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Glutamine increases autophagy under basal and stressed conditions in intestinal epithelial cells

Toshio Sakiyama^{*}, Mark W. Musch^{*}, Mark J. Ropeleski[‡], Hirohito Tsubouchi[§], and Eugene B. Chang^{*}

^{*} Martin Boyer Laboratories, University of Chicago IBD Research Center, Chicago, Illinois

[‡] Gastrointestinal Diseases Research Unit, Department of Medicine, Queen's University, Kingston, Ontario, Canada

[§] Department of Digestive and Life-style related Diseases, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan

Abstract

Background & aims—Glutamine plays a protective role in intestinal cells during physiological stress, however, the protection mechanisms are not fully understood. Autophagy functions in bulk degradation of cellular components, but has been recently recognized as an important mechanism for cell survival under conditions of stress. We therefore sought if glutamine's actions involve the induction of autophagy in intestinal cells and, if so, the mechanisms that underlie this action.

Methods—Formation of microtubule-associated protein light chain 3 (LC3) -phospholipid conjugates (LC3-II) in rat intestinal epithelial IEC18 cells and human colonic epithelial Caco-2_{BBE} cells was determined by Western blotting and localized by confocal microscopy. Activation of mammalian target of rapamycin (mTOR) pathway, mitogen-activated protein (MAP) kinases, caspase-3 and poly (ADP-ribose) polymerase (PARP) were monitored by Western blotting.

Results—Glutamine increased LC3-II as well as the number of autophagosomes. Glutamine-induced LC3-II formation was paralleled by inactivation of mTOR and p38 MAP kinase pathways, and inhibition of mTOR and p38 MAP kinase allowed LC3-II induction in glutamine-deprived cells. Under glutamine starvation, LC3-II recovery after heat stress or increase under oxidative stress was significantly blunted. Glutamine depletion increased caspase-3 and PARP activity after heat stress, which was inhibited by treatment with inhibitors of mTOR and p38 MAP kinase.

Conclusions—Glutamine induces autophagy under basal and stressed conditions, and prevents apoptosis under heat stress through its regulation of the mTOR and p38 MAP kinase pathways. We propose that glutamine contributes to cell survival during physiological stress by induction of autophagy.

Glutamine is the most abundant free amino acid in the body and is a major respiratory fuel and metabolic precursor for many cell types including intestinal epithelial cells and immune cells. 1–3 Although considered non-essential, glutamine becomes conditionally essential during

Correspondence: Eugene B. Chang, MD, Martin Boyer Laboratories, University of Chicago IBD Research Center, 5841 S. Maryland Ave. MC6084, Chicago, Illinois, 60637. fax: (773) 702-2281.

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severe catabolic stress such as major surgery, trauma, or sepsis in which intracellular and plasma glutamine levels decrease rapidly.^{4, 5} Recent studies have demonstrated supplementation of parenteral or enteral glutamine reduces complication rates in critically ill and postoperative patients.^{6–8}

The mechanisms underlying glutamine's protective and trophic actions are incompletely understood. The possibility that it might be essential for the autophagic response of intestinal epithelial cells was therefore considered by this study. Autophagy is a catabolic process that recycles cellular proteins and organelles, an evolutionarily conserved response to metabolic stress.^{9–11} Autophagy is recognized as a cell survival mechanism during period of nutrient deprivation where the bulk degradation of cytoplasmic proteins and non-essential organelles provides an alternative energy source. The process of autophagy is characterized by the formation of double membrane vesicles known as autophagosomes which is mediated by the Atg12-Atg5-Atg16 complex and microtubule-associated protein light chain 3 (LC3) - phospholipid conjugates (LC3-II).^{12, 13} The outer membrane of the autophagosome fuses with the lysosome, and cytoplasm-derived materials are degraded in autolysosome.

