparable levels regardless of the genotype of PERK (Fig. 1.13A). Similar experiments were performed in IRE1^{+/+} and IRE1^{-/-} MEFs and no differences between these cell lines in LC3B-II induction by 2-DG could be detected (Fig. 1.13B). Additionally, we knocked down ATF6 in 1420 cells using siRNAs. The knockdown efficiency was confirmed by the reduced expression of the full-length uncleaved ATF6 as well as by the attenuation of 2-DG-induced increase of Grp78, an ATF6 target. As shown in Fig. 1.13C, knockdown of ATF6 did not reduce LC3B-II levels induced by 2-DG. Collectively, these results demonstrate that none of the UPR transducers are required for autophagy activation by 2-DG. To further corroborate our observations with 2-DG-
induced autophagy, we conducted similar experiments in the above mentioned systems using TM and obtained similar results (Fig. 1.13A-C). These data suggest that the UPR transducers are not required for ER stress-induced activation of autophagy in our experimental systems.

![Diagram](image)

Figure 1.13. 2-DG does not require the UPR transducers to activate autophagy. (A) PERK+/+ and PERK−/− MEFs were treated with 4 mM of 2-DG or 0.5 μg/ml of TM for 16 h, followed by Western blot analysis of LC3B-II expression. (B) IRE1+/+ and IRE1−/− MEFs were treated with 1 or 4 mM of 2-DG or 0.2 μg/ml of TM for 16 h, followed by Western blot analysis of LC3B-II expression. (C) 1420 cells transfected with either siLuc or siATF6 were exposed to 4 mM of 2-DG or 1 μg/ml of TM for 16 h, and the levels of ATF6, Grp78 and LC3B-II were analyzed by Western blot. One representative experiment from at least two is shown.

Both ATP reduction and ER stress induced by GS contribute to autophagy activation

GS is a pathophysiologic stress that occurs during tumorigenesis, and like 2-DG, it also results in both ATP reduction and ER stress. To probe the role of ATP reduction in GS-induced autophagy, the liver kinase B1 (LKB1)-AMPK energy sensing pathway was disrupted by siRNA knockdown of LKB1. Efficient LKB1 knockdown was evidenced by
Figure 1.14. ATP reduction contributes to GS-induced autophagy through the LKB1-AMPK axis. (A) 1420 cells transfected with either siLuc or siLKB1 were subjected to GS or 4 mM of 2-DG for 16 h, and the levels of LKB1, pAMPKα and LC3B-II were analyzed by Western blot. (B) 1420 cells transfected with either non-targeting control siRNA 1 (siNC1) or siAMPKα1 were subjected to GS or 4 mM of 2-DG for 16 h, and the levels of AMPKα1, pACC and LC3B-II were analyzed by Western blot. Top panels show representative blots. Bottom panels show the quantification of the ratios of the blot intensity of LC3B-II to β-actin. Error bars represent SD of at least three independent experiments. #, P<0.05 compared to GS-treated cells transfected with control siLuc (A) or siNC1 (B); NS, statistically not significant compared to 2-DG-treated cells transfected with siLuc (A).

The reduced total LKB1 protein levels as well as its kinase activity measured by pAMPKα. Importantly, in cells transfected with LKB1 siRNAs, GS induced significantly less LC3B-II expression compared to those with control siRNAs (Fig. 1.14A). Moreover, GS-induced LC3B-II levels were also reduced by knocking down AMPKα1 (Fig. 1.14B). These data are consistent with a previous study showing that lowering ATP and subsequently activating the LKB1-AMPK pathway positively regulates GS-induced autophagy (Liang et al., 2007). Notably, in agreement with our previous finding that ATP
reduction does not play a major role in 2-DG-induced autophagy, knocking down LKB1 had no significant effect on LC3B-II induction by 2-DG (Fig. 1.14A).

**Figure 1.15. ER stress is involved in GS-induced autophagy.** (A and B) 1420 cells were subjected to GS for 16 h in the presence or absence of 10 mM of 4-PBA (A) or 10 μM of Sal (B), followed by Western blot analysis of Grp78 and LC3B-II levels. (C) 1420 cells stably expressing Grp78 or empty vectors were subjected to GS for 16 h, followed by Western blot analysis of Grp78 and LC3B-II levels. Top panels show representative blots. Bottom panels show the quantification of the ratios of the blot intensity of LC3B-II to β-actin. Error bars represent SD of at least three independent experiments. #, P<0.05 compared to glucose starved cells without Sal (B) or expressing empty vectors (C); ##, P<0.01 compared to glucose starved cells without 4-PBA (A).

To determine the role of ER stress in autophagy activation by GS, we used the chemical chaperone sodium 4-phenylbutyrate (4-PBA) or overexpressed the molecular chaperone Grp78 to aid in protein folding and relieve ER stress. Moreover, the eukaryotic initiation factor 2α (eIF2α) phosphatase inhibitor salubrinal (Sal) was also utilized to keep eIF2α phosphorylated (inhibited), thereby reducing protein synthesis and the ER burden. As can be seen in Fig. 1.15A and B, both 4-PBA and Sal reduced LC3B-II expression induced by GS in 1420 cells, while simultaneously attenuating the levels of the ER stress marker Grp78. Furthermore, cells stably overexpressing Grp78 also
displayed a blunted LC3B-II increase by GS compared to those bearing empty vectors (Fig. 1.15C). In summary, these observations show that both ATP reduction and ER stress contribute to GS-induced autophagy.

Figure 1.16. Neither CaMKKβ nor the UPR transducers are required for GS-induced autophagy. (A) 1420 cells were subjected to GS for 16 h in the presence or absence of 40 μM of STO, followed by Western blot analysis of LC3B-II expression. (B and C) PERK (B) or IRE1 (C) MEF pairs were subjected to GS for 16 h, followed by Western blot analysis of LC3B-II expression. (E) 1420 cells were transfected with either siLuc or siATF6, and starved of glucose for 16 h. The levels of ATF6, Grp78 as well as LC3B-II were detected by Western blot. Representative blots from at least two independent experiments are shown.

GS-induced ER stress leads to autophagy independently of CaMKKβ or the UPR transducers

Prompted by our observations that CaMKKβ mediates 2-DG-induced autophagy downstream of ER stress, we investigated whether it played a similar role in GS-induced ER stress activation of autophagy. Interestingly, the CaMKKβ inhibitor STO did not alter LC3B-II induction in cells starved of glucose (Fig. 1.16A). Thus, unlike the results shown above with 2-DG and TM, GS-induced ER stress activates autophagy through a mechanism that does not require the CaMKKβ signaling. In examining the role of the UPR transducers in GS-induced autophagy, it was found that neither PERK nor IRE1 knockout in MEFs attenuated GS-induced LC3B-II upregulation (Fig. 1.16B and C).
Similar data were obtained in 1420 cells with ATF6 knocked down (Fig. 1.16D). Therefore, similar to what we found with 2-DG, GS-induced ER stress does not appear to activate autophagy through the UPR transducers.

**GS but not 2-DG activates autophagy through increased ROS and subsequent ERK activation**

In renal proximal tubular cells (Kawakami et al., 2009) and WI38 lung epithelial fibroblasts (Oh and Lim, 2009), it has recently been shown that ER stress induces autophagy via activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2). Based on these findings we performed experiments to determine whether this pathway was involved in autophagy activation by GS-induced ER stress. Indeed, in 1420 cells GS-induced LC3B-II upregulation was found to be accompanied by an increase in ERK1/2 phosphorylation at Thr202/Tyr204 (pERK1/2). Moreover, GS-induced LC3B-II levels were attenuated when ERK1/2 activity was suppressed by either pharmacologic inhibitors PD325901 (Fig. 1.17A) or U0126 (data not shown), or siRNA knockdown (Fig. 1.17B). In contrast, despite upregulation of pERK1/2 in response to 2-DG, blocking ERK1/2 activity had only little (Fig. 1.17B) to non-significant effect (Fig. 1.17A and data not shown) on 2-DG-induced LC3B-II expression. Although these data demonstrate that GS-induced autophagy involves ERK1/2 activation, further experiments reveal that ER stress is not responsible for the observed ERK1/2 activation by GS. In fact, relieving GS-induced ER stress by either 4-PBA, Sal or Grp78 overexpression actually slightly increased pERK1/2 levels (Fig. 1.18A-C), suggesting that ER stress negatively regulates ERK1/2 activity in glucose-starved cells. This is consistent with our finding that in cells treated with the ER stressor TM, pERK1/2 decreased below basal levels observed in
control cells (data not shown). Overall, these results show that ERK1/2 positively regulates GS-induced autophagy by a mechanism independent of GS induction of ER stress.

**Figure 1.17.** ERK activation plays an important role in GS- but not 2-DG-induced autophagy. (A) 1420 cells were subjected to GS or 4 mM of 2-DG for 16 h in the presence or absence of 1 nM of PD325901, followed by Western blot analysis of pERK1/2, total ERK1/2 and LC3B-II levels. (B) 1420 cells transfected with either siLuc or siERK1/2 were subjected to GS or 2-DG for 16 h, followed by Western blot analysis of pERK1/2, total ERK1/2 and LC3B-II levels. Top panels show representative blots. Bottom panels show the quantification of the ratios of the blot intensity of LC3B-II to β-actin. Error bars represent SD of three independent experiments. #, $P<0.05$ compared to GS-treated cells without PD325901 (A) or 2-DG-treated cells transfected with siLuc (B); ##, $P<0.01$ compared to GS-treated cells transfected with siLuc (B); NS, statistically not significant compared to 2-DG-treated cells without PD325901 (C).

