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Authors
Zhou, Haomming
Zhu, Jianjun
Yue, Shi
et al.

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The Dichotomy of Endoplasmic Reticulum Stress Response in Liver Ischemia Reperfusion Injury

Haoming Zhou[1,2], **Jianjun Zhu**[2,3], **Shi Yue**[2], **Ling Lu**[1], **Ronald W. Busuttil**[2], **Jerzy W. Kupiec-Weglinski**[2], **Xuehao Wang**[1,*], and **Yuan Zhai**[2,*]

[1]Liver Transplantation Center, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu Province, China

[2]Dumont-UCLA Transplant Center, Division of Liver and Pancreas Transplantation, Department of Surgery, David Geffen School of Medicine at University of California-Los Angeles, Los Angeles, CA

[3]Department of Liver Surgery, Renji Hospital, Shanghai Jiaotong University, Shanghai, China

Abstract

Endoplasmic reticulum (ER) stress plays critical roles in the pathogenesis of liver ischemia and reperfusion injury (IRI). As ER stress triggers an adaptive cellular response, the question of what determines its functional outcome in liver IRI remains to be defined. In a murine liver partial warm ischemia model, we studied how transient (30m) or prolonged (90m) liver ischemia regulated local ER stress response and autophagy activities and their relationship with liver IRI. Effects of chemical chaperon 4-phenylbutyrate (4-PBA) or autophagy inhibitor 3-methyladenine (3-MA) was evaluated. Our results showed that while the ATF6 branch of ER stress response was induced in livers by both types of ischemia, liver autophagy was activated by transient, but inhibited by prolonged, ischemia. Although 3-MA had no effects on liver IRI after prolonged ischemia, it significantly increased liver IRI after transient ischemia. The 4-PBA treatment protected livers from IRI after prolonged ischemia by restoring autophagy flux, and the adjunctive 3-MA treatment abrogated its liver protective effect. The same 4-PBA treatment, however, increased liver IRI and disrupted autophagy flux after transient ischemia. Although both types of ischemia activated 5′ adenosine monophosphate-activated protein kinase (AMPK) and inactivated protein kinase B (Akt), prolonged ischemia also resulted in downregulations of autophagy-related gene (Atg) 3 and Atg5 in ischemic livers. These results indicate a functional dichotomy of ER stress response in liver IRI via its regulation of autophagy. Transient ischemia activates autophagy to protect livers from IRI, while prolonged ischemia inhibits autophagy to promote the development of liver IRI.

*Address for correspondence: Yuan Zhai, MD, PhD. Dumont-UCLA Transplant Center 77-120 CHS, 10833 Le Conte Ave, Los Angeles, CA 90095. Phone: (310) 825-9426; Fax: (310) 267-2367; yzhai@mednet.ucla.edu. Xuehao Wang, MD, Department of Liver Surgery, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing, P.R. China 210029 Phone: 86-25-68136053; Fax:86-25-84630769; wangxh@njmu.edu.cn.

Authorship: HMZ, JZ, and SY designed experiments and performed in vitro and in vivo experiments; LL, RWB and JWK revised manuscript; XHW, YZ designed, supervise the study and wrote manuscript.

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Introduction

Metabolic disturbance due to hypoxia and nutrient deficiency is the primary consequence of the ischemia process. Redox alteration and ATP deficiency cause dysfunctions of key intracellular organelles and trigger stress responses, such as endoplasmic reticulum (ER) stress response. Cells develop adaptive responses to resolve organelles dysfunctions and survive under stressful conditions. Unfolded protein response (UPR) is activated upon ER stress and three ER transmembrane receptors: PREK, ATF6 and IRE1, transduce signaling cascade to inhibit new protein synthesis and activate transcriptions of selective sets of genes encoding proteins involved in protein folding and protein degradation in ER [1–3]. If UPR fails to resolve ER stress or the stress is sustained, pathological outcomes will follow, including cell death and inflammation. The critical question in ER stress-mediated disease pathophysiology is what mechanism transforms an adaptive stress response to a deleterious one. Liver is the major organ of protein synthesis and rich in ER content. ER stress response has been shown to play key roles in various pathological mechanisms of liver diseases [4], including ischemia and reperfusion injury (IRI) [5–7]. Our previous studies have shown in a murine liver partial warm ischemia model that ischemia-induced liver ER stress triggers mainly the ATF6 signaling pathway and it functions in synergy with TLRs to promote pro-inflammatory immune activation and hepatocyte death [6,8]. The question of how the adaptive stress response is transformed into a pathological process during liver IR, however, has not been defined.