Amino acids have long been known to be regulators of autophagy.¹⁴ The signaling mechanism by which amino acids regulate autophagy appears to involve stimulation of mammalian target of rapamycin (mTOR) kinase,^{15–17} although recent studies showed that they may also use mTOR-independent pathways.^{18, 19} Only certain amino acids are capable of modulating autophagy and their actions are highly cell-specific.²⁰

In the present study, we demonstrate that glutamine induces autophagy in rat intestinal epithelial IEC-18 cells and human colonic epithelial Caco-2_{BBE} cells, a process that is mediated by inhibition of the mTOR and p38 MAP kinase pathways. In IEC-18 cells, glutamine also maintains autophagy under heat and oxidative stressed conditions and prevents apoptosis under heat stressed condition. Our data suggests that glutamine contributes to cell survival during physiological stress by induction of autophagy through its regulation of the mTOR and p38 MAP kinase pathways.

Materials and Methods

Chemicals

Chemicals were obtained from Fisher Scientific (Hanover Park, IL) unless otherwise stated. Media and all cell culture supplements were obtained from Invitrogen (Grand Island, NY), L-leucine and 3-methyladenine from Sigma-Aldrich (St. Louis, MO) and rapamycin and SB203580 from Axxora (San Diego, CA).

Cell Culture

The diploid nontransformed rat small intestinal epithelial IEC-18 cell line (ATCC, Manassas, VA; CRL-1589) was used between passages 20 and 35. IEC-18 cells were grown in high-glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM) containing 2 mM L-glutamine, 5% vol/vol FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, and 0.1 U/ml insulin, and cultured to 90% confluence. The human colon carcinoma cell line Caco-2_{BBE} was used between passages 50 and 75. Caco-2_{BBE} cells were seeded onto cell culture inserts at a density of 10⁵ cells/cm², grown in high-glucose DMEM containing 2 mM L-glutamine, 10% vol/vol FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, and 10 µg/ml transferrin, and allowed to differentiate for 14 days. Both cell lines were then incubated for 24 hours in reduced-serum (1% vol/vol FBS) DMEM containing indicated concentrations of glutamine with all other supplements. Cell cultures were maintained in a humidified 5% CO₂ incubator at 37°C. Heat shock was achieved by sealing dishes and immersing them in a 42°C water bath for 15 or 30

minutes. For confocal microscopy, cells were grown in complete growth medium on glass coverslips. IEC-18 cells were cultured to 90% confluence, and Caco-2_{BBE} cells were cultured for 14 days. Cells were then incubated for 24 hours in reduced-serum DMEM containing either 0 or 0.7 mM glutamine with all other supplements.

RNA silencing of mTOR and p38 MAP kinase

To silence mTOR or p38 MAP kinase, pre-designed siRNA specific for rat mTOR (NM_019906, bases 710–728, ID#s132719) or p38 MAP kinase (NM_031020, bases 666–684, ID#s135448) were purchased from Ambion (Austin, TX). As a negative control, AllStars Negative Control siRNA (Qiagen, Valencia, CA) was used. siRNA (final concentration; 5 nM) was mixed with siLentFect lipid reagent (Bio-Rad, Hercules, CA) in Opti-MEM (Invitrogen) and allow to form complexes for 30 minutes at room temperature. Complexes were added to 70% confluent IEC-18 cells where 15 minutes before the complete medium had been replaced with Opti-MEM. The cells were incubated at 37°C for 30 minutes and then complete medium with 10% vol/vol FBS added. Twenty-four hours after the transfection, cells were incubated for 24 hours in reduced-serum DMEM without glutamine.

