Published in final edited form as:

Gastroenterology. 2011 December ; 141(6): 2188–2199.e6. doi:10.1053/j.gastro.2011.08.005.

Autophagy Suppresses Age-Dependent Ischemia and Reperfusion Injury in Livers of Mice

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Abstract

BACKGROUND & AIMS—As life expectancy increases, there are greater numbers of patients with liver diseases that require surgery or transplantation. Livers of older patients have significantly less reparative capacity following ischemia and reperfusion (I/R) injury, which occurs during these operations. There are no strategies to reduce the age-dependent I/R injury. We investigated the role of autophagy in the age dependence of sensitivity to I/R injury.

METHODS—Hepatocytes and livers from 3- and 26-month-old mice were subjected to in vitro and in vivo I/R, respectively. We analyzed changes in autophagy-related proteins (Atg). Mitochondrial dysfunction was visualized using confocal and intravital multiphoton microscopy of isolated hepatocytes and livers from anesthetized mice, respectively.

RESULTS—Immunoblot, autophagic flux, genetic, and imaging analyses all associated the increase in sensitivity to I/R injury with age with decreased autophagy and subsequent mitochondrial dysfunction, due to calpain-mediated loss of Atg4B. Overexpression of either

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Conflicts of interests Authors have nothing to disclose.

Author Contributions Wang, Ahn, Fischer and Byeon: Data acquisition and analysis

Dunn, Jr.: Study concept and material support

Behrns: Study concept and critical revision of the manuscript

Leeuwenburgh and Kim: Study concept and design, analysis and interpretation of data, preparation for the manuscript, obtained funding and supervision.

Atg4B or Beclin-1 recovered Atg4B, increased autophagy, blocked the onset of the mitochondrial permeability transition, and suppressed cell death after I/R in old hepatocytes. Co-immunoprecipitation analysis of hepatocytes and Atg3-knockout cells demonstrated an interaction between Beclin-1 and Atg3, a protein required for autophagosome formation. Intravital multiphoton imaging revealed that overexpression of Beclin-1 or Atg4B attenuated autophagic defects and mitochondrial dysfunction in livers of older mice after I/R.

CONCLUSION—Loss of Atg4B in livers of old mice increases their sensitivity to I/R injury. Increasing autophagy might ameliorate liver damage and restore mitochondrial function after I/R.

Keywords

MPT; mouse model; aging; liver injury

With advancing age, patients are more likely to acquire primary and secondary hepatic malignancies that are amenable to surgical resection and transplantation. Though the elderly patients may be treated surgically, the aged liver has significantly decreased reparative capacity following I/R injury associated with these operations.¹ The mechanisms underlying I/R injury are multifactorial² and mitochondrial dysfunction is a key event increasing the sensitivity of aged liver to I/R injury.^{1,3} Ischemic preconditioning is the only promising strategy for improving the outcome of liver surgery⁴, but its beneficial effects are limited to young patients. To date, no therapeutic strategy can suppress the age-dependent I/R injury.

Autophagy is a cellular process that efficiently degrades both long-lived cytoplasmic proteins and surplus or dysfunctional organelles by lysosome-dependent machinery.⁵ In liver, macroautophagy (referred to as autophagy hereafter) is a primary catabolic process and confers cytoprotection against prolonged I/R.⁶ Recent evidence supports the presence of selective mitochondrial autophagy, so called mitophagy⁷, that targets and removes only damaged or abnormal mitochondria. Therefore, active enhancement of mitophagy may have a great therapeutic potential for mitochondria-related diseases.

Here we investigate the role of autophagy in the age-dependent sensitivity to I/R injury. We demonstrate both in *in vitro* and *in vivo* models of I/R that Atg4B loss is causatively associated with the increased sensitivity of liver to I/R injury with age.

Materials and Methods

Hepatocyte isolation and culture

Animals received humane care according to protocols approved by the Institutional Care and Use Committee of the University of Florida. Hepatocytes were isolated from male C57BL/6 mice at 3 and 26 months of age by collagenase perfusion method.⁸ The viability after isolation, as judged by trypan blue exclusion, was 90.4 ± 0.40 and 88.2 ± 0.43 in young and old hepatocytes, respectively. Additional details are provided in the Supplemental Materials and Methods section.

Simulation of I/R

To simulate tissue ischemia, hepatocytes were incubated at 37°C in Krebs-Ringer-hydroxyethylpiperazine-N-2 ethanesulfonic acid (KRH) buffer at pH 6.2 in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) for 2 hours.⁸ To simulate reoxygenation and return to physiological pH during reperfusion, anaerobic KRH at pH 6.2 was replaced with aerobic KRH at pH 7.4. Immortalized Atg3 wild type (WT) and knockout (KO) mouse embryonic fibroblasts (MEF, a gift from Dr. Komatsu)⁹ were cultured, exposed to 2 hours of ischemia in KRH at pH 6.2 and reperused in Dulbecco's modified Eagle's

medium (DMEM) for up to 12 hours. After I/R, 20 different fields per each dish were randomly selected and apoptotic morphology were evaluated.

