Mechanisms of glucosamine-induced suppression of the hepatic assembly and secretion of apolipoprotein B-100-containing lipoproteins

Wei Qiu, Rita Kohen Avramoglu, Angela C. Rutledge, Julie Tsai, and Khosrow Adeli[1]

Division of Clinical Biochemistry, Department of Clinical Biochemistry and Pathobiology, Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada

Abstract **Glucosamine-induced endoplasmic reticulum (ER) stress was recently shown to specifically reduce apolipoprotein B-100 (apoB-100) secretion by enhancing the proteasomal degradation of apoB-100. Here, we examined the mechanisms linking glucosamine-induced ER stress and apoB-lipoprotein biogenesis. Trypsin sensitivity studies suggested glucosamine-induced changes in apoB-100 conformation. Endoglycosidase H studies of newly synthesized apoB-100 revealed glucosamine induced *N*-linked glycosylation defects resulting in reduced apoB-100 secretion. We also examined glucosamine-induced changes in VLDL assembly and secretion. A dose-dependent (1–10 mM glucosamine) reduction was observed in VLDL-apoB-100 secretion in primary hepatocytes (24.2–67.3%) and rat McA-RH7777 cells (23.2–89.5%). Glucosamine also inhibited the assembly of larger VLDL-, LDL-, and intermediate density lipoprotein-apoB-100 but did not affect smaller HDL-sized apoB-100 particles. Glucosamine treatment during the chase period (posttranslational) led to a 24% reduction in apoB-100 secretion ($P < 0.01$; n = 4) and promoted post-ER apoB degradation. However, the contribution of post-ER apoB-100 degradation appeared to be quantitatively minor. Interestingly, the glucosamine-induced posttranslational reduction in apoB-100 secretion could be partially prevented by treatment with desferrioxamine or vitamin E. Together, these data suggest that cotranslational glucosamine treatment may cause defects in apoB-100 *N*-linked glycosylation and folding, resulting in enhanced proteasomal degradation. Posttranslationally, glucosamine may interfere with the assembly process of apoB lipoproteins, leading to post-ER degradation via nonproteasomal pathways.**—Qiu, W., R. Kohen 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.**

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Apolipoprotein B-100 (apoB-100) is the major protein component of plasma lipoproteins and is required for the synthesis and secretion of triglyceride-rich circulating lipoproteins such as VLDL (1). ApoB-100 secretion appears to be regulated mainly by intracellular degradative pathways. Ubiquitination and proteasomal degradation of apoB-100 begin cotranslationally and involve the interaction of misfolded apoB with the cytosolic chaperones Hsp70 and Hsp90 (2). Nonproteasomal pathways of apoB-100 degradation have also been described involving both intra-endoplasmic reticulum (ER) and post-ER mechanisms (3–5). A critical factor in deciding the secretory fate of apoB-100 is the availability of neutral lipids (triglyceride, phospholipid, cholesterol, and cholesteryl ester) proximal to the site of apoB-100 synthesis in the ER (6). In the absence of readily available lipid, a significant proportion of newly synthesized apoB-100 in cultured hepatoma cells, as well as in primary hepatocytes from hamsters, rats, and rabbits, is degraded via either the ubiquitin-proteasome-dependent or -independent degradative pathway (7–9).

Although hepatic VLDL assembly in cultured hepatocytes has been intensely investigated, the precise mechanisms regulating this assembly are still controversial (10). The assembly of VLDL is thought to be a two-step process (2). It is, however, unclear in which subcellular compartment the bulk of lipid associates with apoB-100. Mitchell et al. (11) found that triglyceride-rich lipoprotein particles could be formed while apoB-100 is still associated with the translocon, and Yamaguchi et al. (12) found that bulk lipidation could occur in the ER in McA-RH7777 cells. Conversely, Tran et al. (10) showed that enrichment of VLDL with lipid could occur in the Golgi compartment. A

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Abbreviations: ALLN, *N*-acetyl-leucinyl-leucinyl-nor-leucinal; apoB-100, apolipoprotein B-100; BFA, brefeldin A; DFX, desferrioxamine; ER, endoplasmic reticulum; IDL, intermediate density lipoprotein; MTP, microsomal triglyceride transfer protein; PERPP, postendoplasmic reticulum presecretory proteolysis; TBARS, thiobarbituric acid-reactive substances.

[1] To whom correspondence should be addressed.
e-mail: k.adeli@utoronto.ca

great number of observations have led to the suggestion that microsomal triglyceride transfer protein (MTP)-mediated lipid transfer activity is essential to facilitate lipoprotein formation and to prevent apoB-100 degradation during the first step of lipoprotein assembly (13, 14). Conversely, during later stages of VLDL assembly, MTP activity and de novo triglyceride synthesis are not thought to be required for additional lipidation (11, 13, 15, 16).

Interestingly, Fisher and colleagues (5) reported that ω-3 fatty acids act as hypolipidemic agents that can reduce apoB-100 secretion via degradative mechanisms not involving the conventionally understood proteasomal and lysosomal degradative pathways. They further showed that lipid peroxidation and oxidative stress mechanisms are responsible for the observed increase in apoB-100 degradation and the concomitant decrease in apoB-100-containing VLDL secretion. Addition of antioxidants to cells treated with ω-3 fatty acids restored increased levels of VLDL-apoB-100 secretion from both McA-RH7777 cells and rat primary hepatocytes (17).

The mechanisms targeting misfolded apoB-100 to both proteasomal and nonproteasomal degradative pathways are currently incompletely understood. There is, however, strong evidence showing the association of the newly synthesized apoB-100 polypeptide with numerous ER chaperones, including Grp94, Grp78, ERp72, calreticulin, cyclophilin B, Hsp70, Hsp90, and calnexin (18). Several laboratories, including ours, have observed a tight association between apoB-100 and the 78 kDa glucose-regulated protein/immunoglobulin heavy chain binding protein (Grp78/BiP) in the ER of HepG2 cells (19). Interestingly, treatment of HepG2 cells with glucosamine decreased both cellular and secreted apoB-100 in a dose-dependent manner that correlated with increased levels of the ER stress protein Grp78 (20). This glucosamine treatment led to reduced translocational efficiency of apoB-100 in the ER and enhanced ubiquitination and proteasomal degradation of apoB-100. We recently confirmed that overexpression of Grp78 mediated by adenovirus also led to significantly decreased levels of newly synthesized apoB-100 in a dose-dependent manner (20).

There is increasing evidence that high glucose levels in type II diabetes result in increased intracellular glucosamine through the conversion of glucose to glucosamine via the hexosamine pathway (21). Glucosamine has also been proposed to contribute to ER stress and cholesterol accumulation in aortic smooth muscle cells, monocytes, and hepatocytes, all of which could contribute to atherosclerosis (22). We believe the cholesterol accumulation in hepatocytes upon glucosamine treatment may result from an increase in the degradation of apoB-100.

In this report, we have further investigated the mechanisms leading to glucosamine-induced ER stress and alterations in the assembly and secretion of apoB-100-containing lipoproteins in both primary hepatocytes and established hepatocyte cell lines. Our data suggest that glucosamine interferes with apoB-100 *N*-linked glycosylation, leading to defects in protein folding and resulting in enhanced proteasomal degradation cotranslationally. Under certain conditions in which proteasomal degradation is inhibited, glucosamine may also interfere with the VLDL, intermediate density lipoprotein (IDL), and LDL assembly process posttranslationally by inducing post-ER degradation of apoB-100-lipoprotein particles via nonproteasomal pathways.

EXPERIMENTAL PROCEDURES

Cell culture and glucosamine treatment

McA-RH7777 or HepG2 cells were seeded onto collagen-coated 35 or 100 mm plates in DMEM containing 20% FBS for McA-RH7777 cells or 10% FBS for HepG2 cells. Primary hepatocytes from Syrian Golden hamsters were isolated as described previously (8) and seeded onto fibronectin-coated plates in DMEM containing 10% FBS. After allowing 4 h for the cells to adhere, the medium was replaced with fresh DMEM containing 20% FBS (10% FBS for HepG2 cells) in the presence of varying concentrations (0–10 mM) of glucosamine (Sigma, St. Louis, MO) for 16 h at 37°C and 5% CO_2.