It has become clear that cells utilize multiple mechanisms to cope with ER stress [9,10]. Autophagy is an evolutionarily conserved and lysosome-dependent system for degradation and recycling of proteins, organelles and other cellular components [11,12]. It is significantly elevated in response to nutrient deprivation, hypoxia, heat shock, microbial infection, as well as ER stress. Autophagy is able to efficiently remove damaged organelles and protein aggregates, and may serve as a safeguard when UPR fails. We therefore propose that ER stress and autophagy may function coordinately to determine the outcome of stress response in liver IRI. Although autophagy has been implicated in the pathogenesis of IRI, its alterations by IR and subsequent functional significance are highly divergent and controversial [13–15]. Autophagy is constitutively active in most cells for optimal maintenance of homeostasis, IR can either increase or decrease autophagy activities depending on affected organs and ischemia time. Autophagy can either protect cells from necrotic/apoptotic death or promote cell death via autophagic cell death pathway. Both detrimental and beneficial effects of autophagy inhibition have been reported on the development of IRI.

While how autophagy plays dual roles in IRI remain to be clarified, one methodology issue that might have affected conclusions of autophagy studies in vivo has been raised in recent literatures [16,17]. As autophagy is a dynamic process involving multiple steps, static measurement of LC3B-II by either Western blot or GFP-LC3B puncta will not accurately measure autophagy activities. While both autophagy induction and autophagolysosome inhibition can result in increases of LC3B-II, autophagy inhibition or increases in autophagolysosomal activities can lower its levels. Autophagy flux, which is determined by the difference of autophagosome numbers/levels in control vs. lysosome inhibited conditions, is a more reliable indicator of autophagic activities [18,19]. We have measured

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autophagy flux in our liver IRI model. Our results indicate that prolonged ischemia inhibits autophagy flux, which is recovered later during reperfusion. Rapamycin protects livers from IRI by augmenting liver autophagy flux during reperfusion [20].

In this study, we investigated potential dichotomy and underlying mechanisms of stress responses in liver IRI by determining how ER stress and autophagy interacted in response against transient or prolonged ischemia. We tested the hypothesis that ER stress regulated autophagy to affect the development of liver IRI.

Materials and Methods

Mice

Male wide-type (WT) C57BL/6 mice (6–8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in the University of California-Los Angeles animal facility under specific pathogen-free conditions, and received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health (NIH publication 86–23 revised 1985).

Model of warm liver IRI

A model of partial hepatic warm IRI was used as described previously[21]. In brief, after a midline laparotomy, mice were injected with heparin (20U/mouse) and an atraumatic clip was used to interrupt arterial/portal venous blood supply to the cephalad-liver lobes. After 90 min or 30min of ischemia, the clip was removed to initiate liver reperfusion. Mice were sacrificed after 0h, or 1h, or 6h post liver reperfusion. Sham controls underwent the same procedure, but without vascular occlusion. 4-phenylbutyrate (4-PBA, 100 mg/kg, at −2h; Sigma, St. Luis, MO), or 3-Methyladenine (3-MA, 30mg/kg, at −1h; Sigma, St. Luis, MO) were administered intraperitoneally, alone or in combination, prior to the onset of liver ischemia. Serum alanine aminotransferase (sALT) levels were measured by IDEXX Laboratories. Part of the liver specimens were fixed in 10% buffered formalin and embedded in paraffin. Liver sections (4μm) were stained with H&E. The severity of liver IRI was graded blindly using Suzuki's criteria on a scale from 0 to 4. No necrosis, congestion/centrilobular ballooning is given a score of 0, whereas severe congestion and >60% lobular necrosis is given a score of 4 [22].

Western blots

Liver tissue proteins were extracted with ice-cold lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 137mM sodium chloride, 20mM Tris, pH 7.4). Proteins (30 μg) were subjected to 12% SDS-PAGE electrophoresis and transferred to PVDF nitrocellulose membrane. Antibodies against LC3B, β-actin, Phospho-Akt (Thr308), Phospho-Akt (Ser473), Phospho-AMPKα (Thr172), ATG3, ATG5 (Cell Signaling Technology, Danvers, MA), ATF6 (Novus, Littleton, CO), ATF4 (Proteintech Group, Chicago, IL), sXBP1 (Abcam, Cambridge, MA) were used. Membranes were probed with primary antibody (1: 1000) in 10 ml blocking buffer overnight at 4°C. After washing, membranes were further probed with appropriate HRP-conjugated secondary antibody

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(1:1000) in 10 ml of blocking buffer for 1.5 h at room temperature. Clarity™ Western ECL Substrate (Bio-rad, Hercules, CA) were used for chemo-luminescence development. ImageJ 1.47v software was used to quantitate western blot bands.