Cell death assay

Necrosis at 5, 60 and 120 minutes of reperfusion was assessed by propidium iodide (PI) fluorometry.⁸ Caspase 3 expression was determined by immunoblotting after 12 hours of reperfusion in hormonally-defined-medium (HDM).⁸

Adenovirus construction and transfection

Adenovirus encoding Beclin-1 (AdBeclin-1), AdAtg4B and AdmCherry-GFP-LC3 was constructed with corresponding cDNAs using the ViraPower Adenoviral Gateway Expression kit (Invitrogen).⁶ Hepatocytes or MEF were infected with AdBeclin-1 and/or AdGFP-LC3 at the concentration of 10 MOI (multiplicity of infection) in HDM⁶ or DMEM, respectively. AdGFP or Ad β -galactosidase (AdLacZ) was used for viral control.

Confocal microscopy

Confocal images of tetramethylrhodamine methylester (TMRM), calcein, PI and mCherry-GFP-LC3 were collected with a gas-tight chamber (Zeiss) using an inverted Zeiss 510 laser scanning confocal microscope, as previously described.⁶

In vivo I/R

Total hepatic ischemia was induced as previously described⁶, by occluding the portal triad for 20 minutes. Reperfusion was initiated by removing a microvascular clamp. Liver biopsies from the left lateral lobe were collected during I/R and immediately frozen in liquid nitrogen.

Intravital multiphoton microscopy

Old animals were injected with 10^{11} viral particles of AdBeclin-1, AdAtg4B and/or AdGFP-LC3. AdLacZ was used for viral control. After 40 minutes of reperfusion *in vivo*, a midline celiotomy was performed and a 24-gauge catheter was inserted into the carotid artery.¹⁰ Rhodamine 123 (50 ml of 10 μ M/animal), a $\Delta\Psi_m$ -sensitive fluorophore, was infused for 10 minutes. The liver was gently withdrawn from the abdominal cavity and placed over a glass coverslip on the stage of a Zeiss LSM510 equipped with a multiphoton microscope. Images of green fluorescing rhodamine 123 were collected with a 40 $\times$ water-immersion objective lens. Rhodamine 123 was excited with 780 nm from a Chameleon Ultra Ti-Sapphire pulsed laser (Coherent Inc., Santa Clara, CA) and images were collected through 500-550-nm band pass filter. Ten images were randomly collected per each liver. To visualize autophagosomes, z-stacks of GFP-LC3 fluorescence were collected at 0.7- μ m intervals through the thickness of 70 μ m.

Immunoblotting and immunoprecipitation for autophagy proteins

Hepatocyte and liver lysates were prepared and protein expression was detected on the same gel using primary polyclonal antibodies. Immunoprecipitates were incubated with protein A/G Plus agarose (Santa Cruz), eluted in 2 $\times$ Laemmli buffer at 95°C, separated and transferred electrophoretically to nitrocellulose membrane. Additional details are provided in the Supplemental Materials and Methods section.

Data analysis

Differences between groups were compared using ANOVA and *post hoc* Bonferroni analysis (SigmaStat, Ashburn, VA). $P < 0.05$ denotes statistical significance. Data are

expressed as means $\pm$ S.E. All experiments are representative of at least three different cell isolations or animals per group.

Results

Age- and MPT-dependent I/R injury to hepatocytes

Male C57BL/6 mice whose average lifespan is 28 months¹¹, have been chosen in this study. Hepatocytes were isolated from 3-month-old (referred to as “young hepatocytes”) and 26-month-old (“old hepatocytes”) mice. After 2 hours of simulated ischemia, reperfusion was continued for 2 hours. PI fluorometry showed that I/R increased necrosis in an age-dependent manner (Figure 1A).

