Glucosamine-Induced Endoplasmic Reticulum Stress Promotes ApoB100 Degradation
Evidence for Grp78-Mediated Targeting to Proteasomal Degradation

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**Objective**—To investigate the role of glucosamine-mediated endoplasmic reticulum (ER) stress and Grp78 (BiP) in the intracellular degradation of apolipoprotein B100 (apoB100) in cultured hepatocytes.

**Methods and Results**—Glucosamine treatment (2.5 to 10 mmol/L) of HepG2 cells increased levels of the ER chaperones, 78-kDa glucose-regulated protein (Grp78) and Grp94, in a dose-dependent manner and led to significant decreases in both cellular and secreted apoB100 by up to 97% ($P<0.01$). In contrast, no changes were observed in ER resident (ER60, PTP-1B) or secretory (albumin, apoE) control proteins. Glucosamine-induced apoB degradation was similarly observed in primary hamster hepatocytes and McA-RH7777 cells. Glucosamine treatment led to reduced translocation efficiency of apoB100 in the ER and enhanced its ubiquitination and proteasomal degradation. Adenoviral overexpression of Grp78 also led to significantly decreased levels of newly synthesized apoB100 in a dose-dependent manner ($P<0.01$). Grp78-induced downregulation of apoB100 was sensitive to inhibition by the proteasome inhibitor, lactacystin, but not lysosomal protease inhibitors, E64 and leupeptin, suggesting that overexpression of Grp78 selectively induced proteasomal degradation of apoB100.

**Conclusion**—These findings suggest that binding and retention by Grp78 may play a critical role in proteasomal targeting and the ER quality-control of misfolded apoB. Interaction with core lipoprotein lipids may facilitate apoB transport out of the ER by reducing Grp78-mediated ER retention. (Arterioscler Thromb Vasc Biol. 2005;25:571-577.)

**Key Words:** apolipoprotein B ☐ degradation ☐ glucosamine ☐ Grp78 ☐ proteasome

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Hepatic biogenesis of apoB is a complex process involving regulation by multiple post-transcriptional control mechanisms. Intracellular availability of core lipoprotein lipid substrates, particularly triglyceride, appears to dictate the intracellular fate of newly synthesized apoB protein. In the absence of lipid, a significant proportion of newly synthesized apoB100 is degraded in cultured hepatoma cells, as well as in primary hepatocytes from hamsters, rats, and rabbits. The bulk of apoB degradation appears to be mediated by the ubiquitin–proteasome degradative system. High exogenous free fatty acid flux (particularly in cultured hepatocytes) or SREBP1-mediated de novo lipogenesis appear to protect apoB from proteasomal degradation. Ubiquitination and proteasomal degradation of apoB begins cotranslationally and involves the interaction of misfolded apoB with cytosolic chaperones, Hsp70 and Hsp90. Association with these cytosolic chaperones may be important in unfolding and subsequent targeting of the apoB polypeptide to the ubiquitin–proteasome pathway.

Mechanisms that target misfolded apoB or luminal lipoprotein-associated apoB to either proteasomal or nonproteasomal degradative pathways are currently unknown. Ample evidence is available showing the association of newly synthesized apoB polypeptide with endoplasmic reticulum (ER) chaperones. Several laboratories have observed that apoB100 is tightly associated with the 78-kDa glucose-regulated protein/immunoglobulin heavy chain-binding protein (Grp78/BiP) in the ER from HepG2 cells. Despite these findings, the role of Grp78 in hepatic apoB regulation is currently unknown. It is well-established that Grp78 is an ER stress sensor that may be upregulated transcriptionally as a result of an accumulation of misfolded polypeptides in the ER lumen. Grp78 binds transiently to newly synthesized proteins in the ER and more permanently to misfolded unglycosylated proteins whose transport from the ER is blocked. Overexpression of Grp78 is known to cause selective retention of a variety of proteins.
also functions to maintain the permeability barrier of the ER membrane by sealing the luminal side of the translocon pore before and during early translocation.20

Glucosamine treatment has been previously shown to upregulate several ER stress proteins including Grp78,21 suggesting the induction of ER stress. In the current report, we have examined the mechanisms by which Grp78 modulates the hepatic biogenesis of apoB100 under conditions of ER stress induced by glucosamine treatment of HepG2 cells, as well as after specific adenovirus-mediated overexpression of Grp78.

