Glucose starved cells do not engage in pro-survival autophagy*

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Background: Autophagy is a response to nutrient deprivation

Results: Inhibition of autophagy does not sensitize cells to apoptotic or necrotic cell death induced by glucose starvation. Moreover, glucose deprivation inhibits autophagy.

Conclusion: 2-deoxyglucose, but not glucose deprivation, induces autophagy.

Significance: Not all forms of starvation induce cytoprotective autophagy in mammalian cells.

ABSTRACT

In response to nutrient shortage or organelle damage, cells undergo macroautophagy. Starvation of glucose, an essential nutrient, is thought to promote autophagy in mammalian cells. We thus aimed to determine the role of autophagy in cell death induced by glucose deprivation. Glucose withdrawal induces cell death that can occur by apoptosis (in Bax, Bak-deficient MEFs or HeLa cells) or by necrosis (in Rh4 rhabdomyosarcoma cells). Inhibition of autophagy by chemical or genetic means by using 3-methyladenine, chloroquine, a dominant negative form of ATG4B or silencing Beclin-1, Atg7 or p62 indicated that macroautophagy does not protect cells undergoing necrosis or apoptosis upon glucose deprivation. Moreover, glucose deprivation did not induce autophagic flux in any of the four cell lines analyzed, even though mTOR was inhibited. Indeed, glucose deprivation inhibited basal autophagic flux. In contrast, the glycolytic inhibitor 2-deoxyglucose induced pro-survival autophagy. Further analyses indicated that in the absence of glucose, autophagic flux induced by other stimuli is inhibited. These data suggest that the role of autophagy in response to nutrient starvation should be reconsidered.

INTRODUCTION

Autophagy is an evolutionarily conserved cellular process activated upon starvation. In the absence of nutrients, cells engulf their own components in double membrane organelles called autophagosomes. These vesicles fuse to lysosomes, which promotes degradation of the content of the autophagosomes by digestive
enzymes. This process produces new metabolites that can be used as new building blocks and as sources of energy (1,2). For this reason, autophagy promotes cell survival under starvation (3).

Knock-down of genes essential for autophagy has been widely shown to enhance cell death in response to serum and amino acid starvation. However, it is presently unclear whether autophagy can help mammalian cells survive in the absence of glucose. Autophagy protects cancer cells from the glycolytic inhibitor 2-deoxyglucose (2-DG) (4-6), which suggests that autophagy is a pro-survival response to glucose deprivation in mammalian cells. However, we and others have shown that glucose deprivation and 2-deoxyglucose do not exert cytotoxicity through the same pathways (7,8). On the other hand, autophagy is a highly energy-consuming process which involves organelle trafficking and maintenance of the ATP-dependent lysosomal pH, and it is unclear whether under conditions of low ATP autophagy would provide more energy. For this reason, we hypothesized that autophagy could actually be detrimental for cells deprived of glucose, since it may end up consuming more ATP that it can produce by degrading intracellular components.

Nutrient starvation induces autophagy, at least in part, through activation of the AMPK/mTOR energy sensing pathway. Activity of the autophagy-initiating complex containing ULK1 and ULK2 is controlled by mTOR and AMPK (9,10), which are pathways regulated both by amino acids and glucose. This suggests that autophagy would be induced in a similar manner by glucose or amino acid starvation to provide nutrients for survival. Autophagy is protective for cells undergoing energetic stress such as hypoxic/hypoglycemic tumor cells, and the inhibition of autophagy was shown to promote necrotic cell death in apoptosis-deficient cells (11). We and others have previously shown that glucose deprivation kills cells either by apoptosis (caspase-dependent cell death) or necrosis (reviewed in (12,13)). We thus aimed to study the role of autophagy in survival of cells that die by apoptosis and in cells that die by necrosis upon glucose deprivation. Surprisingly, we observed that in contrast to the current view, autophagy does not protect cells from glucose deprivation. Moreover, glucose deprivation did not induce autophagic flux.

EXPERIMENTAL PROCEDURES

Cell culture and treatments
Alveolar rhabdomyosarcoma cell line Rh4, Bak, Bak deficient MEFs immortalized with SV-40 (14), Human Embryonic Kidney (HEK293) and HeLa cells (American Type Culture Collection) were maintained in high-glucose (25 mM), pyruvate-free Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 2 mM L-glutamine, 200 mg/mL penicillin, 100 mg/mL streptomycin sulfate and 10% FBS (Invitrogen).

For treatments, Rh4 cells were plated at a concentration of 200,000/mL and treated in fresh medium 24 hours later at 70%-80% confluence (600,000/mL). HeLa and MEF cells were plated at a concentration of 150,000/mL and treated 24 h later, when they reached the concentration of 500,000/mL (1x10⁶/mL HEK293).

Glucose deprivation was performed by rinsing the cells twice with glucose-free DMEM (Gibco/Invitrogen) and incubating them in glucose-free medium with freshly added 2 mM glutamine and antibiotics, plus 10% FBS dialyzed against PBS. Control cells were incubated in the same medium plus 25 mM glucose. Q-VD-OPH (SM Biochemicals LLC) as caspase inhibitor was used at 20 μM. 2-deoxyglucose (Sigma) was added at 10mM in regular culture medium. Metformin (1-1 Dimethylbiguanide hydrochloride, Sigma) was used at 10mM.