Reperfusion causes onset of the MPT, mitochondrial uncoupling and cell death.^{6,8} The MPT, $\Delta\Psi_m$ and cell death were simultaneously visualized with confocal microscopy of calcein, TMRM and PI, respectively⁶ (Figure 1B). Green fluorescing calcein loads into the cytosol and nucleus but is excluded by normal mitochondria that have closed permeability transition pores. TMRM electrophoretically accumulates into the mitochondria in response to the negative $\Delta\Psi_m$. PI labels nuclei when the integrity of plasma membranes is lost. Since polarized mitochondria take up TMRM while simultaneously excluding calcein, the mitochondria in the green channel appear as dark and round voids where each void represents a single, polarized mitochondrion (top panel, Figure 1B). Before reperfusion, the mitochondria in both young and old hepatocytes depolarized, but excluded calcein, indicating a lack of MPT onset during ischemia. After reperfusion, the mitochondria in old hepatocytes briefly repolarized but lost most TMRM after 5 minutes (bottom panel). As the mitochondria lost $\Delta\Psi_m$, calcein redistributed to the mitochondria, an event signifying the MPT.⁶ Calcein disappeared later and PI labeled nuclei (arrows) due to plasma membrane rupture and necrosis. One noticeable event was that widespread MPT occurred a few minutes after the initial redistribution of calcein to several mitochondria, suggesting that a certain population of mitochondria is more susceptible to MPT induction. Contrarily, in young hepatocytes, the mitochondria sustained $\Delta\Psi_m$ and excluded calcein continuously after reperfusion. As shown by image analysis, the number of TMRM-labeled mitochondria in old hepatocytes, indicative of polarized normal mitochondria, was significantly lower after I/R than that in young hepatocytes (Figure 1B). Thus, old hepatocytes rapidly underwent the MPT and necrosis after I/R, whereas young hepatocytes tolerated I/R rather well.

Altered autophagic flux to I/R with age

Microtubule-associated protein 1 light chain 3-I (LC3-I), a mammalian orthologue of Atg8, is sequentially cleaved, lipidated with phosphatidylethanolamine (PE), targeted to autophagosomes, and later either recycled or degraded in autolysosomes.¹² A dynamic nature of autophagy is assessed by autophagic flux with lysosomotropic reagents such as chloroquine or bafilomycin.^{12,13} Immunoblotting with bafilomycin or chloroquine (Figure 1C and D) showed a similar basal autophagic flux between age groups. Reperfusion of young hepatocytes with lysosomotropic reagents significantly accumulated LC3-II, indicating a strong autophagic response to reperfusion. Addition of lysosomotropic reagents to old cells increased LC3-II at 20 minutes after reperfusion, but the extent of accumulation was substantially lower than young hepatocytes. After 60 minutes, neither bafilomycin nor chloroquine caused LC3-II accumulation, suggesting a defective autophagic response to reperfusion. Next, we visualized autophagic flux with adenoviral tandem mCherry-GFP-LC3¹⁴ (Figure 1E). In the acidic environment of autolysosomes, red fluorescence of mCherry retains its fluorescence, while GFP loses its fluorescence. Therefore, autophagosomes show both red and green fluorescence, generating yellow puncta, whereas autolysosomes exhibit only red puncta. In young cells, bafilomycin markedly increased

yellow puncta (autophagosomes), in parallel with a significant decrease in red puncta (autolysosomes) (Supplementary Figure 1A). However, in old hepatocytes, bafilomycin-induced accumulation of yellow puncta was observed only during 20 minutes of reperfusion and the extent of accumulation was significantly lower than young cells.

To further investigate the effects of aging on hepatocellular autophagy, we examined autophagic flux under the non-ischemic, starvation condition (Figure 1F and Supplementary Figure 1B). Both young and old hepatocytes displayed a robust autophagy, as shown by a marked increase in LC3-II expression by chloroquine, demonstrating that old hepatocytes maintained basal autophagic flux under the non-ischemic condition. The presence of the active autophagy flux was also confirmed under normoxic conditions (Supplementary Figure 1C). Furthermore, confocal imaging of calcein and TMRM demonstrated that the mitochondria from old cells did not undergo MPT onset and sustained $\Delta\Psi_m$ during normoxia (Supplementary Figure 1D). Collectively, these results firmly show that autophagy in aged hepatocytes is functional before I/R but becomes inefficient or defective after I/R.

Causative role of Atg4B loss in old hepatocyte injury

We next explored cellular mechanisms underlying defective autophagy in reperfused old hepatocytes. Atg4B is essential in normal autophagy progression through the reversible modification of LC3.¹⁵ Atg4B expression substantially declined in old cells after I/R (Figure 2A). Reductions in Atg7 and Atg5-Atg12 were similar between age groups. To examine a role of Atg4B loss, we overexpressed Atg4B using adenoviral delivery. Atg4B overexpression in old hepatocytes protected necrosis and MPT onset (Figure 2B and C and Supplementary Figure 2B), but lowered LC3-II expression (Figure 2D). Reduced LC3-II may reflect enhanced lysosomal clearance. Accordingly, we analyzed autophagic flux with tandem mCherry-GFP-LC3. Atg4B overexpression markedly decreased yellow puncta (autophagosomes) but significantly increased the number of red puncta (autolysosomes) (Figure 2E), indicating a rapid lysosomal clearance by Atg4B overexpression. Increased autophagic flux was additionally supported by mCherry-GFP-LC3 analysis with bafilomycin (Supplemental Figure 2A, C and D). Cathepsin D, a lysosomal aspartyl protease, remained unchanged by Atg4B overexpression (Figure 2D).