Western Blot Analysis

Cells were scraped and disrupted in lysis buffer (composition: 50 mM Tris pH 7.4, 150 mM NaCl, 1% vol/vol NP-40, 1 mM Na₃VO₄, 1 mM NaF and the complete protease inhibitor cocktail (Roche Molecular Biosciences, Indianapolis, IN)). An aliquot was removed and protein concentrations were measured using bicinchoninic acid. To the remainder, Laemmli sample buffer was added (composition: 250 mM Tris pH 7.4, 2% wt/vol SDS, 25% vol/vol glycerol, 10% vol/vol 2-mercaptoethanol, and 0.01% wt/vol bromophenol blue) and samples were heated to 70°C for 10 minutes and stored at –80°C until analysis. Equal amounts of protein were separated by SDS-PAGE and transferred immediately onto polyvinylidene difluoride membranes (Polyscreen; Perkin-Elmer, Boston, MA) using 1x Towbin buffer (25 mM Tris pH 8.8, 192 mM glycine, 15% vol/vol methanol). Membranes were subsequently incubated in 3% wt/vol bovine serum albumin (BSA) in Tris-buffered saline (TBST; composition: 140 mM NaCl, 5 mM KCl, 10 mM Tris pH 7.4 with 0.1% vol/vol Tween 20) at room temperature for 1 hour. Antibodies were added and incubated for overnight at 4°C in TBST. Polyclonal rabbit antibodies against LC3 (2775), phospho-mTOR (2971), mTOR (2972), phospho-p70 S6 kinase (9205), phospho-p38 MAP kinase (9211), p38 MAP kinase (9212), phospho-p44/42 MAP Kinase (ERK) (9101), phospho-SAPK/JNK (9251), Caspase-3 (9662) and poly (ADP-ribose) polymerase (PARP) (9542), and monoclonal mouse antibody against phospho-Akt (4051) were purchased from Cell Signaling (Danvers, MA). Polyclonal rabbit antibody against Hsp25 (SPA-801) and monoclonal mouse antibody against Hsp70 (SPA-810) were purchased from Stressgen/Assay Designs (Ann Arbor, MI). Monoclonal mouse antibody against β-actin (AAN01) was purchased from Cytoskeleton (Denver, CO). Membranes were washed three times in TBST and subsequently incubated with species-appropriate peroxidase-conjugated secondary antibodies (1 hour; Jackson ImmunoResearch, West Grove, PA). Blots were washed three times with TBST and once with TBS no Tween and developed using an enhanced chemiluminescence system (Supersignal, Pierce Chemical, Rockford, IL).

Confocal microscopy of autophagosomes

After treatment with 0 or 0.7 mM glutamine in reduced-serum DMEM for 24 hours, cells were washed in PBS and then fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 15 minutes at room temperature. Fixed cells were washed with PBS, permeabilized in 100% methanol for 10 minutes at –20°C, washed in PBS, and blocked in blocking buffer (X909; DAKO, Carpinteria, CA) for 1 hour at room temperature. Cells were subsequently incubated with anti-LC3 antibody in antibody diluent (S3022; DAKO) for

overnight at 4°C. After three TBST washes, cells were incubated with Cy2-anti-Rabbit antibody (Jackson ImmunoResearch) in antibody diluent for 2 hours at room temperature and then washed three times in TBST. Coverslips were mounted on slides using SlowFade Gold anti-fade reagent with DAPI (Invitrogen). Cells were observed with Leica TCS SP2 Laser Scanning Confocal Microscope, photographed at a magnification of x400 and analyzed using Leica Confocal Software (Leica Microsystems Inc., Bannockburn, IL).

Data Analysis

All experiments were repeated at least three times with cells of different passage numbers. Densitometry of autoradiography images was performed using ImageJ (W. Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD) and normalized to the signal intensity of β -actin for equal protein loading control for each sample in each experiment. This quantitation was performed within the linear range of the standard curve defined by the standard sample, β -actin, for all densitometry analysis. For confocal microscopy experiments, the number of LC3 punctate dots and nuclei were counted using ImageJ software. Statistical analysis (ANOVA) was performed by using a Bonferroni correction with StatView 5.0 (SAS Institute, Cary, NC). Data were expressed as means $\pm$ SE.