For induction of autophagy, cells were incubated in amino acid and serum-free, glucose-containing starvation buffers (EBSS or HBSS, Gibco) or with rapamycin (Calbiochem, 2 μM) or NVP-BEZ-235 (Selleck) in regular medium. EBSS was supplemented with Heps 25mM. Autophagy inhibitors as bafilomycin A1 (Calbiochem, dissolved in DMSO, used at 20nM), chloroquine (Sigma), 3-methyladenine (Calbiochem, prepared in glucose-free medium or in starvation buffer), pepstatin A and E-64d (Sigma-Aldrich) were added simultaneously with the treatments unless indicated. An equal amount of DMSO was added to the controls for treatments with bafilomycin.
Measurement of cell death
For analysis of viability, cells were harvested by combining floating cells in the medium and adherent cells detached by trypsinization, and subjected to FACS analysis to detect incorporation of propidium iodide 1 μg/ml (10 min incubation in PBS). For subG1 analysis, cells were washed in PBS, fixed in 70% cold ethanol while vortexing and incubated for 1–10 days at -20°C. Cells were further washed, resuspended in PBS with 40 μg/mL propidium iodide and 100 μg/mL RNase A (Sigma), and incubated for 30 minutes at 37°C before FACS analysis.

For analysis of cell death by incorporation of DAPI in the microscope (Fig.3C), cells were stained with DAPI 0.5μg/ml. 70 transfected (red) cells per condition were analyzed at an inverted Microscope Zeiss Axio Observer.Z1.

Western blotting
Cells were trypsinized, washed with PBS, lysed by resuspending them in Pierce RIPA buffer (Thermo Scientific: 25 mmol/L Tris-HCl (pH 7.6), 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) plus Complete Antiprotease Cocktail (Roche) and Phosphatase Inhibitor Cocktail Tablets PhosSTOP (Roche), and frozen. After sonication, protein concentration was measured with BCA Protein Assay Reagent (bicinchoninic acid; Pierce). Equal amounts of protein were mixed with Laemmli loading buffer. After electrophoresis, protein was transferred to a polyvinylidene difluoride membrane (Millipore) or nitrocellulose blotting membranes (Bio-Rad). PVDF membranes were blocked with 5% nonfat dry milk in Tris-buffered saline–TWEEN (0.1%). Secondary antibodies (1/5000) were HRP conjugated (Sigma) and detected with ECL reagent (Pierce). Equivalent amounts of protein were mixed with Laemmli loading buffer. After electrophoresis, protein was transferred to a polyvinylidene difluoride membrane (Millipore) or nitrocellulose blotting membranes (Bio-Rad).

DNA and RNA transfections, plasmids
For DNA transfections at autophagic flux experiments, cells were incubated in 12-well dishes and the tandem mRFP-EGFP-LC3 plasmid (ptfLC3, (16)) was transfected in antibiotic-free DMEM and incubated overnight with 1 μl of PEI (Polyethylenimine, Polysciences) and 1 μg DNA. Plasmids encoding GFP-LC3 (15), Hit 60 (MoMuLV gag-pol expression plasmid) and pCG (VSV-G envelope protein expression vector) were transfected into HEK293T cells. Cells were incubated in 10-cm dishes in antibiotic-free DMEM and incubated for 6 hours using 2μl of Lipofectamine 2000 (Invitrogen) and 10 μg of DNA. Viruses were collected after 24h (first supernatant) and 48 hours (second supernatant). Then, virus-containing medium was filtered (0.45 μm SFCA membrane filter; Milipore) and aliquots were frozen.

Target cells (Rh4 and HeLa) were plated at 50% confluence and incubated overnight. For infections, the culture medium was replaced by 1 ml of first supernatant supplemented with 8 μg/ml polybrene (Sigma) and 1 μg of DNA. Viruses were collected after 24h (first supernatant) and 48 hours (second supernatant). Then, virus-containing medium was filtered (0.45 μm SFCA membrane filter; Milipore) and aliquots were frozen.
kind gift of Tamotsu Yoshimori (Osaka University). This plasmid was digested with NheI and BamHI to isolate mStrawberry-Atg4B-C74A. This fragment was then blunt-ended and cloned into the SnaBI site of pBabe-Blast to generate pBabe-Blast-mStrawberry-Atg4B-C74A. Insert orientation was again determined by sequencing. For transfections of siRNA, cells were incubated in antibiotic-free DMEM with siRNA 75 nmol/L premixed with DharmaFECT 1 (Dharmacon) in 10-cm dishes. 18 h later cells were trypsinized, plated and 48 h after transfection were treated as indicated. Sense strain sequence for ATG7: 5'-GUUUGUAGCCUCAAGUGUU-3', Beclin-1: 5'-CAGUUUGGCACAAUCAAUA-3'. As a control, a non-matching siRNA oligonucleotide (pBlue: 5'-GUAAGACACGACUUAUCGC-3') was used.

p62/SQSTM1 was downregulated using Dharmacon (Lafayette, CO, USA) On-Target smartpool (cat. no. M-047628-01); Dharmacon ON-Target plus non-targeting pool was used as a control.

Confocal microscopy
Cells were cultured on glass coverslips pre-treated with poly-L-Lysine (Sigma), transfected with fluorescent constructs (if applicable), and treated with the indicated agents. Then they were fixed with a fresh 4% solution of paraformaldehyde for 15 min, washed with PBS twice, mounted in vectashield (Vector laboratories) and visualized at room temperature directly on a Leica TCS SP5 Spectral Confocal microscope with a HCX PL APO lambda blue 63x 1.4 oil objective lenses. Acquisition software was LEICA Application Suite Advanced Fluorescence (LAS AF) version 2.6.0.7266. The projections of Z-stacks are shown. Vesicles (dots) from Z-stacks of whole-field images with multiple cells were analyzed with Fiji/Image J software followed by the Laplacian filter. Results are presented as mean rates and correlate with a measurement of the punctate area in a minimum of 4 independent images and 40 cells.