Liver autophagy flux measurement in vivo

Chloroquine (CQ), ammonium chloride, raises the intralysosomal pH to prevent autophagosome fusion with the lysosome, thus inhibiting autophagy degradation of LC3B II. Groups of mice received either vehicle or CQ (60mg/kg, i.p. at −1h; Tocris Bioscience, Cambridge, UK) were subjected to liver IR with or without 4-PBA pretreatment, as described above. Mice were euthanized at 1h post reperfusion and IR livers were harvested for Western blot analysis of LC3B and β-actin. Liver autophagy flux was measured as the increases of LC3BII/β-actin ratios in the presence and absence of CQ.

Statistical analysis

Results are shown as mean ± SD. Unpaired Student t-test were used for pairwise statistical analyses, with $p<0.05$ (two tailed) considered statistically significant.

Results

1. Liver stress responses against IR

We first tested in vivo whether transient (30m) or prolonged (90m) ischemia triggered different stress responses in livers. To measure ER stress responses, activations of UPR signaling pathways were determined by Western blot analysis of cleaved ATF6, ATF4 and spliced XBP1. Consistent with our previous reports [6,8], upregulation of cleaved ATF6 was evident by both transient and prolonged ischemia, whereas alterations in ATF4 and XBP1 were not as significant at this time point (0h post reperfusion) (Fig. 1A, B). To measure autophagy activities after ischemia, we determined autophagy flux. Separate groups of mice were treated with either PBS or chloroquine (CQ, lysosomal inhibitor) prior to the onset of liver ischemia, liver tissues were harvested at 0h or 1h post reperfusion. Western blot analysis of LC3B-II (Fig. 1C, D) showed that 30m ischemia did increase liver autophagy flux, as indicated by significantly higher ratio of LC3B-II/β-actin in the presence vs. absence of CQ, as compared to that in sham livers. In contrast, 90m ischemia inhibited liver autophagy flux at this early stage of reperfusion. These results indicated that ER stress response was similarly induced by both transient and prolonged ischemia. Autophagy, however, was enhanced by transient ischemia, but inhibited by prolonged ischemia.

2. The dichotomy of ER stress in liver IRI

To determine functions of ER stress response in the disease pathogenesis, we compared effects of small molecule chaperon 4-PBA (ER stress inhibitor) on the development of liver IRI induced by transient vs. prolonged ischemia. Consistent with our previous findings[6], pre-treatment with a single dose of 4-PBA protected livers against prolonged ischemia-induced IRI. However, the same treatment increased liver IRI after transient ischemia, as measured by sALT levels and liver histology at 6h post reperfusion (Fig. 2A, B, C). This result demonstrated a functional dichotomy of ER stress, protective vs. pathogenic, dictated by durations of ischemia, in the pathogenesis of liver IRI.

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As autophagy was differentially regulated by transient vs. prolonged ischemia, we next determined whether ER stress was involved in IR regulation of liver autophagy by analyzing the impact of 4-PBA on autophagy flux after different ischemia times. Administration of 4-PBA prior to the onset of liver ischemia indeed inhibited ATF6 upregulation induced by both transient and prolonged ischemia (Fig. 3A, B). Autophagy flux was restored by 4-PBA in prolonged ischemic livers (Fig. 3C, D). However, the same treatment disrupted liver autophagy flux after transient ischemia, despite that the induction of autophagosome (LC3B-II levels w/o CQ) remained higher, as compared with those in sham (Fig. 3E, F). These results indicated that ER stress played dual roles in regulating autophagy: transient ischemia-induced ER stress augmented, while prolonged ischemia-induced ER stress inhibited, liver autophagy.