To better understand the mechanism by which GS stimulates ERK1/2, which in turn leads to autophagy, we examined the activity of MEK1/2 (measured by MEK1/2 phosphorylation at Ser217/221, pMEK1/2), the upstream kinase of ERK1/2 in the RAS-RAF-MEK-ERK mitogen-activated protein kinase (MAPK) signaling pathway. We found that in 1420 cells, although the levels of pERK1/2 were increased after 8 or 16 h of GS, those of pMEK1/2 were not (Fig. 1.19A). Interestingly however, 2-DG induced a
robust increase in pMEK1/2 at all time points examined (Fig. 1.19A). These results indicate that activation of ERK1/2 in response to GS treatment does not involve an increase in MEK1/2 activity. Based on this result and that GS has been reported to increase reactive oxygen species (ROS), potent regulators of autophagy (Marambio et al., 2010; Scherz-Shouval and Elazar, 2011), we examined the possibility that GS-elicited ROS stimulate ERK1/2 resulting in autophagy induction. Using the intracellular ROS sensor CM-H2DCFDA, we found that GS increased ROS levels in 1420 cells (Fig. 1.19B) and that co-treatment with the ROS scavenger N-acetyl-L-cysteine (NAC) significantly reduced GS-increased pERK1/2 as well as LC3B-II (Fig. 1.19C). In contrast, 2-DG was found not to increase (but actually decrease) ROS and NAC did not attenuate the expression of either pERK1/2 or LC3B-II induced by 2-DG (Fig. 1.19C). Overall, the results presented here show that GS but not 2-DG increases ROS which in turn activate ERK1/2 leading to upregulation of autophagy in the absence of increased MEK1/2 activity.
**Figure 1.19. GS induces ROS-ERK signaling leading to autophagy in a MEK activation-independent manner.** (A) 1420 cells were subjected to GS or 4 mM of 2-DG, and the levels of pMEK1/2 and pERK1/2 were assessed by Western blot at the indicated time points. Representative blots from two individual experiments are shown. (B) 1420 cells were subjected to GS or 4 mM of 2-DG for 16 h. Intracellular ROS levels (FL1-A %) were measured by flow cytometry using the ROS sensor CM-H2DCFDA. Error bars represent SEM of four independent experiments. *, P<0.05 and **, P<0.01 compared to controls. (C) 1420 cells were subjected to GS or 2-DG for 16 h in the presence or absence of 5 mM of NAC, followed by Western blot analysis of pERK1/2 and LC3B-II levels. Top panel shows representative blots. Middle and bottom panels show the quantification of the ratios of the blot intensity of pERK1/2 or LC3B-II to β-actin, respectively. Error bars represent SD of four independent experiments. #, P<0.05 compared to GS-treated cells without NAC; ##, P<0.01 compared to GS-treated cells without NAC; NS, statistically not significant compared to 2-DG-treated cells without NAC.

**mTOR inhibition is required for autophagy activation by 2-DG but not GS**

mTOR is an important negative regulator of autophagy in mammalian cells, which is itself negatively controlled by AMPK through activation of the tuberous sclerosis complex 1 (TSC1)-TSC2 protein complex (Mihaylova and Shaw, 2011; Rabinowitz and White, 2010). Thus, the role of mTOR in both 2-DG- and GS-induced autophagy was
Figure 1.20. TSC-mediated suppression of mTOR is required for 2-DG-induced autophagy and partially contributes to GS-induced autophagy. TSC2+/+ and TSC2−/− MEFs were subjected to GS, 4 mM of 2-DG or 1 μg/ml of TM for 16 h, followed by Western blot analysis of p-p70S6K and LC3B-II levels. Top panel shows representative blots. Bottom panel shows the quantification of the ratios of the blot intensity of LC3B-II to β-actin. Error bars represent SD of three independent experiments. #, $P<0.05$ compared to TM-treated TSC2+/+ cells; ##, $P<0.01$ compared to GS- or 2-DG-treated TSC2+/+ cells.

investigated using TSC2+/+ and TSC2−/− MEFs. We found that in response to either 2-DG, TM or GS, TSC2+/+ but not TSC2−/− cells were able to suppress mTOR activity, as measured by the phosphorylation of the mTOR substrate 70 kDa ribosomal protein S6 kinase (p70S6K) at Thr389 (p-p70S6K). Noticeably, whereas TSC2 deficiency completely prevented 2-DG- or TM-induced LC3B-II upregulation, it had only moderate inhibitory effect on LC3B-II expression induced by GS (Fig. 1.20). These observations are consistent with our previous data which show that 2-DG or TM activates autophagy through the Ca^{2+}-CaMKKβ-AMPK signaling, while GS induces autophagy via pathways that are both dependent (LKB1-AMPK) and independent (ER stress and ROS-ERK1/2) on AMPK activation. These results further support our findings of mechanistic
differences in autophagy induction by therapeutic (2-DG) vs. physiologic (GS) glucose restriction.

1.3 Discussion

Although data from our lab, as well others (DiPaola et al., 2008; Wu et al., 2009) demonstrate that autophagy, an evolutionarily conserved intracellular bulk degradation process, responds to 2-DG treatment, the precise mechanism(s) by which 2-DG-induced autophagy occurs is not fully understood. Here, we find that autophagy activation in response to 2-DG is accompanied by ER stress as well as ATP reduction (Fig. 1.6). Previously, White’s (DiPaola et al., 2008) and Yang’s (Wu et al., 2009) group independently reported that 2-DG activates autophagy in cancer cells; and in the latter study the authors suggested that the activation of eukaryotic elongation factor 2 kinase (eEF2K) is responsible for the induction of autophagy resulting from energy reduction (lowered ATP) due to 2-DG treatment. However, in our experimental systems, addition of exogenous mannose to 2-DG-treated cells, which we previously reported reduces ER stress, abolishes autophagy, but does not restore 2-DG-induced ATP reduction. (Fig. 1.7) Furthermore, results as shown in Fig. 1.8 and 1.9 demonstrate that in the cell lines studied, 2-DG induces autophagy in a similar manner as the classical ER stress inducer TM. In contrast, OX, another glycolytic inhibitor which does not disrupt ER homeostasis, but reduces ATP levels, does not activate autophagy. Thus, our results point to ER stress, and not lowered ATP, as the predominant mechanism by which 2-DG induces autophagy.
The discrepancies between the results from Yang’s group and ours as to how 2-DG stimulates autophagy might be explained by recent results which showed that ER stress induced by TM stimulates eEF2K (Boyce et al., 2008). In a subsequent study the same group reported that ER stress-stimulated eEF2K participates in autophagy induction (Py et al., 2009). Thus, activation of eEF2K could be a downstream event in response to ER stress to induce autophagy independently of ATP reduction. In fact, in one of the cell lines (T98G) used in Yang’s study we find that mannose is also able to reverse 2-DG-induced autophagy as well as ER stress (data not shown), further supporting our conclusion that ER stress is the major activator of autophagy in response to 2-DG.

Next, we explore the detailed signaling pathway(s) that mediates autophagy activation downstream of 2-DG-induced ER stress. We present evidence that supports a mechanism by which 2-DG-induced ER stress results in increased Ca\(^{2+}\) leak from the ER lumen, which subsequently activates AMPK via CaMKK\(\beta\), and ultimately leads to activation of autophagy (Fig. 1.10-1.12). Moreover, we show that TM, which does not reduce ATP, also stimulates autophagy through the Ca\(^{2+}\)-CaMKK\(\beta\)-AMPK pathway, further corroborating the role of this signaling cascade in mediating ER stress-induced autophagy.

The effects of 2-DG and TM in inducing a slow, moderate rise in [Ca\(^{2+}\)]\(c\) as a result of leakage from the ER are in contrast to those of TG, which by blocking ER Ca\(^{2+}\) uptake induces an immediate spike in [Ca\(^{2+}\)]\(c\). In fact, in the one report in which the CaMKK\(\beta\)-AMPK pathway has been shown to be activated by TG leading to autophagy induction, other Ca\(^{2+}\) mobilizers that increase [Ca\(^{2+}\)]\(c\) were shown to do the same (Hoyer-Hansen et al., 2007). However, from that report it was subsequently assumed that ER stress in
general activates the CaMKKβ-AMPK signaling even though it was the dramatically increased levels of intracellular Ca\(^{2+}\) that was stimulating this pathway and not the former. In the case of TG, ER stress was a secondary consequence of ER Ca\(^{2+}\) depletion and it was the subsequent spike in \([\text{Ca}^{2+}]_c\) that led to activation of AMPK. Here, we provide the first evidence that the Ca\(^{2+}\)-CaMKKβ-AMPK pathway can be stimulated as a consequence of ER stress leading to activation of autophagy.

It is well established that AMPK senses energetic stress and is thus activated to modulate metabolism for cellular adaptation (Mihaylova and Shaw, 2011; Shackelford and Shaw, 2009). Given the central role of metabolism in almost every aspect of cellular activities, it is tempting to speculate that the master metabolic regulator AMPK might also be stimulated under other stress conditions (Anderson et al., 2008). Indeed, here we show that AMPK is activated in response to ER stress (Fig. 1.10-1.12). It is known that under resting states, the ER can indirectly affect metabolism by regulating electron transport chain activity through Ca\(^{2+}\) loading into the mitochondria (Cardenas et al., 2010). Additionally, it has been reported that under ER stress inositol-1,4,5-trisphosphate receptor (Deniaud et al., 2008) or ER-localized Bax/Bak oligomerization (Zong et al., 2003) mediates Ca\(^{2+}\) release from the ER. Thus, results presented here suggest that the ER might also regulate cellular metabolism via the Ca\(^{2+}\)-CaMKKβ signaling to activate AMPK under stress conditions. Along this line, it appears that in addition to its canonical role of sensing energy deprivation, AMPK has evolved to sense stress signals from the ER as well.

Although the present study (Fig. 1.14) as well as a previous report provides strong evidence that GS-induced ATP reduction stimulates autophagy via the LKB1-AMPK
pathway (Liang et al., 2007), involvement of GS-induced ER stress in autophagy activation remains unexplored. Here, by using both pharmacologic and genetic methods to alleviate GS-induced ER stress, we show for the first time that GS-induced ER stress contributes to autophagy activation (Fig. 1.15). Interestingly, while the $\text{Ca}^{2+}$-CaMKKβ-AMPK signaling mediates 2-DG-induced autophagy downstream of ER stress, this pathway does not appear to play a role in inducing autophagy in response to ER stress triggered by GS (Fig. 1.16A). Further experimentation will be required to identify the mechanism(s) by which GS-induced ER stress activates autophagy.