Metabolic labeling of glucosamine-treated cultured hepatocytes

After 16 h of treatment with glucosamine, McA-RH7777 cells or primary hamster hepatocytes were incubated with methionine/cysteine-free DMEM at 37°C for 1 h (prepulse methionine/cysteine depletion) and then metabolically labeled with 100 μCi/ml [^{35}S]methionine/cysteine for 2–3 h as described in the figure legends. After metabolic labeling, the conditioned medium was harvested for fractionation. In some experiments, after labeling with 150 μCi/ml [^{35}S]methionine/cysteine for 1 h, 5 mM glucosamine or 0.5 μg/ml brefeldin A (BFA) was added only during the chase period for 3 h.

Lipoprotein fractionation and immunoprecipitation of apoB-containing lipoproteins

Conditioned media were fractionated by rate flotation ultracentrifugation. A total of 4 ml of [^{35}S]methionine/cysteine-labeled sample was adjusted to $d = 1.10$ g/ml with solid NaBr and loaded onto the bottom of a Beckman SW41 centrifuge tube. The sample was overlaid with 3 ml of $d = 1.065$ g/ml NaBr, 3 ml of $d = 1.02$ g/ml NaBr, and 2 ml of $d = 1.006$ g/ml NaBr. After ultracentrifugation at 35,000 rpm (151,000 *g*) at 4°C for 18 h, 1 ml fractions were unloaded from the top of the tube. Each fraction was immunoprecipitated using antiserum against human apoB (Midland Bioproducts) as described previously (23).

Leupeptin, desferrioxamine, and vitamin E treatments

McA-RH7777 cells or primary hamster hepatocytes were pulsed with 100 μCi/ml [^{35}S]methionine/cysteine for 1 h and then treated with or without 5 mM glucosamine or 0.5 μg/ml BFA in a 3 h chase period in the absence or presence of 25 μg/ml leupeptin, 100 μM desferrioxamine (DFX), or 120–200 μM vitamin E as described in the figure legends. Conditioned media were harvested and immunoprecipitated using antiserum against human apoB.

Lipid peroxidation assay

Thiobarbituric acid-reactive substances (TBARS) were used as an indicator of lipid peroxidation and quantified by a method

based on that used by Pan et al. (17). Briefly, primary hamster hepatocytes were plated at near confluence on six-well plates. The next day, the medium was removed from the plates and replaced with phenol red-free medium. DFX was added to some wells at a final concentration of 100 μM. After 5 min of incubation, some wells received glucosamine at a final concentration of 5 mM. After 3 h of incubation, cells were washed twice with cold PBS and collected in 1 ml of PBS containing 150 μM butylated hydroxytoluene. Cells were then centrifuged at 2,000 *g* for 5 min, and the supernatants were discarded. Pellets were resuspended in 150 μl of PBS containing 150 μM butylated hydroxytoluene, and 100 μl of the cell suspensions was mixed with 50 μl of a freshly made solution containing 30% trichloroacetic acid, 0.75% thiobarbituric acid, and 0.5 N hydrochloric acid. Samples were heated at 100°C for 15 min before they were centrifuged for 8 min at 13,600 *g*. The absorbance of the supernatants at 532 nm was then determined. An extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ was used to calculate the amount of TBARS as malondialdehyde equivalents present. Values were normalized to the amount of protein present in each sample by performing a protein assay with a DC protein assay kit (Bio-Rad, Hercules, CA) on the cell suspensions left over after preparing the samples for the lipid peroxidation assay.

Isolation of McA-RH777 microsomes

Microsomes were prepared as described previously. Briefly, McA-RH7777 cells treated with glucosamine or BFA were homogenized with 25 strokes of a Dounce homogenizer in 500 μl of 50 mM sucrose + 3 mM imidazole buffer, pH 7.4, containing 0.1 mM leupeptin, 1 mM PMSF, and 100 kallikrein-inactivating units (KIU)/ml Trasylol (aprotinin). After homogenization, 50 μl of 49% sucrose solution was added, and cells were homogenized with five additional strokes. The homogenate was centrifuged at 2,200 *g* for 10 min at 4°C. The pellet was rinsed with 500 μl of 250 mM sucrose + 3 mM imidazole, pH 7.4, and centrifuged at 2,200 *g* for an additional 10 min at 4°C. Supernatants containing microsomes were pooled and centrifuged at 100,000 *g* for 60 min at 4°C.

Immunoprecipitation, SDS-PAGE, and fluorography

Immunoprecipitation was performed as described previously (24). 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 apoB-100 bands was performed using an imaging densitometer. In some experiments, radiolabeled apoB bands were excised from the gels and dissolved, and the radioactivity (cpm) was measured in a 1219 Rackbeta liquid scintillation counter (LKB Wallac) (20).

Endoglycosidase H digestion of newly synthesized apoB-100

HepG2 cells were treated for 16 h with 5 mM glucosamine. Cells were metabolically labeled with 100 μCi/ml [^{35}S]methionine/cysteine for 5 min, then chased for the indicated times. Cells were harvested and lysed in solubilizing buffer, then immunoprecipitated for 18 h at 4°C using antiserum against human apoB followed by 1 h of incubation at room temperature with 100 μl of a 10% slurry of protein A-Sepharose. Immunoprecipitated proteins were recovered from the protein A-Sepharose by boiling for 5 min in 100 μl of sodium acetate buffer, pH 4.5, containing 10% SDS and 5% β-mercaptoethanol. The denatured samples were incubated for 1 h at 37°C in the presence or absence of 0.005 units of endoglycosidase H (Roche). An equal volume of Laemmli buffer was added, and samples were resolved by 6% SDS-PAGE at 60 V for 16 h.

Trypsin sensitivity studies

Trypsin sensitivity experiments were carried out as described previously (20). Briefly, HepG2 cells were incubated with methionine/cysteine-free DMEM for 1 h followed by the addition of 10 μM *N*-acetyl-leucinyl-leucinyl-nor-leucinal (ALLN) for 1 h. Cells were then pulse-labeled for 3 min with 100 μCi/ml [^{35}S]methionine/cysteine, then chased for 0, 5, 10, 20, or 30 min in complete medium supplemented with 10 mM methionine + 10 μM ALLN. The cells were permeabilized for 10 min at room temperature in cytoskeletal buffer (0.3 M sucrose, 0.1 M KCl, 2.5 mM $MgCl_2$, 1 mM sodium-free EDTA, and 10 mM PIPES, pH 6.8) containing 50 μg/ml digitonin, 10 mM methionine, 150 μM puromycin, 50 μg/ml cyclohexamide, and 1.25 μM ALLN. Permeabilized cells were washed three times with cytoskeletal buffer, and cytoskeletal buffer containing 10 mM methionine, 150 μM puromycin, 50 μg/ml cyclohexamide, and 1.25 μM ALLN as well as 200 μg/ml trypsin were added for 10 min at room temperature followed by 10 min at 0°C. Trypsin was inactivated with the addition of 2 mg/ml soybean trypsin inhibitor for 10 min at 0°C. Triton X-100 was used to allow full trypsin accessibility (acts as a positive control for trypsin digestion). It solubilizes all membranes and allows trypsin to digest apoB in the ER. Cells were solubilized and subjected to immunoprecipitation of apoB as described above.

Statistical analyses

Results are displayed as means ± SD ($n \geq 3$). The unpaired, two-tailed *t*-test was used to assess differences between the experimental group and controls. One-way and two-way ANOVAs were used to determine the statistical significance of the data shown in Fig. 6B, C, respectively. $P < 0.05$ was considered statistically significant.

RESULTS

Glucosamine treatment inhibits the addition of *N*-linked oligosaccharides to apoB-100 in HepG2 cells

We have shown previously that treatment of HepG2 cells with glucosamine induced ER stress, resulting in a decrease in apoB-100-containing lipoprotein secretion that was partly prevented upon lactacystin treatment (20). In this study, we have investigated the precise cellular mechanisms by which glucosamine treatment inhibits the hepatic secretion of apoB-100-containing lipoproteins. As there is evidence that glucosamine interferes with the cotranslational *N*-linked glycosylation of proteins (25), we investigated whether glucosamine treatment caused changes in apoB-100 glycosylation. As expected, in the absence of glucosamine, intracellular radiolabeled apoB-100 over the course of 60 min retained endoglycosidase H sensitivity upon treatment of cell lysates with the enzyme. There was a corresponding mobility shift toward a lower molecular mass on SDS-PAGE, indicating that detectable apoB-100 was from a pre-*trans*-Golgi intracellular compartment and disappeared over time (**Fig. 1A**, top panels). Upon glucosamine treatment, there was a marked decrease in the total amount of immunoprecipitable apoB-100 in addition to a lower apparent molecular mass of all apoB species.