3. Autophagy as the key determinant of ER stress regulation of liver IRI

The dichotomy of ER stress in regulating both liver IRI and autophagy suggested that autophagy played the key role in determining the outcome of ER stress on liver IRI. As transient ischemia-induced ER stress augmented liver autophagy, we tested whether autophagy played a protective role in this type of liver IRI. 3-MA was administered prior to the onset of liver ischemia and liver IRI was evaluated at 6h post reperfusion. Indeed, liver injuries were significantly increased by the 3-MA treatment after transient ischemia, as evidenced by elevated sALT levels and worse preserved liver architectures (H/E staining and Suzuki scores) (Fig. 4A, B, C). Consistent with our observation that liver autophagy had already been inhibited by prolonged ischemia, the same 3-MA treatment neither increased nor decreased liver IRI after prolonged ischemia. Western blot analysis confirmed the inhibitory effect of 3-MA on liver LC3B-II induction (autophagosome formation) by ischemia (Fig. 4D, E). These results confirmed functional significance of autophagy in protecting livers from transient ischemia-induced reperfusion injury.

As alleviation of ER stress by 4-PBA restored liver autophagy flux and protected livers from IRI after prolonged ischemia, we next determined whether autophagy was important for the liver protective effect of 4-PBA in vivo. Co-administration of 3-MA with 4-PBA resulted in a significant increase of liver IRI, as compared with those with 4-PBA alone, as measured by sALT levels and liver histological evaluation (Fig. 5A, B, C). Thus, autophagy was critical for the therapeutic effect of the ER stress-targeted therapy in prolonged ischemia-induced liver IRI.

4. Molecular mechanisms of IR regulation of liver autophagy

To address potential molecular mechanisms of IR regulation of autophagy, we measured activation of two intracellular signaling pathways critical for autophagy induction. Western blot analysis of ischemic liver tissues harvested at 0h post reperfusion showed that phosphorylation of Akt at Ser473 (by mTORC2) was significantly downregulated, while phosphorylation of AMPKα at Thr172 was significantly enhanced, by both 30m and 90m ischemia (Fig. 6A, B). As autophagy involves large numbers of catalytic and structure proteins to form core autophagy machinery, their levels were also measured in IR livers. Western blot results showed that prolonged, but not transient, ischemia resulted in significant downregulations of Atg3 and Atg5 (Beclin-1 was also lower but not statistically

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significant) at 1h post reperfusion, which were prevented by the 4-PBA treatment (Fig. 6C, D). These results suggested that ischemia, regardless of its duration, all triggered mTORC1 inactivation (via inhibition of Akt) and AMPK activation, leading to autophagy induction. However, due to the disruption of core autophagy machinery, autophagy flux was inhibited by prolonged ischemia. Transient ischemia did not disrupt autophagy machinery, thus resulting in enhanced autophagy activities.

Discussion

Our study documented a dichotomy of stress responses in liver IRI dictated by ischemia time. Transient ischemia triggered a protective ER stress response by upregulating autophagy, while prolonged ischemia induced a pathogenic ER stress response with impaired liver autophagy flux due to disrupted autophagy machinery. Thus, amelioration of ER stress may not be always beneficial therapeutically, and cautions should be taken in choosing strategies to target ER stress. Autophagy activities may be measured as an indicator of protective vs. pathogenic nature of ER stress response induced by ischemia.

Although the dichotomy of ER stress in cell death has been established in vitro [1,2], whether ER stress exerts similar dual roles in vivo in the pathogenesis of IRI remains to be fully delineated. In brain and heart models, it has been shown that mild ER stress triggered by either ischemia preconditioning or chemicals or overexpression of UPR signaling molecules could protect organ/tissue from subsequent lethal ischemia insults [23–25]; and severe ischemia induces a pro-pathogenic ER stress, which is characterized by caspase 12 activation [26] and CHOP induction [27], and responsible for neuronal/myocardial cell death and development of IRI [28,29]. Our current study documented in vivo in a single IRI model that ER stress could have either protective or pathogenic roles in the pathogenesis of liver IRI dependent on ischemia times. Furthermore, we found that autophagy activity post ischemia was a key indicator of the nature of ER stress responses. In addition to ischemia, variety of host pre- and post-existing conditions can trigger UPR or regulate severity of ER stress. They include other metabolic stresses (including both acute and chronic types, such as oxidative stress, starvation, diabetes, obesity), genetic mutations involved in protein synthesis/quality control, viral infection, drug toxicity, and inflammatory cytokines, etc [1–4]. Thus, our ability to define the nature of ER stress is important for our study of the general etiology of liver IRI.

