Glucosamine-induced endoplasmic reticulum stress attenuates apolipoprotein B100 synthesis via PERK signaling

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Abstract Glucosamine impairs hepatic apolipoprotein B100 (apoB100) production by inducing endoplasmic reticulum (ER) stress and enhancing cotranslational and posttranslational apoB100 degradation (Qiu, W., R. K. Avramoglu, A. C. Rutledge, J. Tsai, and K. Adeli. Mechanisms of glucosamine-induced suppression of the hepatic assembly and secretion of apolipoprotein B-100-containing lipoproteins. *J. Lipid Res.* 2006. 47: 1749–1761). Here, we report that glucosamine also regulates apoB100 protein synthesis via ER-stress-induced PERK activation. Short-term (4 h) glucosamine treatment of HepG2 cells reduced both cellular (by 62%) and secreted apoB100 (by 43%) without altering apoB100 mRNA. Treatment with proteasomal inhibitors only partially prevented the suppressive effects of glucosamine, suggesting that mechanisms other than proteasomal degradation may also be involved. Glucosamine-induced ER stress was associated with a significantly reduced apoB100 synthesis with no significant change in posttranslational decay rates, suggesting that glucosamine exerted its effect early during apoB biosynthesis. The role of PERK and its substrate, α-subunit of eukaryotic initiation factor 2 (eIF2α), in the suppressive effects of glucosamine on apoB synthesis was then investigated. Coexpression of apoB15 (normally resistant to intracellular degradation) with wild-type double stranded (ds) RNA activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) in COS-7 cells resulted in a dramatic reduction in the levels of newly synthesized apoB15. Interestingly, cotransfection with apoB15 and a kinase inactive PERK mutant (K618A) increased apoB15 expression. In addition, short-term glucosamine treatment stimulated an increase in phosphorylation of PERK and eIF2α. Taken together, these data suggest that in addition to the induction of ER-associated degradation and other degradative pathways, ER stress is associated with suppression of apoB synthesis via a PERK-dependent mechanism.—Qiu, W., Q. Su, A. C. Rutledge, J. Zhang, and K. Adeli. **Glucosamine-induced endoplasmic reticulum stress attenuates apolipoprotein B100 synthesis via PERK signaling. *J. Lipid Res.* 2009.** 50: **1814–1823.**

Supplementary key words α-subunit of eukaryotic initiation factor 2 • glucose-regulated protein 78 • degradation

Hepatic apolipoprotein B100 (apoB100) protein biogenesis is a complex process involving regulation at cotranslational and posttranslational levels (1–6). Intracellular and especially endoplasmic reticulum (ER) availability of core lipoprotein lipid substrates, particularly triglyceride, appears to govern the intracellular fate of newly synthesized apoB100 protein (7, 8). In lipid poor states, a significant proportion of newly synthesized apoB100 is degraded in cultured hepatoma cells (9–12). The bulk of apoB100 degradation appears to be mediated by the ubiquitin-proteasome degradative system (13, 14). The intracellular fate of apoB100 is also critically dependent on its interaction with several key ER factors. Microsomal triglyceride transfer protein (MTP), an ER-localized lipid transfer protein, plays an important role in the lipidation and secretion of apoB100-containing lipoproteins (15, 16). There is ample evidence showing the association of the newly synthesized apoB100 polypeptides with numerous other ER chaperones, including GRP94, GRP78, ERp72, calreticulin, cyclophilin B, and calnexin (17, 18). Perturbation in ER function and interactions of apoB100 with such molecular chaperones would thus be expected to have major consequences on the

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Abbreviations: ALLN, *N*-acetyl-leucinyl-leucinyl-nor-leucinal; apoB, apolipoprotein B; ATF, activating transcription factor; eIF2α, α-subunit of eukaryotic translational initiation factor 2; ER, endoplasmic reticulum; ERAD, endoplasmic-reticulum-associated degradation; IRE1, inositol requirement 1; lactacystin, clasto-lactocystin β-lactone; MTP, microsomal triglyceride transfer protein; PERK, PKR-like endoplasmic reticulum kinase; PKR, double stranded (ds) RNA activated protein kinase; WT, wild-type.

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efficiency of apoB100-containing lipoprotein assembly and secretion.

There is now growing evidence for a link between ER stress and dysregulation of the assembly and secretion of apoB100-containing lipoproteins. During the response to ER stress, unfolded or malfolded proteins retained in the ER lumen are retrotranslocated into the cytoplasm by ER-associated degradation (ERAD) machinery and degraded by the proteasome (19). Oyadomari et al. (20) reported that chaperone protein P58 dramatically increased apoB100 degradation by recruiting the chaperone protein HSP70 to the cytosolic face of the translocon. Ota, Gayet, and Ginsberg (21) recently demonstrated that treatment of McA-RH7777 cells with oleate at a high concentration (1.2 mM) or for a long period of time (16 h) could induce ER stress, which involved an upregulation of GRP78. Increased GRP78 level was accompanied by decreased apoB100 secretion, suggesting that there is an inverse relationship between lipid-loading-mediated ER stress and apoB100 secretion (21). Previous work in our laboratory has also shown a strong inverse association between GRP78 expression and apoB100 stability in ER of HepG2 cells (22). Interestingly, treatment of HepG2 cells, primary hamster hepatocytes, or McA-RH7777 cells with glucosamine increased levels of GRP78 and decreased cellular and secreted apoB100 (22, 23).

In this report, we provide new evidence that glucosamine-induced ER stress is also associated with alterations in apoB100 protein synthesis. The mammalian ER stress responses are mediated by PERK, activating transcription factor 6 (ATF6), and inositol requirement 1 (IRE1) pathways (19). We have found glucosamine-induced ER stress to be associated with increased phosphorylation of double stranded (ds) RNA activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) and α-subunit of eukaryotic initiation factor 2 (eIF2α), leading to impaired apoB100 synthesis.

EXPERIMENTAL PROCEDURES

Cell culture

HepG2 cells (1×10^6) were seeded on collagen-coated six-well plates in MEM containing 10% FBS and allowed to adhere for 4 h. The medium was replaced with high glucose DMEM (4.5 mg/ml; Multicell cat. no. 10013CV) containing 10% FBS and 4 mM glucosamine (Sigma-Aldrich, St. Louis, MO), and cells were incubated for 4 h at 37°C with 5% CO_2.