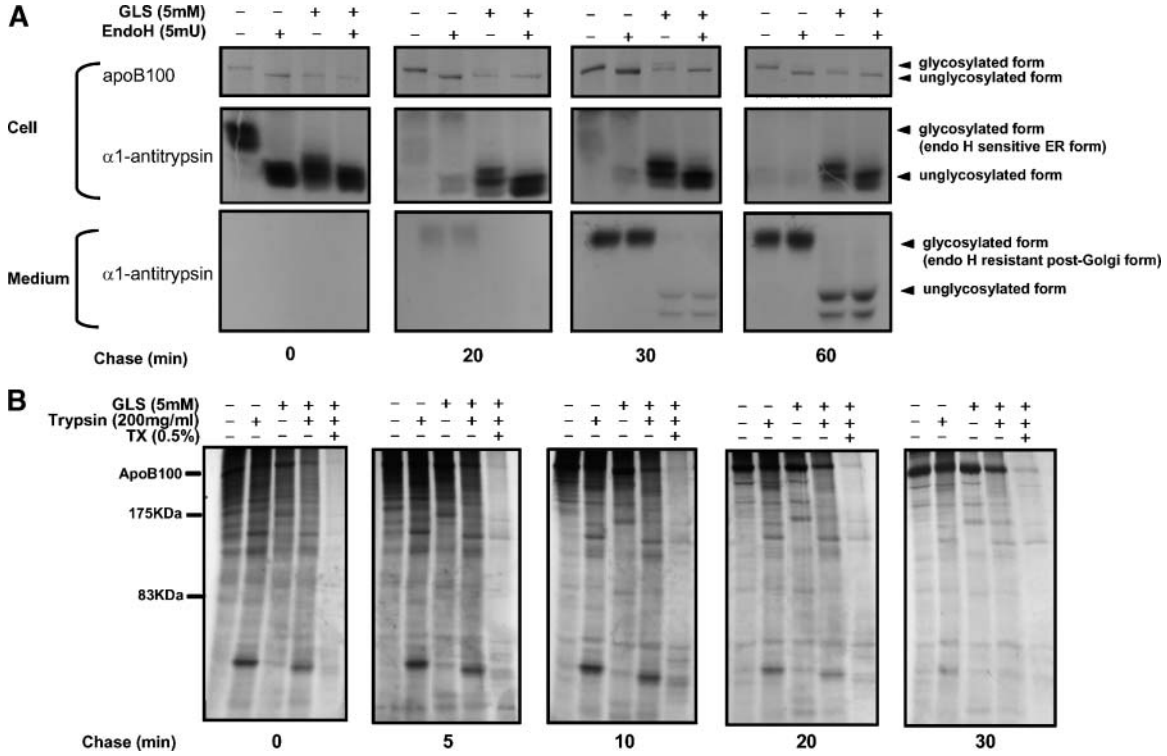

Fig. 1. Evidence for glucosamine (GLS)-induced inhibition of *N*-linked glycosylation of α1-antitrypsin and apolipoprotein B-100 (apoB-100) in HepG2 cells. A: HepG2 cells were treated with or without 5 mM glucosamine for 16 h. The cells were metabolically labeled with 100 μCi/ml [^{35}S]methionine/cysteine for 5 min, then chased for the indicated times. Samples were immunoprecipitated using the antibody indicated; then, half of the sample was incubated for 1 h at 37°C with 5 mU of endoglycosidase H (EndoH), and the other half was incubated in the absence of endoglycosidase H. Results from a representative experiment are shown (n = 4). B: HepG2 cells were pulse-labeled for 3 min with 100 μCi/ml [^{35}S]methionine/cysteine, then chased for the indicated times. The cells were permeabilized, and 200 μg/ml trypsin was added for 10 min at room temperature followed by 10 min at 0°C. Trypsin was inactivated by the addition of 2 mg/ml soybean trypsin inhibitor for 10 min at 0°C. Cells were solubilized and subjected to overnight immunoprecipitation for apoB using anti-apoB antibody, and the samples were resolved on a 6% acrylamide gel. TX, Triton X-100.

Furthermore, endoglycosidase H digestion of glucosamine-treated cells failed to produce a noticeable shift in molecular mass, suggesting that glucosamine treatment may considerably impair the cotranslational addition of *N*-linked oligosaccharides. Glucosamine treatment produced a similar shift toward lower molecular mass of α1-antitrypsin, a secreted glycoprotein, which was used as a control (Fig. 1A, center panels). Because α1-antitrypsin is more stable intracellularly compared with apoB, we were able to follow its secretion into the medium. A large proportion of α1-antitrypsin appeared in the medium after a 30 min chase, where, as expected, it acquired endoglycosidase H resistance after transit and further *N*-linked oligosaccharide trimming in the *trans*-Golgi compartment (Fig. 1A, bottom panels). Surprisingly, there was a small amount of unglycosylated, endoglycosidase H-resistant α1-antitrypsin secreted into the medium upon glucosamine treatment. This suggests that under conditions of glucosamine treatment, some of the protein may bypass or saturate the cellular quality control machinery, which in the case of α1-antitrypsin presumably does not involve rapid proteasomal degradation.

To gauge the effect of glucosamine on apoB translocation, we also performed trypsin accessibility studies in the absence or presence of glucosamine (Fig. 1B). In the absence of glucosamine, and at the early time points (0–10 min of chase), a high percentage of newly synthesized apoB-100 was digested, resulting in a characteristic tryptic profile or laddering effect (more clearly visible at 5, 10, and 20 min). The high sensitivity of apoB-100 to trypsin at these time points is not surprising, because a high percentage of labeled apoB-100 remains associated with the ribosomes and is accessible to trypsin on the cytosolic side of the ER. At later chase periods (20–30 min of chase), a greater proportion of the intact apoB-100 was protected from trypsin digestion. This decrease in trypsin sensitivity of apoB-100 is evidence of the translocation of apoB-100 into the lumen of the ER. By contrast, in cells treated with glucosamine, there appeared to be an overall decrease in the amount of immunoprecipitable apoB-100 and the tryptic fragment profile was slightly different. Together, these data suggest that glucosamine treatment impedes the translocation of apoB-100 into the ER lumen, most likely as a result of the inhibition of *N*-linked glycosylation.

Glucosamine suppresses the secretion of apoB-100-containing lipoproteins from primary hamster hepatocytes and McA-RH7777 cells in a dose-dependent manner

Next, we extended our investigation to determine whether glucosamine treatment could perturb the assembly and

secretion of apoB-100-containing lipoproteins in primary hamster hepatocytes and the rat hepatoma cell line McA-RH7777. We chose to perform these experiments in both primary and McA-RH7777 cells because HepG2 cells are a poor model for the study of VLDL assembly. Primary hamster hepatocytes were treated with 5 mM glucosamine for 16 h (**Fig. 2A**). After a 3 h pulse, there was only a 15% recovery of [^{35}S]apoB-100-containing lipoproteins in the $d <$ 1.006 g/ml fraction (VLDL) relative to untreated control cells. Under these experimental conditions, there was no difference in the incorporation of radiolabel into total protein between glucosamine-treated and untreated control cells (Fig. 2B), ruling out a global inhibition of protein synthesis. We next examined whether glucosamine treatment could affect the secretion of denser apoB-100-containing lipoproteins. Primary hamster hepatocytes were treated with increasing doses of glucosamine for 16 h and then pulse-labeled for 3 h. The media obtained under these different conditions were fractionated by discontinuous gradient rate flotation ultracentrifugation. Compared with untreated cells, glucosamine suppressed the secretion of total [^{35}S]apoB-100-containing lipoproteins in a dose-dependent manner, by 24.3% at 1 mM, 54.8% at 5 mM, and 67.3% at 10 mM (Fig. 2C). In McA-RH7777 cells, the secretion of total [^{35}S]apoB-100-containing lipoproteins was also suppressed by glucosamine in a dose-dependent manner, by 23.2% at 1 mM, 78.7% at 5 mM, and 89.5% at 10 mM compared with the control (Fig. 2D). It is notable that although glucosamine inhibited the secretion of VLDL, IDL, and LDL particles, there appeared to be little effect on HDL-sized apoB-100 particles in primary hamster hepatocytes (Fig. 2C). Similar results were observed with glucosamine treatment of McA-RH7777 cells (Fig. 2D). The formation of [^{35}S]apoB48-containing lipoproteins in McA-RH7777 cells was unchanged by glucosamine treatment (Fig. 2D), suggesting that the glucosamine effect was specific to the assembly of larger apoB-100-containing lipoproteins.