ATP detection Assay
Cells were cultured in 96 well–plates for 20h before treatments. ATP levels were measured using ATPlite 1step Kit (PerkinElmer) following manufacturer’s instructions. Luminescence was measured at a microplate luminescence counter, Victor5 (PerkinElmer). A standard curve of ATPs was set up in the same microplate that was used for the experimental samples.

Statistics
Unless specified, two-tailed, paired Student’s T test was applied. N.S (non significant); one asterisk (*) indicates $p < 0.05$, ** $p<0.01$, *** $p<0.001$.

RESULTS
Inhibition of autophagy does not sensitize cells to apoptosis or necrosis induced by glucose deprivation

We aimed to determine whether autophagy protects from apoptotic or necrotic cell death induced by glucose deprivation. For that aim we subjected different cell lines to glucose deprivation in the presence of two different chemical inhibitors of autophagy. These inhibitors, although not selective, have been widely employed to analyze the role of autophagy in cell death. 3-methyladenine (3-MA) is a PI3-kinase inhibitor which can inhibit the PtdIns kinase VPS34 and thus prevent formation of autophagosomes. Chloroquine blocks lysosomal function and thus inhibits macroautophagy, chaperone-mediated autophagy, degradation of membrane proteins by endocytosis and other lysosome-dependent processes. We subjected cells to glucose deprivation in the presence of 3-MA or chloroquine. We have previously shown that HeLa cells die in part by apoptosis (cell death prevented by caspase inhibitors) and in part by necrosis when subjected to glucose deprivation (17). In these cells it was previously reported that autophagy is a protective mechanism against complete starvation (3). We observed that 3-MA did not sensitize HeLa cells to glucose deprivation, even though 3-MA at doses commonly used to inhibit autophagy is toxic for these cells (Fig. 1A and 1B).

We have previously shown that Bax, Bak deficient MEF die by caspase-8 mediated apoptosis when deprived of glucose (17). Strikingly, these cells are protected from glucose deprivation when incubated in the presence of 3-MA (Fig. 1C and 1D). We analyzed a third cell type, the rhabdomyosarcoma cell line Rh4. These cells die
in a necrotic manner in the absence of glucose, since cell death cannot be rescued by caspase inhibitors (Fig. 1E). Although 3-MA on its own was also quite toxic to these cells, 3-MA prevented cell death of Rh4 cells by glucose deprivation (Fig. 1F, 1G and 1H).

Chloroquine is widely employed to inhibit the last steps of autophagy because of its ability to neutralize the lysosomal pH. We treated the same cell lines with chloroquine in combination with starvation of glucose. The effects were in general quite different from those obtained with 3-MA. In Rh4 cells in which 3-MA markedly protected from cell death, chloroquine did not reduce cell death (Fig. 2A). Chloroquine mildly sensitized Bax, Bak-deficient MEF (Fig. 2B) and HeLa cells (Fig. 2C, D) to glucose deprivation. It should be noted that chloroquine is toxic to every cell line studied in a dose- and time-dependent manner (Fig. 2C, D and data not shown) and the sensitization observed is possibly due to an additive effect on signals involved in cell death.

**Inactivation of autophagy sensitizes cells to 2-deoxyglucose and starvation buffer but not to glucose deprivation**

2-deoxyglucose (2-DG) is a glucose analog that kills tumor cells by apoptosis and is been tested as an anti-tumor drug (12). Its toxic effects are generally attributed to interference with glycolysis and ATP depletion. However, we and others have shown that the effects of 2-deoxyglucose can be attributed to interference with N-glycosylation and endoplasmic reticulum stress rather than to ATP depletion (7,8). 2-DG has been shown to induce autophagy, and toxicity of 2-DG can be enhanced by treatment with 3-MA or with siRNA against Beclin-1 or Atg7 (4,5). We verified that 3-MA but especially chloroquine sensitized Rh4 cells to 2-deoxyglucose (Fig. 2E, F), corroborating previous results that suggest that the effects of 2-deoxyglucose and glucose deprivation are different.

Since these chemical inhibitors are quite unspecific (although we verified that they inhibit autophagy in our cells -data not shown and Fig. 2G), we employed siRNA to knock down genes involved in autophagy. We deliberately avoided the use of cells from mice deficient in autophagy genes because it has been shown that these cells upregulate compensatory protein degradation pathways such as chaperone mediated autophagy, which may protect from apoptosis and complicate interpretation of results (18). We thus transiently silenced Beclin-1, a protein involved in nucleation of the phagophore. Downregulation of Beclin-1 reduced basal and 2-DG-induced autophagy (Fig. 3A) and it clearly enhanced sensitivity of Rh4 cells to amino acid/serum starvation (incubation in EBSS) and to treatment with 2-deoxyglucose. However, only a minor sensitization to glucose deprivation was observed (Fig. 3B). We employed another genetic method that would inhibit autophagy even faster than the use of siRNA: we transfected Rh4 cells with a dominant-negative form of ATG4B that hampers the lipidation of LC3 paralogues (19). Transfected cells were highly sensitized to 2-DG and EBSS, and some basal cell death was observed at longer time points. However, these cells were not sensitized to glucose deprivation (Fig. 3C). We also knocked down transiently ATG7 in Bax, Bak-deficient MEF, which die by apoptosis. The knock down efficient was only modest (Fig. 3D) but sufficient to downregulate basal and EBSS-induced autophagy (Fig. 3E). Since these cells do not die with EBSS or 2-deoxyglucose for many days (not shown) we employed thapsigargin as a control. This drug induces autophagy-dependent cell death in Bax, Bak-deficient MEF (20). Indeed, knockdown of ATG7 reduced cell death induced by thapsigargin, and promoted some cell death on its own at long time points, but it did not alter the response to glucose deprivation (Fig. 3F). We also silenced p62, a molecule involved in delivering cargo to autophagosomes. In the virtual absence of p62 cell death of Bax, Bak-deficient cells proceeded with identical kinetics (Fig. 3G).

