Hepatic Autophagy Mediates Endoplasmic Reticulum Stress–Induced Degradation of Misfolded Apolipoprotein B

Wei Qiu,[1] Jing Zhang,[1] Mark J. Dekker,[1] Huajin Wang,[1] Ju Huang,[2] John H. Brumell,[2] and Khosrow Adeli[1]*

Induction of endoplasmic reticulum (ER) stress was previously shown to impair hepatic apolipoprotein B100 (apoB) production by enhancing cotranslational and posttranslational degradation of newly synthesized apoB. Here, we report the involvement of autophagy in ER stress–induced degradation of apoB and provide evidence for a significant role of autophagy in regulating apoB biogenesis in primary hepatocyte systems. Induction of ER stress following short-term glucosamine treatment of McA-RH7777 cells resulted in significantly increased colocalization of apoB with green fluorescent protein–microtubule-associated protein 1 light chain 3 (GFP-LC3), referred to as apoB-GFP-LC3 puncta, in a dose-dependent manner. Colocalization with this autophagic marker correlated positively with the reduction in newly synthesized apoB100. Treatment of McA-RH7777 cells with 4-phenyl butyric acid, a chemical ER stress inhibitor, prevented glucosamine- and tunicamycin-induced increases in GRP78 and phosphorylated eIF2α, rescued newly synthesized [^{35}S]-labeled apoB100, and substantially blocked the colocalization of apoB with GFP-LC3. Autophagic apoB degradation was also observed in primary rat and hamster hepatocytes at basal conditions as well as upon the induction of ER stress. In contrast, this pathway was inactive in HepG2 cells under ER stress conditions, unless proteasomal degradation was blocked with *N*-acetyl-leucinyl-leucinyl-norleucinal and the medium was supplemented with oleate. Transient transfection of McA-RH7777 cells with a wild-type protein kinase R–like ER kinase (PERK) complementary DNA resulted in dramatic induction of apoB autophagy. In contrast, transfection with a kinase inactive mutant PERK gave rise to reduced apoB autophagy, suggesting that apoB autophagy may occur via a PERK signaling–dependent mechanism. *Conclusion:* Taken together, these data suggest that induction of ER stress leads to markedly enhanced apoB autophagy in a PERK-dependent pathway, which can be blocked with the chemical chaperone 4-phenyl butyric acid. ApoB autophagy rather than proteasomal degradation may be a more pertinent physiological mechanism regulating hepatic lipoprotein production in primary hepatocytes. (HEPATOLOGY **2011; 53:1515-1525**)

Apolipoprotein B100 (apoB), the major protein component of low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL), is constitutively synthesized in the liver and regulated through cotranslational and posttranslational degradation.[1,2] Intracellular degradation of newly synthesized apoB has been shown to involve various mechanisms including endoplasmic reticulum (ER)-associated degradation (ERAD), ER60-associated degradation, LDL receptor–associated degradation, and autophagy. ERAD, an early-stage protein quality control system, is the most extensively studied apoB degradation pathway in cell

Abbreviations: apoB, apolipoprotein B; ALLN, N*-acetyl-leucinyl-leucinyl-norleucinal; ATF, activating transcription factor; eIFα, α-subunit of eukaryotic translational initiation factor 2; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GFP, green fluorescent protein; GLS, glucosamine; IRE1, inositol requirement 1; LC3, microtubule-associated protein 1 light chain 3; PAGE, polyacrylamide gel electrophoresis; PBA, 4-phenyl butyric acid; PBS, phosphate-buffered saline; PERK, protein kinase R–like endoplasmic reticulum kinase; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate; TM, tunicamycin; VLDL, very low density lipoprotein; WT, wild type; Xbp1, x-box binding protein 1.*

From the [1]Molecular Structure and Function; and [2]Cell Biology Programs, Research Institute, The Hospital for Sick Children, University of Toronto, Ontario, Canada.

Received September 7, 2010; accepted February 14, 2011.

This work was supported by an operating grant (T-6658) from the Heart and Stroke Foundation of Ontario to K.A.

culture models such as the HepG2 human hepatoma cell line.[3,4] In lipid-poor conditions or in the absence of microsomal triglyceride transfer protein activity, a large proportion of newly synthesized apoB is rapidly ubiquitinated and degraded by the proteasome.[5] ERAD has also been implicated in apoB degradation in primary hepatocytes, which were shown to ubiquitinate and degrade apoB via the proteasome, although at much lower rate compared to HepG2 cells.[6] Experimental evidence has also suggested that N-terminal cleavage of nascent apoB is another mechanism involved in the proteolysis of apoB within the ER lumen.

Using a permeabilized cell system, we reported the existence of a nonproteasomal degradative pathway that is responsible for specific fragmentation of apoB and generation of a 70-kDa fragment.[7] Permeabilized cells, largely devoid of the cytosolic proteasome components, continued to degrade apoB and generated specific fragments, including a 70-kDa fragment, via a lactacystin-insensitive process.[8] This observation was supported by Du et al. who demonstrated that an N-terminus of 85-kDa apoB fragment was generated in microsomes following transient overexpression of human apoB53 in CHO (Chinese hamster ovary) cells.[9] Studies with LDL receptor–deficient hepatocytes ($Ldlr^{-/-}$) have revealed that LDL receptor plays a critical role in the degradation of newly synthesized apoB.[10] Twisk et al.[10] reported that LDL receptor–deficient hepatocytes ($Ldlr^{-/-}$) secreted more apoB compared to wild-type (WT) hepatocytes, due to reduced degradation of newly synthesized apoB in $Ldlr^{-/-}$ hepatocytes. Recently, more evidence has been obtained showing that apoB turnover is associated with the levels of the LDL receptor. Growing evidence also suggests that autophagy, a late-stage protein quality control system, can mediate apoB degradation.[11-13] Autophagy is a degradation process for cellular components in which double-membrane autophagosomes sequester organelles or portions of cytosol and fuse with lysosomes or vacuoles to facilitate breakdown by resident hydrolases.[14] Ohsaki et al. first observed colocalization of proteasomes, autophagosomes, and apoB in a structure containing lipid droplets, suggesting the involvement of an autophagic mechanism in apoB degradation.[11] Soon after, Pan et al. showed that autophagic degradation of apoB occurred via post-ER presecretory proteolysis, induced by reactive oxygen species generated within hepatocytes from dietary polyunsaturated fatty acids.[12] More recently, Yao and colleagues demonstrated autophagic degradation of an apoB mutant (Ala31Pro substitution), which led to decreased secretion of endogenous apoB and triglycerides.[13] Thus ample evidence now exists for apoB autophagy, although the molecular mechanisms involved in targeting apoB to intracellular autophagy are currently unknown.

Available evidence indicates that ER stress induced by misfolded proteins may trigger insulin resistance, dyslipidemia, and diabetes.[15] We previously reported that induction of ER stress (with glucosamine treatment) leads to misfolding of newly synthesized apoB in the ER and the elimination of apoB via proteasomal and nonproteasomal mechanisms.[16] ApoB stability showed a strong inverse correlation with the expression of glucose-regulated protein 78 (GRP78), a key marker of ER stress.[16] GRP78 overexpression induced rapid degradation of newly synthesized apoB.[16] In line with these observations, Ginsberg and colleagues showed that treatment of McA-RH7777 cells with oleate at a high concentration (1.2 mM) or for a long period of time (16 hours) induced ER stress and upregulated GRP78.[17] Interestingly, GRP78 has been implicated in not only ERAD induction but also stress-induced autophagy.[18]

In this report, we present evidence implicating autophagy in ER stress–induced degradation of misfolded apoB. Under ER stress, apoB autophagy appears to be protein kinase R–like ER kinase (PERK)-dependent and is more pronounced in primary hepatocytes compared to established cell lines. Our data suggest that autophagy may be a physiologically important mechanism for the degradation of misfolded apoB under ER stress conditions.