Immunoblot analysis

Following treatment with 4 mM glucosamine, the cultured cells were washed twice with PBS and lysed using solubilizing buffer (PBS containing 1% Nonidet P-40, 1% deoxycholate, 5 mM EDTA, 1 mM EGTA, 1 mM PMSF, 100 kallikrein-inactivating units/ml aprotinin, and phosphatase inhibitors, as described previously) (24). For experiments involving detection of phosphorylated proteins, cells were lysed in a special solubilizing buffer (solubilizing buffer + 100 mM sodium fluoride and 10 mM sodium pyrophosphate tetra basic decahydrate). Following SDS-PAGE, proteins were transferred electrophoretically for 16 h at 4°C onto polyvinylidene fluoride membranes. The membranes were blocked with a solution of 1% BSA, incubated with the indicated antibodies (see figure legends) and then incubated with appropriate secondary antibodies conjugated to horseradish peroxidase. Membranes were then covered in an enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech, Woodbridge, ON, Canada) for 1 to 5 min and exposed to Kodak Hyperfilm. Films were developed, and quantitative analysis was performed using an imaging densitometer (Bio-Rad, Mississauga, ON, Canada) (24). Anti-human apoB, anti-human apoE, and anti-albumin antibodies were obtained from Midland Bioproducts (Boone, IA). Monoclonal anti-KDEL antibody was from CalBiochem (San Diego, CA). Anti-phosphorylated-PERK, anti-phosphorylated-eIF2α, and anti-eIF2α antibodies were from Oncogene (Boston, MA).

Metabolic labeling of glucosamine-treated cells

After a 3 h treatment of HepG2 cells with 4 mM glucosamine, the cells were preincubated in methionine/cysteine-free MEM with 4 mM glucosamine at 37°C for 1 h, followed by pulse labeling with 100 μCi/ml [^{35}S] methionine for 1 h in the presence of various proteasomal inhibitors (see figure legends). Following the pulse, the medium was harvested for immunoprecipitation of secreted apoB100 or albumin (22). The cells were lysed using 500 μl solubilizing buffer, and cellular apoB100 was immunoprecipitated. In pulse-chase experiments, HepG2 cells were incubated in methionine/cysteine-free MEM in the presence or absence of varying amounts of inhibitors at 37°C for 1 h, labeled with 100 μCi/ml [^{35}S] methionine for 10 min, and then chased for 0, 5, 10, 15, 30, 60, or 120 min under the conditions described in the figure legends.

Transient transfection with PERK constructs

COS-7 or McA-RH7777 cells (5×10^5) were seeded in six-well plates 4 h before the experiments. After washing the cells once with 2 ml of sterile PBS, 1 μg of apoB15 cDNA and 1 μg of wild-type (WT) PERK cDNA or kinase inactive mutant (K618A) PERK cDNA (25) were cotransfected into the cells using 10 μl Lipofectamine™ (Life Technologies, Grand Island, NY) according to the manufacturer's protocol.

RT-PCR analysis of mRNA

Following treatment of HepG2 cells with 4 mM glucoamine for 4 h, total RNA was extracted using a commercially available kit (RNeasy, Qiagen, Mississauga, ON, Canada). First-strand cDNA was synthesized from 5 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen, Burlington, ON, Canada) (26). The resulting cDNA was subjected to 28 cycles of PCR amplification (denaturation at 95°C for 30 s; annealing at 55°C for 60s; extension at 72°C for 90 s). The primer pairs used for detecting mRNA levels are listed in **Table 1**.

Immunoprecipitation, SDS-PAGE, and fluorography

Immunoprecipitation was performed as described previously (22). 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.

Statistical analyses

Results are displayed as mean ± SD, $n \geq 3$. The unpaired, two-tailed *t*-test was used to assess differences between experimental groups and controls. Probability values of < 0.05 were considered to be statistically significant.

TABLE 1. cDNA sequence of primers used in RT-PCR for detecting human mRNAs of apoB, apoE, MTP, GRP78, XBP1(U), XBP1(S), ATF6, and 18SrRNA

	Gene Bank No. and Name		5′→3′	Product Size (bp)
apoB100	M14162 apoB100	F	AGACAGCATCTTCGTGTTTCAA	123
		R	ATCATTTAGTTTCAGCCCAGGA	
apoE	NM 000041 APOE	F	GGTCGCTTTTGGGATTACCT	133
		R	TGTAGGCCTTCAACTCCTTCAT	
MTP	NM 000253 MTTP	F	TCTGAGAGCACAGCGTTTACAT	144
		R	GTTGTTGAAAGCGTCACATGAT	
GRP78	NM 005347 HSPA5	F	ATGATGCTGAGAAGTTTGCTGA	134
		R	GGAAAGTTTACCTCCCAGCTTT	
XBP1(U) XBP1(S)	NM005080 XBP1(U) NM 001079539 XBP1(S)	F	GGAGTTAAGACAGCGCTTGG	XBP1(U) 168
		R	GAGATGTTCTGGAGGGGTGA	XBP1(S) 142
ATF6	NM007348 ATF6	F	GCTGGATGAAGTTGTGTCAGAG	126
		R	TGTTCCAACATGCTCATAGGTC	
18S rRNA	NR 003286 18S rRNA	F	TAAGTCCCTGCCCTTTGTACACA	71
		R	GATCCGAGGGCCTCACTAAAC	

RESULTS

Short-term glucosamine treatment of HepG2 cells induces GRP78 expression and reduces apoB100 secretion

Long-term (16 h) glucosamine treatment of HepG2 cells was previously shown to upregulate GRP78 and decrease apoB100 secretion from HepG2 cells (22). Here, we examined the effects of short-term (4 h) glucosamine treatment. HepG2 cells were treated with 0–16 mM glucoamine for 4 h, and immunoblotting was performed using anti-apoB or anti-KDEL antibodies. As shown in **Fig. 1A**, cellular and secreted apoB100 levels were reduced in a dose-dependent manner. At a concentration of 4 mM glucosamine, cellular and secreted apoB100 were significantly decreased to 62 ± 7% (n = 4, $P < 0.05$) and 43 ± 6% (n = 4, $P < 0.05$), respectively, compared with untreated control cells. The reduction in apoB100 protein mass was associated with a remarkable increase (3.4 ± 0.1-fold, n = 4, $P < 0.05$) in the expression of GRP78. By contrast, the levels of albumin, a major secretory protein of HepG2 cells, did not change following glucosamine treatment. ApoB100 mRNA level was unchanged by glucosamine treatment, indicating that the decrease in apoB100 protein mass was not caused by a drop in the amount of apoB100 mRNA (Fig. 1B). By contrast, the increase in GRP78 protein mass following glucosamine treatment was associated with a higher amount of its mRNA (4.3 ± 0.3-fold, n = 4, $P < 0.05$). These results suggest that similar to the long-term glucosamine treatment, glucosamine treatment for 4 h could interfere with apoB100 production and upregulate GRP78 expression, which is an indication of ER stress.