Degradation of Atg4B by calpain 2

Calpains can be activated with age¹⁶ and hydrolyze autophagy proteins.^{6,17} To investigate a potential involvement of calpains in Atg4B depletion, calpain expression and activity were determined. Upon activation, calpain 1 and 2 rapidly autolyzes the intact catalytic subunit (80-kDa) to 76-kDa- and 43-kDa- fragments, respectively.¹⁸ Thus, loss of the 80-kDa subunit and concomitant increase in autolyzed fragment represents calpain activation. Immunoblotting demonstrated higher basal levels of calpain 1 and 2 in old hepatocytes compared to young hepatocytes (Figure 3A and B). Most calpain 1 existed as an autolyzed form in both groups and the expression of 80-kDa-subunit partially increased after I/R, suggesting a reduced activity upon reperfusion. In contrast, calpain 2 was considerably activated after reperfusion (Figure 3B). Treatment of old hepatocytes with N-acetyl-leucyl-leucyl-methioninal (ALLM), a membrane-permeable calpain inhibitor⁶, suppressed both reperfusion-induced calpain 2 activation and Atg4B loss (Figure 3C), indicating an integral role of calpain 2 in Atg4B depletion. ALLM did not prevent calpain 1 activation (data not shown). ALLM markedly increased autophagic flux (Figure 3E and F and Supplementary Figure 3A) and blocked cell death (Figure 3D) and MPT onset (Supplementary Figure 3B and C). Interestingly, ALLM decreased LC3-II (Supplementary Figure 3D), a pattern reminiscent of our observation made in Atg4B overexpression (Figure 2D). Taken together,

these results suggest that calpain 2 activation contributes to Atg4B depletion in old hepatocytes, and firmly support the key role of Atg4B in I/R injury.

Suppression of I/R injury by Beclin-1

Beclin-1, a mammalian orthologue of Atg6, initiates autophagy¹⁹ and protects young livers against prolonged I/R injury.⁶ We next investigated whether Beclin-1 can confer cytoprotection to old livers. Changes in Beclin-1 after I/R were similar between age groups (Figure 4A and Supplementary Figure 4B). Beclin-1 overexpression in old hepatocytes, however, markedly suppressed reperfusion-mediated necrosis (Figure 4B and C), substantially increased Atg4B, Atg5-Atg12 conjugate and autophagic flux after reperfusion (Figure 4B and D), and prevented the MPT (Supplementary Figure 4A and C).

Mitophagy selectively eliminates abnormal mitochondria. Hence, impaired mitophagy can cause accumulation of dysfunctional mitochondria and cell death. To visualize mitophagy onset, old hepatocytes were treated with AdGFP-LC3⁶, labeled with TMRM, and subjected to I/R. In the control, $\Delta\Psi_m$ was temporarily recovered at 20 minutes of reperfusion with increased number of autophagosomes, as judged by punctate, green fluorescing structures.²⁰ However, we failed to detect the autophagosomes enwrapping the mitochondria, indicating low or lack of mitophagy (top panel, Figure 4E). The mitochondria then depolarized and most autophagosomes disappeared later. In striking contrast, Beclin-1 overexpression persistently increased the number of autophagosomes (middle panel). Importantly, red fluorescing (polarized) mitochondria sequestered in the lumen of autophagosomes were evident under this condition, indicative of mitophagy onset (bottom panel). The number of mitophagic events after 60 minutes of reperfusion was significantly higher in AdBeclin-1-treated cells (11.67 ± 1.76 mitophagy/cell), compared to control cells (0.33 ± 0.33 mitophagy/cell, $p=0.03$).

The MPT not only initiates necrosis but also induces apoptosis by releasing pro-apoptotic factors that are normally sequestered in the mitochondrial intermembrane space.²¹ As Beclin-1 inhibited MPT-dependent necrosis, we reasoned inhibition of apoptosis by Beclin-1. Beclin-1 overexpression markedly suppressed caspase 3 activation in reperfused old hepatocytes (Figure 4F). Together, these results demonstrate that Beclin-1 suppresses Atg4B depletion, which sequentially enhances mitophagy and blocks the MPT and cell death.

Interaction between Atg3 and Beclin-1

Increased Atg4B expression by Beclin-1 in old hepatocytes suggests interaction between autophagy proteins. Atg3 is central for LC3-II formation²² and mitochondrial homeostasis.²³ Immunoprecipitation assay revealed that Beclin-1 was co-immunoprecipitated with Atg3, but neither with Atg4B nor with Atg7 (Figure 5A). Beclin-1/Atg3 interaction was further confirmed in Atg3-null MEF (Figure 5B). While normoxia affected neither WT nor KO cells, Atg3 KO cells were prone to MPT onset and cell death after I/R (Figure 5C). Moreover, Beclin-1 overexpression failed to reverse cell death in Atg3-null cells (Figure 5C and D), suggesting the necessity of Atg3 in Beclin-1-mediated cytoprotection.