Materials and Methods

Cell Culture and Transient Transfection. McA-RH7777 and HepG2 cells were purchased from ATCC (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc.) containing 20% or 10% fetal

Address reprint requests to: Khosrow Adeli, Division of Clinical Biochemistry, DPLM, The Hospital for Sick Children, University of Toronto, Toronto, Ontario M5G 1X8, Canada. E-mail: k.adeli@utoronto.ca; fax: 416-813-6257.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.24269

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

bovine serum, respectively. Isolation of primary hepatocytes from rat or hamster was described previously.[19] The cells (5×10^5) were seeded in six-well plates 4 hours before the experiments, and 1 μg GFP-LC3 (green fluorescent protein–microtubule-associated protein 1 light chain 3) complementary DNA (cDNA)[20] alone, or in addition to 1 μg WT PERK cDNA or kinase inactive mutant PERK (MPERK) cDNA,[21] were cotransfected into the cells, using Lipofectamine 2000 (Life Technologies, Grand Island, NY) according to the manufacturer's protocol.

Immunoblot Analysis. Following treatment with 5 mM glucosamine (GLS), or 5 μg/mL tunicamycin (TM), cultured cells were washed twice with phosphate-buffered saline (PBS) and lysed using solubilizing buffer (PBS containing 1% Nonidet P-40, 1% deoxycholate, 5 mM ethylene diamine tetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 100 kallikrein-inactivating units/mL aprotinin, and phosphatase inhibitors as described).[22] The membranes were blocked with a solution of 1% bovine serum albumin, incubated with the indicated antibodies (see figure legends), and then incubated with appropriate secondary antibodies conjugated to horseradish peroxidase. Monoclonal anti-KDEL antibody was from CalBiochem (San Diego, CA). Anti-phosphorylated α-subunit of eukaryotic translational initiation factor 2 (eIF2α), and anti-eIF2α antibodies were from Oncogene (Boston, MA). Rabbit polyclonal anti-LC3 was from Novus Biologicals, Inc. (Littleton, CO).

Metabolic Labeling, Immunoprecipitation, SDS-PAGE, and Fluorography. After a 2-hour treatment of McA-RH7777 cells or primary rat hepatocytes with 5 mM GLS or 5 μg/mL TM, the cells were preincubated in methionine/cysteine-free minimum essential medium with 5 mM GLS or 5 μg/mL TM at 37°C for 1 hour, followed by pulse-labeling with 100 μCi/mL [^{35}S]methionine for 2 hours in the presence or absence of 1 mM PBA (see figure legends). Following the pulse, the medium was harvested for immunoprecipitation of secreted apoB100 or albumin. The cells were lysed using 500 μL solubilizing buffer and cellular apoB100 was immunoprecipitated under the conditions described in the figure legends. The gels were fixed and saturated with Amplify (Amersham Pharmacia Biotech) before being dried and exposed to Kodak Hyperfilm at −80°C for 1-4 days. Films were developed and quantitative analysis of apoB100 bands was performed using an imaging densitometer.[23]

Reverse Transcription Polymerase Chain Reaction Analysis of Messenger RNA. Following treatment of McA-RH7777 cells with 5 mM GLS or 5 μg/mL TM for 4 hours in the presence or absence of 1 mM PBA, total RNA was extracted using a commercially available kit (RNeasy; Qiagen). First-strand cDNA was synthesized from 5 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen).[24] The resulting cDNA was subjected to 28 cycles of polymerase chain reaction (PCR) amplification (denaturation at 95°C for 30 seconds; annealing at 55°C for 60 seconds; extension at 72°C for 90 seconds). The primer pairs used for detecting messenger RNA (mRNA) levels are listed in Supporting Table 1.

Immunofluorescence Microscopy. Following 24-48 hours transfection, cells were fixed with precooled 100% methanol for 5 minutes and then permeabilized with 0.1% Triton X100 in PBS for 4 minutes. Cells were incubated with rabbit anti-hamster apoB antibody (1:1000) for 1 hour at room temperature or overnight at 4°C in 5% bovine serum albumin. Secondary antibody used in this study was CyTM3-conjugated affini-Pure Donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratory, Inc.), dilute 1:500 for 1 hour. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology; sc3598). Images were captured using a Quorum spinning disk microscope (Leica DMIRE2 inverted fluorescence microscope equipped with a Hamamatsu back-thinned electron multiplying charge-coupled device camera, spinning disc head, and Volocity 5 software [Improvision]). Images were imported into Adobe Photoshop and assembled in Adobe Illustrator software. To quantify the percentage of cells with apoB-GFP-LC3 puncta, at least 200 cells per condition were counted in randomly selected fields. In all cases, only those cells with four or more prominent puncta of apoB-GFP-LC3 were scored positively.

Statistical Analysis. At least three independent experiments were performed for each graph, unless otherwise indicated. The mean ± standard error of the mean is shown in figures. All statistical calculations were completed using GraphPad PRISM software (version 5). For grouped analyses, a two-way ANOVA was used followed by a Bonferroni post-hoc test. To compare control to different treatments a one-way ANOVA was applied followed by a Dunnett's Multiple Comparison Test. Probability values of less than 0.05 were considered to be statistically significant.

Results

Accumulation of apoB in Autophagosomes Following Treatment of McA-RH7777 Cells With GLS and TM. As a first approach to gain insight into the role of autophagy under ER stress conditions, we examined