Results

Glutamine increases autophagy in IEC-18 and Caco-2_{BBE} cells

Autophagosome formation is a complex, multistage process that involves many proteins.^{9–11} The process can be most readily assessed by following the phospholipid conjugation of the protein LC3-I (cytosolic form) to LC3-II (autophagosomal membrane-bound form), which has an increased gel mobility on SDS-PAGE.^{12, 13} Autophagosome formation can also be visualized as LC3-II accumulates in this organelle. To determine if glutamine is required for autophagosome formation, IEC-18 and Caco-2_{BBE} cells were incubated with varying concentrations of glutamine for 24 hours. The concentration range of glutamine ranged from the physiologic concentration normally found in plasma (0.7 mM) to concentrations that were pharmacologic (2.8 mM). In the absence of glutamine, a small degree of LC3-II expression could be detected by Western blotting (Figure 1A and 1B) which was additionally reflected by a small number of autophagosomes observed by confocal microscopy (Figure 1C and 1D). However, with increasing glutamine concentration, a significant increase in both the phospholipid-conjugated LC3-II as well as the number of autophagosomes is observed (Figures 1A to 1D, autophagosome number quantified in 1E and 1F).

Glutamine is essential for the metabolic and nutritional status of the intestinal epithelial cell,² but other amino acids also play a role in this regard. For instance, leucine is believed to be a potent inhibitor of autophagy.^{15–17} Therefore, we examined the effects of leucine on glutamine-stimulated autophagy (see Supplementary Figure 1). As the concentration of leucine in DMEM is 0.8 mM, this concentration was defined as ‘basal’ in assessing LC3-II expression. IEC-18 and Caco-2_{BBE} cells were treated for 24 hours with varying concentrations of leucine (0.8–6.4 mM) with or without 0.7 mM glutamine. Doubling the leucine concentration to 1.6 mM reduced glutamine’s induction of LC3-II by approximately 20% in both cells and increasing leucine further caused an even greater inhibition of glutamine LC3-II induction (see Supplementary Figure 1).

Leucine has been shown to alter activity of a number of proteins in the cell including mTOR.²⁰ mTOR is a kinase which phosphorylates a number of targets, including p70 S6 kinase (S6K). Because Akt (protein kinase B) is a serine/threonine kinase which regulates the activity of mTOR, we used phospho-specific antibodies to examine states of activation of Akt, mTOR, and S6K in conditions of glutamine starvation. Within 6 hours of glutamine depletion, LC3-II

decreased, paralleled by increases in phosphorylated Akt, mTOR and S6K in IEC-18 and Caco-2_{BBE} cells (Figure 2A and 2B).

The identical samples were also analyzed for activation of certain MAP kinase, as they could also be involved in regulation of the autophagic response. In the presence of glutamine, p38 MAP kinase demonstrated a low level of phosphorylation which increased within 6 hours of glutamine deprivation (Figure 2A and 2B), suggesting a role of glutamine to regulate this kinase. Minimal to no changes were observed in phosphorylation of extracellular signal-related kinase (ERK) or c-Jun amino-terminal kinase (JNK) upon glutamine deprivation (data not shown).

To confirm the role of mTOR and p38 MAP kinase in glutamine induction of LC3-II, pharmacologic inhibitors of mTOR (rapamycin) and p38 MAP kinase (SB203580), or siRNAs for mTOR and p38 MAP kinase were used in IEC-18 cells. As previously demonstrated, glutamine depletion decreased the level of LC3-II which is paralleled by an activation of mTOR and p38 MAP kinase (Figure 3). Treatment of the cells with rapamycin (100 nM for 24 hours) and SB203580 (5 μ M for 24 hours) leads to the induction of LC3-II even under condition of glutamine depletion. Silencing mTOR and p38 MAP kinase also restored LC3-II to near normal in glutamine-deprived cells. Therefore, both the mTOR and p38 MAP kinase pathways appear to be involved in the regulation of autophagy by glutamine.