Short-term glucosamine treatment reduces the amount of newly synthesized apoB100, an effect only partially prevented by cotreatment with ALLN or lactacystin

Next, we examined the ability of proteasomal inhibitors, *N*-acetyl-leucinyl-leucinyl-nor-leucinal (ALLN) and clasto-lactocystin β-lactone (lactacystin), to prevent the loss of apoB100 induced by short-term (4 h) glucosamine treatment. The amount of [^{35}S]-labeled apoB100 recovered in the absence of glucosamine treatment was significantly greater in the presence of ALLN or lactacystin in both cell (**Fig. 2A**; 1.85-fold, n = 4, $P < 0.05$; 1.77-fold, n = 4, $P < 0.05$)

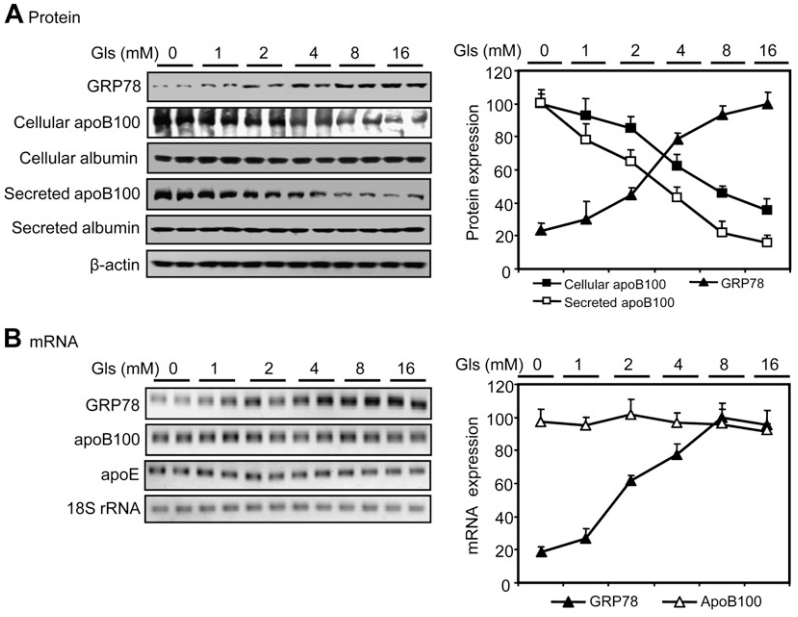

Fig. 1. Short-term glucosamine treatment impairs apoB100 biosynthesis. HepG2 cells (1×10^6) were treated with 0, 1, 2, 4, 8, or 16 mM glucosamine for 4 h. A: The media were collected and cells were lysed. Twenty micrograms of protein from each sample was analyzed by immunoblotting with antibodies against apoB, albumin, KDEL, and β-actin. B: mRNA levels were measured by RT-PCR as described in Experimental Procedures. The RT-PCR products of GRP78, apoB, and apoE from HepG2 cells were quantified and normalized to the amount of 18S rRNA product. In A and B, protein or mRNA levels were expressed as a percentage of the highest value for each factor.