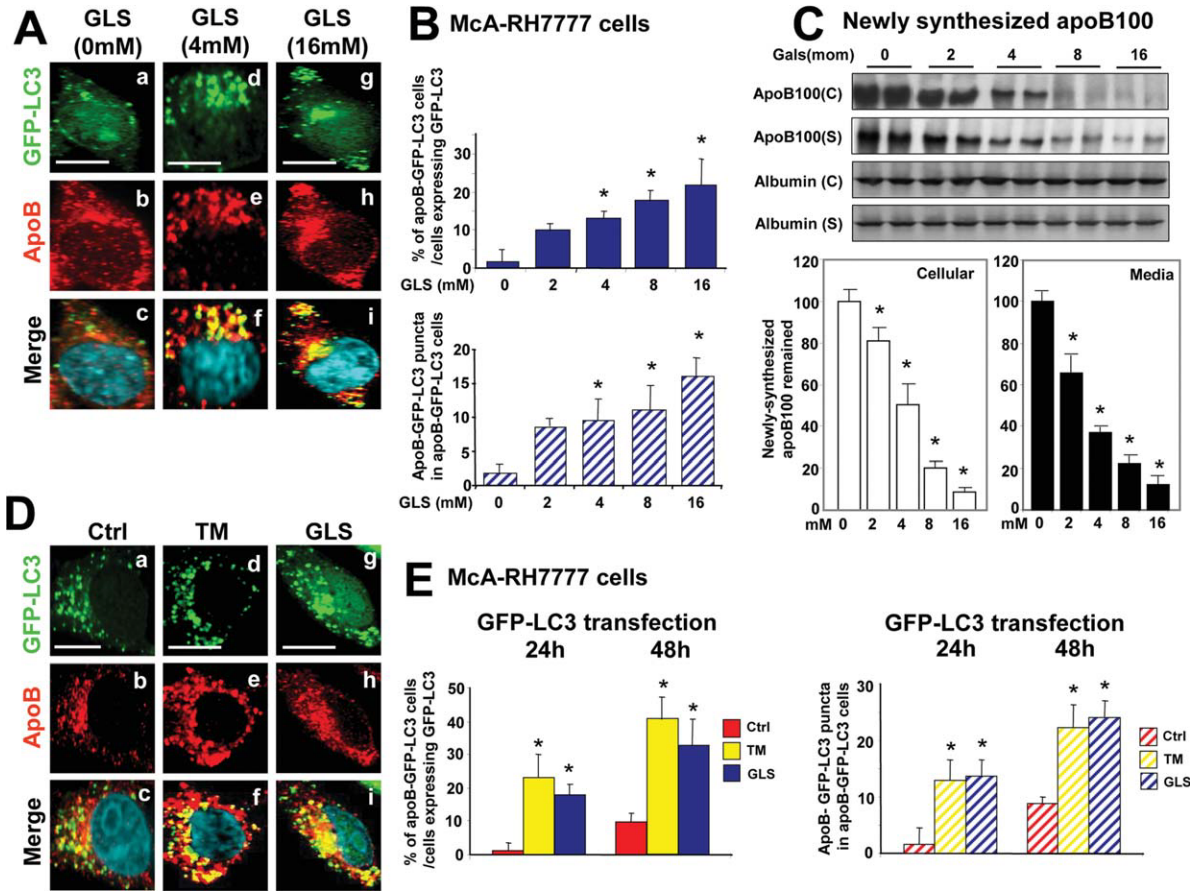

Fig. 1. Glucosamine (GLS) induces colocalization of apoB with GFP-LC3 in a dose-dependent manner. (A) GLS-induced colocalization of apoB (b, e, h; red) with GFP-LC3 (a, d, g; green), referred to as apoB-GFP-LC3 puncta (c, f, i; yellow). Confocal microscopy photographs are shown from three independent experiments. McA-RH7777 cells were transiently transfected with GFP-LC3 cDNA for 24 hours in the presence of GLS (0-16 mM; 4 hours). Scale bar: 7μM. (B) Top panel shows percentage of apoB-GFP-LC3 positive cells, and bottom panel shows the number of apoB-GFP-LC3 puncta in positive cells; *$P < 0.05$ versus untreated (0 mM GLS). (C) GLS treatment (0-16 mM) of McA-RH7777 cells (4 hours) decreased newly synthesized apoB100 in a dose-dependent manner. The samples were first immunoprecipitated (IP) with anti-apoB antibody followed by a second IP with anti-albumin antibody. Data analysis is shown in the bottom panel; n = 4 *$P < 0.05$ versus untreated (0 mM GLS). (D) Tunicamycin (TM)-induced and GLS-induced colocalization of apoB (b, c, e; red) with GFP-LC3 (a, d, g; green), apoB-GFP-LC3 puncta (c, f, i; yellow). McA-RH7777 cells were transiently transfected with GFP-LC3 cDNA for 48 hours. Scale bar: 7μM. (E) Data analysis is shown from cells transfected with GFP-LC3 cDNA for 24 or 48 hours; n = 3, *$P < 0.05$ versus corresponding untreated time point.

the colocalization of apoB with LC3 (the microtubule associated protein 1 light chain 3), an autophagosome marker. Colocalization of apoB with GFP-LC3 was barely detectable (Fig. 1A, panels a-c) under untreated conditions in McA-RH7777 cells transiently expressing GFP-conjugated LC3 (GFP-LC3) for 24 hours. However, the colocalization of apoB with GFP-LC3, referred to as apoB-GFP-LC3 puncta, was markedly enhanced following 4 mM GLS treatment for 4 hours (Fig. 1A, panel d-f). Increasing the GLS concentration to 16 mM led to high levels of apoB-GFP-LC3 puncta concentrated in a juxtanuclear localization, and in the distal area near the plasma membrane (Fig. 1A, panels g-i). The density of apoB-GFP-LC3 puncta–positive cells as well as the number of apoB-GFP-LC3 puncta in each positive cell increased with rising concentrations of GLS (0-16 mM) (*$P < 0.05$) (Fig. 1B). Concomitantly, increased apoB-GFP-LC3 puncta were correlated positively with the degradation of newly synthesized apoB in a GLS dose-dependent manner (*$P < 0.05$) (Fig. 1C). Moreover, as shown in Fig. 1E, under the basal (Fig. 1D, panel c), and TM-treated (Fig. 1D, panel f) or GLS-treated (Fig. 1D, panel i) conditions, the apoB-GFP-LC3 puncta–positive cells, and number of apoB-GFP-LC3 puncta was substantially increased by a longer GFP-LC3 expression time (48 hours).

Links Between ER Stress and apoB Autophagy. We next sought to further investigate links between the induction of ER stress and the autophagic degradation of apoB. Experiments were performed in McA-RH7777 cells treated with TM (5 μg/mL) or GLS (5