Glutamine maintains autophagy and inhibits apoptosis under stressed condition

Autophagy may play a role in cellular maintenance and protection, particularly under conditions of stress.²¹ We therefore examined glutamine-dependent autophagic responses in cells subjected to thermal (heat) stress, a physiological equivalent of fever. To determine if heat stress promotes an autophagic response, IEC-18 cells (grown in 0.7 mM glutamine) were subjected to 42°C for up to 30 minutes. As shown in Figure 4A, heat stress caused a significant decrease in LC3-II, but, after heat stress and during the recovery phase (37°C), this response attenuated and LC3-II abundance returned back to normal levels. Heat stress also stimulated phosphorylation of Akt and mTOR, the latter to a lesser extent. Both changes returned to near basal state after the heat stress removal during the recovery phase. The activity of p38 MAP kinase and expression of β -actin were not affected by heat stress. The induction of heat shock protein (Hsp) 70 and Hsp25, two known heat-inducible stress proteins, could clearly be seen during the recovery phase. To determine if mTOR is responsible for the regulation of autophagy by heat stress, cells were pretreated with rapamycin (100 nM for 24 hours). Rapamycin-treated cells did not show the LC3-II decrease during heat or the LC3-II increase upon return to 37°C (Figure 4B).

To determine whether glutamine plays a role in the autophagy response to heat stress, cells were incubated for 24 hours with 0.7 or 0 mM glutamine. Under conditions of glutamine depletion, the autophagy response to heat stress was minimal and the LC3-II increase after heat stress was small, despite inactivation of mTOR (Figure 5A). In contrast, the robust activation of p38 MAP kinase in glutamine-deprived cells led us to postulate that p38 MAP kinase activation might be involved in inhibition of the normal autophagy response to heat stress. To test this hypothesis, glutamine-deprived cells were treated with the p38 MAP kinase inhibitor SB203580 (5 μ M for 24 hours). Treatment with SB203580 restored the LC3-II levels under basal as well as heat stressed conditions (Figure 5B). Moreover, the changes of LC3-II levels during heat and after heat were similar to those in glutamine-treated cells (compare Figure 5A 0.7 mM glutamine with 0 mM + SB203580 in 5B). These results suggest that glutamine regulates autophagy by suppression of p38 MAP kinase activity under heat stressed condition.

We also examined the glutamine-dependent autophagic responses to oxidative stress (see Supplementary Figure 2). When glutamine was present, 500 μ M H₂O₂ caused a significant

increase in LC3-II, paralleled by a decrease in phosphorylated mTOR. However, treatment with H₂O₂ stimulated the phosphorylation of p38 MAP kinase. Under glutamine deprivation, H₂O₂-induced LC3-II increase was minimal as in the case of heat stress. In the absence of glutamine and despite decreased phosphorylation of mTOR following the addition of H₂O₂, p38 MAP kinase showed a strong activation (see Supplementary Figure 2A). The restoration of the LC3-II level by the treatment with SB203580 (5 μ M for 24 hours) in glutamine-deprived cells suggests that glutamine also increases autophagy under oxidative stressed condition through its inhibitory effect on p38 MAP kinase (see Supplementary Figure 2B).

Heat stress can potentially stimulate an apoptotic response, but could depend on the metabolic state of the cell. In support of this, in the absence of glutamine, heat stress increased the production of cleaved caspase-3, a pivotal aspartic protease in the apoptotic response, and cleaved PARP, one of the main cleavage targets of caspase-3 (Figure 6, middle lane). Glutamine supplementation decreased cleaved caspase-3 and PARP (0.7 mM, Figure 6, lane on far left), indicating the presence of glutamine can direct cell fate and prevent apoptosis during heat stress recovery. Also notable is that when autophagy was blocked with 3-methyladenine (3MA, 10 mM for 24 hours) in glutamine supplemented condition, cleaved caspase-3 and PARP increased, suggesting that, in the absence of the autophagic response, the cell defaults to apoptosis under conditions of heat stress. To determine the involvement of mTOR and p38 MAP kinase, glutamine-depleted cells were treated with rapamycin or SB203580 (100 nM or 5 μ M, respectively) for 24 hours. Both rapamycin and SB203580 inhibited the formation of cleaved caspase-3 and PARP under glutamine deprived, heat stressed conditions (Figure 6, two right lanes). Thus, these inhibitors restore the autophagy response even under glutamine-deprived conditions, supporting the notion of a balance between the autophagy and apoptotic responses in IEC-18 cells that is dependent on glutamine and regulation of mTOR and p38.