The three ER-resident transmembrane UPR transducers, PERK, IRE1 and ATF6 have been shown to mediate ER stress-induced autophagy under different contexts (Kouroku et al., 2007; Lepine et al., 2011; Ogata et al., 2006; Py et al., 2009; Rouschop et al., 2010; Sakaki et al., 2008). However, none of them have been reported to consistently participate in autophagy activation by ER stress. It appears that the type of ER stress incurred largely dictates the involvement of any or all of the UPR transducers. Using knockout and knockdown approaches, we show here that neither 2-DG- nor GS-induced ER stress depends on the UPR transducers to stimulate autophagy (Fig. 1.13 and 1.16B-D). These results further support the idea that responses and signaling pathways elicited by different ER stressors vary depending on the nature of the stress they induce.

In addition to ATP reduction and ER stress, another biological consequence of GS is the increased production of cellular ROS. This occurs by the inhibition of the pentose phosphate pathway (PPP) due to a lack of substrate (G-6-P), and the subsequent failure to maintain a sufficient NADPH level that is critical to the proper functioning of the cellular antioxidant system (Buchakjian and Kornbluth, 2010). Although the major intracellular
metabolite of 2-DG, 2-DG-6-P cannot be further metabolized by the glycolytic pathway, there is evidence that this 2-DG derivative can be utilized by PPP to produce NADPH (Ferretti et al., 1992; Le Goffe et al., 2002). Therefore, moderate concentrations of 2-DG, such as 4 mM used in this study, should not necessarily increase ROS, and only when 2-DG is used at doses high enough to allosterically inhibit HK should a lack of PPP substrate and a subsequent increase in ROS occur. In fact, our results show that not only does 2-DG not increase ROS levels, it actually decreases them (Fig. 1.19B). This latter observation is in agreement with previous reports from several different groups (Duan and Mattson, 1999; Le Goffe et al., 2002; Lee et al., 1999; Park et al., 2009; Zhang et al., 2003). A possible explanation is that at low 2-DG doses, PGI but not HK is inhibited (Kurtoglu et al., 2007b), which would result in rerouting of G-6-P through the PPP thereby increasing NADPH leading to lowered ROS levels. In fact, multiple lines of evidence support the idea that decelerating downstream reactions facilitates the flux of glycolytic intermediates through various biosynthetic pathways including the PPP (Cairns et al., 2011; Vousden and Ryan, 2009).

ROS are known to be important signaling molecules, and in consistent with a previous report (Lee et al., 1998), we find in this study that GS-induced increases in ROS lead to stimulation of ERK, the well-known master regulator that integrates and processes various cellular signals. Moreover, we show that this stimulation does not require activation (above basal levels) of its upstream signal transducer MEK (Fig. 1.17-1.19, and data not shown). This latter result may be explained by previous reports which demonstrated that ROS stimulates ERK through directly inhibiting its phosphatases (Foley et al., 2004; Lee and Esselman, 2002; Levinthal and Defranco, 2005; Traore et al.,
In contrast to GS, the 2-DG activation of ERK as presented in Fig. 1.19 is accompanied by an increase of MEK phosphorylation. However, while the ROS-dependent ERK activation by GS plays an important role in autophagy induction, the increased ERK activity in response to 2-DG (presumably via MEK activation) does not (Fig. 1.17 and 1.19, and data not shown). Although ERK has been shown to positively regulate autophagy (Cagnol and Chambard, 2010), it has also been reported to suppress the TSC1/TSC2 complex and activate mTOR (Laplante and Sabatini, 2012), therefore acting as a potential negative regulator of autophagy. The discrepancies in these reports as well as in our own findings, i.e. GS vs. 2-DG, could be due to the various subcellular localizations ERK resides in (Wortzel and Seger, 2011), responding to different upstream signals which would result in distinct outcomes potentially including modulation of autophagy (Dagda et al., 2008; Zhu et al., 2003).

GS and 2-DG are often thought to be one in the same in terms of investigating biological consequences of interfering with glucose metabolism and the mechanisms involved. However, our results presented here clearly reveal non-overlapping biological responses to GS vs. 2-DG, and distinct molecular mechanisms leading to apparently a similar cellular effect, i.e. autophagy activation. While in 2-DG-treated cells autophagy is mainly activated by ER stress induction through the Ca\(^{2+}\)-CaMKKβ-AMPK pathway (Fig. 1.10-1.12), in GS-treated cells the upregulation of autophagy is only partially attributed to ER stress via a yet unknown mechanism independently of the above mentioned pathway (Fig. 1.15 and 1.16A). Additionally, although both 2-DG and GS reduce ATP and are able to activate AMPK in a LKB1-dependent manner, this LKB1-AMPK energy sensing axis contributes to autophagy stimulation only in cells subjected
Figure 1.21. Model of differential autophagy activation by 2-DG vs. GS. Both 2-DG and GS activate autophagy. However, 2-DG does so mainly through the induction of ER stress and the subsequent Ca\(^{2+}\)-CaMKK\(\beta\)-AMPK signaling, while GS stimulates autophagy via ER stress (CaMKK\(\beta\)-independent), ATP reduction (LKB1-AMPK) as well as ROS (ERK).

to GS but not 2-DG (Fig. 1.14). Further support for this conclusion comes from the findings that GS- but not 2-DG-induced ERK activation plays a critical role in autophagy stimulation (Fig. 1.17). Whereas GS increases ROS which subsequently activate ERK independently of the canonical MAPK signaling, 2-DG decreases ROS and augments ERK activity likely through the MAPK pathway (Fig. 1.19). Overall, our current study strongly suggests that caution should be applied when these two types of glucose
restriction are attempted to be used interchangeably, and that the findings obtained from one do not necessarily apply to the other.

In conclusion, data presented in this chapter support a model (Fig. 1.21) whereby 2-DG activates autophagy predominantly through ER stress and its subsequent activation of the Ca\textsuperscript{2+}-CaMKKβ-AMPK signaling pathway. On the other hand, GS induces autophagy by a number of mechanisms which include activation of the LKB1-AMPK energy sensing pathway, stimulation of the ROS-ERK signaling, and induction of ER stress via a yet to be identified pathway.
CHAPTER 2

Under Hypoxia 2-Deoxyglucose and Glucose Starvation Similarly Inhibits Autophagy

2.1 Overview

Hypoxia is a characteristic microenvironmental condition that most if not all solid tumors encounter during their progression (Bertout et al., 2008; Hockel and Vaupel, 2001). When the rates of rapid tumor growth outpace those of angiogenesis and neovascularization, certain tumor cell populations which commonly located in the center of a tumor face insufficient blood and O₂ supply. These tumor cells therefore experience chronic or permanent conditions of hypoxia. Additionally, due to the highly dynamic angiogenesis as well as aberrant and leaking vasculature of solid tumors, a fraction of tumor cells also undergo cycles of transient or acute hypoxia and reoxygenation. These heterogeneous tumor pathophysiologic conditions result in a wide range of hypoxia levels across different tumors and even within a single tumor, spanning from less than 0.01% to ~2% of O₂ tensions. Regardless of the exact level of O₂ concentrations, tumor hypoxia has been collectively shown to play essential roles during tumorigenesis.

It has been clearly shown that tumor hypoxia correlates with poor patient prognosis, and a number of mechanisms have been proposed to account for the selective advantage of tumor progression and survival favored by hypoxia (Fig. 2.1) (Bertout et al., 2008; Wilson and Hay, 2011). Arguably, hypoxia is the strongest stimulus of angiogenesis, which support tumor growth via supplying necessary nutrients and remodeling the tumor.
niche to create a cancer-promoting microenvironment. Moreover, hypoxia activates multiple pro-survival stress responses thereby suppressing apoptosis and enhancing adaptation capacity of hypoxic tumor cells. Recent evidence also support the notion that hypoxia promotes the maintenance and generation of cancer stem cells (CSCs), a small subset of tumor cells that have been shown in a wide variety of cancers to spawn the whole tumor and be responsible for invasion and metastasis (Keith and Simon, 2007). From an evolution point of view, hypoxia causes genomic instability and thus facilitates the selection and evolution of tumor cells (Bristow and Hill, 2008).

**Figure 2.1. Mechanisms of hypoxia-induced advancement of tumor progression and resistance to therapies.** Tumor hypoxia promotes tumorigenesis via supporting tumor growth through inducing angiogenesis, suppressing apoptosis by activating adaptive stress responses, promoting invasion and metastasis by sustaining CSCs, and facilitating overall tumor evolution via increasing gene mutation and genomic instability. Hypoxia also renders tumor cells resistance to radiotherapy and chemotherapy through inhibition of free radical generation, slowing down cell cycle and altering normal function of the DDR. CSC, cancer stem cell; DDR, DNA damage response.
In addition to the above mentioned intrinsic factors, tumor hypoxia also confers poor clinical outcomes through facilitating resistance to extrinsic anti-tumor therapeutics (Fig. 2.1) (Bertout et al., 2008; Wilson and Hay, 2011). The earliest recognized protective effect of tumor hypoxia against anti-cancer treatment is its reduction of the efficacy of radiotherapy. Since molecular O₂ is required to “fix” the potentially lethal damage on DNA that is elicited from radiation, hypoxia allows the restoration of transient damaged DNA to their original composition. Additionally, the lack of O₂ also suppresses the production of free radicals from H₂O, which prevents the indirect damage to DNA and other macromolecules. Another infamous feature of hypoxia is the chemoresistance it confers on tumor cells. Whereas the rapid proliferation of normoxic tumor cells is exploited for targeting by conventional chemotherapeutics, the slow-growing nature of hypoxic tumor cells help them escape the attack. Furthermore, alteration of DNA damage response and repair pathways can also modulate the effects of both radiotherapy and chemotherapy on hypoxic tumor cells (Bristow and Hill, 2008).

Despite the advantages hypoxia confers on pathophysiologic tumor progression and survival against therapeutic manipulations, it opens up a window of selectively targeting hypoxic tumor cells using glycolytic inhibitors such as 2-DG (Denko, 2008; Kurtoglu et al., 2007b). This latter feature is a result of the switch from aerobic to anaerobic glucose metabolism. When glycolysis is inhibited by 2-DG in the presence of O₂, cells can still produce ATP through their mitochondria using alternative energy sources, i.e. amino and fatty acids. Thus, 2-DG only induces sublethal levels of ATP reduction and does not kill most tumor cell types under normal O₂ tensions. However, under hypoxic conditions, OXPHOS in mitochondria is largely inhibited and hence cells rely mainly on glycolysis.
for energy production. Under these conditions, in contrast to normoxia, 2-DG severely depletes ATP, eventually leading to massive cell death (Fig. 2.2).