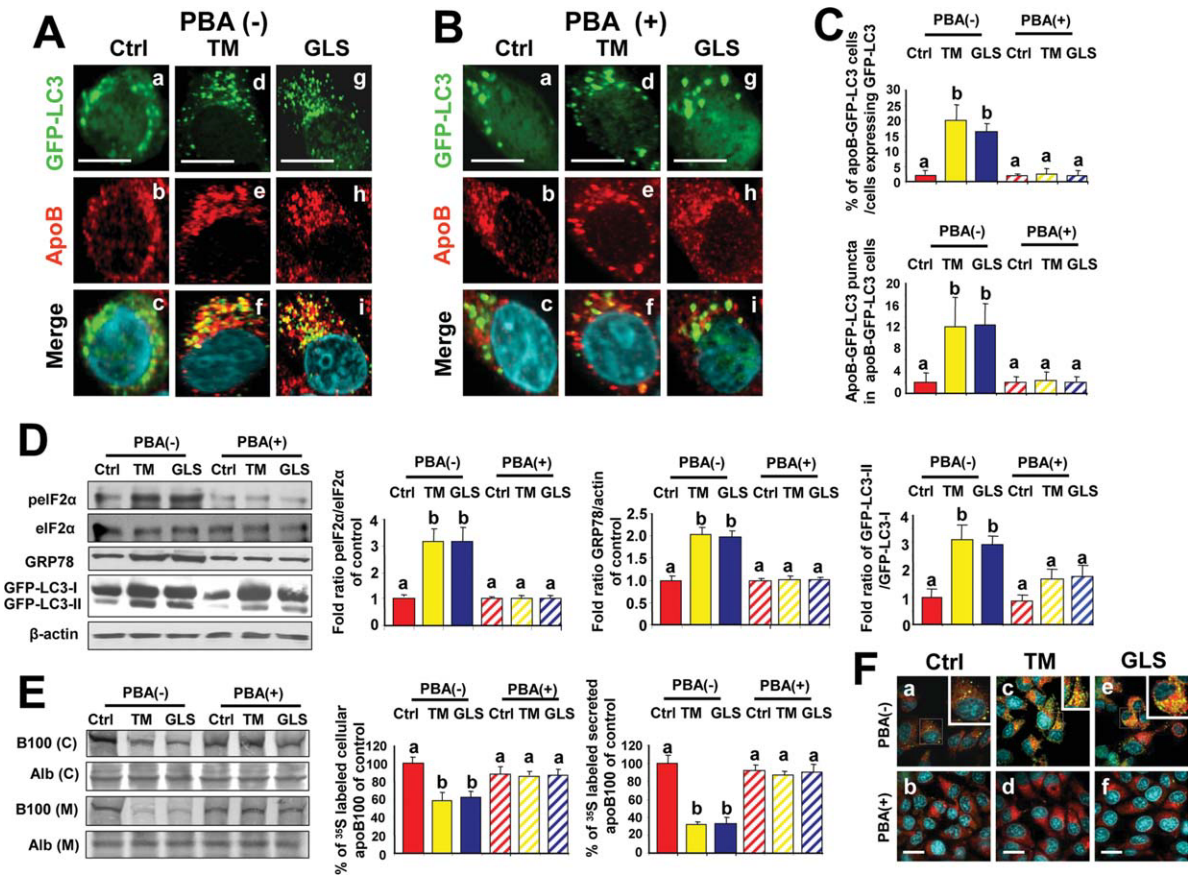

Fig. 2. PBA reduces ER stress–induced apoB autophagy in McA-RH7777 cells. (A,B) PBA treatment of McA-RH7777 cells blocks tunicamycin (TM)-induced and glucosamine (GLS)-induced colocalization of apoB with GFP-LC3. ApoB is labeled red (b, e, h); and GFP-LC3 is labeled green (a, d, g); apoB-GFP-LC3 puncta are labeled yellow (c, f and i). Scale bar: 7 μM. (C) Data analysis from (A and B), top panel shows percentage of apoB-GFP-LC3–positive cells, and bottom panel shows the number of apoB-GFP-LC3 puncta in positive cells; n = 3, with different letters indicating significance $P < 0.05$. (D) PBA treatment of McA-RH7777 cells blocks TM- and GLS-induced levels of GRP78, phosphorylation of eIF2α, and ratio of GFP-LC3-II/GFP-LC3-I conversion. Representative western blots are shown as well as data analysis from three independent experiments, with different letters indicating significance; $P < 0.05$. (E) PBA treatment of McA-RH7777 cells normalizes TM- and GLS-induced decreases in newly synthesized apoB100; data analysis is shown; n = 3, with different letters indicating significance, $P < 0.05$. (F) Confocal images showing colocalization of apoB with endogenous LC3 (a, c, e, yellow) in McA-RH7777 cells following treatment with TM or GLS.

mM) for 4 hours in the presence or absence of 4-phenyl butyric acid (PBA, 1 mM), a chemical inhibitor of ER stress.[25] Treatment with TM or GLS resulted in increased apoB-GFP-LC3 puncta–positive cells and a higher number of apoB-GFP-LC3 puncta in each cell (Fig. 2A, panels f and i; and analysis of data shown in Fig. 2C; different letters indicate significance, $P < 0.05$). Similar results were obtained when colocalization of apoB and endogenous LC3 was examined in nontransfected cells (Fig. 2F, and Supporting Fig. 1). Treatment with TM or GLS also led to elevated levels of GFP-LC3-II conversion, an autophagsome membrane-associated lipidated protein conjugated to GFP (Fig. 2D; different letters indicate significance, $P < 0.05$). PBA treatment significantly reduced ER stress–induced formation of apoB-GFP-LC3–positive cells and number of apoB-GFP-LC3 puncta (Fig. 2B, and analysis data shown in Fig. 2C; $P < 0.05$), and decreased apoB-GFP-LC3-II conversion (Fig. 2D; $P < 0.05$). Furthermore, PBA treatment markedly inhibited ER stress based on reduced cellular levels of GRP78 and phosphorylated eIF-2α (Fig. 2D). PBA treatment also prevented the loss of newly synthesized cellular and secreted apoB-100 (Fig. 2E) following TM and GLS treatment (different letters indicate significance, $P < 0.05$). These results strongly indicate that the induction of ER stress augments autophagic degradation of apoB, whereas suppression of ER stress blocks apoB autophagy.

Evidence of ER Stress–Induced Autophagy in Primary Rat Hepatocytes. We next assessed whether autophagic degradation of apoB also occurs in primary hepatocytes. Primary rat hepatocytes were transiently transfected with GFP-LC3 cDNA for 40 hours, and

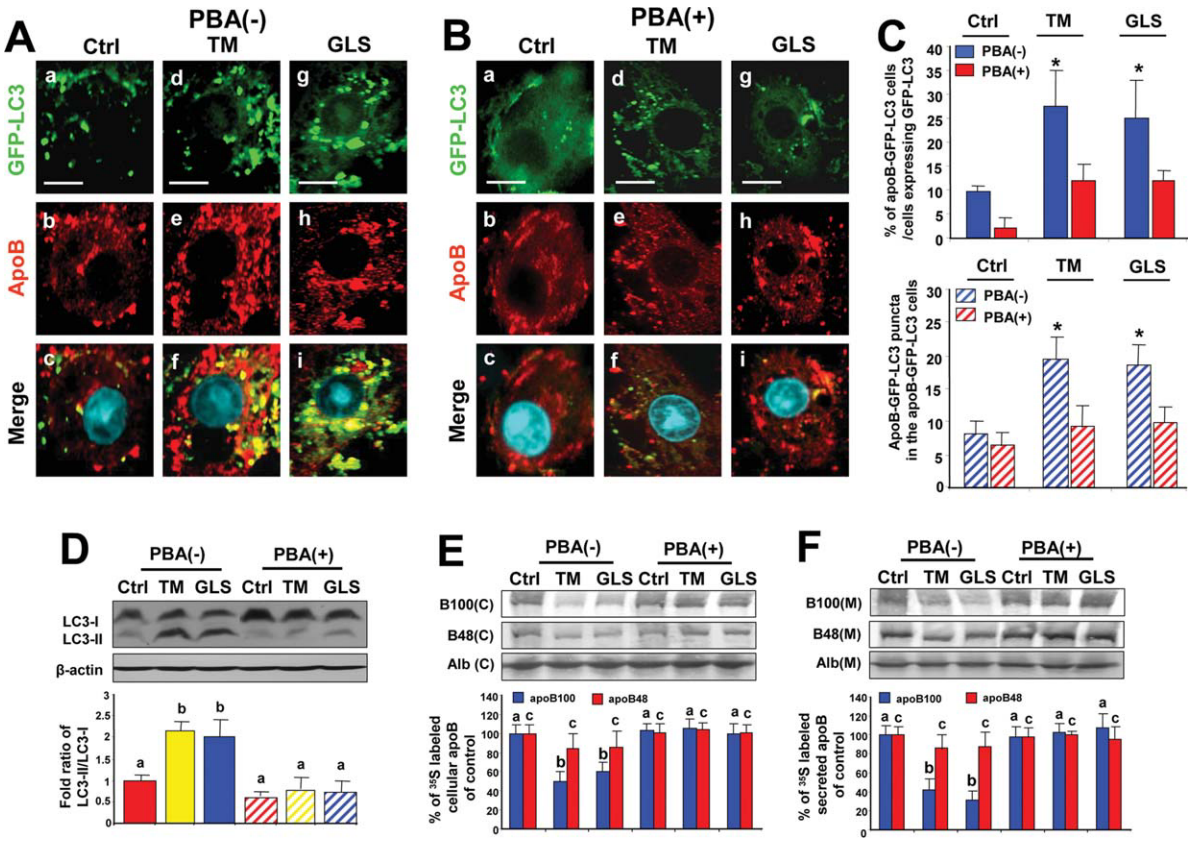

Fig. 3. PBA reduces ER stress–induced apoB-autophagy in primary rat hepatocytes. (A,B) Confocal microscopy images showing colocalization of apoB (b, e, h, red) with GFP-LC3 (a, d, g, green), and apoB-GFP-LC3 puncta (c, f, i, yellow), in primary rat hepatocytes following treatment with tunicamycin (TM) or glucosamine (GLS). Scale bar: 11 μM. (C) Data analysis is shown in the top panel showing percentage of apoB-GFP-LC3–positive cells, and the bottom panel shows the number of apoB-GFP-LC3 puncta in positive cells; n = 3, $^*P < 0.05$ versus corresponding control. PBA also reduced the conversion of endogenous LC3-II/LC3-1 (D), and increased recovery of newly synthesized apoB100 and apoB48 (E,F), in cells treated with TM or GLS; data are from three independent experiments, with different letters indicating statistical significance; $P < 0.05$.