The relationship between ER stress response and autophagy has been studied in vitro and in vivo. ER stress could augment autophagy [10] at multiple stages. Cytosolic calcium release from ER activated Ca++/calmodulin-dependent kinase β, which subsequently activated AMPK [30]. ER stress could inactivate Akt, resulting in the decrease of mTORC1 activity [31,32]. CHOP negatively regulated Bcl-2 at the transcriptional level, which led to increased activities of Beclin-1 [33]. Additionally, death-associated protein kinase 1 was activated in ER stress response, which promoted autophagy induction by Beclin-1 phosphorylation [34]. The PERK-ATF4-CHOP pathway had been shown to transcriptionally upregulate ATG5, ATG12, and promoted LC3 I to II conversion [35,36]. In IRI models, AMPK was activated, accompanied by mTORC1 inactivation, in cardiomyocytes in response to ischemia and its inhibition reduces autophagy induction and increases cell death [37]. In brain, ischemia preconditioning upregulated tuberous sclerosis complex 1 gene

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(TSC1) expression, which protected neurons from cell death in subsequent ischemia by inducing productive autophagy [38]. In our model, the constitutive activity of mTORC1 was relatively low (measured by pS6K) and autophagy flux was constitutively active in sham livers [20]. Prolonged ischemia, although upregulated LC3B-II levels, actually inhibited autophagy flux early after reperfusion. Current study further showed that autophagy flux was enhanced only by transient ischemia, which did play a protective role in liver IRI.

Issues regarding to autophagy in IRI are complicated with controversies [13,39]. In heart models, there were studies shown that autophagy was induced in myocardium by IR [40] via AMPK or Beclin-1-dependent mechanisms, depending on stages of the disease process [37]. However, the opposite, i.e., diminished autophagy in ischemic heart tissues, had also been observed [41]. The resolution of the controversy was attempted in an *in vitro* cell culture study with simulated ischemia, which showed that mild ischemia enhanced autophagy in myoblasts and severe ischemia triggered rather apoptotic/necrotic cell death without increased autophagy [42]. Our results derived from in vivo IR setting confirmed this conclusion. The question remains to be determined was what mechanisms were responsible for the inhibition of autophagy by prolonged/severe ischemia. As no differences were found in AMPK and Akt signaling pathways in response to transient and prolonged ischemia in our liver IRI model, we measured autophagy machinery proteins in ischemic livers. Indeed, levels of Atg3 and atg5 were significantly lower in livers after prolonged ischemia, and 4-PBA treatment, which alleviated ER stress, prevented these Atg downregulation, restored autophagy flux, and protected livers from IRI. These results suggested that disruption of autophagy machinery might be responsible for the impaired autophagy flux in livers after prolonged ischemia. Consistent with our finding, a previous report has documented that autophagy was impaired in hepatocytes exposed to anoxia/reoxygenation and in livers post warm IR, which was due to degradation of Atg7 and Beclin-1 by a calpain 2-dependent mechanism [43]. Along the same line, hepatocytes in aged mice had decreased abilities of autophagy due to loss of Atg4B, which increased their susceptibilities to IR-induced cellular damages[44]. Importantly, restoration of these degraded autophagy proteins by either calpain inhibitors or viral vector-mediated overexpression protected hepatocytes or aged livers from IR damages.

In summary, our current study has established a functional relationship between ER stress and autophagy in the pathogenesis of liver IRI. The functional dichotomy of stress responses, dictated by the duration/severity of ischemia, is dependent on their interactions. Autophagy activity is a key determinant of the outcome of stress responses in the disease process.

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Abbreviations

4-PBA	4-Phenylbutyrate
3-MA	3-Methyladenine
AMPK	5′ Adenosine monophosphate-activated protein kinase
Akt	Protein kinase B
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATG	Autophagy-related gene
CQ	Chloroquine
ER	Endoplasmic reticulum
IRE1	Inositol requiring kinase 1
IRI	Ischemia reperfusion injury
LC3	Microtubule-associated protein 1A/1B-light chain 3
mTORC	Mammalian (mechanical) target of Rapamycin complex
PBS	Phosphate buffered saline
PREK	Protein kinase R (PKR)-like endoplasmic reticulum kinase
sALT	Serum Alanine aminotransferase
TLR	Toll-like-receptor
XBP-1	X-box binding protein 1