**Figure 2.2. 2-DG selectively kills hypoxic tumor cells.** In aerobic normal cells, 2-DG only moderately lowers ATP since the mitochondria can produce sufficient energy from fatty acids and amino acids through OXPHOS. However, in hypoxic tumor cells, the mitochondria can no longer generate adequate energy with limited O$_2$ availability. Hence, 2-DG severely depletes ATP in such cells and results in selective cytotoxicity. Taken from Maher et al., 2004.
In fact, a series of work from our lab have provided strong evidence demonstrating this selective toxicity of 2-DG in three models of hypoxia -- the chemical model where the mitochondrial ATP synthase inhibitor oligomycin (OM) is used to block ATP production from mitochondria; the genetic model where the so-called $\rho^0$ cells are used which lack mitochondrial DNA (mtDNA); and the environmental model where cells are cultured under low O$_2$ tensions (Liu et al., 2001; Liu et al., 2002; Maher et al., 2004). Moreover, our animal studies have also demonstrated 2-DG’s hypoxia-targeting property in a retinoblastoma model (Boutrid et al., 2008; Pina et al., 2010) as well as its enhancement of adriamycin and paclitaxel therapeutic efficacy in human osteosarcoma and non-small cell lung cancers (Maschek et al., 2004). These studies have led to a phase I clinical trial using 2-DG in combination with conventional chemotherapy to target slow-growing hypoxic and fast-proliferating normoxic tumor cells, respectively (Raez et al., 2012).

Since hypoxic cells comprise of an important subpopulation of tumor cells that 2-DG specifically targets, we investigate in this chapter the mode and mechanisms of autophagy regulation by 2-DG in cells subjected to the different models of hypoxia mentioned above. Moreover, as hypoxia is often accompanied by GS occurred in the tumor microenvironment, here we also explore how autophagy is regulated by GS under hypoxic conditions.
2.2. Results

2-DG suppresses autophagy in the chemical model of hypoxia

Previously, our lab has established a model that chemically mimics hypoxia to evaluate 2-DG’s efficacy in selectively targeting hypoxic tumor cells. In this model, OM is used to inhibit mitochondrial ATP synthase and thus the ATP production from the mitochondria (Liu et al., 2001). As expected, in 1420 cells 2-DG severely depleted ATP levels in the presence of OM, as compared to the moderate lowering of ATP achieved by 2-DG alone (Fig. 2.3A). To our surprise, the 2-DG-induced increase of LC3B-II was abolished when OM was present, and in fact, even below the basal levels of cells treated with vehicle only (Fig. 2.3B). This decrease of LC3B-II reflected a slowing-down of autophagic flux rather than an acceleration of autophagic proteolysis since blockage of lysosomal degradation by EST did not restore LC3B-II to basal levels (Fig. 2.3B). Moreover, the LC3B immunofluorescence data further showed that there was a slight reduction of autophagosome formation in 1420 cells treated with 2-DG in this chemical model of hypoxia (Fig. 2.3C). To better understand this reduction of autophagy in response to 2-DG under chemical hypoxia, the changes of LC3B-II expression were monitored over 24 h. Whereas no change was detected after 8 h of treatment, LC3B-II levels began to decrease at 16 h, and continued to diminish during the remainder of the experiment both in the presence and absence of EST (Fig. 2.3D). Similar to the results obtained in 1420 cells, 2-DG also induced a severe ATP drop, and reduced LC3B-II levels in the presence of OM in MDA-MB-435 cells (Fig. 2.3E and F). Overall, these data show that in contrast to the stimulation of autophagy by 2-DG in cells grown
aerobically, autophagy activity was diminished by this glucose analog under chemically-mimicked hypoxic conditions where mitochondrial ATP production is blocked.

**Figure 2.3. 2-DG decreases autophagy activity in the chemical model of hypoxia.** (A) 1420 cells were treated with 4 mM of 2-DG for 5 h in the presence or absence of 0.1 μg/ml of OM, followed by measurement of intracellular ATP levels. Results are representative of three independent experiments. Error bars indicate SD of triplicate samples. ###, P<0.001 compared to 2-DG treated alone. (B) 1420 cells were treated with 2-DG or OM alone or 2-DG plus OM with or without 4 μg/ml of EST. LC3B-II expression was measured by Western blot 16 h after treatment. (C) 1420 cells were treated with vehicle or 2-DG plus OM for 16 h, and the endogenous LC3B (green) distribution was observed by fluorescent microscopy. Nuclei were counterstained using DAPI (blue). One of two individual experiments is shown. (D) 1420 cells were treated with 2-DG or OM alone or 2-DG plus OM with or without 4 μg/ml of EST. LC3B-II expression was measured by Western blot at the indicated time points. (E and F) MDA-MB-435 cells were treated and analyzed similarly as in (A) and (B), respectively, except that 2-DG was used at 10 mM. All Western blot results are representative of at least three independent experiments.
2-DG reduces autophagy in the genetic model of hypoxia

In addition to the chemical model, a genetic model of hypoxia has also been used in our previous studies to probe in vitro the anti-tumor effects of 2-DG (Liu et al., 2001).

### Figure 2.4. 2-DG suppresses autophagy activity in the genetic model of hypoxia.

(A) 143B cells and the mtDNA deficient \( \rho^0 \) derivatives 206 cells were treated with 10 mM of 2-DG for 16 h in the presence or absence of 10 \( \mu \)g/ml EST/Pep A, followed by Western blot analysis of LC3B-II expression. (B) MDA-MB-435 cells (435) and the corresponding \( \rho^0 \) cells (435\( \rho^0 \)) were similarly treated and analyzed as in (A). Results are representative of at least two independent experiments.

This genetic model makes use of the so-called \( \rho^0 \) cells, which are depleted of their mitochondrial DNA (mtDNA) compared to their parental counterparts via long-term culture in the presence of ethidium bromide, a compound which inhibits mtDNA replication. Here, we extended the above mentioned findings in the chemical hypoxia model to the genetic one. As shown in Fig. 2.4A, whereas in human osteosarcoma cell line 143B 2-DG increased LC3B-II expression, this increase was abrogated in the corresponding \( \rho^0 \) cells (206) treated with 2-DG. In fact, when the lysosomal protease
inhibitors EST and pepstatin A (EST/Pep A) were used, LC3B-II levels in 2-DG-treated 206 cells were much lower than those in vehicle-treated parental 143B cells. Similar observations were made in another cell pair, MDA-MB-435 (435) and 435p0, respectively (Fig. 2.4B). These data indicate that in cells lack of mtDNA and hence cannot undergo mitochondrial-mediated ATP synthesis, 2-DG inhibits both autophagosome formation and degradation, thereby resulting in a strong reduction of autophagy activity.

2-DG and GS inhibit autophagy in the environmental model of hypoxia

In order to determine how 2-DG regulates autophagy under more physiologic conditions, we utilized an environmental model of hypoxia (Liu et al., 2002; Maher et al., 2004) where 1420 cells were placed under low levels of O2 and assayed for autophagy activity. We found that in cells grown under 1% O2 (mild hypoxia), 2-DG showed a similar upregulation of LC3B-II as compared to 2-DG-treated cells grown under 21% O2 (normoxia). However, at a lower O2 concentration of 0.1% (moderate hypoxia), 2-DG-induced LC3B-II upregulation was largely attenuated and under <0.1% O2 (severe hypoxia) completely abrogated (Fig. 2.5A, top panel). Notably, when EST/Pep A was included in these experiments, 2-DG lost its LC3B-II inducing ability at moderate hypoxia, and even lowered the levels of this autophagy marker in cells grown under severe hypoxia compared to those under normoxia without drug exposure (Fig. 2.5A, bottom panel). These results suggest that 2-DG inhibits autophagy activity in cells cultured under moderate to severe hypoxic conditions. Moreover, the increased ability of 2-DG to reduce LC3B-II expression along with decreasing O2 levels was found to be well
correlated with its depletion of intracellular ATP under different hypoxic conditions (Fig. 2.5B).

**Figure 2.5.** 2-DG inhibits autophagy activity in the environmental model of hypoxia. (A) 1420 cells were treated with 10 mM of 2-DG under different O₂ tensions for 24 h in the presence or absence of 10 μg/ml EST/Pep A, followed by Western blot analysis of LC3B-II expression. (B) 1420 cells were treated with 10 mM of 2-DG under different O₂ tensions for 16 h. Intracellular ATP levels were measured and normalized to protein contents. (C) 1420 cells were treated with 2-DG at increasing doses (0, 0.5, 1.5, 5, 12 and 24 mM) under 21% or <0.1% O₂ tensions for 24 h in the presence or absence of EST/Pep A, followed by Western blot analysis of LC3B-II expression. (D) 1420 cells were treated with 2-DG at increasing doses (0, 0.5, 1.5, 5, and 12 mM) under 21% or <0.1% O₂ tensions for 16 h. Intracellular ATP levels were measured and normalized to protein contents. Results are representative of at least two independent experiments. Error bars indicate SD of triplicate samples.
To determine the concentration of 2-DG required to block autophagy under conditions of severe hypoxia, we treated cells with doses of this sugar analog ranging from 0.5 to 24 mM. While cells were found to be able to upregulate LC3B-II at all 2-DG doses under normoxia, under severe hypoxia this was only observed with low (0.5 and 1.5 mM) but not high doses (5, 12 and 24 mM) (Fig. 2.5C, top panel). In fact, when EST/Pep A was present, high doses of 2-DG under severe hypoxia reduced LC3B-II expression below basal levels (Fig. 2.5C, bottom panel), indicating that high doses of 2-DG markedly impair autophagy activity under this condition. Notably, this impairment is associated with the dramatically depleted ATP levels achieved only by high doses of 2-DG under severe hypoxia (Fig. 2.5D). Although due to the sensitivity of the technique we used to measure ATP, it is difficult to reach an exact number of ATP levels required to sustain autophagy activity, our data (Fig. 2.5B and D) indicate that an ATP reduction more than 50% might be a threshold to switch autophagy from activation to inhibition. It is also important in the future to determine the autophagy regulating roles of ATP produced from different cellular compartments, e.g. mitochondria vs. glycolysis.