**Glucose deprivation does not induce autophagic flux**

Inhibition of autophagy sensitized cells to 2-deoxyglucose but not to glucose deprivation. However, many studies had reported signs of autophagy in mammalian cells upon glucose deprivation (for instance (21-23)). This prompted us to analyze whether glucose deprivation actually induced autophagic markers, and especially,
autophagic flux under our conditions of selective glucose deprivation. We generated HeLa and Rh4 cells stably expressing the autophagosomal marker LC3-GFP and we starved them of glucose. Noticeable but modest puncta are observed in HeLa cells either growing under normal conditions or subjected to glucose deprivation (Fig. 4A). The fact that glucose deprivation does not induce an obvious translocation of LC3 could suggest that glucose deprivation does not induce autophagy; however, it is also possible that it is inducing autophagy but autophagosomes are rapidly cleared by fusion with lysosomes. In order to distinguish between these two possibilities, we employed bafilomycin A1 to block lysosomal degradation of autophagosomal content. Bafilomycin A1 alone induced accumulation of LC3-GFP puncta, which indicates a high level of basal autophagy. However, incubation with bafilomycin A1 in the absence of glucose did not enhance the formation of the puncta (Fig. 4A, B). Similar results were observed in Rh4 cells (Fig. 4C, D). As controls, starvation buffers or the mTOR inhibitor rapamycin were used. Bafilomycin A clearly enhanced the formation of puncta triggered by these treatments (Fig. 4A-D). We employed another method to analyze autophagic flux based on the lysosomal neutralization of GFP but not RFP fluorescence when these two molecules are fused to LC3. When LC3 is inside autophagolysosomes with acidic pH, only the red fluorescence is observed (16). We verified that incubation in starvation buffer EBSS or treatment with rapamycin induced autophagy in Rh4 cells, but glucose deprivation did not (Fig. 4E).

A different method to analyze autophagic flux is to measure the levels of p62 (an LC3-binding protein degraded by autophagy) and of lipidated (autophagosomal, LC3-II) LC3 by western blot. We analyzed p62 and LC3-II after depriving cells of glucose. In HeLa cells, while levels of p62 are not reduced, LC3-II accumulates after treatment, which could indicate activation of autophagy (Fig. 5A). However, LC3-II accumulation may also mean autophagy is reduced compared to basal autophagy. When cells were incubated in regular culture medium in the presence or absence of inhibitors of the last stages of autophagy (the mixture of the protease inhibitors pepstatin A and E64D), LC3-II was accumulated at a much faster rate than in glucose-free medium. Moreover, the combination of glucose deprivation and protease inhibitors promotes similar or even lower accumulation (at long time points) than protease inhibitors alone. This indicates that while basal autophagy is high, glucose deprivation does not induce autophagy, and moreover, it seems to reduce it. In contrast, incubation in Earle’s starvation buffer (EBSS), induced autophagy in HeLa (Fig. 5A) and in Bax, Bak deficient cells (Fig. 5B). Intriguingly, glucose deprivation promotes the accumulation of p62 in these cells, suggesting that either glucose deprivation inhibits autophagic flux –while the rate of synthesis of p62 may remain the same- or that glucose regulates p62 levels independently of autophagy. LC3-II accumulated strongly upon depriving cells of glucose. However, the presence of bafilomycin A did not increase the levels of LC3-II. Altogether, these results suggest that both in HeLa and in Bax, Bak deficient MEF (that can be kept alone for almost two days without apparent toxicity (17)) glucose deprivation inhibits, rather than induces autophagy. We then compared the effects of 2-deoxyglucose and glucose deprivation in Rh4 cells (Fig. 5C,D). Glucose deprivation induced autophagy in Rh4 cells, whereas 2-deoxyglucose clearly induced autophagy.

Glucose deprivation engages starvation signals but it inhibits autophagy

Two possibilities are non-exclusive and compatible with the results described above. Glucose deprivation may not engage the same pro-autophagic signals triggered by amino acid starvation or by 2-DG. Or glucose may be required for completion of autophagy even if starvation signals occur. In order to examine these possibilities, we first analyzed whether the cell types that we used do not properly inactivate mTOR in response to glucose deprivation due, for instance, to constitutive hyperactivation of the mTOR pathway. We observed that in Rh4 cells (Fig. 6A) and in HeLa and Bax, Bak deficient MEFs (not shown), mTOR is inactivated upon
glucose deprivation, as measured by S6 and 4EBP1 dephosphorylation. 2-deoxyglucose, which induces autophagy in Rh4 cells, inhibited mTOR with similar kinetics (Fig. 6A). Inhibition of mTOR is usually considered sufficient to trigger autophagy, and mTOR inhibitors are bona-fide autophagy inducers. However, it is possible that, if the signal to inhibit mTOR in the absence of glucose was not sufficiently strong, AMPK activation was also required to induce autophagy by phosphorylating ULK1, Vps34 and Beclin-1 (9,10,24). HeLa cells cannot activate AMPK upon energy stress because they lack the kinase LKB1, and we could not consistently detect phosphorylation of its substrate phospho-Acetyl-CoA carboxylase (ACC) in Rh4 cells (not shown). For this reason, we analyzed AMPK and autophagy activation in a cell line which has been used to study induction of autophagy by glucose deprivation, HEK293 (10). Glucose removal inactivated mTOR, and as reported by Kim et al, it activated AMPK (Fig. 6B). However, autphagic flux was not induced (Fig. 6C). These data together with results described in figure 5 regarding accumulation of p62 suggest that even though glucose deprivation may engage the right signals to promote autophagy, it cannot proceed.