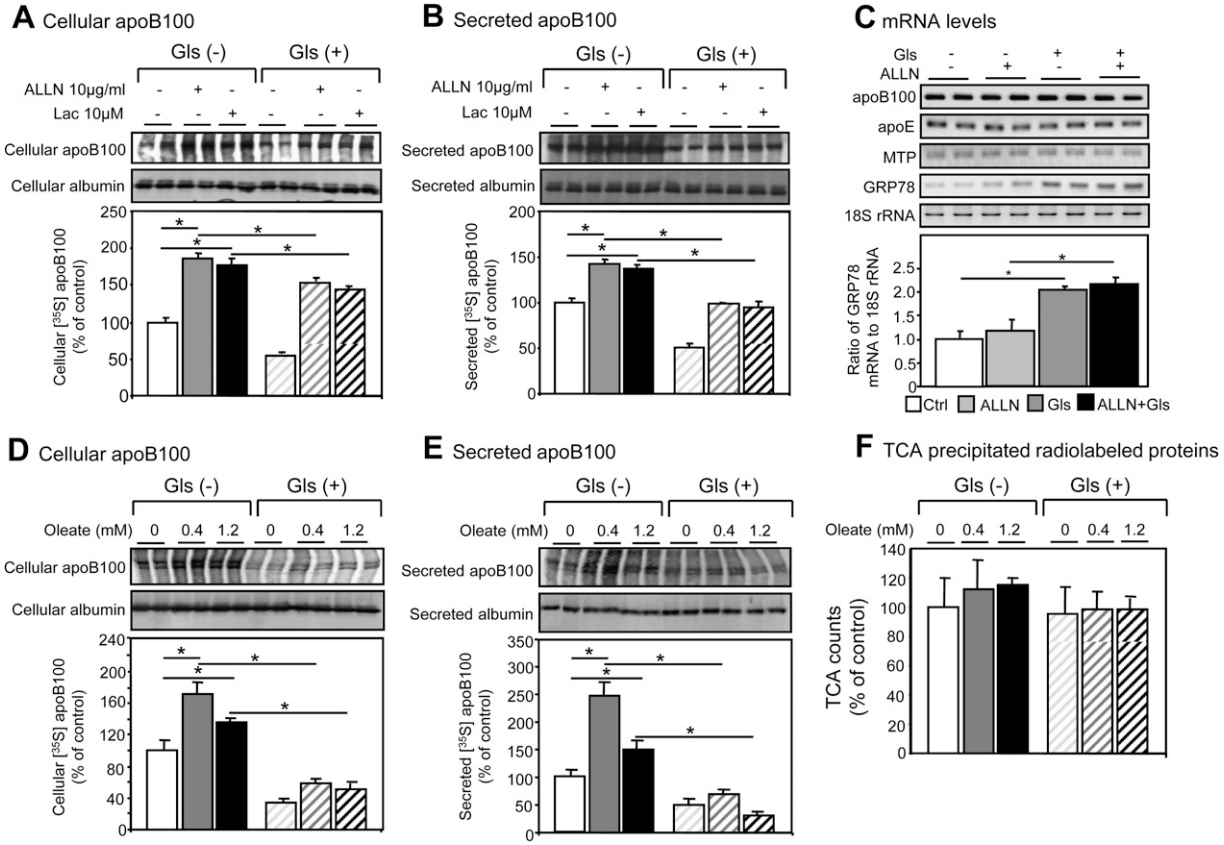

Fig. 2. Proteasomal inhibitors can only partially prevent the reduction in the amount of newly synthesized apoB100 induced by short-term glucosamine treatment. HepG2 cells (1×10^6) were untreated or treated with 4 mM glucosamine for 3 h, deprived of methionine and cysteine for 1 h in the presence or absence of 4 mM glucosamine and 20 μg/ml ALLN or 10 μM lactacystin, and then incubated with 100 μCi/ml [^{35}S] methionine for 1 h. A: Immunoprecipitated cellular [^{35}S]-labeled apoB100. B: Immunoprecipitated secreted [^{35}S]-labeled apoB100. C: mRNA levels were measured by RT-PCR as described in Experimental Procedures. The RT-PCR products of apoB, apoE, MTP, and GRP78 from HepG2 cells treated with 4 mM glucosamine for 4 h and then treated with 20 μg/ml ALLN in addition to the glucosamine for another 2 h were quantified, normalized to the amount of 18S rRNA, and expressed as a percentage of the control group. HepG2 cells (1×10^6) were also treated with 4 mM glucosamine in the presence of 0, 0.4, or 1.2 mM oleate for 3 h, incubated with methionine/cysteine depleted DMEM for 1 h, and then pulsed with 100 μCi/ml [^{35}S] methionine for 1 h. D: Immunoprecipitated cellular [^{35}S]-labeled apoB100. E: Immunoprecipitated secreted [^{35}S]-labeled apoB100. F: TCA precipitable radioactivity upon treatment with 0, 0.4, or 1.2 mM oleate. *$P < 0.05$.

and media fractions (Fig. 2B; 1.42-fold, n = 4, $P < 0.05$; 1.37-fold, n = 4, $P < 0.05$), respectively, compared with untreated control cells. The amount of [^{35}S]-labeled apoB100 recovered was also significantly greater in glucosamine-treated cells following the addition of ALLN or lactacystin. This increase was seen in both the cell (Fig. 2A; 1.52-fold, n = 4, $P < 0.05$; 1.44-fold, n = 4, $P < 0.05$) and media fractions (Fig. 2B, 0.98-fold, n = 4, $P < 0.05$; 0.94-fold, n = 4, $P < 0.05$). Importantly, however, the glucosamine-induced apoB100 loss could not be completely prevented by proteasome inhibition, suggesting that mechanisms other than proteasomal degradation may also be involved. These results are consistent with the inability of MG132, another proteasomal inhibitor, to completely block the loss of apoB100 observed with long-term (16 h) glucosamine treatment (22). As shown in Fig. 2C, no changes in apoB100, apoE, or MTP mRNA levels were observed in the presence of ALLN and/or glucosamine. The only change in mRNA detected was the increase in GRP78 mRNA upon glucosamine treatment that was also shown in Fig. 1B.

Pulse-labeling experiments were also conducted in HepG2 cells pretreated with 4 mM glucosamine and/or 0.4 or 1.2 mM oleate for 4 h. As expected, oleate treatment increased both cellular (Fig. 2D) and secreted (Fig. 2E) apoB100 levels following a 1 h pulse compared with untreated cells (n = 4, $P < 0.05$). However, in the presence of glucosamine, oleate treatment could not increase the cellular or secreted levels of apoB100 to the same extent seen without glucosamine treatment (n = 4, $P < 0.05$). Therefore, addition of fatty acids, which could increase triglyceride production, was not able to overcome the effects of glucosamine. Oleate treatment has been shown previously to promote production of apoB100-containing lipoproteins by decreasing the amount of poorly lipidated and misfolded apoB100 that would be targeted for proteasomal degradation (1). These results provide further evidence that induction of proteasomal degradation is not entirely responsible for the decrease in apoB100 levels caused by glucosamine treatment. In addition, cotreatment with 4 mM glucosamine and 0.4 or 1.2 mM oleate for 4 h had no

effect on total protein synthesis by HepG2 cells, as determined by total TCA-precipitable radioactity (Fig. 2F), indicating that there is no global effect on protein synthesis at the concentrations tested.

Glucosamine exerts its effect early during apoB biosynthesis

Detailed pulse-chase experiments using a 10 min pulse and chase times of 0 to 120 min were carried out in the presence of ALLN and glucosamine to assess the effects of these treatments on the production and loss of apoB100. As shown in **Fig. 3A**, when proteasomal degradation was blocked by ALLN, apoB100 decay rates did not significantly differ between control and glucosamine-treated cells. Importantly, however, the total accumulation of [^{35}S]-labeled apoB100 was substantially reduced at early chase time points (0 to 15 minutes) in glucosamine-treated cells (the asterisk indicates differences with the control values for each time point), suggesting that the effect of glucosamine on apoB100 levels may occur during its synthesis (translationally). In contrast, glucosamine treatment did not affect the levels of albumin, a control secretory protein, under the same experimental conditions (Fig. 3B). Although glucosamine treatment has been shown to promote proteasomal degradation of apoB100, which is a cotranslational and posttranslational process, this pathway was inhibited by the addition of ALLN, suggesting that in these experiments glucosamine was causing an early loss of apoB100 by a mechanism other than proteasomal degradation. To further investigate this possibility, we next