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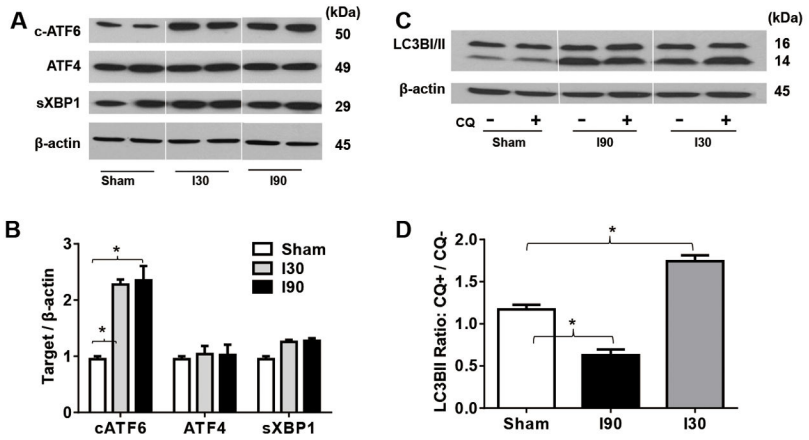

Figure 1.

Western blot analysis of liver autophagy flux and UPR response against IR. Liver tissues were harvested after either 30m or 90m ischemia and 0h or 1h of reperfusion. Activation of liver UPR response by IR was determined by Western blot analysis of cleaved ATF6, ATF4 and spliced XBP-1 at 0h post reperfusion (A). Average ratios of UPR signaling molecules/β-actin were plotted (B). To measure autophagy flux, separate groups of mice were received either vehicle or CQ treatment (60mg/kg, i.p. at −1h), as described in the Material and Methods. Represented LC3B II and β-actin Western blots were shown (C). Average ratios of LC3B II/β-actin in the presence and absence of CQ were calculated and plotted (D). Representative results of at least 2 different experiments. n=2–3/group. *p<0.05.

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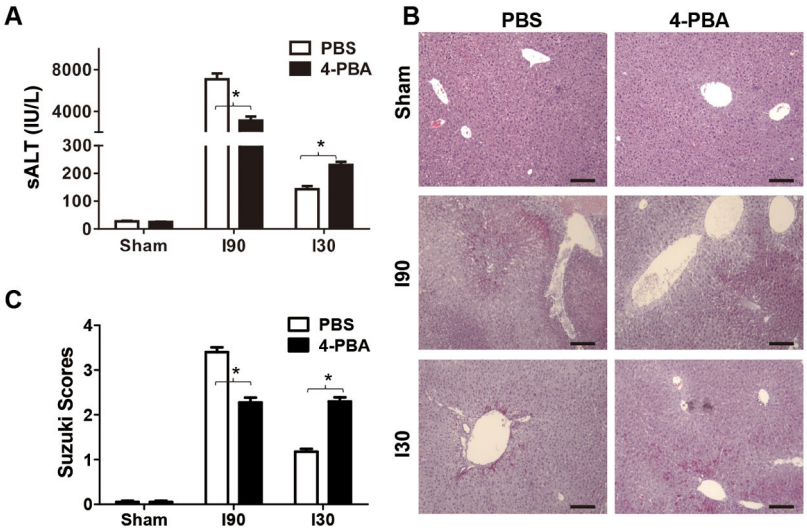

Figure 2.

Dichotomy of ER stress in liver IRI. 4-PBA (100 mg/kg, i.p. at −2h) was administered in different groups of mice prior to the onset of either transient (I30) or prolonged (I90) liver ischemia, as described in the Materials and Methods. Liver IRI was evaluated at 6h post reperfusion (R6h). Average sALT levels of different experimental groups were plotted (A. n=4mice/group), as well as representative liver histology (H/E, B, x100, Scale bars indicate 100μM) with Suzuki scores (C). Representative results of 2 different experiments. n=3–4/group. *p<0.05.