We then performed an experiment to verify inhibition of basal autophagy (Fig. 6D). Bax, Bak-deficient MEFs accumulated LC3-II upon EBSS treatment. If 3-MA was added in order to block the initial steps of autophagy, LC3-II was cleared because autophagy keeps occurring but no new LC3-II is produced (Fig. 6D). However, this was not the case with glucose deprivation. Cells accumulated LC3-II, and after addition of 3-MA, levels of LC3-II remained high, indicating that clearance of autophagosomes does not occur.

It is thus possible that glucose deprivation inhibits autophagic flux by not allowing completion of all steps from initiation to lysosomal degradation of autophagolysosomal content and recycling. If this was the case, glucose deprivation should inhibit autophagy induced by other stimuli. We thus incubated cells with an autophagy-inducing compound, the mTOR and Akt inhibitor BEZ-235 (25), in the absence or presence of glucose. We observed that, although mTOR was inhibited by this compound in both conditions, glucose deprivation inhibited autophagic flux induced by the drug (Fig. 6E and 6F). Thus, we conclude that glucose deprivation inhibits, rather than induce autophagy.

In order to gather more insight about the mechanism, we analyzed ATP levels of cells treated by removing glucose or by addition of 2-deoxyglucose. Results described in Figure 7A indicate that, surprisingly, glucose deprivation does not trigger the loss of ATP in Rh4, which may be obtaining ATP from glycogen or amino acids under these conditions. On the other hand, 2-deoxyglucose promotes an early decrease in ATP levels. However, both treatments promote cell death starting at about 20-24h, and both treatments inactivate mTOR with similar kinetics. This could suggest a correlation between a loss of ATP and induction of autophagy. However, we analyzed loss of ATP in other cell lines employed in this study, and glucose deprivation reduced ATP levels at short time points (Fig. 7B), which did not correlate with autophagic flux (Figs. 4A, 5A, 5B). We could not observe a significant effect of glucose deprivation on modulation of ATP levels by BEZ-235 or vice versa (Fig. 7C). We also tested the hypothesis that glucose deprivation, due to modulation of energy or metabolite levels, could be impairing lysosomal pH and thus, its function. However, measurement of lysosomal pH using Lysosensor indicated that glucose deprivation reduces lysosomal pH (or enlarges the lysosomal compartment) in Bax, Bak deficient MEFs like EBSS does (Fig. 7D). Bafilomycin was used as a control of pH neutralization. In Rh4 cells, 2-DG, which induces autophagy, and glucose deprivation, which does not, did not significantly alter lysosomal pH (Fig. 7E).

DISCUSSION

Glucose deprivation is thought to be a macroautophagy-inducing stimulus. We present data here that demonstrates that glucose depletion does not induce autophagy in a variety of cell lines, and that it can actually inhibit basal autophagy and autophagic flux induced by a drug. Accumulation of lipidated (autophagosomal) LC3 upon treatment with a stimulus can mean that it...
induces autophagy, but also that it inhibits basal autophagy, because LC3-containing autophagosomes would accumulate. For this reason it is necessary to compare punctate (or lipidated) LC3 in cells treated with and without lysosomal inhibitors, or treated with these inhibitors on their own to determine the rate of basal autophagy. We have observed accumulation of lipidated (autophagosomal) LC3 upon glucose deprivation, which has probably led other authors to conclude that this is an autophagy-inducing stimulus like other forms of starvation. In some articles glucose deprivation was combined with starvation of other nutrients and growth factors contained in serum, or it was performed under hypoxia. It is possible that it was the deprivation of these other nutrients or oxygen what triggered autophagy (11,21,26,27).

Physiologically, conditions that accompany low blood glucose (possibly, reduction of insulin levels) may induce autophagy in some tissues. Liver autophagy has been shown to contribute to the maintenance of blood glucose and amino acid levels (28). Autophagy in newborn mice is essential for their survival upon weaning, and mice in which mTOR cannot be inactivated show the same phenotype (neonatal cell death) than mice deficient in Atg5 (29,30). Moreover, these mice can be rescued by providing glucose or gluconeogenic amino acids. Our results are not incompatible with the possibility that autophagy contributes to regulate glucose homeostasis via gluconeogenesis. Autophagy produces amino acids, which could potentially be converted to glucose by gluconeogenic cells. Additionally, autophagy can participate in digestion of lipid droplets and production of free fatty acids that could be used to make ATP (1). So it is possible that under some circumstances, autophagy can contribute to glucose and ATP generation. However, it remains to be determined what is the cause for the induction of autophagy upon fasting: low blood glucose or low amino acids or other hormonal signals that follow hypoglycemia. Our results suggest that glucose is not the nutrient that regulates autophagy, and that ATP loss does not correlate with autophagy induction.

We report here that glucose deprivation actually inhibits autophagy, although the mechanism is unclear. Glucose may alter multiple steps of autophagy, which is an ATP-demanding process. It has recently been reported that glucose deprivation does not stimulate production of WIP12-containing membranes, which suggests that it fails to induce VPS34 activity (31). Experiments shown in figure 5 suggest that at long time points, autophagy is inhibited at the earlier steps. However, other experiments indicate that glucose deprivation inhibits the latest steps of autophagy: we observe accumulation of p62 even at short time points, and figure 6D indicates that upon treatment with 3-MA autophagic vesicles are not cleared. Ammonia is produced under conditions of glucose deprivation (23). Since ammonia is a potent inhibitor of lysosomal function, it is possible that this is the reason why glucose deprivation inhibits autophagy. Lampidis and colleagues have recently reported that under hypoxia, glucose deprivation inhibits, rather than induce autophagy (32), and Knecht and colleagues have reported that glucose promotes autophagy under starvation, in agreement with our data (33). Moreover, it had been observed that raising glucose concentration enhanced autophagy and clearance of mutant Huntingtin (34). In this regard, it should be noted that the buffers commonly employed to mimic starvation and induce autophagy in culture (HBSS, EBSS) contain glucose.