then treated with TM (5 μg/mL) or GLS (5 mM) for 4 hours in the presence or absence of PBA (1 mM). Treatment with TM or glucosamine resulted in substantially increased colocalization of apoB with GFP-LC3 and increased number of apoB-GFP-LC3 puncta (Fig. 3A, panels f and i; analysis of data shown in Fig. 3C; $^*P < 0.05$ versus corresponding control). Similar results were obtained when colocalization of apoB and endogenous LC3 was examined in non-transfected cells (Supporting Fig. 2). Increased apoB-GFP-LC3 puncta were observed together with elevated endogenous LC3-II conversion (Fig. 3D; $^*P < 0.05$). Importantly, treatment with TM and glucosamine decreased [^{35}S]-labeled cellular and secreted apoB100 (Fig. 3E,F; different letters indicate significance, $P < 0.05$), apoB48 was also slightly reduced but this change did not reach statistical significance suggesting that ER stress induces autophagy of apoB100 in primary rat hepatocytes. Importantly, PBA treatment inhibited colocalization of apoB with GFP-LC3 (Fig. 3B, panels f and i; analysis of data shown in Fig. 3C), reduced the endogenous LC3-II conversion (Fig. 3D; different letters indicate significance, $P < 0.05$), and led to a significantly increased recovery of [^{35}S]-labeled cellular or secreted apoB100 (Fig. 3E,F; different letters indicate significance, $P < 0.05$), suggesting that blocking ER stress prevents apoB autophagy. Interestingly, PBA was also found to significantly block colocalization of apoB with GFP-LC3 in primary rat hepatocytes under basal conditions (Fig. 3C; top panel, $^*P < 0.05$ versus corresponding control). However, under basal conditions, PBA did not significantly alter the number of apoB-GFL-LC3 puncta in positive cells (Fig. 3C, bottom panel), or endogenous LC3-II conversion (Fig. 3D).

ER Stress–Induced Autophagy of apoB-GFP-LC3 Is Decreased by the Autophagy Inhibitor 3-MA and Enhanced by the Lysosomal Protease Inhibitor E64d. In order to explore the mechanisms by which apoB is degraded by autophagy, primary rat

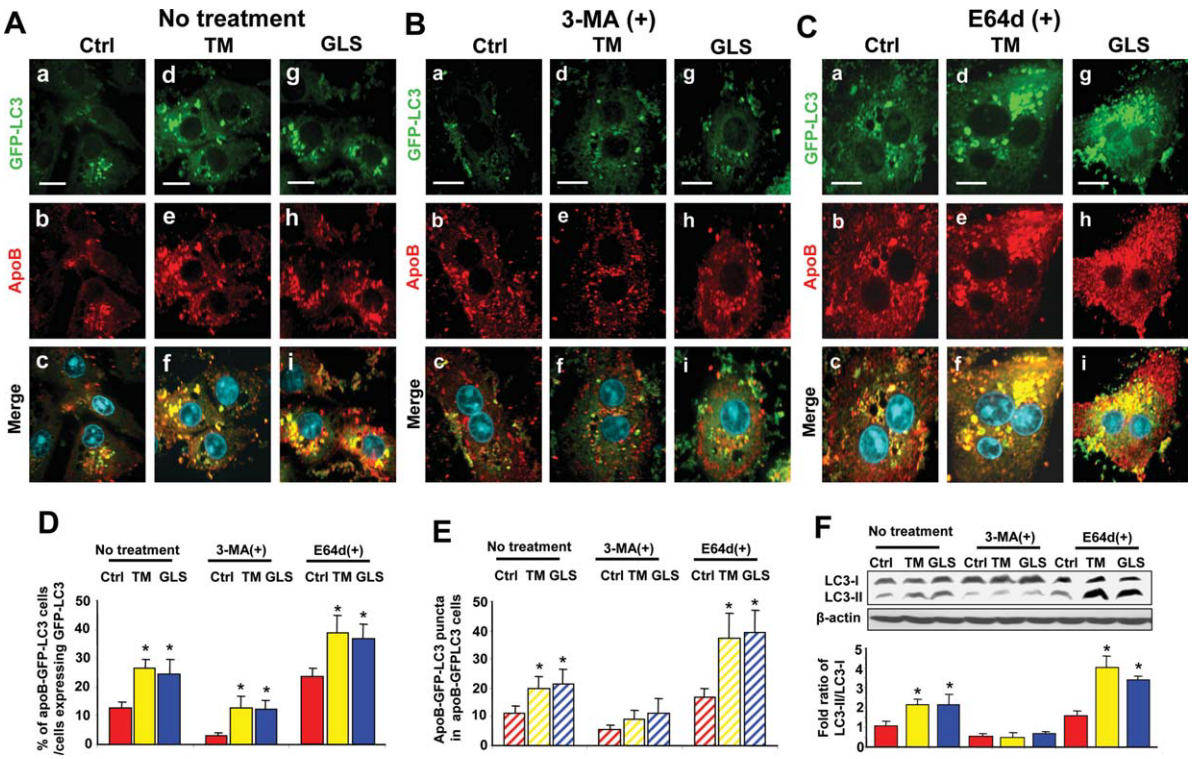

Fig. 4. Presence of 3-methyadenine (3-MA) blocks, whereas E64d enhances, apoB-GFP-LC3 puncta induced by ER stress. (A-C) Confocal microscopy photographs of primary rat hepatocytes treated with tunicamycin (TM, 5 μg/mL) or glucosamine (GLS, 5 mM) in the presence of 3-MA (5 mM) or E64d (5 μg/mL). Colocalization of apoB (b, e, h, red) with GFP-LC3 (a, d, g, green), apoB-GFP-LC3 puncta (c, f, i, yellow). Scale bar: 17 μM. Data analysis from (A-C) is shown in (D), percentage of apoB-GFP-LC3–positive cells, and (E), the numbers of apoB-GFP-LC3 puncta in positive cells; three independent experiments, $^*P < 0.05$. The 3-MA blocked the conversion of endogenous LC3-II, and E64d reduced LC3 turnover (F) when exposed to TM or GLS treatment, n = 3, $^*P < 0.05$.

hepatocytes were transiently transfected with GFP-LC3 cDNA for 44 hours then treated with TM (5 μg/mL) or glucosamine (5 mM) in the presence or absence of 3-methyadinine (5 mM), an autophagy inhibitor,[14] or E64d (5 μg/mL), a lysosome protease inhibitor[26] for 4 hours. Chemical induction of ER stress significantly increased apoB-GFP-LC3 positive cells and the number of apoB-GFP-LC3 puncta (Fig. 4A; panels f and i; analysis of data shown in Fig. 4D,E; $^*P < 0.05$ versus corresponding control). Endogenous LC3-II conversion (Fig. 4F; $^*P < 0.05$ versus corresponding control) was significantly increased as compared to basal controls. The addition of 3-MA significantly decreased the number of apoB-GFP-LC3 positive cells and the number of apoB-GFP-LC3 puncta (Fig. 4B, panels c, f, and i; and analysis of data shown in Fig. 4D,E; $^*P < 0.05$ versus corresponding control), and endogenous LC3-II conversion (Fig. 4F; $P < 0.05$) under basal and ER stress conditions. By contrast, addition of the lysosomal protease inhibitor E64d, markedly increased the number of apoB-GFP-LC3 positive cells and the number of apoB-GFP-LC3 puncta (Fig. 4C, panels c, f, and i; analysis of data shown in Fig. 4D,E; $P < 0.05$), as well as blocked endogenous LC3-II turnover (Fig. 4F; $^*P < 0.05$ versus corresponding control). Taken together, these data further support the induction of apoB autophagy in a process that involves the formation of autophagosomes and accumulation in lysosomes for eventual proteolysis.