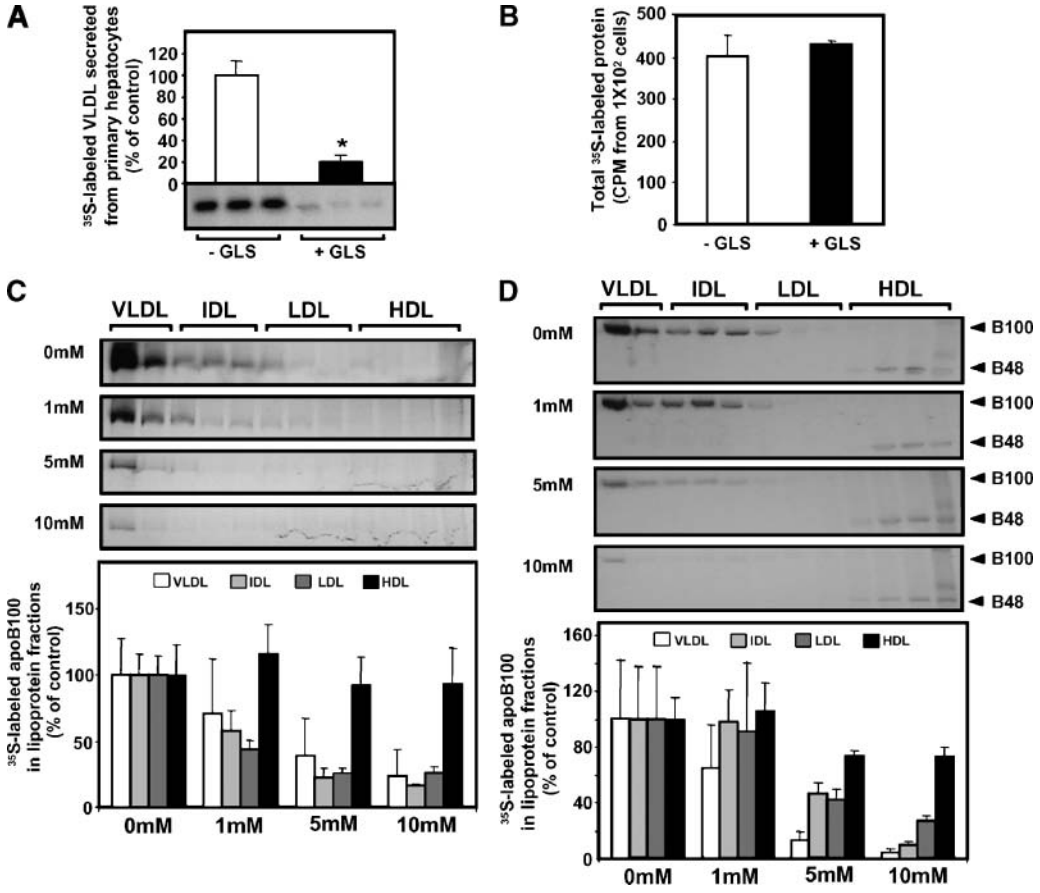

Fig. 2. Glucosamine (GLS) inhibits the secretion of apoB-100-containing lipoproteins from cultured hepatocytes in a dose-dependent manner. Primary hamster hepatocytes were treated with or without 5 mM glucosamine for 16 h and then pulsed with 100 μCi/ml [^{35}S]methionine/cysteine for 3 h. Total VLDL in media (A) and total cellular protein (B) (cpm) are shown. Primary hamster hepatocytes (C) and McA-RH7777 cells (D) were treated with 0–10 mM glucosamine for 16 h and labeled with 100 μCi/ml [^{35}S]methionine for 3 h. Media were then fractionated using discontinuous NaBr gradient ultracentrifugation. The sample was overlaid with 3 ml of d = 1.065 g/ml, 3 ml of d = 1.020 g/ml, and 2 ml of d = 1.006 g/ml NaBr and centrifuged at 151,000 g for 18 h at 4°C. The fractions were immunoprecipitated with anti-apoB antibody and resolved by SDS-PAGE. IDL, intermediate density lipoprotein. * $P < 0.01$. Error bars represent SD.

The proteasomal inhibitor MG132 does not reverse the glucosamine-induced reduction in secretion of apoB-100-containing lipoproteins

Our previous studies showed that treatment of HepG2 cells with glucosamine could induce ER stress, as indicated by an increase in chaperone molecules such as Grp78 and the rapid intracellular degradation of apoB-100 in a proteasome-dependent manner (20). We thus investigated whether the proteasomal inhibitor MG132 could block the glucosamine-induced reduction in secretion of apoB-100-containing lipoproteins. McA-RH7777 cells were treated with 5 mM glucosamine for 16 h and then pulse-labeled for 2 h. The media from different incubation conditions were fractionated by discontinuous gradient rate flotation ultracentrifugation (**Fig. 3A**). Immunoprecipitated ^{35}S-labeled apoB-100 associated with VLDL, IDL, and LDL particles was significantly decreased to 6.8 ± 2.0% (n = 3; $P < 0.05$), 12.9 ± 3.4% (n = 3; $P < 0.05$), and 16.4 ± 5.2%, (n = 3; $P < 0.05$) respectively, compared with untreated controls, suggesting that glucosamine treatment affects the assembly of larger VLDL, IDL, and LDL particles (Fig. 3B, panels a, b). Supplementation of the medium with 25 μM MG132 during the pulse period resulted in no significant increase in immunoprecipitated ^{35}S-labeled apoB-100 associated with VLDL, IDL, or LDL particles [5.6 ± 1.5% vs. 6.8 ± 2.0% (n = 3; $P > 0.05$); 16.3 ± 5.5% vs. 12.9 ± 3.0% (n = 3; $P > 0.05$); and 16.4 ± 5.5% vs. 24.3 ± 8.8% (n = 3; $P > 0.05$), respectively], suggesting that the proteasomal inhibitor MG132 may not block the glucosamine-induced decrease in the secretion of apoB-100-containing VLDL, IDL, or LDL particles (Fig. 3B, panel c). By contrast, treatment with glucosamine for 16 h before the prepulse methionine/cysteine depletion and removal of glucosamine from the prepulse methionine/cysteine depletion and pulse periods caused a significant recovery in immunoprecipitated ^{35}S-labeled apoB-100 associated with VLDL or IDL [VLDL-apoB, 31.7 ± 8.0% vs. 6.8 ± 2.0% (n = 3; $P < 0.05$); IDL-apoB, 49.3 ± 12.9% vs. 12.9 ± 3.0% (n = 3; $P < 0.05$)] compared with treatment with glucosamine for 16 h plus the prepulse and pulse periods, suggesting that the glucosamine effect on VLDL and IDL secretion is transient and may be partially reversible (Fig. 3B, panel d). Posttranslational treatment with glucosamine also disrupts the assembly and secretion of apoB-100-containing lipoproteins from McA-RH7777 cells.

To explore posttranslational mechanisms by which glucosamine disrupts the secretion of apoB-100-containing lipoproteins from McA-RH7777 cells, we developed a protocol whereby cells were treated with glucosamine only during the chase period after metabolic labeling (Fig. 3C).