It is possible that some forms of starvation or drugs commonly used to promote autophagy transduce signals that glucose deprivation does not. In this regard, 2-deoxyglucose has been shown to induce autophagy by a pathway more related to endoplasmic reticulum stress than to ATP depletion, since mannose could prevent it but it could not revert ATP loss (5). Classical "starvation" in buffers is achieved by depriving cells simultaneously of growth factors, vitamins and all amino acids, which may regulate signaling molecules such as reactive oxygen species, or activate other signaling pathways besides mTOR inactivation. In this sense, it has been shown that rapamycin requires Ca²⁺ signals to induce autophagy (35) and complete starvation triggers DNA damage and PARP activation which are required for autophagy to proceed (36). Alternatively, it is possible that glucose engages an anti-autophagic signal (33), or glucose, acetate
or some other glucose-derived molecule is required for vesicle trafficking or recycling.

Our results indicate that chloroquine, 3-MA and genetic blockade of autophagy have different effects on cell survival. Possibly, 3-MA blocks class I PI3-kinases which may contribute to the observed effects. Alternatively, chloroquine is likely altering other lysosomal processes such as chaperone-mediated autophagy or endocytosis, which could potentially sensitize cells to 2-deoxyglucose or glucose deprivation. However, it is difficult to conclude that lysosomal blockade sensitizes cells specifically to glucose deprivation, because it is toxic by itself, and we only observed some sensitization in two cell lines. However, protection by 3-MA was very reproducible, and suggest that inhibitors of early steps of autophagy may be used to treat ischemic diseases, as previously suggested (37). Altogether, our data prompt for a reevaluation of the role of autophagy in starvation.

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**FOOTNOTES**

Abbreviations used are: AMPK, 5′ adenosine monophosphate-activated protein kinase; 2-DG, 2-deoxyglucose; 3-MA, 3-methyladenine; HBSS, Hank's Balanced Salt Solution; EBSS, Earle's Balanced Salt Solution; DMEM, Dulbecco's modified Eagle's medium; MEF, Mouse embryonic fibroblasts; PI, propidium iodide.
FIGURE LEGENDS

Figure 1. 3-methyladenine inhibits apoptosis or necrosis induced by glucose deprivation

A, B. HeLa cells were deprived of glucose in the absence or presence of 3-MA at indicated concentrations. Cell death was analyzed by propidium iodide incorporation at 16h (A) or 24h (B). Figure shows average and SEM of 3 experiments.
C. Bax,Bak (-/-) MEFs were subjected to glucose deprivation for the indicated times, in the absence or presence of 3-MA at indicated concentrations. Cells were collected for subG1 analysis at times shown. Figure shows average and SEM of 3 experiments.
D. Bax/Bak -/- MEFs were treated with 3-MA 10mM for 48h in the presence or absence of glucose. Photographs show 80% of the field and were taken using a 20x objective. Note that 3-MA prevents cell shrinkage induced by glucose deprivation.
E. Rh4 were grown in glucose-free medium in the presence of caspase inhibitors (Q-VD) or DMSO as vehicle control. Cells were collected at indicated times and subjected to PI uptake analysis. Data represent average and SEM of 3 experiments. Untreated control cells (labeled as “C”) were cells incubated in DMEM for 16h.
F, G. Rh4 cells were deprived of glucose in the absence or presence of 3-MA at indicated concentrations. Cell death was analyzed by propidium iodide incorporation at 24h (F) or 37h (G). Figure shows average and SEM of minimum 3 experiments.
H. Rh4 cells were treated with 3-MA 10mM for 30h in the presence or absence of glucose. Photographs showing 25% of the field were taken using a 20x objective.

Figure 2. Inhibition of lysosomal function with chloroquine promotes mild sensitization to glucose deprivation. 3-MA and chloroquine sensitize to 2-deoxyglucose.

A. Rh4 cells were deprived of glucose in the absence or presence of chloroquine (CQ) at indicated concentrations. Cell death was analyzed by propidium iodide incorporation after 24 hours. Figure shows average and SEM of minimum four experiments.
B. Bax/Bak -/- MEFs were subjected to glucose deprivation for the indicated times, in the absence or presence of chloroquine 10 µM. Cell death was analyzed by propidium iodide incorporation after 48 h. Figure shows average and SEM of three experiments.
C, D. HeLa cells were deprived of glucose in the absence or presence of chloroquine at indicated concentrations. Cell death was analyzed by propidium iodide incorporation at indicated times. Figure shows average and SEM of minimum three experiments.
E. Rh4 cells were treated with 2-DG at 2mM in the absence or presence of chloroquine at indicated concentrations. Cell death was analyzed by propidium iodide incorporation after 48 and 72 h. Figure shows average and SEM of 6 experiments.
F. Rh4 cells were treated with 2-DG 2mM in the absence or presence of 3-MA at indicated concentrations. Cell death was analyzed by propidium iodide incorporation after 48 and 72 h. Figure shows average and SEM of minimum four experiments.
G. Rh4 cells were incubated with the protease inhibitors E64d and Pepstatin A (20µM each) for 6h in regular medium (-) or EBSS, in the presence or absence of 3-MA (10mM), and blotted for LC3 and actin.