Discussion

The gut mucosa continually faces physiological stresses, including large changes in luminal pH and osmolarity, luminal bacteria, and physiological state of immune and inflammatory activation.²² Several factors are important in maintaining gut homeostasis. Glutamine, for example, may regulate proliferation of intestinal epithelial cells by modulating responsiveness to growth factors.^{23, 24} Small intestinal mucosa becomes atrophic when the gut is deprived of glutamine, as during total parenteral nutrition.²⁵ Glutamine depletion can increase permeability of the gut which promotes translocation of luminal bacteria and toxins.²⁶ Glutamine has been shown to protect intestinal epithelial cells during physiological stress because it is required for stress-induced heat shock protein expression.^{27, 28} It has also been shown to attenuate cytokine expression as well as NF- κ B activation,^{29–31} enhance glutathione synthesis,³² and prevent apoptosis.^{33–35}

Autophagy is generally considered a survival process during periods of metabolic stress.^{36, 37} However, more recently, its role as a survival pathway under other forms of stress, including oxidative and toxic (anticancer drug), has become increasingly recognized.^{21, 38} Autophagy also plays a role in clearing intracellular microbes and bacterial toxins.^{39, 40} Polymorphisms of two related autophagy genes, ATG16L1 and IRGM, were shown to be associated with increased risk for inflammatory bowel diseases.^{41–43} When ATG16L1 and IRGM expression were silenced by siRNA, cellular ability to form autophagosomes was compromised as was autophagic clearance of *Salmonella typhimurium* and *Mycobacteria*, respectively.^{42, 44} In this study, we demonstrate that glutamine is essential for autophagy in intestinal epithelial cells. Glutamine depletion, on the other hand, compromises this process, both under basal and stressed conditions, and, in the latter case, cell fate defaults to apoptosis.

Several cell signaling pathways are involved in the regulation of autophagy which are a cell type-specific and signal-dependent.⁴⁵ mTOR is involved in the negative control of autophagy and has been proposed as a 'nutrient sensor', although the way by which amino acids, leucine in particular, modulate mTOR activity is not fully understood.²⁰ In rat hepatocytes, glutamine is able to activate p70 S6K, a downstream target of mTOR, alone or in combination with leucine.⁴⁶ By contrast, glutamine reverses the activation of p70 S6K induced by leucine in IEC-18 cells.⁴⁷ Antagonistic effects of leucine and glutamine on the mTOR pathway have also reported in myogenic C₂C₁₂ cells.⁴⁸ Likewise, glutamine's effects on p38 MAP kinase vary depending on the cell type. Glutamine induces p38 MAP kinase activation in rat hepatocytes,⁴⁹ but in IEC-18 and Caco-2_{BBE} cells, it inhibits p38 MAP kinase activity. The role of the p38 MAP kinase as a negative regulator of autophagy has also been described in hepatocytes following stimulation by insulin, ethanol and amino acids such as glutamine and glycine,⁴⁹ and induction of autophagy by inhibition of p38 MAP kinase pathway was recently reported in colorectal cancer cells.⁵⁰

We believe our study has several important clinical implications. Under conditions of critical illness, post-surgical stress, chronic inflammation, or starvation, glutamine is rapidly depleted from the body.^{4, 5} Organs such as the gastrointestinal tract which are highly dependent on glutamine as a fuel source are particularly susceptible to injury under these conditions. If sustained, glutamine depletion could impair the gut's ability to mount an autophagic response. That would have at least two potential complications. First, the inability to mount an autophagic response could increase mucosal injury to stress (heat (fever), oxidant (ischemia-reperfusion), etc), resulting in increased cellular apoptosis, enhanced mucosal permeability, and dysfunction of transport mechanisms. This could explain the observed transmigration of luminal organisms and microbial-derived products such as lipopolysaccharide and peptidoglycan. Second, impairment of autophagy could impair normal clearance of intracellular organisms or enterotoxins, particularly opportunistic infectious pathogens.^{40, 42, 44} We speculate that this may contribute to the poor outcomes and increased susceptibility of malnourished populations during epidemics of infectious diarrheal diseases.