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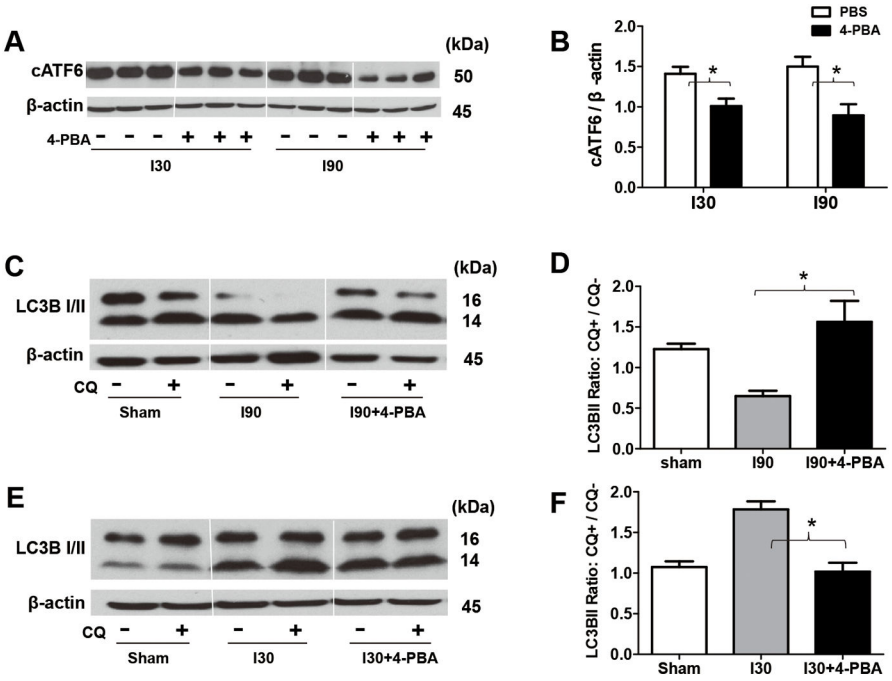

Figure 3.

ER stress regulation of autophagy in liver IRI. 4-PBA (100 mg/kg, i.p. at −2h) was administered with or without CQ (60mg/kg, i.p. at −1h) in different groups of mice prior to the onset of either transient (I30) or prolonged (I90) liver ischemia, as described in the Materials and Methods. Ischemic liver tissues were harvested at 1h post reperfusion. Western blots of ATF6 (A, B) and LC3B-II (C, D, E, F), as well as their calculated ratios vs. β-actin in different experimental groups were shown. Representative results of 2 different experiments. n=3–4/group. *p<0.05.

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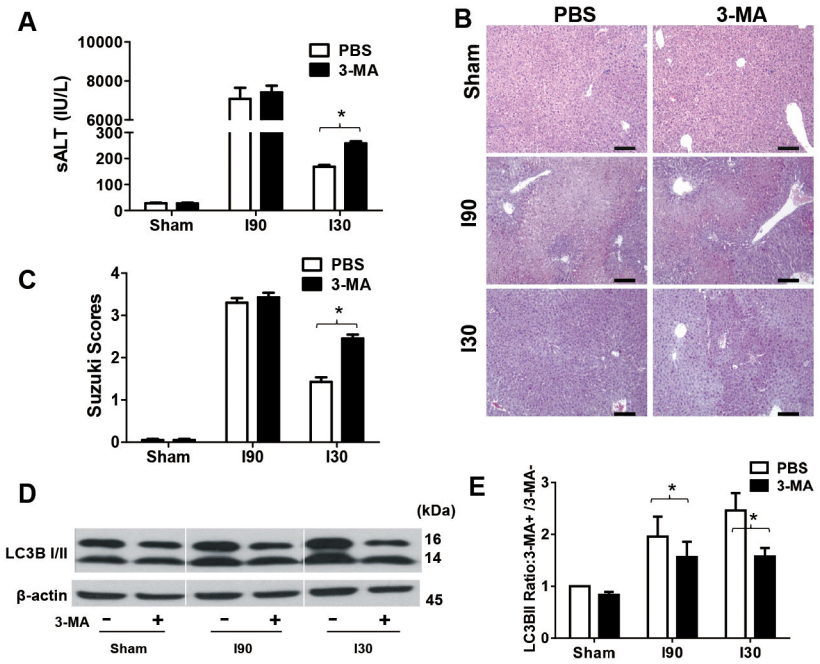

Figure 4.

Autophagy regulation of liver IRI. 3-MA (30mg/kg, i.p. at −1h) or PBS (vehicle control) was administered into mice prior to the onset of liver ischemia, as described in the Materials and Methods. Treated mice were subjected to 30m or 90m ischemia followed by 6h of reperfusion. Average sALT levels of different experimental groups were plotted (A), as well as representative liver histology (H/E, B, x100, Scale bars indicate 100μM) with Suzuki scores (C). Representative Western blot of liver LC3B-II was shown (D). Ratios of LC3B-II/β-actin were calculated and plotted (E). Representative results of at least 2 different experiments. n=3–4/group. *$p<0.05$.