<table>
<thead>
<tr>
<th></th>
<th>- EST/Pep A</th>
<th>+ EST/Pep A</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS</td>
<td>21%</td>
<td>&lt;0.1%</td>
</tr>
<tr>
<td>LC3B-II</td>
<td>- +</td>
<td>- +</td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.6. GS inhibits autophagy activity under severe hypoxia.** 1420 cells were glucose starved under 21% or <0.1% O₂ tensions for 16 h in the presence or absence of 10 μg/ml of EST/Pep A, followed by Western blot analysis of LC3B-II expression. Results are representative of three independent experiments.
Since GS is often accompanied by hypoxia in solid tumors, we next aimed to determine how autophagy was modulated by GS under hypoxic conditions. Similar to the effects of 2-DG as shown above, under severe hypoxia GS was incapable of increasing LC3B-II, and in the presence of EST/Pep A further reduced its levels as compared to those in unstarved cells under normoxia (Fig. 2.6). Moreover, in a chronic hypoxia model where cells were grown under moderate hypoxia 24 hrs before subjected to treatment, 2-DG and GS elicited a similar pattern of LC3B-II expression (Fig. 2.7) that is reminiscent of our previous findings obtained under acute moderate hypoxia (Fig. 2.5A). Overall, our data presented here provide strong evidence that both 2-DG and GS suppress autophagy activity under hypoxia, which is well correlated with severe ATP depletions.

![Western blot image showing LC3B-I, LC3B-II, and β-Actin levels under different conditions](image)

**Figure 2.7. Both 2-DG and GS suppress autophagy activity under a model of chronic hypoxia.** 1420 cells were incubated under 21% or 0.1% O2 tensions for 24 h. Then, the cells were subjected to 10 mM of 2-DG or GS in the presence or absence of 10 μg/ml of EST/Pep A without disturbance of the O2 levels. After another 16 h incubation, LC3B-II expression was measured by Western blot. Results are representative of two independent experiments.

2-DG and GS block autophagy under severe hypoxia at multiple levels

To better understand the mechanism(s) by which 2-DG decreases autophagy activity under severe hypoxia, an autophagy PCR array was used to examine the mRNA
expression of autophagy-associated genes. It is noteworthy that 2-DG decreased the mRNA levels of the majority of the core autophagy machinery components in 1420 cells grown under severe hypoxia compared to those under normoxia without drug treatment (Fig. 2.8). This result indicates that under severe hypoxia, glucose restriction may inhibit autophagy at various stages.

Figure 2.8. 2-DG decreases the expression of the core autophagy machinery components under severe hypoxia. 1420 cells were either left untreated under 21% O₂ or treated with 10 mM of 2-DG under <0.1% O₂ for 24 h. The mRNA levels of the indicated core autophagy machinery components were measured on an autophagy PCR array using qPCR. Bars represent the average fold change from three independent experiments of the mRNA levels in 2-DG-treated cells grown under <0.1% O₂ compared to those in untreated cells under 21% O₂. *, statistically significant with $P<0.05$ or less.

Accordingly, autophagy initiation, expansion and degradation were examined in cells treated with either 2-DG or GS under severe hypoxia. The interaction between Beclin1 and PI3K III is critical for the latter’s autophagy-specific enzyme activity and the initiation of autophagy (He and Klionsky, 2009). While neither 2-DG nor GS interfered with the functional PI3K III levels in normoxic cells as assessed by the amount of the
PI3K III proteins co-immunoprecipitated with Beclin1, both treatments reduced this amount in cells under severe hypoxia (Fig. 2.9A). Next, the covalent conjugation of Atg12 to Atg5, an indispensable step during autophagosome expansion (He and Klionsky, 2009), was investigated. Although autophagy PCR array data showed no significant decreases in Atg5 and Atg12 transcripts in 2-DG-treated cells under severe hypoxia (Fig. 2.8), Western blot analysis clearly revealed a reduction of the Atg5-Atg12 conjugate formation under this condition (Fig. 2.9B). Our previous autophagy flux data obtained in the presence of EST/Pep A (Fig. 2.5A and C, and 2.6) suggest that autophagy degradation is impaired in cells subjected to 2-DG or GS under severe hypoxia. Here, we used two additional assays to further corroborate these findings and better understand the mechanism of autophagy degradation impairment under this condition. Firstly, due to the relative stability of EGFP within the autolysosome degradative environment, we monitored the amount of free EGFP resulting from cleavage of EGFP-LC3B fusion proteins as a marker of autophagy degradative activity (Klionsky et al., 2012). Under normoxia, both 2-DG and GS upregulated the free EGFP levels, consistent with increased autophagy activity under this condition. However, when cells were grown under severe hypoxia, 2-DG and GS could no longer increase its amount (Fig. 2.9C). Secondly, LysoTracker Green (LTG) was used to measure the functional lysosome compartment. Flow cytometric analysis showed that under normoxia, both 2-DG and GS enhanced the dye staining. This indicates an increased lysosome number/activity, and is in agreement with an upregulated degradation demand during autophagy stimulation (Fig. 2.9D). However, when 2-DG or GS was applied to cells under severe hypoxia, LTG staining was reduced as compared to that in untreated control
cells under normoxia, suggesting a decreased functional lysosomal compartment and autophagy degradative capacity (Fig. 2.9D). Taken together, our results show that 2-DG and GS disrupt autophagy under severe hypoxia at multiple steps, including initiation, expansion as well as degradation.

Figure 2.9. 2-DG and GS inhibit autophagy at multiple stages. (A) 1420 cells were subjected to 10 mM of 2-DG or GS under 21% or <0.1% O₂ tensions. Protein lysates were harvested after 24 h of treatment, and part of the lysates were immunoprecipitated with an anti-Beclin1 primary antibody. Lysates from untreated cells grown under 21% O₂ were also immunoprecipitated with a species- and isotype-matched control IgG. Western blot was used to measure PI3K III and Beclin1 levels in the immunoprecipitated (IP Beclin1) as well as the whole cell lysates (WC). (B) 1420 cells were similarly treated as in (A), and the Atg5-Atg12 conjugate levels were analyzed by Western blot. (C) 1420 cells stably expressing EGFP-LC3B fusion proteins were similarly treated as in (A), and the free EGFP levels were analyzed by Western blot. All blots show one representative experiment from three independent ones. (D) 1420 cells were similarly treated as in (A), and the functional lysosomes (FL1-A %) were measured by flow cytometry using the lysosome trophic dye LysoTracker Green. Error bars represent SEM of three independent experiments. *, P<0.05, and ***, P<0.001 compared to untreated cells under 21% O₂; ^, P<0.05 compared to untreated cells under 21% O₂.
2.3 Discussion

Under normal O₂ conditions the moderate reduction of ATP by 2-DG does not appear to play a significant role in autophagy activation. Since 2-DG is currently under clinical investigation directed at selectively targeting the hypoxic malignant cell populations in solid tumors (Raez et al., 2012), we addressed the question of how autophagy responds to 2-DG when cells are grown under anaerobic hypoxic conditions. As expected, ATP levels are severely depleted in three different models -- chemical, genetic and environmental hypoxia. However, to our surprise, in contrast to the upregulation of autophagy activity in cells grown under normoxia, 2-DG decreases autophagy even below basal levels when cells are grown in either of these hypoxia models (Fig. 2.3-2.5 and 2.7). Interestingly, these results are consistent with a report where the complex I inhibitor metformin was used to block mitochondrial respiration and ATP generation (Ben Sahra et al., 2010a; Ben Sahra et al., 2010b). Thus, it appears that one of the advantages of using 2-DG as an anti-cancer drug could be the lack of autophagy activation or even a blockage of this pro-survival process in certain hypoxic tumor cell populations. Similar to 2-DG, GS also suppresses autophagy activity under hypoxia (Fig. 2.6 and 2.7). Since GS is often accompanied by hypoxia found in solid tumors, our findings with autophagy inhibition by GS under severe hypoxia may have pathophysiologic relevance toward a more comprehensive understanding of the tumor microenvironment.

Although previous reports demonstrated a generally positive correlation between in vivo tumor hypoxia (and potentially lack of glucose) and autophagy markers (Degenhardt
et al., 2006; Karantza-Wadsworth et al., 2007), these analyses were not designed to measure autophagy flux. Based on our autophagy activity assays with GS under moderate and severe hypoxia, some of the increase of the markers might actually reflect a blockage of autophagic degradation and a decrease of autophagy flux. Additionally, due to tumors’ tremendous heterogeneity in their hypoxia extents (from about 2% to less than 0.01%) and regions, it is also very likely that the small amount of severe hypoxic tumor cells, which bear low autophagy markers, could be overlooked by the relatively greater number of surrounding mild hypoxic cells displaying increased marker levels. Future development of more accurate in vivo autophagy flux assays, higher-resolution analytical methods on a per cell basis, as well as more sensitive hypoxia probes that can distinguish between subtle differences in O₂ tensions, would be necessary to resolve the above mentioned issues and clarify the modulation of autophagy by GS in severe hypoxic tumors cells in vivo.