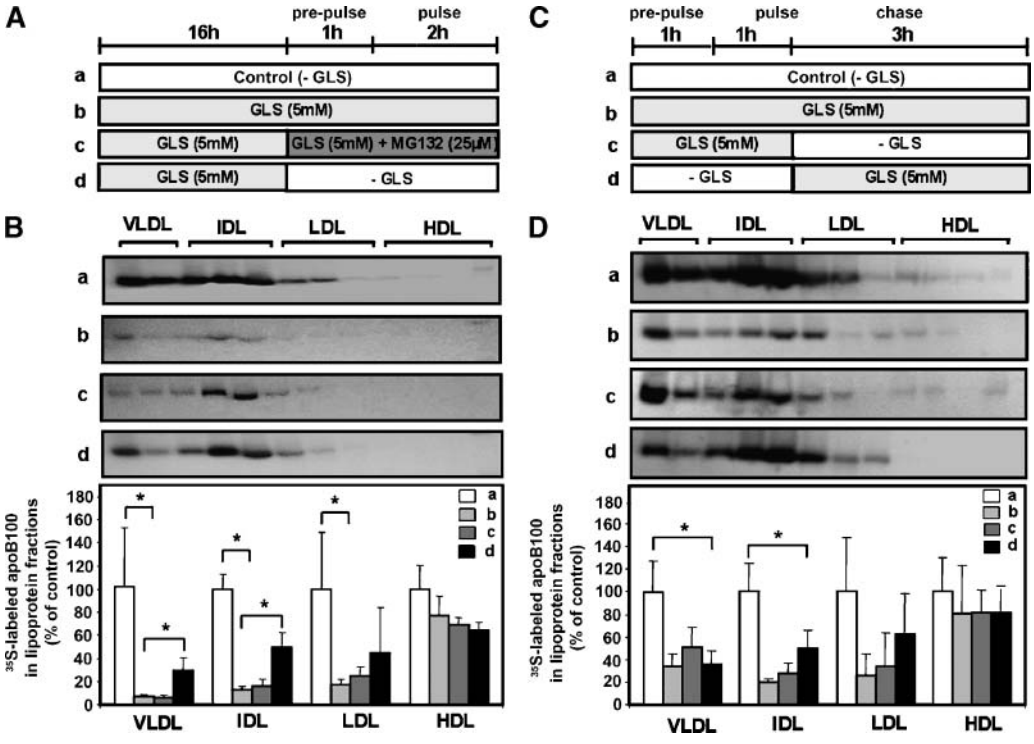

Fig. 3. Cotranslational and posttranslational effects of glucosamine (GLS) on the formation and secretion of apoB-100-containing lipoproteins from McA-RH7777 cells. A, C: Protocols used in the experiments. B: McA-RH7777 cells were pulsed with 100 μCi/ml [^{35}S]methionine/cysteine for 2 h in the absence of glucosamine (a), with 5 mM glucosamine treatment for 16 h (b), in the presence of 25 μM MG132 (c), or with washing away of glucosamine during prepulse and pulse periods (d). D: McA-RH7777 cells were pulsed with 150 μCi/ml [^{35}S]methionine/cysteine for 1 h and chased for 3 h in the absence (a) or presence (b) of glucosamine, with washing away of glucosamine during the chase time (c), or with glucosamine present only during the chase period (d). The lipoprotein fractionations were as described for Fig. 2D. * $P < 0.05$. Error bars represent SD.

McA-RH7777 cells were pulse-labeled with [^{35}S]methionine/cysteine for 1 h and then chased for 3 h in the absence or presence of 5 mM glucosamine. Media were collected and subjected to density gradient ultracentrifugation to resolve the different lipoprotein subclasses. Immunoprecipitated ^{35}S-labeled apoB-100 associated with VLDL and IDL particles was significantly decreased to 35.5 ± 11.9% (n = 3; $P < 0.05$) and 49.6 ± 15.5% (n = 3; $P < 0.05$) compared with untreated controls, suggesting that glucosamine treatment may affect the posttranslational assembly or secretion of larger VLDL and IDL apoB-100-containing particles (Fig. 3D, panels a, d).

Oleate does not reverse the glucosamine-induced decrease in apoB-100-containing lipoprotein secretion in McA-RH7777 cells

We next investigated whether oleate could reverse the glucosamine-induced decrease in apoB-100-containing lipoproteins observed post-ER. To block possible proteasomal or nonproteasomal degradative pathways for newly synthesized apoB and monitor the effect of glucosamine on apoB-100 only in the chase period, ALLN was added during the prepulse and pulse periods. McA-RH7777 cells were pulse-labeled for 1 h and chased for 3 h with 5 mM glucosamine in the presence or absence of 360 μM oleate (**Fig. 4A**). As expected, immunoprecipitated ^{35}S-labeled apoB-100 associated with VLDL and IDL lipoproteins secreted into the media was significantly decreased to 75.8 ± 5.9% ($P < 0.05$) and 68.8 ± 11.2% ($P < 0.05$) with glucosamine treatment compared with untreated controls (Fig. 4B). The glucosamine-induced decrease in ^{35}S-labeled apoB-100 associated with VLDL and IDL lipoproteins was not prevented with the addition of 360 μM oleate [52.0 ± 15.1% ($P < 0.05$) and 40.0 ± 9.3% ($P < 0.05$), respectively] (Fig. 4C). In fact, oleate treatment actually augmented the effect of glucosamine on loss of apoB-100 from the VLDL and IDL fractions (compare lanes 2 in Fig. 4B, C and lanes 4 in Fig. 4B, C) ($P < 0.05$).

Glucosamine reduces the secretion of apoB-containing lipoproteins by perturbing the post-ER trafficking of apoB by McA-RH7777 cells

To examine whether glucosamine disturbs the post-ER trafficking of apoB-100, we repeated the experiments described above using a different protocol. ALLN, a proteasome and protease inhibitor, was used in the pulse and chase periods to block the degradation of apoB-100 in the secretory pathway. BFA was used as a control because we initially hypothesized that glucosamine decreased the secretion of apoB-100 by blocking the transport of apoB-100 from the ER to the Golgi. As depicted in **Fig. 5A**, McA-RH7777 cells were pulse-labeled with 150 μCi/ml [^{35}S]methionine/cysteine for 1 h and chased for 3 h with 5 mM glucosamine or 0.5 μg/ml BFA. Total immu-

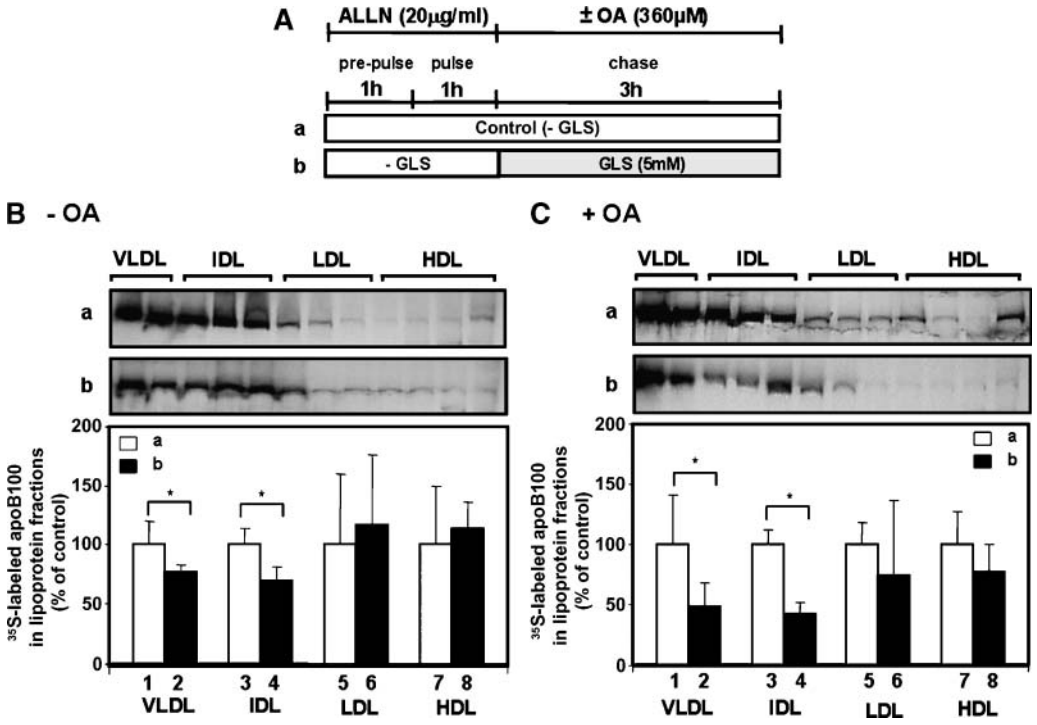

Fig. 4. Effect of oleate (OA) on the secretion of apoB-100-containing lipoproteins in glucosamine (GLS)-treated McA-RH7777 cells. A: Protocol used in the experiments. B: McA-RH7777 cells were pulsed with 150 μCi/ml [^{35}S]methionine/cysteine for 1 h and then chased for 3 h in the absence (a) or presence (b) of 5 mM glucosamine. C: Oleate (360 μM) was added during the chase period. The lipoprotein fractionations were as described for Fig. 2D, and the radioactivity of the corresponding [^{35}S]apoB-100 bands is shown in Fig. 4B, C (bottom panels). ALLN, *N*-acetyl-leucinyl-leucinyl-nor-leucinal. * $P < 0.05$. Error bars represent SD.