**Methods**

**Cell Culture and Glucosamine Treatment**

HepG2 cells (8×105) were seeded on collagen-coated 6-well plates in α-MEM medium containing 10% FBS and allowed to adhere for 4 hours. The medium was replaced by serum-free low-glucose DMEM (Multicell catalog number10014CV), and the cells were incubated for 16 hours at 37°C with 5% CO2. The medium was replaced with 10% fetal bovine serum high glucose DMEM (4.5 mg/mL, Multicell catalog number10013CV) in the presence of a varying amount of glucosamine (Sigma, St. Louis, Mo) for 16 hours at 37°C in 5% CO2.

**Infection of HepG2 Cells With Recombinant Adenovirus**

Three recombinant adenoviruses were used in this study; AdGrp78, encoding full-length human Grp78 cDNA;22 AdrER-60, encoding rat ER-60 cDNA;23 and Adβ-gal, encoding β-galactosidase cDNA.23 Adenoviruses were amplified and titrated in HEK293 cells by multiplicity of infection (moi) test. Purified adenoviruses were aliquoted and stored at −80°C until use. HepG2 (5×105) cells were seeded on collagen-coated 6-well plates. Four hours after seeding, cells were infected with AdGrp78 at moi from 1 to 20. AdrER-60 or Adβ-gal infection were used as adenovirus control and phosphate-buffered saline used as a noninfected control.

**Immunoblot Analysis**

After infection with recombinant adenovirus or treatment with glucosamine, the cultured cells were washed twice with phosphate-buffered saline and lysed using solubilizing buffer A (phosphate-buffered saline containing 1% Nonidet P-40, 1% deoxycholate, 5 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L PMSF, 100 kallikrein-inactivating units/mL aprotinin).23 Immunoblot analysis was performed as described.23 Anti-human apoB, anti-KDEL, anti-albumin, and anti-apoE antibodies were obtained from (Midland Bioproducts, Boone, Iowa). Monoclonal anti-KDEL was from Calbia (San Diego, Calif). Anti-PTP-1B was from Oncogene (Boston, Mass). Anti-ubiquitin antibody was from Stressgen (Victoria, BC, Canada). Anti-MTP antibody was a gift from Bristol Myers Squibb. Anti-rat ER-60 antibody was a gift from Dr Reiko Urade.

**Metabolic Labeling of Glucosamine-Treated or Adenovirus-Infected Cells**

In pulse-chase experiments, HepG2 cells were incubated in methionine/cysteine-free MEM in the presence or absence of varying amount of inhibitors at 37°C for 1 hour and labeled with 50 to 100 μCi/ml [35S]methionine/cysteine for 15 to 30 minutes, then chased for 0, 5, 10, 15, 30, 60, and 120 minutes under the conditions described in the Figure legends. HepG2 permeabilization and trypsin sensitivity assays were performed as previously described.24

**Immunoprecipitation, SDS-PAGE, and Fluorography**

Immunoprecipitation was performed as described previously.23 The gels were fixed and saturated with Amplify (Amersham Pharmacia Biotech) before being dried and exposed to Kodak Hyperfilm at −80°C for 1 to 4 days. Films were developed and quantitative analysis of apoB100 bands was performed using an imaging densitometer.

**Results**

**Glucosamine-Induced Grp94 and Grp78 Expression Results in a Dose-Dependent Decrease in Cellular and Secreted ApoB100 in HepG2 Cells**

After 16-hour glucosamine treatment, the mass of both Grp94 and Grp78 was significantly increased in a dose-dependent manner. Grp94 and Grp78 were increased in a dose-dependent manner by up to 97.2±3.9% (P<0.01) and 97.2±4.0% (P<0.01), respectively, with 10 mmol/L glucosamine treatment. In contrast, both secreted apoB100 mass and cellular apoB100 mass were respectively decreased to 3.0±0.7% (P<0.01) or 14.3±1.6% (P<0.01) of control levels at 10 mmol/L glucosamine treatment (Figure 1A). There were no significant changes in the mass of control proteins, albumin, the ER resident protein ER-60,23 and protein tyrosine phosphatase 1B (PTP-1B) on treatment of HepG2 cells with up to 10 mmol/L glucosamine (Figure 1B).