In summary, we report that glutamine is essential for maintaining autophagy and mounting an autophagic response under conditions of stress in intestinal epithelial cells. A model for glutamine regulation of autophagy is shown in Supplementary Figure 3. Under conditions of glutamine depletion, intestinal epithelial cells are unable to mount an autophagic response to stress, resulting in apoptosis. Glutamine contributes to cell survival during physiological stress by induction of autophagy through its regulation of the mTOR and p38 MAP kinase pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LC3	microtubule-associated protein light chain 3
mTOR	mammalian target of rapamycin

MAP

mitogen-activated protein

DMEM

Dulbecco's Modified Eagle Medium

PARP

poly (ADP-ribose) polymerase

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Figure 1.

Glutamine enhances autophagy in IEC-18 and Caco-2_{BBE} cells. (A) IEC-18 cells were incubated with varying concentrations of glutamine for 24 hours and analyzed by Western blotting. The ratio of LC3-II to β -actin density was calculated using ImageJ software and set to one for the glutamine-deprived condition. Images are representative of four separate experiments. (B) Caco-2_{BBE} cells were incubated with varying concentrations of glutamine for 24 hours and analyzed by Western blotting. The ratio of LC3-II to β -actin density was calculated using ImageJ software and set to one for the glutamine-deprived condition. Images are representative of four separate experiments. (C) Autophagosome formation in IEC-18 cells was observed using confocal microscopy in the absence and presence of glutamine. IEC-18

cells were incubated with 0 or 0.7 mM glutamine for 24 hours, fixed, and stained for LC3 (*green*) as well as nuclei were stained using DAPI (*blue*). Images are representative of three separate experiments. (D) Autophagosome formation in Caco-2_{BBE} cells was observed using confocal microscopy in the absence and presence of glutamine. Caco-2_{BBE} cells were incubated with 0 or 0.7 mM glutamine for 24 hours, fixed, and stained for LC3 (*green*) as well as nuclei were stained using DAPI (*blue*). Images are representative of three separate experiments. Autophagosome formation, visualized by accumulation of LC3, was quantified and counted using ImageJ software in at least 300 cells in each experiment. The number of autophagosomes per cell of IEC-18 cells is shown in (E) and that of Caco-2_{BBE} cells is shown in (F). Data are as means $\pm$ SE. * $P < 0.05$ compared with 0 mM glutamine by ANOVA using a Bonferroni correction.

Figure 2.

Glutamine inhibits mTOR and p38 MAP kinase activity in IEC-18 and Caco-2_{BBE} cells. (A) IEC-18 cells were incubated with 0.7 mM glutamine for 24 hours. Cells were subsequently deprived of glutamine for varying times up to 24 hours. Samples were collected at designated times and analyzed by Western blotting. Images are representative of three separate experiments. (B) Caco-2_{BBE} cells were incubated with 0.7 mM glutamine for 24 hours. Cells were subsequently deprived of glutamine for varying times up to 24 hours. Samples were collected at designated times and analyzed by Western blotting. Images are representative of three separate experiments. Densitometric values were obtained using ImageJ software and normalized to β-actin, set to one for condition immediately after glutamine deprivation. Data

are as means $\pm$ SE. * $P < 0.05$ and ** $P < 0.01$ compared with cells at time point 0 hour by ANOVA using a Bonferroni correction.