As Beclin-1 interacts with Bcl-2 homologues²⁴, cytoprotection by Beclin-1 could stem from its antagonizing effect on Bcl-2 homologues. To test this, old hepatocytes were treated with ABT-737, a BH3 mimetic competitively inhibiting the interaction between Beclin-1 and Bcl-2/Bcl-X_L.²⁵ ABT-737 failed to reverse reperfusion-induced cell death (data not shown), suggesting that Beclin-1-mediated cytoprotection is independent of its interaction with Bcl-2 homologues.

Clearance of autophagosomes through fusion with lysosomes is the final step in autophagy. We next asked whether I/R affect lysosomes. Despite higher baseline levels in old cells, changes in cathepsin D after I/R were similar between age groups (Figure 6A and Supplementary Figure 5A and B), suggesting a minor role of lysosomal proteases in reduced autophagy. While Atg4B overexpression did not affect cathepsin D after reperfusion (Figure 2D), Beclin-1 overexpression enhanced cathepsin D (Figure 6B), implying a distinct lysosomal impact by Beclin-1. Together, lysosomal integrity and activity in old cells appears to be well-maintained after I/R, which further supports our conclusion that impaired autophagosome formation, but not defective lysosomal clearance, is causal to the age-mediated autophagic failure after I/R.

I/R *in vivo*

To validate our findings to *in vivo* I/R, livers at different ages were subjected to 20 minutes of ischemia and 40 minutes of reperfusion *in vivo*. Old animals were treated with AdBeclin-1 or AdAtg4B before I/R. Immunoblotting showed that Atg4B expression in old liver decreased substantially after reperfusion. Consistent with hepatocyte data, Beclin-1 overexpression reversed Atg4B loss (Figure 6C). Likewise, Atg4B overexpression in old livers suppressed Atg4B loss and increased LC3 expression (Figure 6D). Intravital multiphoton images with rhodamine 123, a $\Delta\Psi_m$ indicator¹⁰, revealed that reperfusion of young liver exhibited punctate, bright green fluorescence predominantly in hepatocytes, denoting polarized mitochondria and lack of mitochondrial injury (Figure 6F). In striking contrast, most rhodamine 123 disappeared in old liver with some diffuse cytosolic staining, indicating widespread mitochondrial depolarization and dysfunction. Administration of AdBeclin-1, however, suppressed reperfusion-induced autophagosome loss (Figure 6E) and $\Delta\Psi_m$ loss (Figure 6F). Atg4B overexpression also blocked mitochondrial depolarization. The number of hepatocytes with polarized mitochondria was significantly higher in either Beclin-1- or Atg4B-overexpressed livers (Figure 6F). Therefore, *in vivo* results not only confirm our *in vitro* findings, but further support that depletion of Atg4B is directly associated with mitochondrial dysfunction in I/R injury to old liver.

Discussion

Hepatic I/R injury profoundly impacts the burden of liver diseases. As life expectancy continues to rise and since the aged liver does not tolerate pathological stresses well, we are facing a drastic increase in the number of elderly patients with liver diseases that require surgical resection and transplantation surgery. Here we demonstrate that defective autophagy as a consequence of Atg4B loss, is a causal mechanism for the age-dependent hepatic reperfusion injury and that enhancement of autophagy has a therapeutic potential for ameliorating the age-mediated liver I/R injury.

Aging is closely linked to many pathological conditions and functional decline in heart, brain and muscle²⁶. With immunoblotting, genetic and imaging approaches, we uncover here that Atg4B loss is a key event culminating in reduced autophagic response in reperfused aged hepatocytes. Consequently, aged cells exhibit defective autophagosome formation and inefficient or insufficient autophagic flux (Figure 1C-E), impaired mitophagy (Figure 4E) and mitochondrial failure (Figure 1B). Reversal of cell death and MPT onset by specific overexpression of Atg4B firmly supports a causative role of Atg4B depletion in the age-dependent I/R injury (Figure 2). The physiological relevance of such a relationship was confirmed and extended in I/R *in vivo*, as evidenced by restoration of $\Delta\Psi_m$ and Atg4B by Atg4B and Beclin-1 overexpression (Figure 6C-F). We also show that Beclin-1 exerts cytoprotection against the age-dependent I/R injury, as evident by the following observations. First, specific overexpression of Beclin-1 blocked cell death (Figure 4C and F). Second, Beclin-1 markedly suppressed reperfusion-induced Atg4B loss (Figure 4B).