**Glucosamine Treatment Inhibits the Accumulation of Newly Synthesized ApoB100 in HepG2 Cells**

The direct effect of glucosamine on newly synthesized apoB100 accumulation in HepG2 cells was examined using cells that were treated in the presence or absence of 5 mmol/L glucosamine for 16 hours. After 1 hour of metabolic labeling (Figure 2A, top), both radiolabeled secreted apoB100 and cellular apoB100 were significantly reduced in cells treated with glucosamine compared with those in untreated HepG2 cells (P<0.05). In contrast, there was no effect on the 2 secreted control proteins, albumin or apoE (P>0.05), on treatment with 5 mmol/L glucosamine (Figure 2B and 2C). There was also no difference in the incorporation of radiolabel into total protein between treated and untreated HepG2 cells (data not shown). A pulse-chase experiment was also performed in HepG2 cells treated with 5 mmol/L glucosamine and apoB100 accumulation monitored for up to 2 hours. ApoB100 accumulation was significantly lower.
throughout the chase beginning from early (5 minutes; \(P<0.01\)) to late (120 minutes; \(P<0.05\)) chase times (Figure 2D), suggesting that glucosamine treatment may inhibit apoB100 synthesis and enhance its intracellular degradation.

Pulse-chase experiments were also performed using HepG2 cells pretreated in the presence or absence of 5 mmol/L glucosamine and in the absence or presence of 360 \(\mu\)mol/L oleate (Figure 2E). As expected, glucosamine treatment caused decreases in cellular and secreted apoB at the 1-hour and 2-hour chase times compared with untreated cells (n=4; \(P<0.05\)). However, treatment with oleate partially restored apoB secretion. When primary hamster hepatocytes were pretreated for 2 hours with 360 \(\mu\)mol/L oleate before the addition of glucosamine (Figure 2F), intracellular apoB was also protected against degradation. Similar results were observed under the same experimental condition using rat McA-RH7777 cells (data not shown).

Figure 2. Effect of glucosamine treatment on apoB100 turnover in HepG2 cells. HepG2 cells were treated with 5 mmol/L glucosamine for 16 hours and then pulsed with 100 \(\mu\)Ci/mL \[^{35}S\] methionine/cysteine, (A) immunoprecipitated with anti-human apoB antibody, (B) with anti-human apoE antibody, or (C) with anti-human albumin antibody. D, A pulse-chase experiment. Upper panel, The cell lysates and medium immunoprecipitated with anti-human apoB antibody. Lower panel, Total radiolabeled apoB100 (cells plus medium); n=3. *\(P<0.05\); **\(P<0.01\). E, HepG2 cells were treated for 16 hours in the absence or presence of glucosamine. Cells were then pretreated for 2 hours in the presence of oleate, pulsed, and chased 1 or 2 hours in the presence and absence of 360 \(\mu\)mol/L oleate. F, Primary hamster hepatocytes were pretreated with 360 \(\mu\)mol/L oleate for 1 or 2 hours and incubated in the absence or presence of glucosamine before pulse-chase as described.

Glucosamine Treatment Increases Trypsin Sensitivity of ER-Associated/Ubiquinated ApoB100 in HepG2 Cells

To determine a possible effect of glucosamine on translocation efficiency of newly synthesized apoB100 across the ER membrane, we used a previously published protocol involving trypsin digestion of digitonin-permeabilized HepG2 cells.\(^{24}\) As shown in Figure 3A, although newly synthesized apoB100 was sensitive to trypsin digestion in cells treated in the presence or absence of glucosamine, there was a significantly higher level of sensitivity in glucosamine-treated cells (trypsin-resistant apoB100 recovered after trypsin digestion was increased).