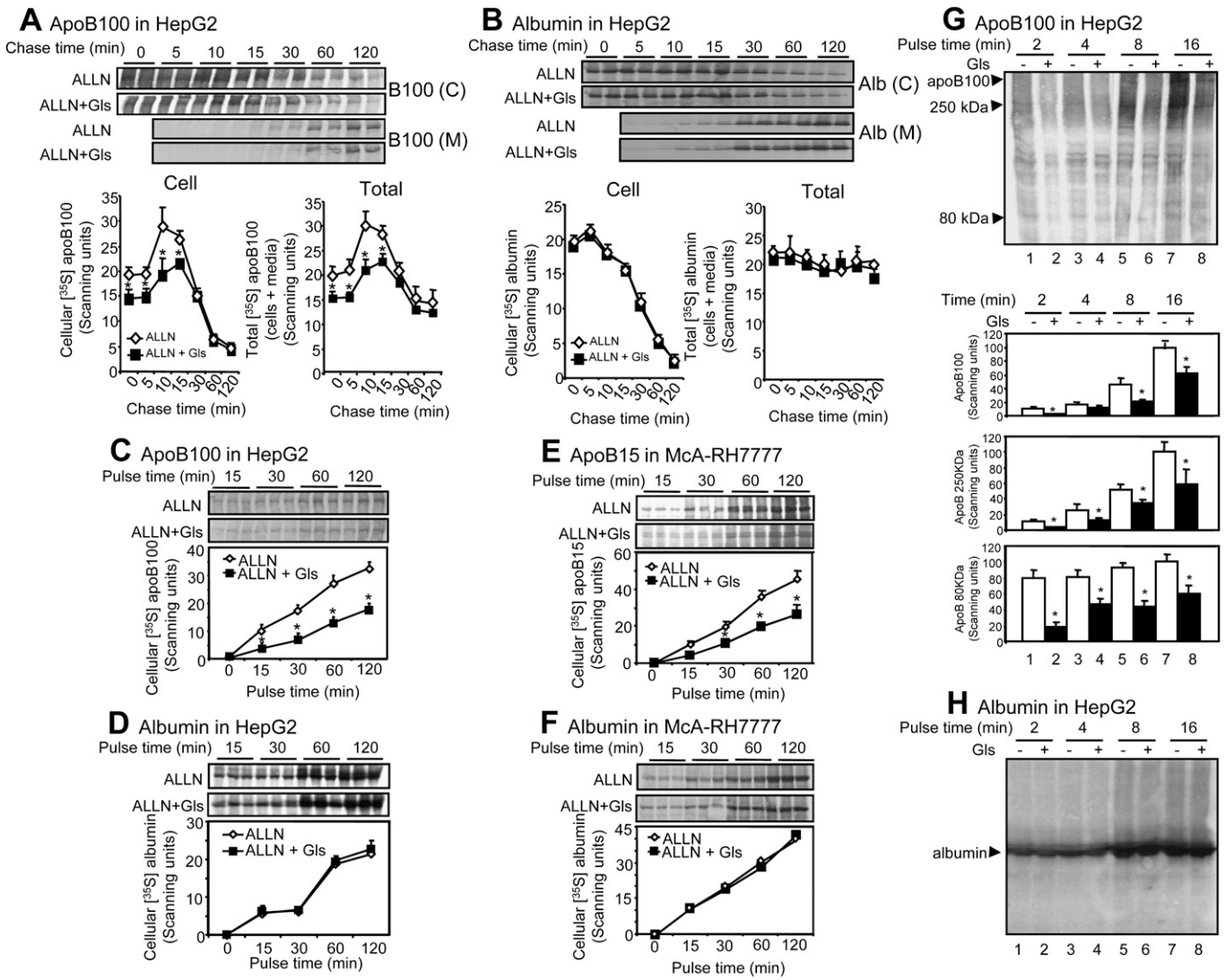

Fig. 3. Glucosamine exerts its effect early during apoB100 biosynthesis. HepG2 or McA-RH7777 cells (1×10^6) were untreated or treated with 4 mM glucosamine for 3 h and then incubated with methionine/cysteine-depleted DMEM with or without glucosamine for 1 h in the presence of 20 μg/ml ALLN. A, B: HepG2 cells were labeled with 100 μCi/ml [^{35}S] methionine for 10 min and then chased for 0, 5, 10, 15, 30, 60, or 120 min. ALLN with or without glucosamine was present during the entire pulse-chase period. C, D: HepG2 cells were labeled with 100 μCi/ml [^{35}S] methionine for 0, 15, 30, 60, or 120 min in the presence of ALLN. E, F: McA-RH7777 cells were transiently transfected with apoB15 for 40 h and then treated similarly to the HepG2 cells in C and D. G, H: HepG2 cells were labeled with 100 μCi/ml [^{35}S] methionine for 2, 4, 8, or 16 min in the presence of ALLN. The cell lysates were first immunoprecipitated with anti-human apoB antibody (A, C, E, and G) and then with anti-human albumin antibody (B, D, F, and H). *$P < 0.05$.

performed a detailed pulse experiment using pulse times of 0 to 120 min to determine whether glucoamine treatment was affecting apoB synthesis. As demonstrated in Fig. 3C, the accumulation of [^{35}S]-labeled apoB100 was significantly reduced in glucosamine-treated HepG2 cells beginning at 15 min and continuing up to 120 min of radiolabeling (n = 3, $P < 0.05$), despite addition of ALLN in both the prepulse and pulse periods. In contrast, glucosamine treatment did not affect the levels of albumin, a control secretory protein, under the same experimental conditions (Fig. 3D). Similar results were obtained in McA-RH7777 cells transiently transfected with apoB15 and treated with glucosamine (Figs. 3E, F). ApoB15 was used because such small forms of apoB have been shown to be resistant to proteasomal degradation (27). These results indicate further that proteasomal degradation is unlikely to be responsible for the early decrease in apoB100 levels that has been observed upon short-term glucosamine treatment in the presence of ALLN. These results led to the hypothesis that glucosamine treatment might also be impairing translation of apoB100. To further investigate this possibility, radiolabeling experiments with short pulse periods were conducted. As shown in Fig. 3G in the presence of ALLN, the appearance of newly synthesized (nascent) apoB polypeptide chains at 80 or 250 kDa was reduced in glucosamine-treated cells within the first 2 to 4 min of radiolabeling (bars 1 and 3 vs. 2 and 4). The amount of newly synthesized apoB100 was also markedly suppressed in glucosamine-treated cells after 8 to 16 min of radiolabeling (bars 5 and 7 vs. 6 and 8). No changes were observed in the amount of the control protein, albumin, with glucosamine treatment (Fig. 3H). These results may suggest that translation initiation rate of apoB (or possibly elongation rate) is reduced and are consistent with apoB100 translation being attenuated by glucosamine treatment.

Short-term glucosamine treatment activates the PERK pathway

Further experiments were conducted to determine the mechanism mediating glucosamine-induced ER stress and attenuation of apoB100 synthesis. As shown in **Fig. 4A**, following 4 h of glucosamine treatment, the levels of phosphorylation of PERK and eIF2α were significantly increased by 3.6-fold (n = 4, $P < 0.05$) and 2.1-fold (n = 4, $P < 0.05$), respectively. However, if the treatment was continued for 16 h, the levels of phosphorylation of PERK and eIF2α were significantly decreased to 38% ($P < 0.05$) and 65% ($P < 0.05$), respectively, compared with the 4 h treatment. These results suggest that short-term (4 h) glucosamine treatment activated the PERK pathway but that the activation was not maintained with long-term glucosamine treatment. We also assessed two other ER stress response pathways, ATF6 and IRE1. As shown in Fig. 4B, the ATF6 mRNA level began to increase following 4 h of 4 mM glucosamine treatment, but the increase was not statistically significant until 16 h of treatment. With 4 h of glucosamine treatment, the amount of the spliced form of XBP1 mRNA, which is generated upon activation of the IRE1 pathway of the ER stress response, was only 42% ($P < 0.05$) of the amount formed with 4 h of tunicamycin treatment (a positive control), and even less of the spliced form of XBP1 mRNA was present following 16 h of glucosamine treatment. The GRP78 mRNA level was significantly increased in HepG2 cells treated with either 5 μg/ml tunicamycin for 4 h or 4 mM glucosamine for 4 or 16 h. These results suggest that both 4 and 16 h treatments with 4 mM glucosamine induced ER stress. The upregulation of