Figure 3. Inhibition of autophagy sensitizes to 2-deoxyglucose and starvation buffer but not to glucose deprivation.

A, B. Rh4 cells were transfected with siRNA against Beclin-1 or control siRNA. A: 48h after transfection cells were treated with or without 2-DG for 48h and Bafilomycin for the last 3h of incubation. Protein was collected for western blot. B: 48h after transfection cells were treated with 2-DG, EBSS or subjected to glucose deprivation for times shown. Figure shows average and SEM of 5 experiments.
C. Rh4 cells were transfected with mStrawberry (Control) or mStrawberry-Atg4B (C74A) plasmids and incubated for 24h. The cells were then cultured in EBSS, Glc+, Glc- or 2-DG for the indicated times, and fluorescent (transfected) cells were scored as dead or alive with a low concentration of DAPI (see methods).

D, E. Bax, Bak (-/-) MEF were transfected with siRNA against ATG7 or control siRNA (NT, 5-UAAGGCUAUGAGAGAUACtt). D: samples were collected for western blot at times indicated. E: After 48h of transfection cells were cultured in regular culture medium or EBSS 95% + 5% medium and bafilomycin for 3h and collected for western blot. F: 48h after transfection cells were cultured in Glc+, Glc- or thapsigargin (1µM) and collected for FACS analysis. Figure shows average and SD of 3 experiments.

G. Bax, Bak (-/-) MEF were transfected with siRNA against p62 or control siRNA. 48h later they were deprived of glucose. Cell death was analyzed by SubG1. Figure shows average and SD of two experiments. Right panel: cells were collected at times indicated after transfection for western blot analysis.

Figure 4. Glucose deprivation does not induce autophagic flux

A-D. HeLa (A,B) or Rh4 (C,D) stably transfected with GFP-LC3 (HeLa-LC3, Rh4-LC3) were treated with DMEM with glucose (Glc+), glucose deprivation (Glc-), amino acid deprivation (HBSS/EBSS) or Rapamycin (Rapa) for 6h with or without bafilomycin (Baf) for the last 3h. The expression pattern of GFP-LC3 was examined under the confocal microscope. Punctate LC3 in cells was measured as described in methods. B and D show averages+SEM from two (HeLa, B) or four (Rh4, D) independent experiments.

E. Rh4 were transfected with mRFP-GFP-LC3 plasmid and treated 24h post-transfection for 15h. Representative images from 3 independent experiments are shown. Arrows indicate red points (autophagolysosomes).

Figure 5. Induction of autophagic flux by 2-deoxyglucose and starvation but not glucose deprivation

A. HeLa cells were deprived of glucose or incubated in 95% EBSS+5% culture medium for the indicated times, in the presence or absence of E64d and Pepstatin A (EP) (10µM each). Proteins were resolved by SDS-PAGE and immunoblot. Bands immunoreactive with anti-LC3 antibody and with anti-p62 are shown. Untreated control cells (labeled as “c”) were cells incubated in DMEM for 3h. Lower panel shows quantification of relative LC3 II levels as described in methods (average and SEM of minimum 3 independent experiments).

B. Bax, Bak DKO MEF were subjected to glucose deprivation or incubated in 95% EBSS+5% culture medium for the indicated times, in the absence or presence of 20nM bafilomycin (Baf) for the last 3 h to avoid toxicity. Untreated control cells (labeled as “c”) were cells incubated in DMEM for 6h in the presence (B) or absence (C) of bafilomycin for the last 3h. Bands immunoreactive with anti-LC3 antibody and with anti-p62 are shown. Lower panel shows quantification of relative LC3 II levels as described in methods (average and SEM of 3 independent experiments).

C. Rh4 cells were subjected to glucose deprivation or incubated in 90% EBSS+10% culture DMEM for the indicated times, in the absence or presence of 20nM bafilomycin for the last 3 h to avoid toxicity. Untreated control cells were cells incubated in DMEM for 3h in the presence (“B”) or absence (“C”) of bafilomycin for the last 3h. Proteins were resolved by SDS-PAGE and immunoblot. Lower panel shows quantification of relative LC3 II levels as described in methods. Results are representative of 3 independent experiments; 2 for EBSS.

D. Rh4 cells were treated with 2-DG for the indicated times, in the absence or presence of 20nM bafilomycin for the last 3 h. Untreated control cells were cells incubated in DMEM for 3h in the presence...
(‘‘B’’) or absence (‘‘C’’) of bafilomycin for the last 3h. Lower panel shows quantification of relative LC3 II levels as described in methods (3 independent experiments).

Figure 6. Glucose deprivation engages starvation signals but it inhibits autophagy

A. Rh4 cells were treated with glucose free medium, 2-DG or metformin for the indicated times. Proteins were resolved by SDS-PAGE and immunoblot. Phospho-4E-BP1, 4E-BP1, phosphoS6, S6 and actin were detected using secondary infrared antibodies.

B. HEK293 cells were incubated in glucose-free medium in the presence or absence of glucose. Proteins were resolved by SDS-PAGE and immunoblot. Phosphorylated and total ACC and S6 proteins, 4E-BP1 and actin were detected using infrared antibodies.

C. HEK293 cells were deprived of glucose for the times shown, with or without E64d and Pepstatin A (EP) at the concentration of 20µM each, collected and subjected to western blot.

D. Bax, Bak -/- MEFs were treated with or without glucose (Glc-) or EBSS for the indicated times (expressed in hours), followed by 2h of treatment with 3-MA where indicated and collected for western blot.

E. Rh4 cells were incubated in glucose-free or glucose-rich medium for 15h, in the presence or absence of E64d and Pepstatin A (EP) at 20µM each, and in the presence or absence of the mTOR inhibitor BEZ235 at concentrations shown. Proteins were resolved by SDS-PAGE and immunoblot. Bands immunoreactive with LC3, phospho4E-BP1, 4E-BP1, phosphoS6, S6 and p62 are shown.