Figure 3. Trypsin-sensitivity and ubiquitination status of apoB100 in cells treated with glucosamine. A, HepG2 cells were treated in the absence or presence of 5 mmol/L glucosamine (16 hours) and 10 \(\mu\)g/mL ALLN (1 hour). Cells were pulsed with \[^{35}S\] methionine/cysteine for 5 minutes and chased for 5 minutes. Cells were permeabilized with 75 \(\mu\)g/mL digitonin, followed by trypsin treatment. Trypsin digestion was halted by the addition of protease inhibitors and cells were subjected to immunoprecipitation with a specific anti-apoB antibody. The level of trypsin sensitive apoB is shown in the lower graph (expressed as percent of control). B, HepG2 cells were treated with/without 5 mmol/L glucosamine for 16 hours and then incubated with/without lactacystin or MG132 for 3 hours. The cell lysates were immunoprecipitated with anti-human apoB antibody, then immunoblotted with anti-ubiquitin antibody (upper panel), and then re-probed with anti-human apoB antibody (lower panel) (n=4). The ratio of ubiquitinated apoB over total apoB mass is shown in the lower graph, C, Immunoblotting with an anti-human MTP antibody (n=4).
of glucosamine-treated HepG2 cells was 27.46±1.43% (P<0.01) of that in untreated control cells (Figure 3A, lower graph). Interestingly, there were considerably fewer protected apoB fragments recovered after trypsin digestion of glucosamine-treated cells, suggesting a significantly greater level of trypsin sensitivity and thus reduced translocation efficiency across the ER membrane.

Next, we investigated whether newly synthesized apoB100 was ubiquitinated in glucosamine-treated HepG2 cells. The transient ubiquitin-conjugated apoB100 pool was examined in HepG2 cells treated in the presence or absence of 5 mmol/L glucosamine. In the presence of the proteasomal inhibitors clasto-Lactocystin β-Lactone or MG132 (Figure 3B), the protected apoB100 pool was found to be ubiquitinated in glucosamine treated cells, and the ratio of ubiquitinated apoB over total apoB mass was considerably higher in glucosamine treated cells compared with untreated controls.

We also examined the effect of glucosamine treatment on MTP protein mass to determine whether the suppressive effects of glucosamine on apoB100 could be partially related to MTP inhibition. After treatment of HepG2 cells with 5 mmol/L glucosamine, cell lysates were subjected to immunoblotting using an anti-MTP antibody (Figure 3C). No significant changes in MTP protein levels could be detected by Western blotting using an anti-MTP antibody (Figure 3C). No significant changes in MTP protein levels could be detected by Western blotting using an anti-MTP antibody (Figure 3C).

Adenovirus-Mediated Grp78 Overexpression Reduces ApoB100 Secretion From HepG2 Cells
To examine the specific effect of Grp78 on apoB100 secretion, HepG2 cells were infected with AdGrp78, an adenoviral vector encoding the full-length human Grp78 cDNA. The adenoviral vector Adβ-gal, encoding β-galactosidase cDNA was also used, in addition to a no-virus control. The 78-kDa protein identified by the anti-KDEL antibody and corresponding to Grp78 was increased in a dose-dependent manner (Figure 5A) and culture media apoB100 were immunoprecipitated with anti-human apoB antibody (upper panels). Corresponding bands were scanned and quantified as shown (lower panel). The apoB-depleted samples were re-immunoprecipitated with anti-human albumin antibody (middle panels). The radiolabeled cellular and media apoB100 were immunoprecipitated with anti-human apoA1 antibody (D) or anti-human apoE antibody (E); n=3; **P<0.05; ***P<0.01.