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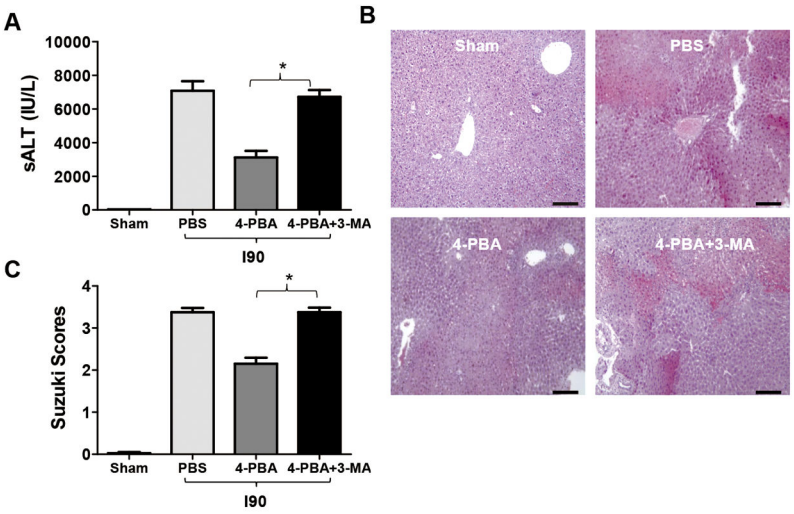

Figure 5.

Autophagy is required for 4-PBA protection of livers from IRI. 4-PBA (100 mg/kg, i.p. at −2h) was administered with or without 3-MA (30mg/kg, i.p. at −1h) in different groups of mice prior to the onset of prolonged liver ischemia. Liver IRI was evaluated at 6h post reperfusion. Average sALT levels of different experimental groups were plotted (A), as well as representative liver histology (B, H/E, x100, Scale bars indicate 100μM) with Suzuki scores (C). Representative results of 2 different experiments. n=3–4/group. *p<0.05.

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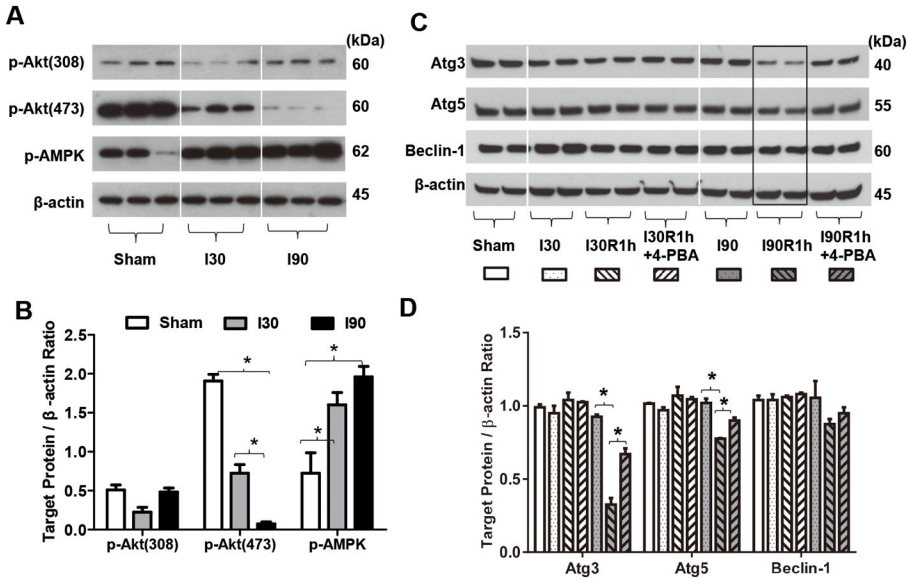

Figure 6.

Autophagy signaling pathways and machinery proteins in liver IRI. Liver tissues were harvested after either 30m or 90m ischemia and 0h or 1h of reperfusion. (A) Representative Western blots of phosphorylated Akt (at Thr308 and Ser473), phosphorylated AMPK (Thr172), and β-actin (at 0h post reperfusion) were shown. (B) Average ratios of p-Akt and p-AMPK vs. β-actin were plotted. (C) Representative Western blots of Atg3, Atg5, Beclin-1 and β-actin (at 0h and 1h post reperfusion) were shown. (D) Average ratios of Atg3, or Atg5, or Beclin-1 vs. β-actin were plotted. Representative results of 2 different experiments. n=3–4/group. *$p<0.05$.