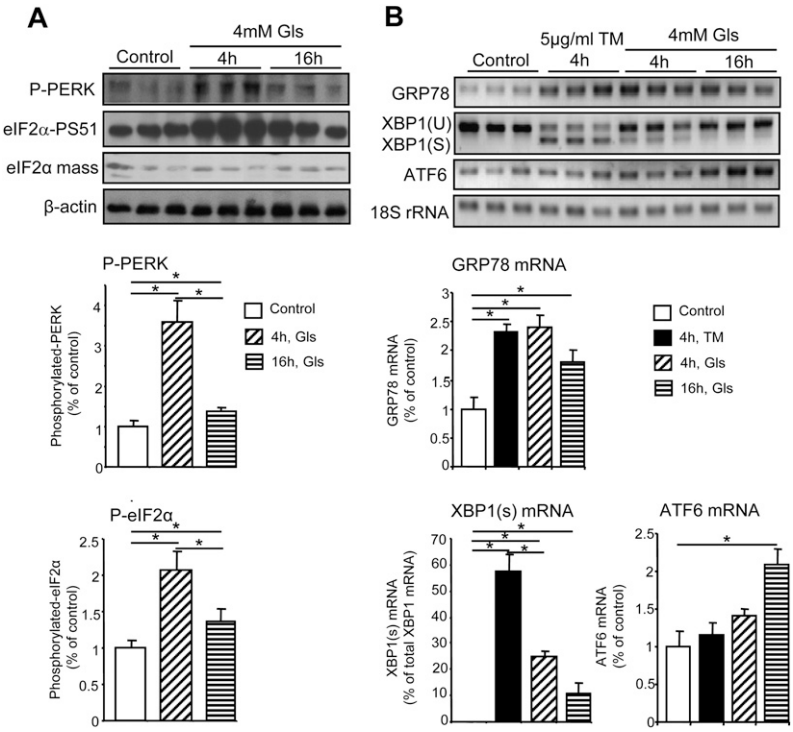

Fig. 4. Short-term glucosamine treatment induces ER stress responses. A: HepG2 cells (1×10^6) were treated with or without 4 mM glucosamine for 4 h. The cells were lysed and 40 μg of total protein from each sample was analyzed by immunoblotting with antibodies against phosphorylated PERK, phosphorylated eIF2α, eIF2α, and β-actin. B: HepG2 cells (1×10^6) were treated with 4 mM glucosamine for 4 or 16 h. Tunicamycin treatment (5 μg/ml) was used as a positive control. mRNA levels were measured by RT-PCR as described in Experimental Procedures. The RT-PCR products of spliced XBP-1, ATF6, and GRP78 from HepG2 cells were quantified and normalized to the amount of 18S rRNA. *$P < 0.05$.

GRP78 could have been induced by a combination of the ER stress pathways, most likely the IRE1 and ATF6 arms of the pathway, which play a role in the upregulation of chaperone proteins (19). However, the inhibition of apoB100 translation observed with 4 h of glucosamine treatment was likely a consequence of activation of the PERK pathway since there was little activation of the IRE1 and ATF6 pathways at this time point and since it is the PERK pathway that plays a role in inhibiting general translation through phosphorylation of eIF2α to try to reduce the burden on the ER under ER stress conditions (19).

Glucosamine-induced PERK activation attenuates apoB100 synthesis

To verify that the glucosamine-induced reduction of apoB100 synthesis was associated with PERK activation, we cotransfected COS-7 cells with plasmids encoding apoB15 cDNA and Myc-tagged WT PERK cDNA, Myc-tagged kinase inactive mutant (K618A) PERK cDNA, or no cDNA (mock) to evaluate the effects on the synthesis of apoB15. As demonstrated in the left panel of **Fig. 5A**, the mass of the WT and mutant forms of PERK was notably increased following transient expression of the constructs in both control and glucosamine-treated COS-7 cells. By contrast, there was no change in β-actin, a control protein. Interestingly, increased phosphorylation of eIF2α on serine 51 (Fig. 5A, right panel, bar 2) following transient expression of WT PERK was associated with a 35% reduction in the amount of newly synthesized apoB15 ($P < 0.05$) compared with mock-transfected cells (Fig. 5B, bar 1 vs. 2), suggesting that the translation of apoB15 may have been attenuated due to impaired eIF2α function. In contrast, transient expression of kinase inactive PERK led to a significant increase in newly synthesized apoB15 (178% of control, $P < 0.05$) (Fig. 5B, bar 1 vs. 3). This likely occurred due to the dominant-negative property of the mutant PERK con-