Grp78-Induced Downregulation of ApoB100 Secretion in Intact HepG2 Cells Is Sensitive to the Proteasome Inhibitor, clasto-Lactocystin β-Lactone
We also monitored apoB100 turnover in HepG2 cells overexpressing Grp78 and treated with the proteasomal inhibitor, clasto-Lactocystin β-Lactone. As expected, lactacystin treatment of control HepG2 cells (no virus or Adβ-gal infected) significantly increased radiolabeled apoB100 accumulated in cells (P<0.01; Figure 5A) and culture media (P<0.05 or P<0.01; Figure 5B). Interestingly, lactacystin treatment of AdGrp78-infected cells (cells overproducing Grp78) also led to significantly higher accumulation of radiolabeled apoB100 in both the cells (P<0.01) (Figure 5A).
and media ($P<0.01$) (Figure 5B), suggesting that proteasomal inhibition could block Grp78-induced degradation of apoB100. In pulse-chase experiments (Figure 5C), total radiolabeled apoB100 accumulation was significantly lower starting after 5 minutes of chase ($P<0.05$) and continuing up to 2 hours after the chase period ($P<0.05$) in cells infected with AdGrp78 compared with that of control Adβ-gal-infected cells. However, pretreatment with 10 μmol/L lactacystin resulted in a much greater recovery of radiolabeled apoB100 in cells overexpressing Grp78 starting after 5 minutes of chase ($P<0.01$) and continuing up to 2 hours after the chase period ($P<0.05$) (Figure 5D). Neither of 2 lysosomal inhibitors, E64 (25 μg/mL) or leupeptin (25 μg/mL) could block apoB100 degradation stimulated by Grp78 overexpression (Figure 1A, available online at http://atvb.ahajournals.org).

**Discussion**

Several lines of evidence suggest that newly synthesized apoB associate with the ER chaperone, Grp78, during transit through the secretory pathway. Grp78 was found to associate intracellularly with apoB100 in HepG2 cells, as well as with VLDL–apoB48 in the secretory pathway of rat liver. However, the functional consequences of this interaction have not been well-defined. Two experimental approaches were used in the present study to examine whether modulating apoB100–Grp78 interaction can influence the biogenesis of apoB during its transit through the secretory pathway. We first examined the effect of glucosamine-induced ER stress that induces the expression of a number of ER chaperones, including Grp78, on apoB100 biogenesis. Under these conditions, both cellular and secreted apoB100 levels were significantly downregulated. This correlated with a dose-dependent increase in levels of the chaperone proteins Grp78 and Grp94 in the ER of glucosamine-stimulated HepG2 cells. The mass of 3 control proteins—albumin, PTP-1B and ER-60—was not significantly changed in glucosamine-treated HepG2 cells, suggesting that the effect of glucosamine was specific to apoB100. These data suggest that Grp78 promotes apoB100 degradation by interfering with the ER-associated protein degradation machinery.
secretion in glucosamine-treated HepG2 cells, although they did not address the underlying mechanisms or the involvement of ER chaperones. To determine whether the effect of glucosamine-induced ER stress on apoB production was mediated by Grp78, we specifically overexpressed Grp78 in HepG2 cells and examined the effect on apoB, as well as a number of control proteins. Adenoviral-mediated overexpression of Grp78 led to a significant decline in intracellular accumulation and secretion of apoB100, an effect that correlated closely with Grp78 expression level. These data clearly support the notion that enhanced expression of Grp78 may be a key underlying mechanism for ER stress-induced down-regulation of apoB100. Interestingly, glucosamine-induced degradation of apoB could be partially reversed by addition of exogenous oleate suggesting that enhanced lipid availability may partially rescue misfolded apoB chains retained in the ER. Glucosamine-induced apoB degradation and partial protection by oleate were observed in not only HepG2 cells but also primary hamster hepatocytes and McA-RH 7777 rat hepatoma cells. Interestingly, recent data from our laboratory has shown no significant effect of glucosamine on apoB48 secretion in McA-RH7777 cells (data not shown).

Experimental evidence suggests that binding of Grp78 to target proteins promotes proteasomal degradation. Because Grp78-apoB association has been well-documented, changes in apoB100 stability in the ER may occur through prolonged binding of overexpressed Grp78 to apoB100. Prolonged binding between Grp78 and apoB100 may hinder translocation of apoB100 across the translocon channel leading to induction of proteasome-dependent degradation of apoB100 on the cytosolic side of ER membrane. Our trypsin sensitivity experiments support an effect of glucosamine on translational efficiency of apoB100, leading to its proteosomal degradation. Pariyarath et al. have suggested a model in which apoB translation is not completed until lipoprotein assembly terminates, and assembly with lipids or entry into the ubiquitin–proteasome pathway occurs, whereas apoB polypeptides remain associated with the translocon and attached to the ribosome. According to this model, increased Grp78 levels may lead to an increased Grp78/apoB ratio in the ER lumen, which might impede normal apoB100 exit from the ER and result in increasing apoB degradation via the ubiquitin–proteasome pathway. However, whether prolonged Grp78–apoB100 interaction leads to retrograde translocation of apoB chains out of the ER lumen is unknown and requires further investigation. Interestingly, measurement of the ratio of ubiquitinated apoB-to-total apoB mass clearly showed a considerable increase in the ubiquitinated apoB in glucosamine-treated cells, suggesting increased susceptibility to proteosomal degradation.