Third, Beclin-1 significantly elevated autophagic flux (Figure 4D). Fourth, confocal microscopy visualized MPT inhibition (supplementary Figure 4A and C) and reversal of defective mitophagy by Beclin-1 (Figure 4E). Finally, intravital multiphoton imaging of anesthetized mice after I/R *in vivo* revealed that enhancing hepatic Beclin-1 in old animals substantially suppressed reperfusion-mediated Atg4B depletion, defective autophagy, mitochondrial depolarization and failure (Figure 6C and E-F). Furthermore, we have identified a novel Atg3/Beclin-1 interaction (Figure 5). A critical role of Atg3 in Beclin-1-mediated protection was verified in that 1) Atg3-null cells were highly prone to the MPT and cell death after I/R and 2) cytoprotection by Beclin-1 overexpression was manifested only in Atg3-WT cells, but not in Atg3-deficient cells. Cumulatively, these findings strongly suggest that I/R in aged liver causes Atg4B loss, which in turn impairs autophagy and accumulates dysfunctional mitochondria, leading to the MPT and ultimately cell death (Figure 7).

After I/R, aged cells exhibited a marked reduction of autophagic flux, which was reversed by Atg4B overexpression. In agreement with this finding, Fujita *et al.* have shown a critical role of Atg4B in LC3 lipidation and autophagic degradation.²⁷ Recently, in Atg4B knockout mice, Atg4B depletion in liver severely reduces LC3-II conversion and autophagic flux.²⁸ Thus, Atg4B not only activates and recycles LC3 but regulates formation of both autophagosome and autolysosomes, consistent with our results.

Manifestations of cellular aging have been associated with concomitant increase in calpain activity.²⁹ Unregulated calpains can result in the unwanted cleavage of key functional proteins. Notably, calpains preferentially hydrolyze some autophagy proteins.^{6,17} Calpain 2 activity elevated after reperfusion and ALLM suppressed Atg4B depletion, MPT onset and cell death in old hepatocytes after I/R. Hence, the calpain-mediated depletion of Atg4B may be a critical contributing mechanism to age-related autophagy defects and increased sensitivity to I/R injury. It is nevertheless possible that events other than calpain activation may attribute to Atg4B reduction. For instance, aged tissues have higher basal levels of reactive oxygen species (ROS) than young tissues.²⁶ As I/R further increases reactive oxygen species²¹ and Atg4 is highly vulnerable to oxidative stress³⁰, oxidative stress in reperused aged cells could irreversibly damage Atg4B. Future studies are warranted for a complete understanding of the mechanisms underlying Atg4B loss.

The baseline levels of Beclin-1 were indistinguishable between age groups. Interestingly, hepatocytes at both ages exhibited a notable decrease in Beclin-1 during ischemia, but recovered partially after reperfusion, which was also observed in hepatocytes overexpressing Beclin-1, suggesting the intrinsic sensitivity of Beclin-1 to ischemia. Reduction of Beclin-1 by ischemia has been shown in liver.⁶ Beclin-1 overexpression both in hepatocytes and in livers not only increased Atg4B expression but suppressed reperfusion-induced Atg4B loss. Recently, caspase-3 has been shown to cleave Atg3, Beclin-1 and Atg4.³¹ However, administration of caspase inhibitor to old cells did not affect Atg4B (Supplementary Figure 4D), suggesting a minor role of caspase-3 in Atg4B loss.

Our results provide the first evidence to our knowledge that Atg3 is a direct target of Beclin-1 and that its physical interaction with Atg3 promotes autophagic responsiveness to I/R. The importance of Atg3 in mitochondrial homeostasis and viability has been reported in mammalian cells.²³ Since Atg3 is part of the conjugate system downstream to Beclin-1, it remains to be determined whether Beclin-1-dependent cytoprotection is directly caused by its interaction with Atg3. Our findings of increased sensitivity of Atg3-null cells to the MPT and cell death, and lack of Beclin-1-mediated cytoprotection suggest that Beclin-1/Atg3 interaction, at least in part, contributes to the cytoprotective effects in old hepatocytes.

Additionally, we provided evidence that the cytoprotection by Beclin-1 was independent of Bcl-2/Bcl-X_L interactions.

Beclin-1 enhances mitophagy and blocks onset of the MPT, in agreement with our previous study.⁶ We repeatedly observed that widespread MPT was preceded by the initial MPT onset to several mitochondria, suggesting that some mitochondria are more susceptible to MPT induction. Signals triggering $\Delta\Psi_m$ loss initiate in discrete mitochondrial pool and then propagate to neighboring mitochondria.³² Hence, it would be plausible that interventions blocking the initial MPT induction may attenuate subsequent signal propagation to adjacent mitochondria and ultimately widespread MPT onset. With Beclin-1 overexpression, many repolarized mitochondria coexisted with mitophagic autophagosomes and, importantly, these mitochondria remained polarized during reperfusion. Thus, we speculate that the mitochondria entrapped in autophagosome may represent the discrete population that could initiate the MPT. Timely clearance of injury-prone mitochondria by autophagy would be an indispensable step for survival of neighboring mitochondria and cell.