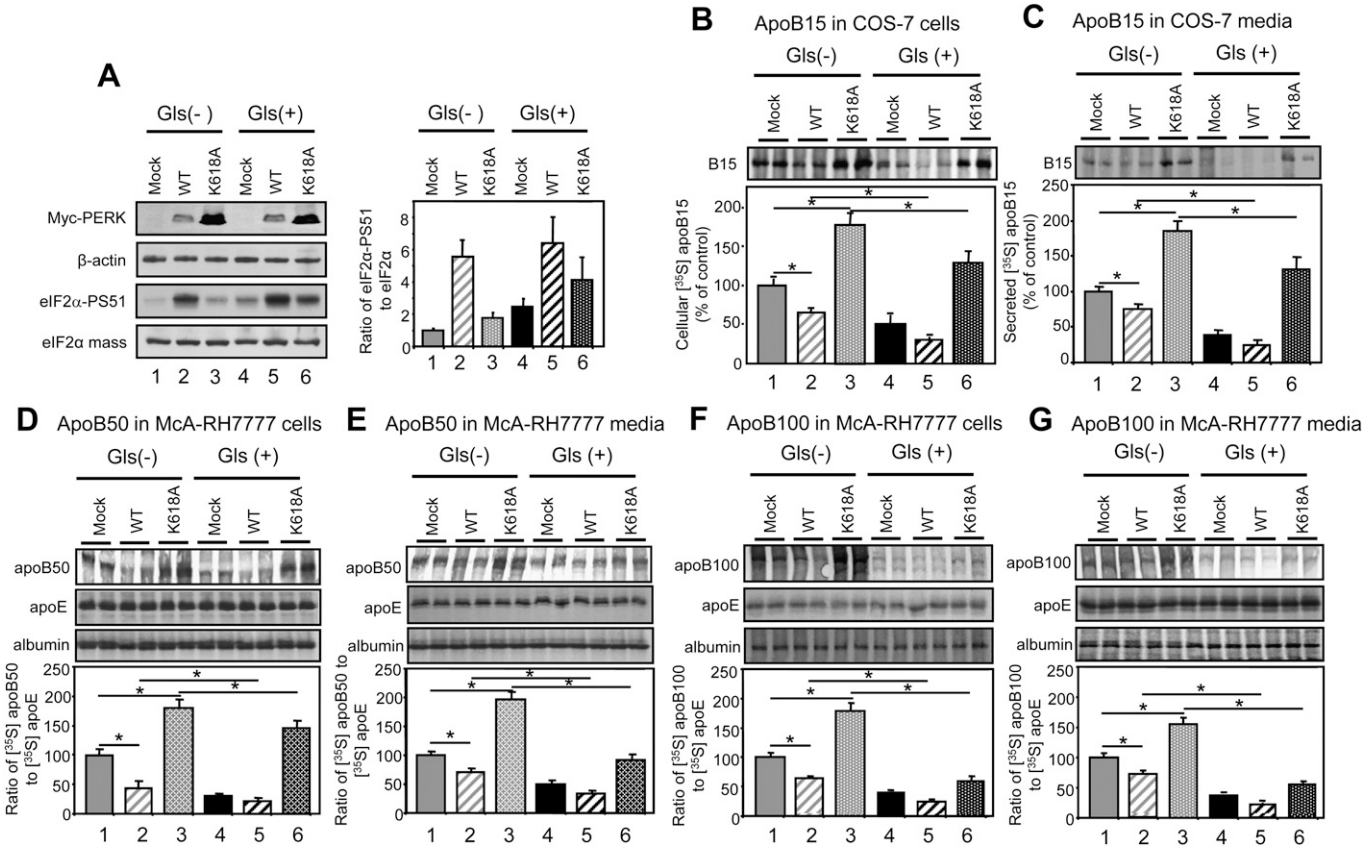

Fig. 5. Glucosamine-induced PERK activation attenuates apoB100 synthesis. A–C: COS-7 cells (1×10^6) were cotransfected with 1 μg of a plasmid encoding apoB15 cDNA and 1 μg of a plasmid encoding WT PERK cDNA, kinase inactive mutant (K618A) PERK cDNA, or no cDNA (mock). Cells were incubated for 40 h and then treated with or without 4 mM glucosamine for 3 h. A: The cells were incubated in the presence or absence of 4 mM glucosamine for 1 h. Cell lysates were analyzed by immunoblotting with antibodies against the myc-tag on the PERK constructs, phosphorylated eIF2α, eIF2α, and β-actin ($P < 0.05$). B, C: The cells were incubated with methionine/cysteine-depleted DMEM for 1 h and then radiolabeled with 100 μCi/ml [^{35}S] methionine for 1 h in the presence of 20 μg/ml ALLN. The prepulse and pulse steps were performed in the presence or absence of 4 mM glucosamine. The cell lysates (B) and conditioned media (C) were immunoprecipitated with anti-human apoB antibody. McA-RH7777 cells (1×10^6) stably expressing human apoB50 (D and E) (28) or normal McA-RH7777 cells (F and G) were transfected with 1 μg of WT PERK or K618A PERK cDNA, incubated for 40 h, and then incubated with or without 4 mM glucosamine for 3 h. The cells were then starved of methionine/cysteine for 1 h and labeled with 100 μCi/ml [^{35}S] methionine for 1 h in the presence of 20 μg/ml ALLN with or without 4 mM glucosamine. The cell lysates (D and F) and conditioned media (E and G) were first immunoprecipitated with anti-human apoB100 antibody, followed by a second immunoprecipitation with anti-apoE antibody and a third immunoprecipitation with anti-albumin antibody. Bands corresponding to proteins of interest were scanned and quantified. $P < 0.05$.

struct, which should have inhibited any basal activation of the pathway, thereby removing translation inhibition and increasing the translation of apoB15. Unexpectedly, there was a slight increase in phosphorylation of eIF2α upon expression of the mutant form of PERK. The reason for this is unclear. Short-term glucosamine treatment enhanced activation of the PERK pathway and eIF2α phosphorylation in cells overexpressing WT PERK, which was associated with a 30% reduction ($P < 0.05$) in apoB15 level (Fig. 5B, bar 2 vs. 5). In contrast, the activation of the PERK pathway by glucosamine treatment appeared to negate the inhibition of the pathway by the kinase inactive form of PERK. This is supported by lower apoB15 production in cells treated with glucosamine and expressing K618A PERK compared with that in cells expressing K618A PERK alone (Fig. 5B, bar 3, 178% vs. bar 6, 129%, $P < 0.05$). ApoB15 secretion was also similarly affected (Fig. 5C). These experiments were repeated in McA-RH7777 cells stably expressing human apoB50 (28) (Fig. 5D, E) and also in normal McA-RH7777 cells expressing endogenous apoB100 (Fig. 5F, G). Transient expression of WT PERK cDNA significantly suppressed the synthesis apoB50 or apoB100 but had no effect on control proteins, apoE or albumin (Fig. 5D–G). These results provide further evidence that glucosamine may impair all apoB forms tested, including apoB15, apoB50, and apoB100 synthesis via a PERK-dependent mechanism.

DISCUSSION

In response to ER stress in mammalian cells, three pathways regulate induction of chaperone expression, ERAD components, and translational attenuation (19). These coordinated signaling events allow the cell to respond to excessive accumulation of misfolded proteins in the ER by increasing the folding capacity, reducing protein synthesis, and enhancing proteasomal degradation. Previous work in our laboratory demonstrated that 16 h glucosamine treatment of HepG2 cells and other hepatocyte cell models can induce ER stress, which is associated with increased levels of GRP78 and a decreased rate of apoB100 secretion due to increased proteasomal and nonproteasomal degradation (22, 23). In this study, we report that glucosamine-induced ER stress is also associated with suppression of apoB100 synthesis via a mechanism involving increased PERK signaling. We first found that short-term (4 h) glucosamine treatment of HepG2 cells significantly reduced both cellular and secreted apoB100. Proteasomal inhibitors, ALLN or lactacystin, could only partially protect against the suppressive effects of glucosamine on total apoB100 levels, suggesting involvement of other cotranslational or posttranslational mechanisms. Monitoring of apoB100 biosynthesis using detailed pulse-chase experiments revealed a significantly reduced level of apoB100 accumulation at early time points, indicating that glucosamine exerted its suppressive effects very early in apoB100 biosynthesis. Importantly, with short-term glucosamine treatment in the presence of ALLN, the rate of disappearance of apoB100 from the cells during the 2 h chase period was similar between control and glucosamine-treated cells, despite significantly lower accumulation of apoB100 with glucosamine treatment. These observations suggest that under these experimental conditions, the predominant effect of glucosamine may have been at the level of apoB100 protein synthesis.