Although Grp78–stimulated apoB100 degradation in HepG2 cells was inhibited by the proteasomal inhibitor, lactacystin, this inhibition appeared to be incomplete, suggesting that apoB100 synthesis may also be affected by Grp78 overexpression. Pan et al. has recently shown that translocation arrest of apoB in the ER channel can exert a selective and negative effect on the synthesis of apoB at the stage of elongation. Grp78 overexpression may also affect apoB100 translation in HepG2 cells via this mechanism.

Increased Grp78-apoB interaction may obstruct the luminal face of the translocon pore before and during early translocation thus arresting the newly synthesized protein in an elongation state.

Although glucosamine treatment induced the overexpression of ER chaperones, Grp78 and Grp94, we cannot exclude the possibility of additional mechanisms being affected by glucosamine treatment and that this may, in turn, regulate apoB100 secretion. The profound inhibitory effect of glucosamine on cellular and secreted levels of apoB100 may also be mediated by other co-translational or post-translational mechanisms such as an effect on N-linked and/or O-linked glycosylation of apoB100. Changes in glycosylation pattern of apoB would be expected to induce misfolding and destabilization of apoB100. Further studies are needed to examine any change in apoB glycosylation induced by glucosamine. However, available evidence does appear to rule out glucosamine-induced changes in MTP as mediating the inhibition of apoB100 secretion, because no changes in MTP protein mass could be detected in glucosamine-treated cells.

In summary, Grp78 may play an important role in regulation of hepatic apoB100 secretion initially assisting apoB100 in achieving correct folding and secretion. However, prolonged interaction with Grp78 may lead to ER retention of newly synthesized and partially translocated apoB100 molecules, leading to enhanced proteasomal degradation. It is widely believed that Grp78 binds more strongly to incompletely folded proteins rather than mature native proteins. Because apoB appears to be recognized as an incompletely folded protein throughout most of its transit through the secretory pathway, binding and retention by Grp78 may play a critical role in proteasomal targeting and the ER quality-control of misfolded apoB molecules. Interaction of apoB chains with core lipoprotein lipids may reduce Grp78-mediated ER retention and facilitate transport out of the ER.

Acknowledgments
This work was supported by operating grants to KA (T-4301) and RCA (T-5385) from the Heart and Stroke Foundation of Ontario. R.K.A. is a recipient of a Heart and Stroke Foundation of Canada postdoctoral fellowship. R.C.A. is a Career Investigator of the Heart and Stroke Foundation of Ontario.

References


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Arterioscler Thromb Vasc Biol. 2005;25:571-577; originally published online December 23, 2004;
doi: 10.1161/01.ATV.0000154142.61859.94
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Figure I. Grp78 does not induce non-proteasomal apoB100 degradation in intact or permeabilized HepG2 cells overexpressing Grp78 (please see www.ahajournals.org). (A) 48 h after infection of HepG2 cells with AdGrp78 or Adβ-gal, the cells were preincubated with 25 mg/ml E64 or leupeptin and pulsed with 50 mCi/ml [35S] methionine/cysteine. The radioactivity was chased for 0, 1, and 2 h. Chase media and cell lysates were collected for immunoprecipitation with anti-human apoB antiserum. n=3, ** p < 0.01. (B) HepG2 cells were infected with AdGrp78, AdER-60 or Adβ-gal. 48 h after infection, the cells were pulsed for 15 min, chased for 10 min, permeabilized with digitonin, and incubated for 0, 1 or 2 h. Cell lysates were immunoprecipitated with anti-human apoB (B, upper panel) followed by a second immunoprecipitation using anti-human albumin anti-serum (B, lower panel), respectively.