Energy deprivation is generally considered a universal autophagy stimulator. However, our current findings show in three different models of hypoxia that when glucose is restricted and ATP is markedly depleted, autophagy activity is decreased instead of increased. Interestingly, the inhibition of autophagy by 2-DG and GS under severe hypoxia occurs even when there are robust upstream signals for autophagy induction, i.e. strong AMPK activation and near complete mTOR inhibition (data not shown). These observations suggest that drastic ATP depletion acts as a common mechanism to block autophagy activity downstream of the autophagy induction stage. Indeed, our findings are in agreement with earlier studies showing an energy dependence of autophagy as a process that involves highly dynamic and energy-consuming events.
Hypoxia

2-DG & GS

↓

ATP↓↓↓

PI3K III-Beclin1

ATG12-ATG5

Lysosome

Autophagy

Figure 2.10. Model of similar autophagy inhibition by 2-DG and GS under hypoxia. Both 2-DG and GS inhibit autophagy under hypoxic conditions. This inhibition occurs at multiple steps of the autophagy process, including initiation (PI3K III-Beclin1 complex formation), expansion (ATG12-ATG5 conjugation) and degradation (functional lysosomes), and is likely a consequence of severely depleted ATP levels under this condition.

including membrane/vesicle flow and lysosome acidification (Meijer, 2009; Plomp et al., 1987; Plomp et al., 1989; Schellens and Meijer, 1991). Therefore, evidence provided by us and others raises questions concerning the linear relationship between energy depletion and autophagy activation, and indicates a bi-modal regulation of autophagy by ATP deprivation. Thus, it appears that in a cell type- and stress-dependent manner,
moderate ATP depletion activates autophagy when cells still have sufficient energy to execute this process, while severe ATP depletion inhibits autophagy through preventing the ATP-dependent autophagy steps from functioning.

Taken together, we show in this chapter that glucose restriction inhibits autophagy activity under different models of hypoxia, which likely results from severe ATP depletion observed under these conditions. Specifically, the autophagy blockage by 2-DG and GS under severe hypoxic occurs through disruption of the PI3K III-Beclin1 complex for autophagy initiation, the conjugation of ATG12 to ATG5 for autophagosome expansion, as well as inhibition of the functional lysosomal compartment for autophagic degradation (Fig. 2.10).
CHAPTER 3

Autophagy Protects Tumor Cells against 2-Deoxyglucose Cytotoxicity via Relieving Endoplasmic Reticulum Stress

3.1 Overview

UPR, as described above in Chapter 1, initially serves as an adaptive response to cope with ER stress for cells to survive. However, if the insults are too severe or persist for too long time, UPR will lead to apoptosis through upregulation of CHOP, activation of c-Jun NH$_2$-terminal kinase (JNK) and/or cleavage of caspase 4 (caspase 12 in murine cells). Moreover, ER stress will also results in cell death via Ca$^{2+}$-mediated mechanisms such as mitochondria overloading and calpain activation (Ferrari et al., 2002; Schroder and Kaufman, 2005; Szegedi et al., 2003).

Although the major therapeutic targets of 2-DG are the hypoxic tumor cells where this sugar analog severely depletes cellular ATP levels, our lab has recently found that 2-DG also preferentially kills a subset of tumor types even in the presence of normal O$_2$ levels (Kurtoglu et al., 2007a). This selective sensitivity to 2-DG in these cells are associated with their heightened susceptibility to 2-DG’s inhibitory effect on N-linked glycosylation and its induction of ER stress/UPR-mediated CHOP expression. Moreover, the addition of exogenous mannose not only reverses 2-DG-elicited interference with N-linked glycosylation and ER stress/UPR induction, but abolishes 2-DG’s apoptotic and cytotoxic effects. These findings demonstrate that 2-DG kills selected tumor types under
normoxia via interference with N-linked glycosylation and induction of ER stress/UPR-mediated apoptosis.

Autophagy has been best recognized as a cytoprotective process activated in response to starvation, whereby it digests and recycles bulk cytoplasmic contents to provide biosynthetic precursors and energy for cell survival. Recently, the role of autophagy in targeting specific cellular components and organelles has also been well documented (Klionsky et al., 2011). This includes the sequestration and degradation of damaged mitochondria (mitophagy), superfluous peroxisomes (pexophagy) and ribosomes (ribophagy), accumulated protein aggregates (aggrephagy) and lipid droplets (lipophagy), as well as expanded ER compartment during ER stress (reticulophagy). Particularly, autophagy of ER has been demonstrated to counterbalance the deleterious effects of ER stress induced in yeasts (Bernales et al., 2006).

Although autophagy is in general considered as a pro-survival mechanism in response to most developmental cues and physiologic level of stresses, it has also been shown to play a pro-death role in certain circumstances. For example, there is evidence that autophagy is necessary for the complete tissue remodeling and cell loss during Drosophila salivary gland development (Levine and Klionsky, 2004). Moreover, a variety of chemicals have been shown to induce autophagic cell death, or type II programmed cell death (Maiuri et al., 2007). For example, Ding et al. have reported that autophagy induced by a number of ER stressors confers cytoprotection to cancer cells while enhances cell death in untransformed normal cells (Ding et al., 2007a).

So far, the exact mechanisms that determine whether autophagy plays a pro- or anti-survival role in a certain circumstance remain largely unknown. Nevertheless, it appears
that the life-or-death decision depends on a combination of multiple factors including the nature of the stresses, the extent and duration of the sustained autophagy activity, the substrates of autophagy degradation, as well as the genetic contexts of the cells and tissues. Based on the current literature, autophagy appears to either promote cell survival through facilitating adaptation or execute cell death due to excessive self-cannibalism and -destruction. In addition, autophagy has also been shown to either excluded from or coexist with apoptosis, and to suppress or accelerates the later process resulting in cell survival or death (Fig. 3.1) (Maiuri et al., 2007).

**Figure 3.1. Mode of autophagy determination of life or death.** In a context specific manner, stress-induced autophagy can either facilitates cellular adaptation and/or blocks apoptosis thereby promoting cell survival, or execute massive self-destruction and/or enhances apoptosis thus accelerating cell death.

Therefore, using both pharmacologic and genetic approaches, we investigated in this chapter the functional role of autophagy in cell death/apoptosis induced by 2-DG
treatment. Moreover, we also studied how autophagy activation modulates 2-DG-induced ER stress/UPR.

3.2 Results

_Inhibition of autophagy exacerbates whereas enhancement of autophagy ameliorates 2-DG cytotoxicity_

2-DG has shown anti-tumor activity both in vitro and in vivo, and has entered two phase I clinical trials (Raez et al., 2012; Stein et al., 2010). In this regard, we investigated the role autophagy plays in 2-DG-elicited cytotoxicity in cancer cells. In agreement with previous reports (DiPaola et al., 2008; Wu et al., 2009), we found that autophagy protected cells from 2-DG-induced cell death in various cancer cell lines. On one hand, 3-methyladenine (3-MA), an autophagy inhibitor by blocking PI3K III, increased 2-DG-induced cell death in 1420 cells (Fig. 3.2A) and sensitized MDA-MB-435 cells to 2-DG treatment (Fig. 3.2B). On the other hand, the mTOR inhibitor and autophagy promoter rapamycin reduced cell death in both 1420 (Fig. 3.2C) and SKBR3 (Fig. 3.2D) cells treated with 2-DG.

_Blockage of autophagy increases 2-DG-induced ER stress and apoptosis_

Previously, we have reported that 2-DG causes cell death through ER stress/UPR-mediated apoptosis in selected cancer cell lines under normoxia, including 1420 cells (Kurtoglu et al., 2007a). To investigate whether autophagy reduces 2-DG-induced cytotoxicity through modulating ER stress, we examined autophagy, ER stress/UPR and apoptosis markers in response to 2-DG treatment in 1420 cells where Atg7 was
Figure 3.2. 3-MA increases and rapamycin decreases 2-DG cytotoxicity. (A) 1420 cells were treated with different doses of 2-DG (0, 2, 4, 8, and 16 mM) for 48 h in the presence or absence of 10 mM of 3-MA. (B) MDA-MB-435 cells were treated with different doses of 2-DG (0, 8, 16, 25, and 32 mM) for 48 h in the presence or absence of 3-MA. (C) 1420 cells were treated with different doses of 2-DG (0, 2, 4, 8, and 16 mM) for 72 h in the presence or absence of 0.1 μg/ml rapamycin (Rapa). (D) SKBR3 cells were treated with different doses of 2-DG (0, 8, 16, 25, and 32 mM) for 48 h in the presence or absence of Rapa. In all experiments cell death was measured after drug exposure by trypan blue exclusion assay. Error bars indicate SD of triplicate samples. #, P<0.05, and ##, P<0.01 compared to cells treated with the corresponding doses of 2-DG without 3-MA (A and B) or Rapa (D).

specifically knocked down by siRNAs. As shown in Fig. 3.3A, siRNAs against Atg7 successfully reduced the protein levels of Atg7 compared to those against luciferase, which was used as a siRNA control in this set of experiments. Knocking down of Atg7 attenuated the induction of LC3B-II by 2-DG, which correlates with the increases in the expression of the ER stress/UPR markers Grp78 and CHOP (Fig. 3.3A). Additionally, the levels of the apoptosis marker cleaved caspase 3 assessed 16 h after drug exposure were
Figure 3.3. Atg 7 knockdown enhances 2-DG-induced ER stress, apoptosis and cell death. (A) 1420 cells were transfected with either siLuc or siAtg7 and treated with different doses of 2-DG (0, 0.5, 1.5, 4 and 8 mM) for 16 h. Atg7 knockdown efficiency as well as markers for autophagy, ER stress and apoptosis were analyzed by Western blot. (B) 1420 cells were transfected with either siLuc or siAtg7 and treated with different doses of 2-DG (0, 2, 4, 8 and 16 mM) for 48 h. Cell death was measured after drug exposure by trypan blue exclusion assay. Error bars indicate SD of triplicate samples. #, P<0.05, and ##, P<0.01 compared to cells transfected with siLuc and exposed to the corresponding doses of 2-DG. All results show one representative experiments from two independent ones.

significantly increased above those in cells transfected with siLuc (Fig. 3.3A), which corresponds with increased cell death assayed at 48 h of 2-DG treatment (Fig. 3.3B). Moreover, knocking down Atg7 elicited similar increases of ER stress, apoptosis and cell death in cells treated by TM (data not shown). These observations support the findings from other groups that autophagy acts as a survival mechanism against ER stress-induced toxicity in both yeast and mammalian cells (Bernales et al., 2006; Ding et al., 2007b;
Fujita et al., 2007; Kouroku et al., 2007; Ogata et al., 2006). In summary, our data presented here demonstrate that autophagy protects cancer cells from 2-DG-induced cytotoxicity, and indicate that this is likely achieved through relieving 2-DG-elicited ER stress.