F. Quantification of LC3 II levels of cells treated as in E. Values were normalized to actin and to the control of cells treated in the presence of E64d and Pepstatin (Glc+EP). Results show average and SEM of 3 independent experiments. Asterisk indicates significance >0.05 when compared to the same treatment in the presence of glucose.

Figure 7. ATP levels or lysosomal pH do not explain the effects of glucose deprivation.

A. Rh4 cells were incubated in glucose free medium or in the presence of 2-DG for the indicated times. ATP levels were measured as described in Experimental procedures. Values shown are relative to untreated controls for each time point, and represent average and SEM of 3 (2-DG) or 6 (Glc-) independent experiments.

B. Bax, Bak -/- MEFs and HeLa cells were treated with glucose free medium in the presence or absence of Q-VD for the indicated times. Error bars represent the average of 3 (Bax, Bak -/- MEF) or 5 (HeLa) independent experiments. ATP levels were normalized to cell numbers in each well.

C. Rh4 cells were treated with glucose free medium and BEZ 100nM for the indicated times. ATP levels were measured as described in Materials and Methods. Values shown are relative to untreated controls for each time point. Data represent the average and SEM of 3 independent experiments.

D. E. Bax, Bak -/- MEFs (D) or Rh4 cells (E) were cultured in Glc+, Glc+ Bafilomycin (20nM), EBSS, Glc- or 2-DG (10mM) for the indicated times. After trypsinization cells were stained with 2.5µM of LysoSensor (LifeTechnologies) for 10 minutes at 37ºC. Mean LysoSensor intensity of live cells was analyzed by FACS. Data shown are relative to untreated controls and represent the average and SEM of 3 independent experiments.
Figure 1

A

HeLa - 16h

% Death (PI)

mM 3-MA 0 1 10 0 1 10

Glc-

N.S

B

HeLa - 24h

% Death (PI)

mM 3-MA 0 1 10 0 1 10

Glc-

N.S

C

Bax, Bak -/- MEF - 72h

% subG1

mM 3-MA 0 1 10 0 1 10

Glc-

N.S

D

- 10mM 3MA

Glc+

Glc-

E

% Death (PI)

mM 3-MA 0 1 10

Glc-

DMSO

Q-VD

C 16 24 48 h

F

Rh4 - 24h

% Death (PI)

mM 3-MA 0 1 10

Glc-

***

N.S

G

Rh4 - 37h

% Death (PI)

mM 3-MA 0 1 10

Glc-

*

H

- 10mM 3MA

Glc+

Glc-
Fig. 2

A. Rh4 - 24h

B. Bax, Bak -/- MEF 48h

C. HeLa - 16h

D. HeLa - 24h

E. Rh4 - 48h, 72h

F. Rh4 - 48h, 72h

G. Western blot for LC3-I and LC3-II with Actin as a control.
Fig. 3

A

|          | Glc+ | 2-DG Baf |
|----------|------|----------|
| control  | Beclin-1 | LC3-I   |
| Bec-1    | LC3-II  | Actin   |

B

![% Death (PI)](image)

C

![% Death](image)

D

![% Death](image)

E

| Glc+Baf | EBSS Baf |
|---------|----------|
| control | Atg7     | control |
| Atg7    | control | Atg7    |

LC3-I

LC3-II

Actin

F

![% Death (PI)](image)

G

![% SubG1](image)

[p62 siRNA](image)
Figure 4

A  HeLa-LC3  Bafilomycin

Glc+

Glc-

HBSS

Rapa

B

LC3 punctate

0 2 4 6 8

- Baf - Baf - Baf - Baf

Glc+ Glc- HBSS Rapa

C  Rh4-LC3  Bafilomycin

Glc+

Glc-

EBSS

Rapa

D

LC3 punctate

0 2 4 6

- Baf - Baf - Baf - Baf

Glc+ Glc- EBSS Rapa

E

Glc+  Glc-  EBSS  Rapamycin
Figure 5

A  
HeLa

B  
Bax, Bak -/- MEF

C  
Rh4

D  
Rh4

EBSS

Glc+EP  Glc-  Glc- EP  EBSS  EBSS EP

Glc+EP  Glc-  Glc- EP  EBSS  EBSS EP

2-DG  2-DG-Baf

HeLa  Rh4

LC3-I

LC3-II

Actin

LC3 II (relative)

Glc-  Glc- Baf  EBSS  EBSS-Baf

EBSS

EBSS

EBSS-Baf

LC3

Actin

EBSS

EBSS

EBSS-Baf

LC3 II (relative)

Glc-  Glc- Baf  EBSS  EBSS-Baf

2-DG  2-DG-Baf

EBSS

EBSS

EBSS-Baf

LC3 II (relative)
Figure 7

A

ATP (relative to control)

Glc- 2-DG

0 0.2 0.4 0.6 0.8 1

C 1 3 6 18 h

Rh4

B

Bax, Bak -/- MEF

ATP (relative to control)

Glc- Glc- Q-VD

0 0.2 0.4 0.6 0.8 1

C 3 6 24 3 6 24 h

HeLa

C

ATP (relative to control)

Glc- Glc- BEZ

0 1 3 6 18

p=0.1

p=0.9

Glc-

D

Bax, Bak -/- MEF

LysoSensor intensity

Glc+ Baf EBSS Glc-

6 h 16 h

E

Rh4

LysoSensor intensity

Glc+ Baf 2-DG Glc-

6 h 16 h
Glucose starved cells do not engage in pro-survival autophagy
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