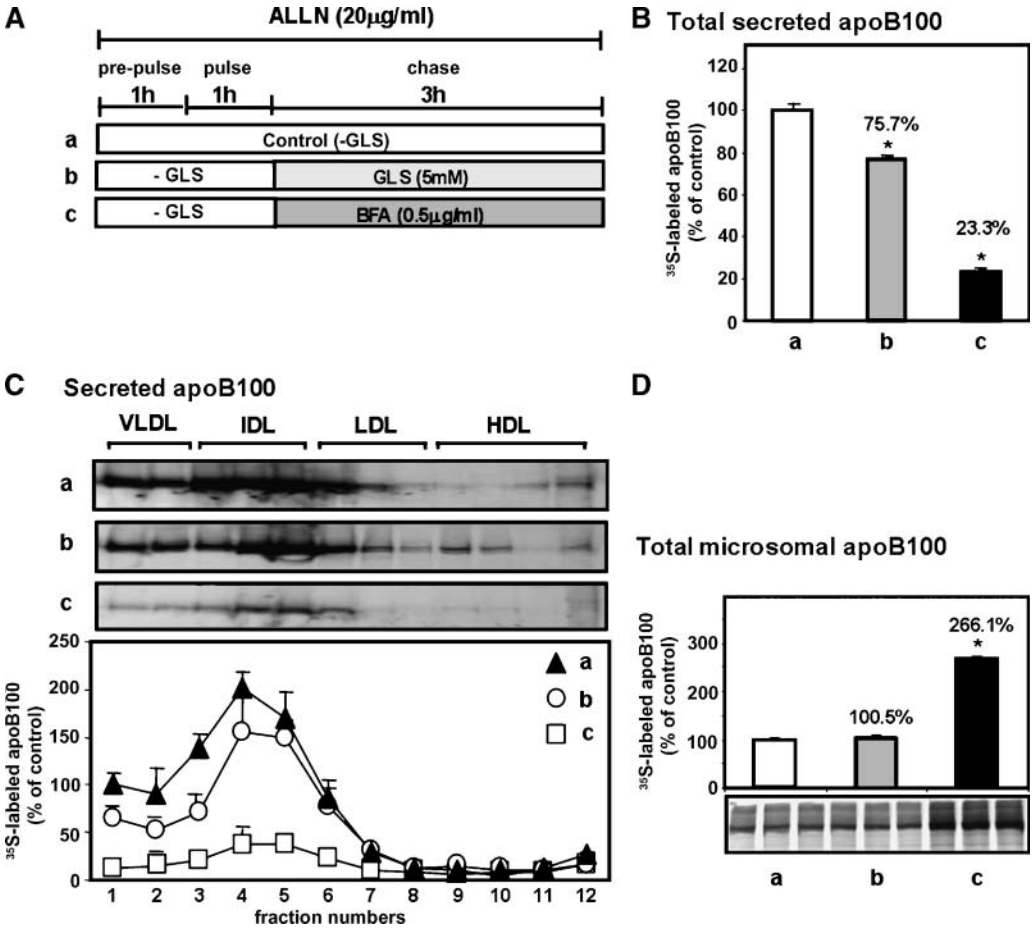

Fig. 5. Glucosamine (GLS) decreases the secretion of apoB-100-containing lipoproteins posttranslationally by perturbing the postendoplasmic reticulum trafficking of apoB-100 from McA-RH7777 cells. A: General protocol used in the experiments. McA-RH7777 cells were pulsed with 150 μCi/ml [^{35}S]methionine/cysteine for 1 h and then chased for 3 h in the absence (a) or presence (b) of 5 mM glucosamine or in the presence of 0.5 μg/ml brefeldin A (BFA) (c). B: Total ^{35}S-labeled apoB-100 secreted into the media. C: Media lipoproteins were fractionated as described for Fig. 2D. Lipoprotein distribution (top panel) and the corresponding radioactivity (bottom panel) are shown. D: ApoB-100 was isolated from total microsomes prepared using ^{35}S-labeled McA-RH7777 cells. Homogenates were centrifuged at 2,200 *g* for 10 min at 4°C in 250 mM sucrose + 3 mM imidazole, pH 7.4. Supernatants containing microsomes were centrifuged at 100,000 *g* for 60 min at 4°C. ^{35}S-labeled microsomal apoB-100 is shown. * $P < 0.01$. Error bars represent SD.

noprecipitated apoB-100 associated with lipoproteins secreted in the media was significantly decreased to 75.7 ± 3.3% (n = 4; $P < 0.01$) of the control in the presence of glucosamine and to 23.3 ± 1.3% (n = 4; $P < 0.01$) of the control in the presence of BFA (Fig. 5B). Most of the loss in newly synthesized apoB-100 appeared to occur in the VLDL and IDL fractions (Fig. 5C). Total immunoprecipitated ^{35}S-labeled apoB-100 isolated from microsomes was significantly increased to 266.1 ± 3.3% (n = 4; $P < 0.01$) of the control with BFA treatment, suggesting that BFA was likely effective in blocking intracellular apoB-100 transport from the ER to the Golgi compartment. By contrast, total ^{35}S-labeled microsomal apoB-100 was not changed by treatment with 5 mM glucosamine compared with untreated controls [100.5 ± 2.6% (n = 4; $P > 0.05$)], suggesting that glucosamine induced decreases in the secretion of apoB-100-containing lipoproteins by disrupting posttranslational events that may include the degradation of apoB by McA-RH7777 cells (Fig. 5D).

An inhibitor of lipid peroxide formation, DFX, but not the lysosomal inhibitor, leupeptin, affects the posttranslational apoB-100 degradation induced by glucosamine

We explored whether the mechanism by which glucosamine reduced the secretion of apoB-100-containing lipoproteins involved lysosomal degradation. As depicted in **Fig. 6A**, McA-RH7777 cells were pulse-labeled with 100 μCi/ml [^{35}S]methionine/cysteine for 1 h and chased for 3 h with 5 mM glucosamine or 0.5 μg/ml BFA in the absence or presence of 25 μg/ml leupeptin. Total immunoprecipitated ^{35}S-labeled apoB-100 from the media was significantly decreased to 81.01 ± 0.22% ($P < 0.05$) with 5 mM glucosamine and to 44.82 ± 15.3% ($P < 0.05$) with 0.5 μg/ml BFA compared with untreated controls. How-