Whether hepatic macroautophagy declines with age is the subject of on-going debate. While early studies using a pulse-chase proteolysis assay proposed a decreased autophagy in aged liver^{33,34}, Wohlgemuth *et al.* recently demonstrated that the expression of Atg and lysosomal-associated membrane protein (LAMP) and the occurrence of autophagic vacuoles in rat livers are not influenced by normal aging.³⁵ Despite reduced blood flow and volume, changes in hepatic parenchymal structure and function are minimal with advancing age.³⁶ In agreement with this view, our results show that the baseline levels of Atg and the basal autophagic flux are indiscernible between ages. Consistently, hepatocytes from both ages displayed a robust autophagic flux under the non-ischemic condition (Figure 1F). Additionally, the number of polarized mitochondria in old cells is comparable to that in young cells (supplementary Figure 1D). Since mitochondrial turnover is mediated by autophagy, these results further suggest equivalent levels of basal autophagy between age groups. Notably, we repeatedly observed that the baseline levels of LAMP-2 (data not shown) and cathepsin D are, indeed, higher in aged hepatocytes compared to young cells, suggesting a protective or adaptive strategy of autophagy in aged cells. A constitutive activation of autophagy with aging is reported in other tissues.³⁷ A striking reduction in autophagy occurred only in the old cells subjected to a severe stress such as I/R. Besides macroautophagy, aging alters lysosomal membrane stability and decreases chaperone-mediated autophagy (CMA) in liver, an event mainly accountable for cytosolic protein clearance³⁸. How I/R affects CMA is unknown. A compensatory cross-talk between macroautophagy and CMA has been recently reported in fibroblasts.³⁹

In conclusion, we show both *in vitro* and *in vivo* that the loss of Atg4B attributes to the increased sensitivity of old liver to lethal I/R injury. Enhancing autophagy could be a novel strategy to improve liver function of the elderly patients after liver resection and transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Dr. Beth Levine, Dr. Xiao-Ming Yin and Dr. Masaaki Komatsu for providing us with Beclin-1 cDNA, AdGFP-LC3 and Atg3 KO MEF cell lines, respectively.

Grant support. This work was supported in part by US National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases grant DK079879 (J-S Kim) and National Institute on Aging AG 17994 (C.

Leeuwenburgh), AG 21042 (C. Leeuwenburgh) and the University of Florida Institute on Aging, Claude D. Pepper Older Americans Independence Center (1 P30 AG028740).

List of Abbreviations

I/R	ischemia/reperfusion
Atg	autophagy-related protein
MPT	mitochondrial permeability transition
ROS	reactive oxygen species
KRH	Krebs-Ringer-hydroxyethylpiperazine-N-2 ethanesulfonic acid
HDM	hormonally-defined medium
WT	wild type
KO	knockout
MEF	mouse embryonic fibroblasts
DMEM	Dulbecco's modified Eagle's medium
AdBeclin-1	adenovirus encoding Beclin-1
AdAtg4B	adenovirus encoding Atg4B
AdGFP-LC3	adenovirus encoding green fluorescence protein-labeled microtubule-associated protein 1 light chain 3
AdmCherry-GFP-LC3	adenovirus encoding monomeric Cherry and green fluorescence protein-labeled microtubule-associated protein 1 light chain 3
MOI	multiplicity of infection
AdLacZ	adenovirus encoding β -galactosidase
TMRM	tetramethylrhodamine methylester
PI	propidium iodide
$\Delta\Psi_m$	mitochondrial membrane potential
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
PE	phosphatidylethanolamine
CMA	chaperone-mediated autophagy

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Figure 1.

Age- and MPT-dependent cell death after I/R. (A) Hepatocytes from 3(Young)- and 26-month (Old)-old mice were subjected to simulated I/R. Necrosis at 5, 60 and 120 minutes of reperfusion was measured by PI fluorometry. (B) After 2 hours of ischemia, young (top panel) and old hepatocytes (bottom panel) were reperfused and confocal images of calcein, TMRM and PI (arrows) were simultaneously collected. Scale bar: 10 μ m. The number of polarized mitochondria per cell was counted at 5 and 20 minutes of reperfusion. To determine autophagic flux, lysates from young (Y) and old (O) hepatocytes were collected at 0 minute of ischemia and at 20 and 60 minutes after reperfusion. LC3 was immunoblotted with 50 nM bafilomycin (C) or 10 μ M chloroquine (D). (E) Hepatocytes infected with AdmCherry-GFP-LC3 were subjected to I/R. Confocal images of yellow (autophagosomes) and red (autolysosomes) puncta were collected after reperfusion with and without bafilomycin (Baf). Arrows indicate PI-labeled nuclei (necrosis). (F) After 2 hours of starvation in amino acid- and serum-free KRH, LC3 was immunoblotted with chloroquine (CQ). LC3-II expression was assessed relative to the level in control young hepatocytes and expressed as percent change. * $P < 0.05$.