3.3 Discussion

It has been previously shown that autophagy can act either as a pro- or anti-survival mechanism (Rubinsztein et al., 2007). Here, we find that rapamycin which is known to stimulate autophagy, alleviates 2-DG toxicity (Fig. 3.2C and D), whereas 3-MA which blocks autophagy, aggravates it (Fig. 3.2A and B). In addition to these chemical compounds, specific knocking down of Atg7 by siRNAs also dramatically increased 2-DG-induced cell death (Fig. 3.3B). These data collectively show that autophagy acts as a pro-survival adaptive response to 2-DG.

Our above mentioned findings are in agreement with two recent reports showing that inhibition of autophagy increases the anti-tumor effect of 2-DG in vitro (DiPaola et al., 2008; Wu et al., 2009). In one of these studies (Wu et al., 2009), 2-DG induced autophagy was inhibited with siRNAs against eEF2K and ATP levels were lower than in cells treated with non-targeting siRNAs (Wu et al., 2009). Thus, the authors concluded that greater ATP reduction via autophagy blockade leads to increased cell death. However, previous findings from our lab have demonstrated that moderate energy reduction achieved by 2-DG under normoxia is not sufficient to kill most tumor cells and the mechanism by which 2-DG kills a subset of cancer cells in the presence of O₂ is via
ER stress/UPR-mediated apoptosis (Kurtoglu et al., 2007a). Moreover, our results which show that inhibiting 2-DG-induced autophagy through siRNAs targeting Atg7 increases the levels of ER stress/UPR markers as well as apoptosis (Fig. 3.3A), are consistent with reports from other groups demonstrating that autophagy promotes cell survival in response to ER stress (Bernales et al., 2006; Ding et al., 2007b; Fujita et al., 2007; Kouropoulu et al., 2007; Ogata et al., 2006).

Supplying ATP from autophagy relies on breaking down of the macromolecules inside the autolysosomes. Therefore, functional autophagic degradation is expected to be required for the autophagy-mediated pro-survival response if the latter effect is due to enhanced ATP production through autophagy. However, we find that suppression of autophagic degradation by the lysosome protease inhibitor EST does not enhance 2-DG cytotoxicity. Interestingly, Bernales et al. have reported that ER stress-induced autophagy protects yeast cells to a similar extent in either degradation-sufficient or -deficient strains, indicating that autophagic sequestration of expanded ER instead of its degradation is the critical step in the autophagy-mediated survival response (Bernales et al., 2006). These observations further supports that autophagy protects tumor cells against 2-DG via reducing ER stress rather than by increasing ATP.

In the previous reports, it was shown that autophagy inhibition increased cell death only when 2-DG was used at relatively high doses, i.e. 10 mM (DiPaola et al., 2008) and 6.3 to 50 mM (Wu et al., 2009), respectively. However, our data show that blocking autophagy by Atg7 knockdown greatly enhances cytotoxicity of 2-DG at a low dose of 2 mM (near 30%, Fig. 3.3B), which is within the concentration used in animal studies and close to the maximal does that could be administered in patients. In addition,
pharmacologic inhibition by 3-MA also significantly increases cell death induced by this low dose of 2-DG, albeit to a lesser extent (more than 20%, **Fig. 3.2A**).

Overall, results presented in this chapter show that autophagy plays a protective role against 2-DG-induced cytotoxicity, which is apparently through relieving ER stress. Moreover, our data also suggest that combining autophagy inhibition with 2-DG holds great promise in achieving therapeutic gain in clinic.
MATERIALS AND METHODS

Cell culture

Human pancreatic tumor cell line 1420 (MIA PaCa-2) and glioblastoma multiforme cell line T98G were purchased from ATCC, and maintained in DMEM with 1 g/L of glucose (Mediatech) and MEM with 1 g/L of glucose and 2 mM of L-glutamine (Life Technologies), respectively. Human breast cancer cell line SKBR3 was a gift from Dr. Joseph Rosenblatt (University of Miami), and maintained in RPMI with 2 g/L of glucose, 2 mM of L-glutamine and 25 mM of HEPES buffer (Life Technologies). Human osteosarcoma cell line 143B and its ρ⁰ cell pair 206 were obtained from Dr. Carlos Moraes (University of Miami) and maintained in DMEM with 4.5 g/L of glucose (Mediatech) and 50 μg/ml of uridine (Sigma-Aldrich). Human melanoma cell line MDA-MB-435²⁸ and 435ρ⁰ were provided by Dr. Keshav Singh (Roswell Park Cancer Institute) and similarly maintained. The PERK⁺/+ and PERK⁻/- as well as the IRE1⁺/+ and IRE1⁻/- MEFs were kindly provided by Dr. David Ron (University of Cambridge). The TSC2⁺/+ and TSC2⁻/- MEFs, both with p53 deleted, were gifts from Dr. Brendan Manning (Harvard University). All MEFs were cultured in DMEM with 4.5 g/L of glucose. All DMEM media for cell passage contained 4 mM of L-glutamine and 1 mM of sodium pyruvate, and were supplemented with penicillin/streptomycin (AMRESCO) and 10% FBS (Life Technologies). For glucose starvation, no glucose DMEM (Life Technologies) containing 4 mM of L-glutamine was used, with supplementation of 1 mM of sodium
pyruvate (Life Technologies), the antibiotics listed above and dialyzed FBS (Life Technologies).

**Stable cells**

pEGFP-C1 vector expressing the EGFP-LC3B fusion protein was a kind gift from Dr. Enrique Mesri (University of Miami). The plasmids were transfected into 1420 cells using Optifect (Life Technologies), and monoclonal colonies were obtained by serial dilution. Polyclonal 1420 cells stably expressing Grp78 were generated by Katherine Philips and Howard Leung (University of Miami, manuscript under preparation). Both 1420 derivatives were selected and maintained by G418 (Mediatech) at 1.5 mg/ml.

**Hypoxia**

For acute hypoxia at 1% (mild) and 0.1% (moderate) O₂ tensions, cells were treated under normal O₂ conditions and then placed in a humidified Pro-Ox *in vitro* hypoxia chamber attached to a model 110 oxygen controller (Reming Bioinstruments). A gas mixture of 95% N₂ and 5% CO₂ was used to perfuse the chamber to achieve the desired oxygen levels. For acute hypoxia at <0.1% (severe) O₂, cells were placed in a humidified pile plate following treatment, and the plate was perfused with the above mentioned gas mixture for 30 min. The pile plate was then sealed and cells were incubated for the indicated time. Experiments involving chronic hypoxia at 0.1% O₂ were performed in a humidified hypoxia glove box (Coy Laboratory Products). After pre-incubation under hypoxia for 24 h, cells were treated within the glove box to prevent fluctuation of the O₂ levels. Additionally, media and drug solutions used for treatment were also placed inside
the glove box, along with the cells, to be equilibrated to the low oxygen environment 24 h before use.

**Drugs and antibodies**

2-Deoxyglucose, 3-methyladenine, mannose, oligomycin, oxamate, N-acetyl-L-cysteine, rapamycin and tunicamycin were purchased from Sigma-Aldrich. Sodium 4-phenylbutyrate, BAPTA-AM, EST, pepstatin A, STO-609 were obtained from EMD Millipore. Salubrinal and U0126 were purchased from Enzo Life Sciences and Promega, respectively. PD325901 was a kind gift from Dr. Mark Pegram (Stanford University). The following rabbit primary antibodies were from Cell Signaling Technology: pACC (Ser79), AMPKα, pAMPKα (Thr172), Atg7, Beclin1 (Western blot), cleaved caspase 3, Grp78, LC3B (#2775 which preferentially detects LC3B-II and #3868 which detects both forms), LKB1, p-p70S6K (Thr389) and PI3K III. Mouse primary antibody against CHOP was also purchased from Cell Signaling Technology. Mouse anti-β-actin and anti-ATF6 primary antibodies were from Sigma-Aldrich and IMGENEX, respectively. Mouse anti-Beclin1 antibody and normal mouse IgG used for immunoprecipitation were obtained from Santa Cruz Biotechnology. The ERK1/2 and pERK1/2 (Thr202/Tyr204) rabbit primary antibodies as well as the GFP (EGFP) mouse primary antibody were gifts from Dr. Enrique Mesri. Rabbit primary antibodies against Atg12 and pMEK1/2 (Ser217/221) were kindly provided by Dr. Balakrishna Lokeshwar (University of Miami) and Dr. Mark Pegram, respectively. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibody were purchased from Promega. Alexa Fluor 488 anti-rabbit IgG was obtained from Life Technologies.
**ATP quantification**

Intracellular ATP levels were measured with the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer’s directions. Briefly, cells were lysed after 5 h of drug exposure with the reagent included in the assay kit for 10 min. Then the mixtures were transferred into opaque-walled 96-well plates, and luminescence produced from ATP-mediated chemical reaction was read by the luminescence module of the FLUOstar OPTIMA microplate reader (BMG LABTECH). For long-term treatment (16 h), ATP levels were normalized to protein contents measured by a Micro BCA Protein Assay (Thermo Scientific).

**Western blot**

Following drug exposure for the indicated times, cells were lysed with RIPA buffer (Cell Signaling Technology) supplemented with 1:100 phosphatase inhibitor cocktail 2 and protease inhibitor cocktail (both from Sigma–Aldrich). Protein concentrations of each sample were determined using a Micro BCA Protein Assay Kit according to the manufacturer’s directions, and equal amounts of proteins were loaded onto Tris-HCl gels (Bio-Rad). After SDS-PAGE, proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore), blocked with 5% milk and probed with corresponding primary antibodies overnight at 4°C (except 1 h for β-actin at RT). The membrane was washed and probed with secondary antibodies for 1 h at RT. Then, the membrane was incubated with SuperSignal West Pico or Femto Chemiluminescent Substrate (Thermo Scientific) and signals were visualized on Blue Lite Autorad Films (ISCBioExpress).
Quantification of blot intensity was performed using ImageJ (National Institutes of Health).

**Immunoprecipitation**

Cells were harvested using non-denaturing cell lysis buffer with 1% Triton (Cell Signaling Technology) supplemented with 1:100 phosphatase inhibitor cocktail 2 and protease inhibitor cocktail. Equal amounts of protein lysates were incubated with primary antibody overnight at 4°C. The next day, protein A/G Plus agarose beads (50% slurry) (Santa Cruz Biotechnology) were added and the mixture was incubated for another 2 h at 4°C. After centrifugation, pellets were washed with lysis buffer, and resuspended with blue loading buffer (Cell Signaling Technology). All further steps were preceded following regular Western blot procedures.