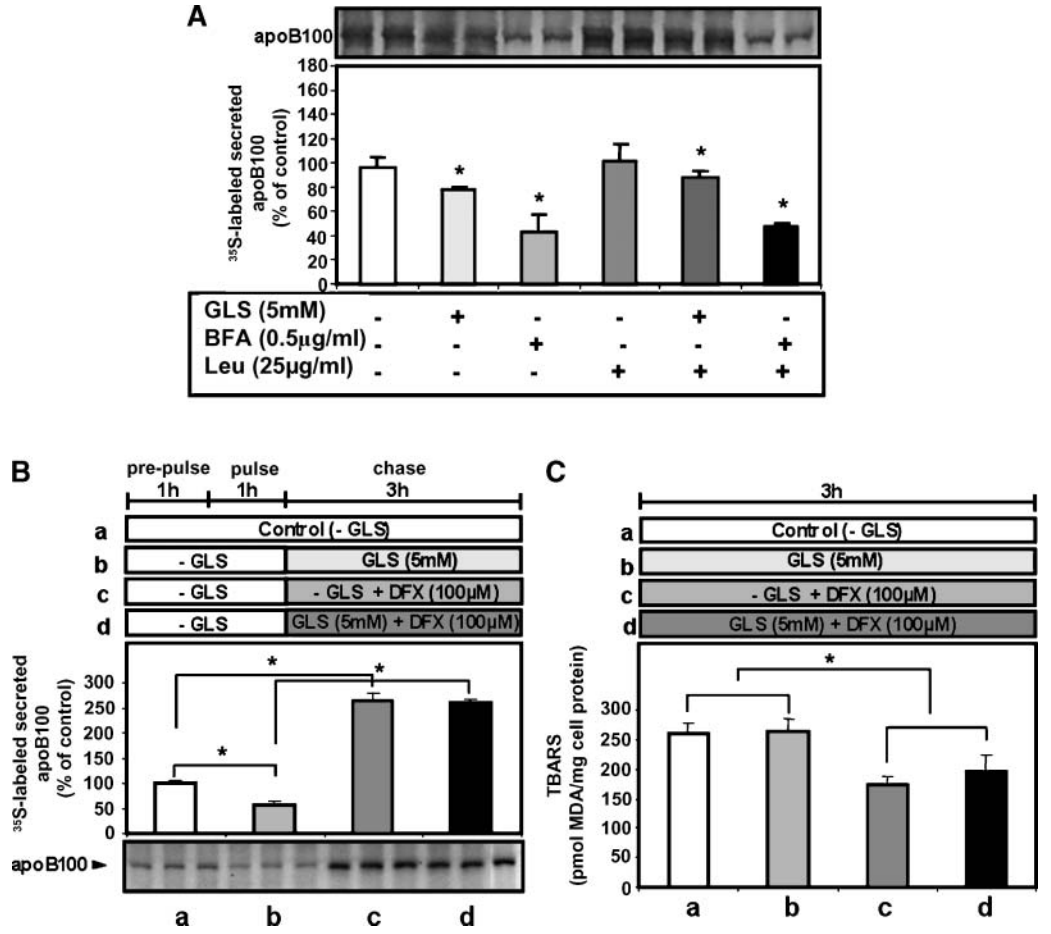

Fig. 6. Effects of a lysosomal protease inhibitor and an antioxidant on apoB-100 degradation in control and glucosamine (GLS)-treated hepatocytes. A: McA-RH7777 cells were labeled with 100 μCi/ml [^{35}S]methionine/cysteine for 3 h in the absence or presence of 5 mM glucosamine, 0.5 μg/ml BFA, and 25 μg/ml leupeptin (Leu). The media were immunoprecipitated with anti-apoB antibody. ApoB-100 bands are shown in the top panel, and radioactivity (cpm) is shown in the bar graph. B: Primary hamster hepatocytes were labeled with 100 μCi/ml [^{35}S]methionine/cysteine for 1 h and chased for 3 h in the absence or presence of 5 mM glucosamine and/or 100 μM desferrioxamine (DFX) (protocol shown in top panel). ApoB-100 bands are shown in the bottom panel, and the corresponding radioactivity is shown in the bar graph. C: Primary hamster hepatocytes were incubated in the absence or presence of 5 mM glucosamine and/or 100 μM DFX for 3 h before harvesting for a thiobarbituric acid-reactive substances (TBARS) assay, as described in Experimental Procedures, to assess the levels of lipid peroxides. * $P < 0.05$. Error bars represent SD.

ever, leupeptin treatment could not reverse the reduced secretion of apoB-100 induced by glucosamine.

Next, we investigated whether glucosamine might inhibit the secretion of apoB-100-containing lipoproteins by a mechanism involving increased postendoplasmic reticulum presecretory proteolysis (PERPP). Primary hamster hepatocytes were pulse-labeled with 100 μCi/ml [^{35}S]methionine/cysteine for 1 h (in the absence of glucosamine) and then chased for 3 h with 5 mM glucosamine in the absence or presence of 100 μM DFX (Fig. 6B). Total immunoprecipitated ^{35}S-labeled apoB-100 from conditioned media was significantly decreased to 57.7 ± 7.4% ($P < 0.05$) of the control with 5 mM glucosamine treatment (Fig. 6B, panels a, b). Interestingly, inclusion of DFX in the 3 h chase period not only increased the basal level of apoB-100 secretion but also protected apoB against the effects of the glucosamine treatment (Fig. 6B, panels c, d). Similar results were obtained when vitamin E was included with the glucosamine treatment of primary hamster hepatocytes (data not shown). These experiments were also performed in both HepG2 and McA-RH7777 cells, but the effects were less pronounced (data not shown).

We also examined the effect of glucosamine and DFX on intracellular lipid peroxides. As shown in Fig. 6C, DFX treatment was able to significantly decrease the amount of lipid peroxides present in both glucosamine-treated and untreated primary hamster hepatocytes (n = 9; $P < 0.05$). This decrease in lipid peroxides coincided with the increased levels of apoB-100 observed in Fig. 6B. Glucosamine treatment did not significantly increase lipid peroxidation levels (Fig. 6C).

Glucosamine induces mainly cotranslational degradation of apoB-100 and has a minor effect on the posttranslational degradation of apoB-100 in primary hamster hepatocytes

Finally, to estimate the effect of glucosamine treatment on proteasomal or PERPP apoB-100 degradation pathways, we carried out pulse-chase experiments in primary hamster hepatocytes in which cells were treated with 5 mM glucosamine for 16 h (**Fig. 7B**, total radiolabeled apoB-100, cellular and secreted; Fig. 7C, secreted radiolabeled apoB-100; Fig. 7D, cellular radiolabeled apoB-100). Of note, total radiolabeled apoB-100 was significantly increased to 138 ± 7.3% [979 ± 52 cpm (n = 3; $P < 0.05$)] in the presence of 25 μM MG132 compared with controls treated with glucosamine alone (707 ± 54 cpm) in a 3 h chase period, suggesting that glucosamine treatment induced the proteasomal degradation of apoB-100. On the other hand, although secreted radiolabeled apoB-100 was significantly increased to 129 ± 13.1% [403 ± 41 cpm (n = 3; $P < 0.05$)], total radiolabeled apoB-100 was only slightly and nonsignificantly increased to 109 ± 2.2% [772 ± 16 cpm (n = 3; $P > 0.05$)] in the presence of 100 μM DFX. Thus, under these conditions (glucosamine treatment during both pulse and chase periods), the induction of apoB-100 degradation by glucosamine was largely mediated by the proteasome, with only a small, nonsignificant contribution by PERPP.

DISCUSSION

Our previous studies have demonstrated that glucosamine-induced ER stress promotes apoB-100 degradation (20). In this report, we extended our studies by further examining the mechanisms by which glucosamine impaired the hepatic assembly and secretion of apoB-100-containing lipoproteins in cultured hepatocytes. Our data appear to suggest that glucosamine destabilizes the assembly and secretion of apoB-100-containing lipoproteins by multiple mechanisms. First, defective *N*-linked glycosylation of apoB-100 induced by glucosamine treatment could be an early event that leads to impairment of the assembly and intracellular biogenesis of apoB-containing lipoproteins. Our data clearly demonstrated that newly synthesized apoB-100 was resistant to endoglycosidase H digestion, suggesting a glucosamine-induced block in *N*-linked glycosylation of newly synthesized apoB-100 molecules. It is possible that the impaired glycosylation induced by glucosamine causes a conformational change in the apoB-100 molecule, resulting in increased availability of apoB-100 for ubiquitin-proteasomal degradation (3). This observation is well supported by Vukmirica et al. (26), who found that the *N*-glycans at the N terminus of several apoB constructs are required for secretion. In addition, improperly folded apoB-100 molecules could stimulate ER-stress chaperones such as Grp78 (27). Because apoB-100 is known to associate with Grp78 (19), this increased ratio of Grp78 to apoB-100 could result in prolonged retention of apoB-100 in the ER and promote the proteasomal degradation of apoB-100 (20).

Glucosamine treatment only during the chase period of radiolabeling experiments appears to disturb the posttranslational second step in the formation of apoB-100-containing lipoproteins. Our data showed that glucosamine inhibited the assembly of larger VLDL and IDL particles