Figure 2.

Atg4B loss in reperused old hepatocytes. (A) Immunoblots at 0 and 120 minutes of ischemia (I) and at 20 and 60 minutes after reperfusion (R). The expression of Atg4B relative to the level at 0 hour of ischemia in young hepatocytes was determined. ** $P < 0.01$. (B) Old hepatocytes were incubated with different titers of AdAtg4B and overexpression was determined by immunoblotting. Necrosis with AdAtg4B (10 MOI) or AdGFP (viral control) was evaluated by PI fluorometry. (C) Confocal analysis of the MPT. (D) Immunoblots with Atg4B overexpression. (E) Confocal analysis in mCherry-GFP-LC3-labeled old hepatocytes. The number of puncta was counted after 20 minutes of reperfusion. * $P < 0.05$.

Figure 3.

Atg4B loss by calpain 2. Expression of calpain 1(A) and calpain 2(B) was determined by immunoblotting. (C) Old hepatocytes were subjected to I/R with 50 μ M ALLM and protein expressions were compared to control. (D) Necrosis in reperfused old cells was assessed with or without ALLM. Autophagic flux in old cells was determined by mCherry-GFP-LC3 analysis (E and F). * $P < 0.05$.

Figure 4.

Cytoprotection by Beclin-1. (A) Immunoblots of Beclin-1 after I/R. Beclin-1 expression was expressed relative to the level at 0 hour of ischemia in young hepatocytes. (B) Atg expression in Beclin-1-overexpressed old hepatocytes. (C) Suppression of necrosis by Beclin-1. (D) Changes in autophagic flux by Beclin-1 overexpression were determined by immunoblotting (top panel) and mCherry-GFP-LC3 analysis (bottom panel). * $P < 0.05$. (E) Old hepatocytes were infected with either AdGFP-LC3/AdLacZ or AdGFP-LC3/AdBeclin-1. Confocal images of GFP-LC3 and TMRM were collected before and after reperfusion. Magnified images represent AdBeclin-1-treated cells after 60 minutes of reperfusion (bottom panel). Scale bar: 3 μ m. (F) Hepatocytes at different ages were subjected to either 2 hours of ischemia/12 hours of reperfusion (I/R) or normoxia (Nor). Caspase-3 activation was evaluated by immunoblotting.

Figure 5.

Interaction of Beclin-1 with Atg3. (A) Cell lysates at different ages were first immunoprecipitated (IP) with Beclin-1 and subsequently immunoblotted (IB). Bcl-xL and normal rabbit IgG were used for a positive and negative control, respectively. Some old hepatocytes were treated with AdBeclin-1(AdB). (B) Cell lysates from Atg3 WT and KO MEF were immunoblotted with viral control (Con) or AdBeclin-1. Atg3 was immunoblotted after Beclin-1 immunoprecipitation (right panel). (C) Atg3 WT and KO cells were subjected to either 6 hours of normoxia (left panel) or 2 hours of ischemia/4 hours of reperfusion (right panel). Note a lack of the MPT and cell death after normoxia. While WT cells tolerated I/R, mitochondrial dysfunction and cell death (arrows) were evident in KO cells with or without Beclin-1 overexpression. (D) Apoptosis was morphologically evaluated after 4 hours of reperfusion in Atg3 cells (left panel). Cell death was assessed after reperfusion (right panel).

Figure 6.

Cathepsin D expression. (A) Immunoblotting of cathepsin D. (B) Immunoblotting in AdBeclin-1-treated old hepatocytes. Cytoprotection by Atg4B or Beclin-1 in *in vivo* I/R. After *in vivo* I/R, tissue lysates were collected at given times. Immunoblotting analysis was performed with Beclin-1 (C) and Atg4B (D) overexpression. (E) Old mice were injected with either AdGFP-LC3/AdLacZ or AdGFP-LC3/AdBeclin-1 and intravital multiphoton images of autophagosomes after *in vivo* I/R were collected at 0.7- μ m intervals. (F) Livers were labeled with rhodamine 123 after *in vivo* I/R and multiphoton images of the mitochondria were collected. Punctate bright green fluorescence of rhodamine 123 represents polarized mitochondria. The number of cells containing polarized mitochondria, indicative of viable cells, was counted after 40 minutes of reperfusion. *P < 0.05.

Figure 7. Scheme of MPT induction and cell death after I/R

Reperfusion of ischemic old hepatocytes increases calpain 2 activity, which in turn hydrolyzes Atg4B. Atg4B loss subsequently impairs autophagy and promotes onset of the MPT and cell death. This chain reaction is prevented by calpain inhibitor (ALLM) and overexpression of Atg4B. Beclin-1 overexpression increases autophagy and blocks MPT onset by its interaction with Atg3.