Figure 3.

mTOR and p38 MAP kinase pathways are involved in glutamine's regulation of autophagy in IEC-18 cells. IEC-18 cells were incubated in 0.7 mM glutamine supplemented medium for 24 hours, or glutamine-deprived media without or with mTOR inhibitor rapamycin (Rap; 100 nM) and p38 MAP kinase inhibitor SB203580 (SB; 5 μ M) for 24 hours. For silencing of mTOR and p38 MAP kinase with siRNA, silencing oligonucleotides or nonsilencing siRNA (control) were introduced into 70% confluent IEC-18 cells. Twenty-four hours after the transfection, cells were incubated for 24 hours in glutamine-deprived media. Total cell lysates were analyzed by Western blotting. Images are representative of three separate experiments. The ratio of LC3-II to β -actin density was calculated using ImageJ software, and set to one for no glutamine, inhibitors and siRNA. Data are as means $\pm$ SE. * P < 0.05 compared with 0 mM glutamine, no inhibitors and siRNA by ANOVA using a Bonferroni correction.

Figure 4.

Heat stress regulates autophagy via the mTOR pathway. (A) IEC-18 cells were incubated with 0.7 mM glutamine for 24 hours followed by heat shock at 42°C for 15 or 30 minutes, collected at designated times and analyzed by Western blotting. The ratio of LC3-II to β -actin density was calculated using ImageJ software, set to one for the sample before heat shock. Images are representative of three separate experiments. Data are as means $\pm$ SE. * P < 0.05 compared with unstimulated condition by ANOVA using a Bonferroni correction. (B) Inhibition of the mTOR pathway blocks the autophagy response to heat stress. IEC-18 cells were incubated in 0.7 mM glutamine supplemented media without rapamycin (left three lanes) or with 100 nM rapamycin (right three lanes) for 24 hours followed by heat shock at 42°C for 30 minutes. Cells were collected before, immediately after, and 90 minutes after heat shock, and analyzed by Western blotting. The ratio of LC3-II to β -actin density was calculated using ImageJ software, set to

one for the unstimulated condition without rapamycin. Images are representative of three separate experiments. Data are as means $\pm$ SE. * $P < 0.05$ compared with unstimulated condition and no rapamycin by ANOVA using a Bonferroni correction.

Figure 5.

Glutamine depletion decreases the autophagy response to heat stress by the activation of p38 MAP kinase. (A) Decreased autophagy response to heat stress in glutamine-depleted cells. IEC-18 cells were incubated with 0.7 mM glutamine (left three lanes) or without glutamine (right three lanes) for 24 hours followed by heat shock at 42°C for 30 minutes. Cells were collected before, immediately after, and 90 minutes after heat shock, and analyzed by Western blotting. Images are representative of three separate experiments. Densitometric values were obtained using ImageJ software and normalized to β-actin, set to one for unstimulated condition in 0.7 mM glutamine. Data are as means ± SE. * $P < 0.05$ and ** $P < 0.01$ compared with unstimulated condition and 0.7 mM glutamine by ANOVA using a Bonferroni correction. (B) Inhibition of p38 MAP kinase restores the normal autophagy response to heat stress in glutamine-depleted cells. IEC-18 cells were incubated in glutamine-depleted media without

SB203580 (left three lanes) or with 5 μ M SB203580 (right three lanes) for 24 hours followed by heat shock at 42°C for 30 minutes. Cells were collected before, immediately after, and 90 minutes after heat shock, and analyzed by Western blotting. Images are representative of three separate experiments. The ratio of LC3-II to β -actin density was calculated using ImageJ software, and set to one for unstimulated condition without glutamine and SB203580. Data are as means $\pm$ SE. * P < 0.05 compared with unstimulated condition, 0 mM glutamine and no SB203580 by ANOVA using a Bonferroni correction.

Figure 6.

Enhanced autophagy decreases apoptosis in IEC-18 cells. IEC-18 cells were incubated in 0.7 mM glutamine supplemented media without or with 3-methyladenine (3MA; 10 mM) for 24 hours (left two lanes), or incubated in glutamine-deprived media without or with rapamycin (Rap; 100 nM) and SB203580 (SB; 5 μM) for 24 hours (right three lanes). The cells were heat shocked at 42°C for 30 minutes, followed by one hour recovery, and analyzed by Western blotting. Images are representative three separate experiments. β-actin was used as a loading control.