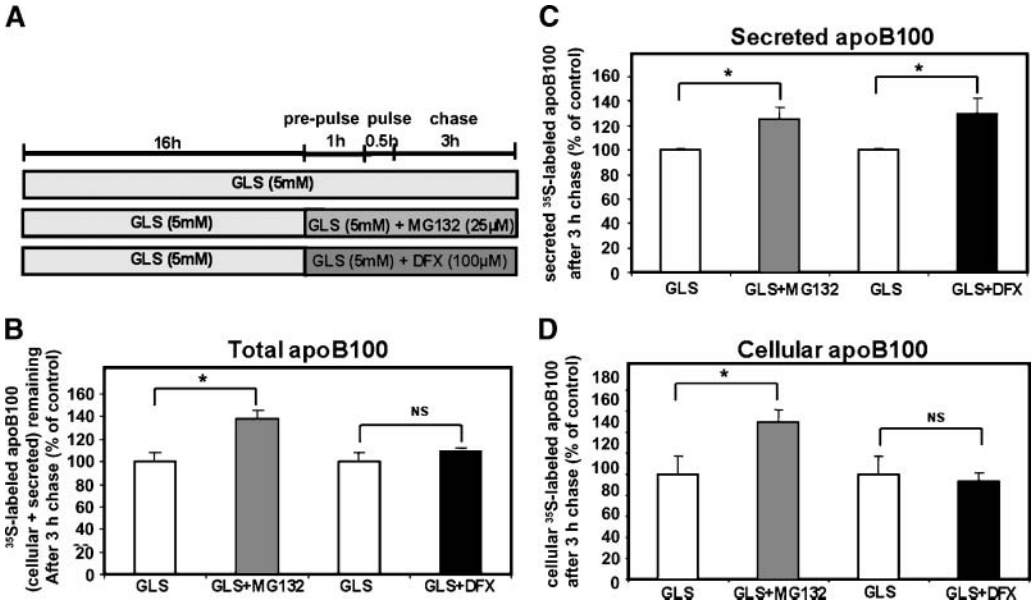

Fig. 7. Effect of glucosamine (GLS) on cotranslational and posttranslational apoB-100 degradation in primary hamster hepatocytes. A: The protocol used in the experiments. Primary hamster hepatocytes were treated with 5 mM glucosamine for 16 h, labeled with 100 μCi/ml [^{35}S]methionine/cysteine for 30 min, and chased for up to 3 h in the absence or presence of 25 μM MG132 or 100 μM DFX. Radiolabeled apoB-100 bands were analyzed. B–D: Total amounts of apoB-100 (secreted + cellular) (B), secreted apoB-100 (C), and cellular apoB-100 (D) remaining after the 3 h chase are shown (percentage of control). * $P < 0.05$. Error bars represent SD.

but did not affect HDL-sized apoB-100 particles. The formation of apoB-48-containing lipoproteins in McA-RH7777 cells was also unchanged by glucosamine treatment, suggesting that the effect of glucosamine was specific to the assembly of larger apoB-100 molecules. Based on the experimental results shown in Fig. 5, the effects of glucosamine added only during the chase period on the secretion of apoB-100 were insensitive to ALLN treatment. This suggests that the degradation of apoB under this condition is nonproteasomal in nature and does not involve ALLN-sensitive proteases. MG132, a proteasomal inhibitor, could not block the glucosamine-induced inhibition of the assembly of the larger VLDL and IDL apoB-100-containing particles. These findings strongly suggest that glucosamine can inhibit the assembly and secretion of apoB-100-containing lipoproteins posttranslationally.

Our previous studies (20) demonstrated that glucosamine treatment increases the ratio of ubiquitinated apoB-100 to total apoB-100, indicating that more apoB-100 is being targeted for proteasomal degradation upon glucosamine treatment. Proteasomal inhibitors, however, were unable to completely inhibit the effects of glucosamine on apoB-100, suggesting that glucosamine also induced the loss of apoB through another mechanism(s). We believe that this might involve a post-ER degradative pathway.

Fisher et al. (5) have previously reported that PUFAs could stimulate the degradation of apoB-100 by a post-ER presecretory pathway. This was concluded based on the lack of effects of PUFAs on the lipidation or degradation of apoB-100 at the ER but the loss of apoB from the Golgi without heightened apoB-100 secretion. It has been proposed that this mechanism involved increased lipid peroxidation and oxidative stress, as indicated by the increased appearance of lipid peroxidation products and the ability of the antioxidant vitamin E and the iron chelator DFX to protect apoB-100 against the degradation (17). We propose that a similar mechanism may result in the loss of apoB-100 upon glucosamine treatment during the chase, as the effect appeared to be posttranslational and could be prevented with vitamin E or DFX treatment but was insensitive to the lysosomal protease inhibitor leupeptin. DFX treatment did reduce lipid peroxide levels in parallel with the protection of apoB-100, demonstrating that lipid peroxidation could potentially affect apoB-100 levels. However, because glucosamine treatment did not significantly increase lipid peroxidation according to the TBARS assay used, it is unclear whether the mechanism of glucosamine-induced posttranslational apoB-100 degradation involves oxidative stress.

It is likely that the effect of glucosamine on the secretion of VLDL, IDL, and LDL may involve a failure of apoB lipidation. In our previous studies (20, Fig. 2E), the addition of oleic acid began during the prepulse and was continued throughout the subsequent pulse and chase periods. The data suggested that oleic acid could increase newly synthesized apoB-100 translocation across the ER membrane and partially decrease the apoB-100 degradation resulting from glucosamine treatment, indicating that glucosamine treatment could (cotranslationally) inhibit apoB-100 lipidation. In this study (Fig. 4C), oleic acid was added after the pulse and only during the chase period, when newly synthesized apoB-100-containing lipoproteins have likely completed the initial lipidation process and are ready to be secreted. The oleic acid treatment was included to determine whether or not it could overcome the effects of posttranslational glucosamine treatment on apoB-100 levels. Surprisingly, it was found that oleic acid treatment significantly augmented the effects of glucosamine on the loss of apoB-100 from the VLDL and IDL fractions. As polyunsaturated fatty acids have been found to induce oxidative stress and promote the post-ER degradation of apoB-100 (17), oleic acid might have increased the loss of apoB-100 in glucosamine-treated cells by exaggerating the post-ER apoB-100 degradation.

We attempted to quantitatively assess the relative contribution of the proteasomal pathway and PERPP to glucosamine-induced apoB degradation. Pulse-chase experiments were performed in primary hamster hepatocytes. This cell model was chosen because it exhibits both proteasomal and nonproteasomal degradation of apoB, with proteasomal degradation being less predominant than in other cell types. Our data suggest that under conditions in which glucosamine is present during both the pulse and chase periods, the bulk of glucosamine-induced degradation is proteasomal in nature. This is likely attributable to cotranslational inhibition of the *N*-linked glycosylation of apoB-100, leading to considerable destabilization of newly synthesized apoB-100 molecules. Under these conditions, there is likely little substrate available for PERPP. However, in experiments in which glucosamine was added only during the chase period (and not present during the pulse, when it would interfere with synthesis and cotranslational *N*-linked glycosylation), we were able to observe a significant effect of DFX on apoB-100 recovery (Fig. 6B), suggesting the involvement of post-ER degradation under conditions in which *N*-linked glycosylation is not blocked and proteasomal degradation is inhibited/minimized.

Glucosamine, widely synthesized endogenously and also sold commercially for the treatment of arthritis (28, 29), has recently been linked, controversially, to insulin resistance in both cultured cells and animal models (30, 31). Increased plasma levels of apoB-100-containing lipoproteins are an important risk factor in the development of atherosclerosis and are commonly found in insulin-resistant states and type 2 diabetes (32). Our initial hypothesis was that glucosamine treatment could induce insulin resistance and increase apoB-100 production. Instead, we observed a significant inhibition of apoB-100 secretion. Thus, glucosamine appears to have a direct inhibitory effect on apoB secretion. Glucosamine is being used as a research tool to examine the intracellular biogenesis of apoB-100. We are not suggesting that glucosamine can be used clinically to decrease apoB production.

The glucosamine dosage used in our experiments was based on two criteria. First, we consulted previous publications. For instance, to induce Grp78 in CHO cells, Lin et al. (33) used a 36 h, 10 mM glucosamine treatment. Second, we based these dosages on our own optimization

experiments. No toxicity, morphological changes, or effects on the synthesis of control non-*N*-linked glycosylated proteins such as apoE or albumin were observed with glucosamine concentrations up to 10 mM.

The inhibition of apoB-100 secretion by hepatocytes induced by glucosamine under our experimental conditions suggests that glucosamine-induced ER stress decreases the secretion of apoB-100 from the liver and perturbs lipoprotein metabolism. These results could partially explain recent work from Werstuck et al. (22) showing that glucosamine-induced ER stress deregulates lipid metabolism, leading to the accumulation of cholesterol in hepatocytes. Secretion of cholesterol-containing VLDL particles from hepatocytes would be impaired by the increased degradation of apoB-100. The effects of the high intracellular glucosamine levels that occur in diabetes on lipoprotein metabolism are unclear (22). Further in vivo studies are needed to clarify the role of glucosamine in the lipoprotein biosynthetic pathway.

In summary, glucosamine may serve as an important tool in the study of the regulation of hepatic apoB-100 secretion. Cotranslationally, glucosamine treatment may cause defects in apoB-100 folding and glycosylation, resulting in enhanced proteasomal degradation. Posttranslationally, glucosamine may interfere with the lipoprotein assembly process in general, leading to decreased VLDL, IDL, and LDL secretion and to post-ER degradation via nonproteasomal pathways.

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