ER Stress Induction of apoB Autophagy Is Linked to the Activation of PERK and IRE1. To examine underlying mechanisms, mRNA levels of key molecules in ER stress pathways were determined following 0, 2, 4, and 16 hours of treatment with glucosamine (5 mM) or TM (5 μg/mL) in the presence or absence of PBA (1 mM) in McA-RH7777 cells. the mRNA levels of GRP78, PERK and ratio of spliced/unspliced form of Xbp-1 were significantly increased by 1.7-fold ($^*P < 0.05$), 1.45-fold ($^*P < 0.05$), and 4.23-fold ($^*P < 0.05$), respectively, following glucosamine treatment (Fig. 5A,B). ATF6 mRNA level remained unchanged. PBA treatment markedly inhibited increases in mRNA levels of GRP78, PERK and ratio of spliced/unspliced form of Xbp-1 ($P < 0.05$), suggesting that under our

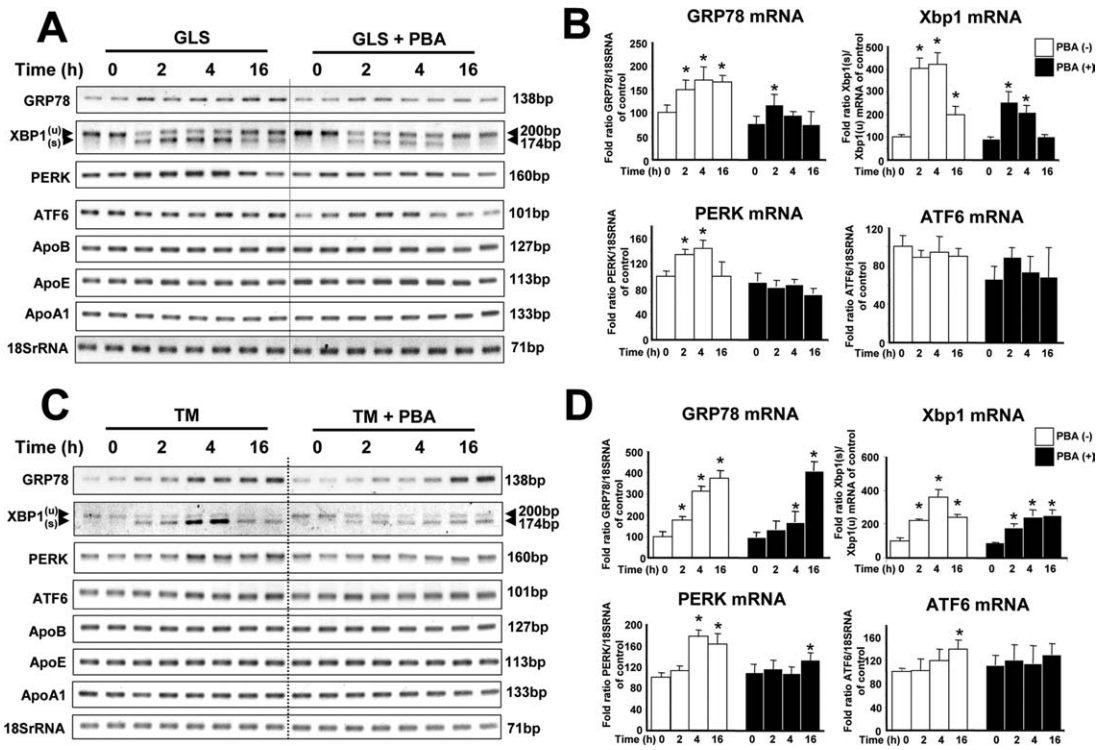

Fig. 5. ER stress induction is linked to the activation of PERK and IRE1. Messenger RNA levels of GRP78, PERK, and Xbp1 (spliced) in McA-RH7777 cells treated with (A,B) glucosamine (GLS) or (C,D) tunicamycin (TM). (A) The blots of RT-PCR products in GLS-treated cells (0, 2, 4, and 16 hours; 5 mM) in the presence or absence of PBA (1 mM) are shown. (B) Data analysis from (A); three independent experiments. $^*P < 0.05$ versus 0 hours. (C) Shown are the blots of RT-PCR products from McA-RH7777 cells treated with TM (0, 2, 4, and 16 hours; 5 μg/mL) in the presence or absence of PBA (1 mM). (D) Data analysis is from (C); three independent experiments. $^*P < 0.05$ versus 0 hours.

experimental conditions, PERK and IRE1, but not ATF6 signaling may be linked to apoB-autophagic degradation. Similar results were observed in cells treated with TM (Fig. 5C,D).

ER Stress Induces apoB Autophagy Via a PERK-Dependent Mechanism. To investigate the role that PERK activation may play in ER stress–induced apoB autophagic degradation, we cotransfected McA-RH7777 cells with GFP-LC3 cDNA and WT PERK cDNA, or kinase inactive (K618A) mutant (M) PERK cDNA, or control (mock), and examined the colocalization of apoB with GFP-LC3 following TM or glucosamine treatment. Under basal conditions (in the absence of ER stress–inducing agents), transfection with PERK WT cDNA led to a significantly increased number of apoB-GFP-LC3–positive cells and the number of apoB-GFP-LC3 puncta (Fig. 6A, panels c and f; analysis of data shown in Fig. 6D,E; $^*P < 0.05$ versus mock), as well as elevated GFP-LC3-II conversion (Fig. 6F; $^*P < 0.05$ versus mock), compared with mock-transfected cells. In contrast, transfection with the kinase inactive mutant PERK (M PERK) had an opposite effect (Fig. 6A, panels c and i; analysis of data shown in Fig. 6D-F, $^*P < 0.05$ versus mock). Similar effects were observed following the induction of ER stress produced by TM or glucosamine. As demonstrated in Fig. 6B,C, in the presence of either TM or glucosamine, overexpression of WT PERK led to increases in apoB-GFP-LC3–positive cells and the number of apoB-GFP-LC3 puncta (analysis of data shown in Fig. 6D,E; $^*P < 0.05$ versus mock), and higher GFP-LC3-II conversion (analysis of data shown in Fig. 6F; $^*P < 0.05$ versus mock) when compared to mock-transfected cells. By contrast, transfection with the kinase inactive mutant PERK significantly blocked ER stress–induced apoB autophagy (analysis of data shown in Fig. 6D-F; $^*P < 0.05$ versus mock). Taken together, these data suggest that ER stress–induced apoB-autophagic degradation is PERK signaling–dependent.

Discussion

In response to ER stress, mammalian cells initially react by attenuating protein synthesis which prevents further accumulation of unfolded proteins in the ER.[27] This response is followed by transcriptional induction of ER chaperone genes to increase protein