**Fluorescent microscopy**

Cells were seeded in 8-well Lab-Tek II Cc chamber slides (Thomas Scientific). Following drug exposure, cells were fixed with 10% neutral buffered formalin solution containing 4% formaldehyde (Sigma-Aldrich), and permeabilized with methanol at -20°C. Following blocking with 5% normal goat serum (Dako), cells were incubated with anti-LC3B antibody (1:400) overnight at 4°C. Then cells were washed and incubated with Alexa Fluor 488 anti-rabbit IgG (1:500) for 1 h at RT, mounted with ProLong Gold antifade reagent with DAPI (Life Technologies) and visualized with a Laborlux fluorescent microscope (Leitz) equipped with a DFC 340 FX digital camera (Leica Microsystems).
Confocal microscopy

Cells cultured in 8-well Lab-Tek II Cc chamber slides were fixed with 10% neutral buffered formalin solution containing 4% formaldehyde (VWR), washed with PBS (Mediatech) and mounted with ProLong Gold antifade reagent with DAPI. Samples were visualized with a Leica TCS SP5 confocal microscope (Leica Microsystems). Five fields were taken and used for calculating dots per cell ratios for each sample using ImageJ “Particle Analysis” function, and three of them (excluding the ones with highest and lowest ratios) were used to obtain the average ratios.

qPCR and autophagy PCR array

Total cellular RNAs were extracted by the RNeasy Plus Mini Kit (QIAGEN) and mRNAs were reverse-transcribed using the ImProm-II Reverse Transcription System (Promega). qPCR experiments were performed on an ABI 7300 Real-Time PCR System (Life Technologies) using PerfeCTa SYBR Green FastMix, ROX (Quanta Biosciences). The primer sequences for CaMKKβ are as follows: 5’ GCTGACTTTGTTGTTGAGCA 3’ (F) and 5’ AGAAGATCTTTGAGGCTTCTCA 3’ (R). The RT² Profiler Human Autophagy PCR Array (384-well plate format) was purchased from QIAGEN. Total RNAs were extracted as mentioned above and the RT² First Strand Kit (QIAGEN) was used to reverse-transcribe mRNAs. Gene expression was measured via qPCR using the RT² qPCR Mastermix (QIAGEN) on an ABI 7900HT Fast Real-Time PCR System (Life Technologies). PCR array data were analyzed using the RT² Profiler PCR Array Data Analysis tools available on the manufacturer’s website.
**ROS detection**

After drug exposure, cells were trypsinized and resuspended in PBS. Equal numbers of cells were labeled with 1.5 μM of ROS indicator CM-H₂DCFDA (Life Technologies) in PBS for 30 min at 37°C. The C6 Flow Cytometer with the accompanying Accuri CFlow software (BD Biosciences) was used to measure and analyze the CM-H₂DCFDA fluorescence signals.

**Lysosome measurement**

Cells were similarly harvested as for ROS detection. Equal numbers of cells were incubated with 80 nM of LysoTracker Green (Life Technologies) in PBS for 5 min at 37°C, followed by flow cytometric analysis.

**[Ca²⁺]ᵋ measurement**

After drug exposure, cells in 24-well plates were washed with assay buffer consisting of DPBS containing 0.1 g/L CaCl₂ (Mediatech) supplemented with 1 g/L of glucose and 10 mM of HEPES (Life Technologies). Cells were then incubated at 37°C for 45 min with assay buffer containing Indo-1-AM (Life Technologies) at a final concentration of 4 μM. After washing with assay buffer, the baseline Indo-1 fluorescence ratios (emission at 405 nm to 485 nm, with excitation at 355 nm) were measured for 10 min (read at every 20 sec) with a SynergMx microplate reader (BioTek). Cells were then treated with 1 μM of thapsigargin (Sigma-Aldrich) to deplete [Ca²⁺]ᵋ and induce [Ca²⁺]ᵋ increase, and the Indo-1 ratios were immediately measured for another 30 min.
siRNA transfection

Cells were transfected with siRNAs using DharmaFECT siRNA transfection reagent #2 (Dharmacon) in 25 cm² flasks. Twenty four hours after transfection, cells were collected and re-seeded into 6-well or 24-well plates and drug-treated for Western blot or cytotoxicity analysis, respectively. For confocal microscopy experiments, cells were first transfected as described above in 6-well plates, followed by transferring into chamber slides. Anti-Luc siRNA 1 (siLuc, Thermo Scientific) and Silencer Negative Control #1 siRNA (siNC1, Life Technologies) were used as non-targeting controls. siRNAs specifically targeting ERK1/2 (siERK1/2) were purchased from Cell Signaling Technology, and those targeting AMPKα1 (siAMPKα1) and CaMKKβ (siCaMKKβ) from Life Technologies. ON-TARGETplus SMARTpool siRNAs against ATF6 (siATF6), Atg7 (siAtg7) and LKB1 (siLKB1) were obtained from Thermo Scientific.

Cytotoxicity assay

After drug exposure for 48 or 72 h, attached cells in each well of a 24-well plate and their respective culture medium were collected and centrifuged at 400×g for 5 min. The pellets were then resuspended in Hanks Balanced Salt Solution (HBSS) (Mediatech) and analyzed with a Vi-Cell cell viability analyzer (Beckman Coulter) based on trypan blue exclusion.
Statistics

Statistical analyses were performed by two-tailed Student’s $t$-test, and a $P$ value less than 0.05 was considered significant.
Recent progress in the understanding of cancer has revealed reprogrammed metabolism as a hallmark of this disease. This has led to the consensus that increased tumor utilization of glucose can be exploited as a universal target for therapeutic gain. Thus, a comprehensive appreciation of how tumor cells respond to metabolic interventions will be important for designing effective therapeutic strategies. In this dissertation we focus on an evolutionarily conserved stress-adaptive mechanism, autophagy, and investigate its mechanisms of regulation and functional roles in response to therapeutic (2-DG) and physiologic (GS) glucose restriction under normoxic as well as hypoxic conditions.

We find that under normoxia 2-DG activates autophagy predominantly through induction of ER stress rather than by reduction of ATP. We further find that this occurs through the ER stress-mediated Ca^{2+}-CaMKKβ-AMPK pathway. Since autophagy has been shown to act as a cytoprotective mechanism, clinical and pre-clinical investigations are currently underway to improve the efficacy of various anti-tumor drugs through inhibition of this process. However, physiologic levels of autophagy are critical for maintaining homeostasis and loss of autophagy function has been implicated in numerous human diseases such as neurodegeneration. Moreover, homeostatic autophagy activity has been demonstrated to prevent early stage tumorigenesis. Therefore, a general inhibition of autophagy might disrupt the beneficial roles of this process causing
unwanted clinical complications. Since autophagy has been suggested to be potentially involved in maintaining genomic stability, inhibition of it might also result in long-term side effects that may be difficult to be detected during treatment periods. In this regard, our findings of the detailed signaling pathway that mediates 2-DG-induced autophagy could provide potential targets to inhibit drug-specific autophagy induction for enhancing treatment efficacy while minimizing unnecessary side effects.

Another key finding derived from this work is that 2-DG- as well as TM-induced ER stress is able to activate AMPK. This result suggests that in addition to the well-established role of AMPK for sensing energetic stress, it might also have evolved to act as a sensor of ER stress. Thus, it will be important to determine whether AMPK activation is a general consequence of ER stress or is limited to specific types of stresses. Since AMPK is a master regulator of cellular metabolism, our work might also open a new field of investigation focused on determining the biological effects of AMPK activated by ER stress as compared to stress induced by energy deprivation. Considering the close physical and functional interaction between the ER and mitochondria, it is conceivable that mitochondrial regulation could be a key factor connecting ER stress, AMPK and metabolism.

In addition to therapeutic glucose restriction by 2-DG, in this thesis we also study how autophagy is regulated by GS, a physiologic form of glucose restriction. We find that GS stimulates autophagy through both ER stress (CaMKKβ-independent) and ATP reduction (LKB1-AMPK). Moreover, only GS but not 2-DG increases ROS which in turn activate ERK contributing to autophagy induction. These findings highlight the phenotypic and mechanistic differences between 2-DG and GS, and raise caution in the
application of these two types of glucose restriction, which are often used interchangeably.

It is known that hypoxia, a characteristic of most, if not all, solid tumors, renders fundamental changes in tumor cell behaviors. Indeed, our results clearly demonstrate a switch from autophagy activation to autophagy inhibition in response to 2-DG when cells are placed under hypoxia. Thus, our findings provide additional support for the rationale of using 2-DG to kill hypoxic tumor cells since this sugar analog is able to prevent the adaptive activation of autophagy. In addition, 2-DG can also block the basal activity of this pro-survival process in certain targeted hypoxic cell populations. As GS and hypoxia are often observed in areas of solid tumors, our finding that GS inhibits autophagy under severe hypoxia might add new insights to the regulation of autophagy by the pathophysiologic tumor microenvironment.

In addition to the mechanistic studies, we show that functionally, autophagy is activated by 2-DG as an adaptive response to relieve ER stress. Moreover, we also find that inhibiting 2-DG-induced autophagy significantly enhances the cytotoxic effects of 2-DG in tumor cells. Most notably, we find that when autophagy is inhibited, a low, near clinical achievable dose of 2-DG, which by itself has only minimal effect in killing cancer cells, displays a dramatic increase in its cytotoxicity. Future studies should be focused on whether specifically blocking 2-DG-induced autophagy based on the signaling pathway involved could yield similar effects. Furthermore, animal studies will be required to determine the therapeutic efficacy of this combinatorial strategy before moving it to clinical trials.
Overall, by delineating the differential autophagy regulation by therapeutic and physiologic glucose restriction under different environmental conditions, our study contributes to a better knowledge of the interactions among autophagy, glucose metabolism and tumor microenvironment. Furthermore, this work also provides useful information for combining autophagy inhibition with 2-DG to achieve improved clinical outcomes.
REFERENCES