To further investigate ER-stress-associated alterations in apoB100 protein synthesis, experiments were conducted using a shorter apoB15 construct. ApoB15 is known to be relatively stable and resistant to intracellular degradation. Stability of apoB molecules has previously been shown to be inversely proportional to the length of the nascent polypeptide (27). Experiments involving cotransfection of apoB15 cDNA with WT PERK cDNA or kinase inactive mutant PERK cDNA indicate that apoB15 synthesis and cellular accumulation can be modulated by altering PERK activity. ApoB15 levels were markedly reduced in cells overexpressing WT PERK, while apoB15 expression was increased in cells overexpressing kinase inactive PERK. The same results were obtained following transient transfection of PERK cDNA into McA-RH7777 cells stably expressing apoB50 or normal McA-RH7777 cells expressing endogenous apoB100. It is interesting that although activation of the PERK pathway is associated with a global decline in translation initiation via phosphorylation of eIF2α, translation of apoB100 appears to be particularly sensitive to modulation by the PERK pathway. In our studies, two other hepatic-specific control proteins, albumin and apoE, appeared to be less affected under the same experimental conditions (at glucosamine concentrations of 4 or 16 mM). At higher concentrations (32 mM for 4 h), down-regulation of secretion of all three secretory proteins (apoB100, apoE, and albumin) was observed with a greater effect observed on apoB100 (data not shown), suggesting that at higher concentrations, glucosamine has a global inhibitory effect on all secretory proteins. Ota, Gayet, and Ginsberg (21), also reported that oleate (at concentrations >1.2 mM) induced hepatic ER stress in rat McA-RH7777 cells leading to increased apoB100 degradation but appeared to have no effects on apoB48, albumin, or apoA-I levels. The underlying mechanisms for this high sensitivity of apoB100 to ER stress are unknown. We postulate that at lower concentrations of glucosamine (up to 16 mM), apoB100 may be more sensitive to glucosamine treatment (compared with other hepatic secretory proteins, such as apoE and albumin) possibly due to the presence of an N-linked glycosylation site at its N terminus and its large size. N-linked glycosylation has been shown to intrinsically accelerate folding and enhance stability (29). As previously shown (23), glucosamine can interfere with N-linked glycosylation of apoB cotranslationally and induce misfolding, possibly leading to inhibition of translation and subsequent proteasomal degradation. Some proteins, such as apoE, appear to be less dependent on N-linked glycosylation for folding and secretion (30), possibly due to their smaller size.

MTP is an important ER resident chaperone that lipidates the nascent apoB100 protein and facilitates VLDL

assembly (16). Pan et al. (31) reported that treatment of HepG2 cells with an MTP inhibitor (CP-10447) led to translocation arrest of apoB100 at the ER and had a negative effect on the synthesis of apoB100 at the stage of peptide elongation. However, Liao et al. (32) showed that blocking MTP activity interferes with the secretion of apoB100-containing proteins without causing retention of apoB at the ER or ER stress, suggesting that MTP activity is disassociated from ER stress. These experiments and previous studies have demonstrated that suppression of apoB100 biosynthesis with glucosamine treatment did not involve changes in MTP mRNA or protein expression (22). Therefore, MTP was unlikely to be involved in the loss of apoB100 observed with glucosamine treatment.

We previously found that not only GRP78 but also GRP94 was upregulated in HepG2 cells following glucosamine treatment (22). We postulate that the ATF6 pathway is activated upon glucosamine-induced ER stress, leading to increased expression of GRP78 and GRP94. ATF6, a transmembrane transcription factor, is translocated to the Golgi apparatus and cleaved by proteases, such as S1P and S2P, upon ER stress, leading to enhanced transcription of ER chaperones, such as GRP78 and GRP94 (33). Interestingly, lipidated nascent apoB100 molecules are transported to the Golgi associated with network of ER molecular chaperones, including GRP78 and GRP94 (18). Alterations in ER concentrations of these chaperones are thus expected to exert major effects on ER biogenesis of apoB100 and its nascent lipoprotein particles. Our current results suggest that XBP-1 and ATF6 may also play a role in the increased GRP78 mRNA and protein levels observed with short-term glucosamine-induced ER stress.

The ER-stress-induced IRE1 pathway may also play a role in regulating apoB100 biogenesis at the ER. IRE1, an ER transmembrane RNase, senses ER stress via its ER lumen domains and splices XBP1 premRNA via its cytosolic domains. The spliced form of XBP1 mRNA is translated into a transcription factor that induces the expression of proteins involved in lipid synthesis and ER biogenesis and enhances the expression of ER chaperones such as GRP78, p58IPK, ERdj4, PDI-P5, and HEDJ (34–36). XBP1 also activates transcription of ERAD component genes, such as EDEM, HRD1, Derlin-2, and Derlin-3, through a *cis*-acting, unfolded protein response element (21). XBP1 was recently shown by Ota, Gayet, and Ginsberg (21) to mediate the ER stress inducing effects of oleate at high concentrations and the resulting suppression of apoB100 secretion. The XBP1 pathway may also mediate some of the effects of glucosamine-induced ER stress. The IRE1 pathway via XBP1 likely played a role along with ATF6 in the upregulation of GRP78 mRNA and protein observed with glucosamine treatment. GRP78 upregulation has been found previously to promote proteasomal degradation of apoB100 (22) and therefore under conditions where proteasomal inhibitors were not present, could have played a role in the loss of apoB100 that occurred.

In summary, induction of ER stress by glucosamine appears to alter apoB100 biogenesis in the ER via multiple mechanisms. Induction of ER stress by glucosamine treatment appears to lead to *a*) PERK phosphorylation and inactivation of eIF2α, causing translational attenuation of apoB100 synthesis; *b*) activation of ATF6 and IRE1 pathways, thus inducing increased ER chaperone capacity and an increase in ERAD components; *c*) accelerated degradation of misfolded apoB100 molcules via the proteasomal pathway (22); and *d*) increased posttranslational, nonproteasomal degradation of apoB100 via the postendoplasmic reticulum presecretory proteolysis pathway (23).

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