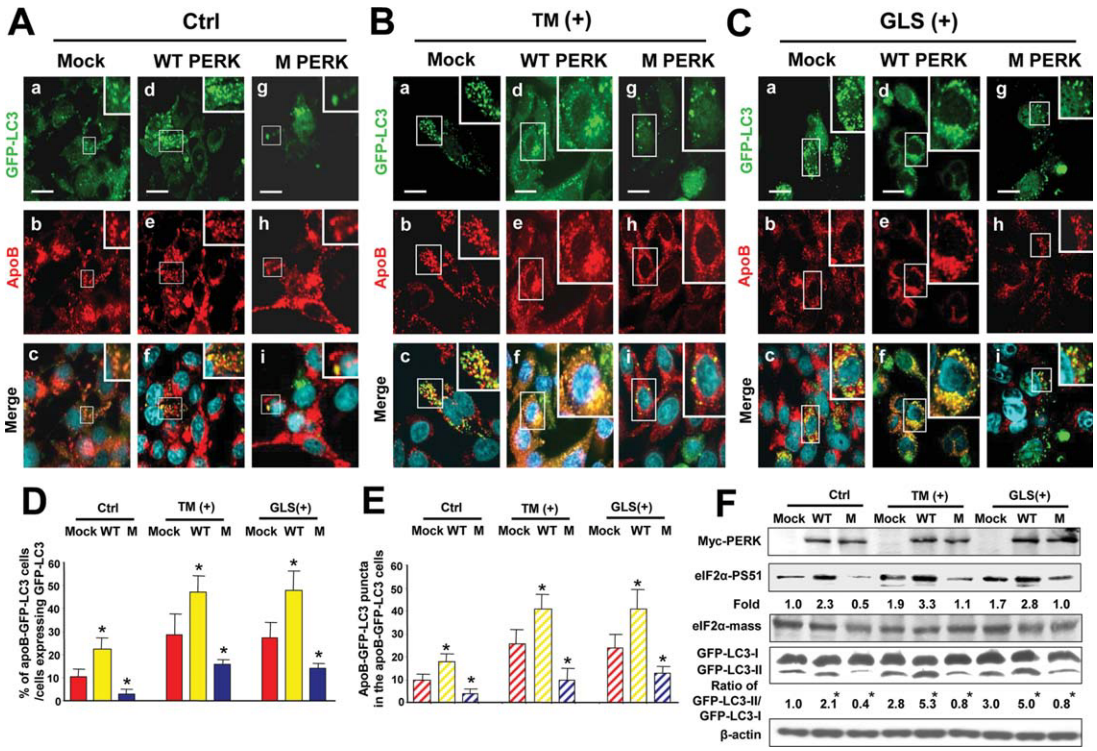

Fig. 6. ER stress–induced apoB autophagy is PERK signaling–dependent. McA-RH7777 cells were transiently transfected with GFP-LC3 cDNA alone or with wild-type PERK (WT-PERK) or kinase inactive (K618A) PERK (M-PERK) for 48 hours in the absence (A) or presence of (B) tunicamycin (TM) or (C) glucosamine (GLS). (A-C) Confocal microscopy images showing colocalization of apoB (b, e, h, red) with GFP-LC3 (a,d,g, green), and apoB-GFP-LC3 puncta (c, f, i, yellow). Scale bar: 17 μM. (D,E) Data analysis from (A-C); $^*P < 0.05$ versus corresponding mock treatment. (F) Western blots showing the levels of myc-PERK, phosphorylated eIF2α-PS51, eIF2α-mass, and GFP-LC3-I and GFP-LC3-II; β-actin as a protein loading control; and the fold ratio of GFP-LC3-II/GFP-LC3-1; n = 3, $^*P < 0.05$ versus corresponding mock treatment.

folding capacity and transcriptional induction of ERAD component genes to increase ERAD. The activation of autophagic degradation and induction of apoptosis are late defensive and surveillance systems to safely dispose of organelles and cells injured by ER stress to ensure the survival of the organism.[28] Numerous studies have now demonstrated a direct link between induction of ER stress and autophagy[14] and have proposed this pathway as an essential component of the unfolded protein response.[29]

Among mammalian proteins, apoB is particularly prone to misfolding under ER stress conditions due to its large size and its requirement for lipid binding to facilitate folding and lipoprotein assembly. Interest in ER stress–induced apoB degradation has also arisen because of the important role of apoB in cardiovascular disease and recent data implicating apoB as a potential factor linking hepatic ER stress and insulin resistance.[30] Early work in our laboratory demonstrated that apoB protein synthesis was attenuated,[21] and proteasomal degradation was increased following glucosamine-induced ER stress.[16] These studies also suggested the involvement of a posttranslational degradative mechanism responsible for ER stress related late stage degradation of misfolded apoB.[19] Evidence obtained in the present study suggests that ER stress induced autophagy may be responsible for the posttranslational loss of misfolded apoB.

Coimmunoprecipitation of LC3 with apoB in both McA-RH7777 and primary rat hepatocytes were also attempted, however, we were unable to detect a direct interaction between LC3 and apoB under our experimental conditions (data not shown). It appears that autophagosome membrane resident LC3-II may not directly bind apoB within the autophagosomes. Whether LC3-II indirectly associates with ubiquitinated apoB mediated by p62/SQSTM1 needs be further investigated. LC3 is a key factor in formation of autophagosomes but a direct interaction with substrate proteins is not essential to induce autophagy.

To assess whether autophagy is a common mechanism for ER stress–induced apoB turnover in hepatic cells, we monitored this process in two liver cell lines, namely, HepG2 and McA-RH7777 rat hepatoma cells

and primary hepatocytes isolated from rats and hamsters. ApoB-autophagic degradation was not detected in HepG2 cells following the induction of ER stress, unless proteasomal degradation was inhibited by ALLN and cells were supplemented with oleic acid (Supporting Fig. 3). This was not unexpected because we previously reported that the predominant mechanism of apoB degradation following ER stress in HepG2 cells was proteasomal in nature.[16] Our current data appears to suggest that blocking proteasomal degradation in ER stressed HepG2 cells leads to the activation of apoB autophagy, which may act to clear apoB aggregates accumulating in the ER in the absence of proteasome activity. These data also suggest that proteasomal and autophagic degradative pathways may in fact be coordinately regulated. Proteasomal degradation is perhaps an early quality control system, whereas, apoB-autophagic degradation may be a late quality control mechanism. It is likely that newly synthesized apoB molecules that escape the early-stage proteasomal degradation may become substrates for autophagy if not properly lipidated and removed from the secretory pathway. This hypothesis is supported by a recent study by Zhong et al. who showed that expression of A31P, an apoB mutant, leads to rapid proteasomal degradation, but a significant proportion of A31P escapes the ER quality control and is present in the Golgi compartment. However, post-ER degradation of A31P was found to occur via autophagy.[13] In addition, our data also suggests that apoB autophagy is more active in primary hepatocytes compared to that in McA-RH7777 cells suggesting that this pathway may be more physiologically relevant *in vivo*.

Importantly, we have presented evidence of apoB autophagy in both primary rat and primary hamster hepatocytes under basal and ER stress–induced conditions (Supporting Fig. 3). ApoB-GFP-LC3 puncta was clearly detectable in both rat and hamster primary hepatocytes under basal conditions, and was considerably enhanced following the induction of ER stress. These data suggest that apoB autophagy is likely an important mechanism of apoB turnover in primary hepatocytes and is active in unstressed and stressed conditions. Interestingly, apoB autophagy was robustly inhibited when cells were treated with PBA, a chemical agent that facilitates protein folding in the cell. Fisher and coworkers were first to demonstrate DHA-induced apoB-autophagic degradation in McA-RH7777 cells due to accumulation of lipid peroxides in or after the Golgi apparatus. ApoB was shown to undergo oxidative damage, to form aggregates, and to subsequently be diverted out of the secretory pathway by autophagosomes for delivery to lysosomes for destruction.[12] In the present study, although PBA could prevent ER stress–induced apoB-autophagic degradation, it was unable to inhibit DHA-induced or ALLN-induced apoB autophagy in rat primary hepatocytes (Supporting Fig. 4), suggesting that the mechanisms mediating apoB-autophagic degradation under ER stress may be different from that induced by DHA or ALLN.

Although a large body of evidence suggests that ER stress regulates autophagic degradation,[29] the underlying mechanisms remain to be elucidated. Three pathways (PERK, ATF6, and IRE1 pathways) regulate the mammalian ER stress response.[28] PERK, a transmembrane kinase, phosphorylates eIF2α to attenuate translation, and to up-regulate expression of ATF4, leading to enhanced transcription of target genes such as CHOP. ATF6, a transmembrane transcription factor, is translocated to the Golgi apparatus and cleaved by proteases such as S1P and S2P, leading to enhanced transcription of ER chaperone genes. IRE1, a transmembrane ribonuclease, splices Xbp1 pre-mRNA, and pXbp1(S) translated from mature Xbp1 mRNA activates transcription of ERAD component genes. In the present study, we found that the ATF6 pathway is inactive upon acute ER stress (induced by TM or glucosamine) perhaps because ATF6 has been suggested to regulate chronic ER stress.[31] By contrast, PERK activation appeared to be critical to ER stress–induced activation of apoB-autophagic degradation. Our observations are consistent with a previous report that PERK/eIF2α phosphorylation plays a critical role in mediating autophagosome associated LC3-II conversion during ER stress induced by polyglutamine 72 repeat (polyQ72) aggregates.[32] It remains to be defined whether Xbp1 also plays a role in apoB-autophagic degradation.

In summary, these data collectively suggest that apoB-autophagic degradation in hepatic cells is largely dependent on the cell type used and cell culture conditions. This pathway is inactive in HepG2 cells but can be activated if proteasomal degradation is inhibited by ALLN and supplemented with oleate. ApoB-autophagic degradation is however highly active in primary hepatocytes under both normal and ER stress conditions. Ameliorating ER stress with chemical chaperones such as PBA abolishes apoB-autophagic degradation under ER stress conditions. Finally, induction of PERK signaling may be essential to apoB autophagy.

Acknowledgment: We acknowledge Mark Naples and Chris Baker for expert technical assistance with isolation of rat and hamster primary hepatocytes.

References

1. Rutledge AC, Su Q, Adeli K. Apolipoprotein B100 biogenesis: a complex array of intracellular mechanisms regulating folding, stability, and lipoprotein assembly. Biochem Cell Biol 2010;88:251-267.
2. Sundaram M, Yao Z. Recent progress in understanding protein and lipid factors affecting hepatic VLDL assembly and secretion. Nutr Metab (Lond) 2010;7:35.
3. Liao W, Yeung SC, Chan L. Proteasome-mediated degradation of apolipoprotein B targets both nascent peptides cotranslationally before translocation and full-length apolipoprotein B after translocation into the endoplasmic reticulum. J Biol Chem 1998;273:27225-27230.
4. Fisher EA, Zhou M, Mitchell DM, Wu X, Omura S, Wang H, et al. The degradation of apolipoprotein B100 is mediated by the ubiquitin-proteasome pathway and involves heat shock protein 70. J Biol Chem 1997;272:20427-20434.
5. Zhou M, Fisher EA, Ginsberg HN. Regulated co-translational ubiquitination of apolipoprotein B100. A new paradigm for proteasomal degradation of a secretory protein. J Biol Chem 1998;273:24649-24653.
6. Taghibiglou C, Rudy D, Van Iderstine SC, Aiton A, Cavallo D, Cheung R, et al. Intracellular mechanisms regulating apoB-containing lipoprotein assembly and secretion in primary hamster hepatocytes. J Lipid Res 2000;41:499-513.
7. Adeli K. Regulated intracellular degradation of apolipoprotein B in semipermeable HepG2 cells. J Biol Chem 1994;269:9166-9175.
8. Cavallo D, Rudy D, Mohammadi A, Macri J, Adeli K. Studies on degradative mechanisms mediating post-translational fragmentation of apolipoprotein B and the generation of the 70-kDa fragment. J Biol Chem 1999;274:23135-23143.
9. Du EZ, Kurth J, Wang SL, Humiston P, Davis RA. Proteolysis-coupled secretion of the N terminus of apolipoprotein B. Characterization of a transient, translocation arrested intermediate. J Biol Chem 1994;269:24169-24176.
10. Twisk J, Gillian-Daniel DL, Tebon A, Wang L, Barrett PH, Attie AD. The role of the LDL receptor in apolipoprotein B secretion. J Clin Invest 2000;105:521-532.
11. Ohsaki Y, Cheng J, Fujita A, Tokumoto T, Fujimoto T. Cytoplasmic lipid droplets are sites of convergence of proteasomal and autophagic degradation of apolipoprotein B. Mol Biol Cell 2006;17:2674-2683.
12. Pan M, Maitin V, Parathath S, Andreo U, Lin SX, St Germain C, et al. Presecretory oxidation, aggregation, and autophagic destruction of apoprotein-B: a pathway for late-stage quality control. Proc Natl Acad Sci U S A 2008;105:5862-5867.
13. Zhong S, Magnolo AL, Sundaram M, Zhou H, Yao EF, Di Leo E, et al. Nonsynonymous mutations within APOB in human familial hypobetalipoproteinemia: evidence for feedback inhibition of lipogenesis and postendoplasmic reticulum degradation of apolipoprotein B. J Biol Chem 2010;285:6453-6464.
14. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet 2009;43:67-93.
15. Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, et al. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 2004;306:457-461.
16. Qiu W, Kohen-Avramoglu R, Mhapsekar S, Tsai J, Austin RC, Adeli K. Glucosamine-induced endoplasmic reticulum stress promotes ApoB100 degradation: evidence for Grp78-mediated targeting to proteasomal degradation. Arterioscler Thromb Vasc Biol 2005;25:571-577.
17. Ota T, Gayet C, Ginsberg HN. Inhibition of apolipoprotein B100 secretion by lipid-induced hepatic endoplasmic reticulum stress in rodents. J Clin Invest 2008;118:316-332.
18. Li J, Ni M, Lee B, Barron E, Hinton DR, Lee AS. The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. Cell Death Differ 2008;15:1460-1471.
19. Qiu W, Avramoglu RK, Rutledge AC, Tsai J, Adeli K. Mechanisms of glucosamine-induced suppression of the hepatic assembly and secretion of apolipoprotein B-100-containing lipoproteins. J Lipid Res 2006;47:1749-1761.
20. Huang J, Canadien V, Lam GY, Steinberg BE, Dinauer MC, Magalhaes MA, et al. Activation of antibacterial autophagy by NADPH oxidases. Proc Natl Acad Sci U S A 2009;106:6226-6231.
21. Qiu W, Su Q, Rutledge AC, Zhang J, Adeli K. Glucosamine-induced endoplasmic reticulum stress attenuates apolipoprotein B100 synthesis via PERK signaling. J Lipid Res 2009;50:1814-1823.
22. Qiu W, Federico L, Naples M, Avramoglu RK, Meshkani R, Zhang J, et al. Phosphatase and tensin homolog (PTEN) regulates hepatic lipogenesis, microsomal triglyceride transfer protein, and the secretion of apolipoprotein B-containing lipoproteins. HEPATOLOGY 2008;48:1799-1809.
23. Qiu W, Kohen-Avramoglu R, Rashid-Kolvear F, Au CS, Chong TM, Lewis GF, et al. Overexpression of the endoplasmic reticulum 60 protein ER-60 downregulates apoB100 secretion by inducing its intracellular degradation via a nonproteasomal pathway: evidence for an ER-60-mediated and pCMB-sensitive intracellular degradative pathway. Biochemistry 2004;43:4819-4831.
24. Qiu W, Avramoglu RK, Dubé N, Chong TM, Naples M, Au C, et al. Hepatic PTP-1B expression regulates the assembly and secretion of apolipoprotein B-containing lipoproteins: evidence from protein tyrosine phosphatase-1B overexpression, knockout, and RNAi studies. Diabetes 2004;53:3057-3066.
25. Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, et al. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science 2006;313:1137-1140.
26. Tanida I, Minematsu-Ikeguchi N, Ueno T, Kominami E. Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. Autophagy 2005;1:84-91.
27. Mori K. Signalling pathways in the unfolded protein response: development from yeast to mammals. J Biochem 2009;146:743-750.
28. Yoshida H. ER stress and diseases. FEBS J 2007;274:630-658.
29. Yorimitsu T, Nair U, Yang Z, Klionsky DJ. Endoplasmic reticulum stress triggers autophagy. J Biol Chem 2006;281:30299-30304.
30. Su Q, Tsai J, Xu E, Qiu W, Bereczki E, Santha M, et al. Apolipoprotein B100 acts as a molecular link between lipid-induced endoplasmic reticulum stress and hepatic insulin resistance. HEPATOLOGY 2009;50:77-84.
31. Wu J, Rutkowski DT, Dubois M, Swathirajan J, Saunders T, Wang J, et al. ATF6alpha optimizes long-term endoplasmic reticulum function to protect cells from chronic stress. Dev Cell 2007;13:351-364.
32. Kouroku Y, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H, et al. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell Death Differ 2007;14:230-239.