Tunicamycin induces ubiquitination and degradation of apolipoprotein B in HepG2 cells

Wei LIAO and Lawrence CHAN¹

Departments of Molecular and Cellular Biology and Medicine, 1 Baylor Plaza, Baylor College of Medicine, Houston, TX 77030-3498, U.S.A.

Apolipoprotein (apo) B-100 is an essential component of atherogenic plasma lipoproteins. Previous studies have demonstrated that the production of apoB-100 is regulated largely by intracellular degradation at both the co-translational and post-translational levels and that proteasome-mediated and non-proteasome-mediated pathways are involved in this process. ApoB-100 is a glycoprotein. The present study was undertaken to address the question of whether the inhibition of N-linked glycosylation with tunicamycin would interfere with apoB-100 production. We demonstrated that the treatment of HepG2 cells with tunicamycin decreased the net production of apoB-100 by enhancing co-translational degradation of the protein. This effect of tunicamycin was partly prevented by lactacystin, a specific proteasome inhibitor. Because lactacystin only partly reversed

the effects of tunicamycin on apoB biogenesis, tunicamycin seemed also to induce apoB co-translational degradation in HepG2 cells by one or more non-proteasomal pathways. Furthermore, tunicamycin increased apoB ubiquitination approx. 4-fold. The proportion of the newly synthesized apoB-100 that was secreted and incorporated into the nascent lipoprotein particles was unaffected by tunicamycin. Thus the tunicamycin-mediated inhibition of N-linked glycosylation interferes with the production of apoB-100 that is mediated by both proteasomal and non-proteasomal pathways.

Key words: atherosclerosis, lactacystin, lipoproteins, proteasome, ubiquitin.

INTRODUCTION

The net hepatic output of apolipoprotein (apo) B-100, an essential protein component of plasma lipoproteins, very-low-density, intermediate-density, low-density lipoproteins and lipoprotein (a), is regulated at the translational and post-translational levels by degradation in a pre-Golgi compartment [1,2]. ApoB-100 biogenesis in HepG2 cells has been used as a model for secretory proteins regulated by intracellular degradation. Multiple factors [3–6], including the presence and absence of low-density lipoprotein receptor in the liver cells [7], seem to modulate the amount of apoB degraded in the cell.

Almost a decade after Borchardt and Davis [1] described the intracellular degradation of apoB, the ubiquitin–proteasome pathway was identified as a major mechanism for the intracellular degradation of apoB-100 [8]. Contributions from many laboratories have provided substantial detail to the role of the proteasome in apoB-100 degradation [9–17]. ApoB-100 is one of the largest proteins known and has a complex structure [18,19]. The hypothesis for the involvement of such a wasteful pathway for an important protein such as apoB-100 is that it is a mechanism by which the cell rids itself of misfolded apoB-100.

ApoB-100 undergoes translational and post-translational modification in the form of glycosylation; up to approx. 10 % by weight of apoB-100 is carbohydrate [20]. Both N-linked and O-linked glycosylation occur in apoB-100 molecules. The N-linked glycosylation of glycoproteins begins co-translationally in the endoplasmic reticulum and might have a role in determining the conformational properties of some secretory proteins [21,22]. Little is known about whether alterations in the glycosylation of apoB-100 might affect its production by the liver. In the present

study we examined the effect of tunicamycin-mediated inhibition of the N-linked glycosylation of apoB-100 on its production in HepG2 cells, a human hepatoma cell line that is widely used for investigating the production of apoB. Tunicamycin inhibits the formation of *N*-acetyl-glucosamine-containing lipid intermediates involved in the generation of the N-linked oligosaccharide chains [23]. We found that treatment with tunicamycin decreases apoB-100 biogenesis by increasing the co-translational degradation of apoB-100. This degradation was partly protected by the specific proteasome inhibitor lactacystin. Treatment with tunicamycin also increased apoB ubiquitination. Therefore the inhibition of N-linked glycosylation induces intracellular degradation of apoB-100 via mechanisms that involve both proteasomal and non-proteasomal pathways.

EXPERIMENTAL

Materials

Nitrocellulose membrane was from Schleicher and Schuell. Silica (fumed), *N*-ethylmaleimide, tunicamycin and *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal were from Sigma. Gamma-Bind G Sepharose was from Amersham. Lactacystin was obtained from CalBiochem. Mouse monoclonal antibody against ubiquitin and goat polyclonal antibodies against human apoB and apoA-I were from Chemicon. Rabbit polyclonal antibody against human albumin was from Research Diagnostic. Monoclonal antibody against human apoB (1D1) was kindly provided by Dr R. W. Milne (Ottawa Heart Institute, Ottawa, Ontario, Canada). Peroxidase-conjugated goat IgG fraction against

Abbreviations used: apo, apolipoprotein; FCS, fetal calf serum.

¹ To whom correspondence should be addressed (e-mail lchan@bcm.tmc.edu).

human albumin, [^{35}S]methionine, methionine, RPMI 1640 and methionine-free RPMI 1640 were from ICN. Tris/glycine gradient gels were from Novex.

Cell culture

HepG2 cells were from A. T. C. C. and were maintained at 37 °C under an air/CO₂ (19:1) atmosphere in RPMI 1640 medium (Gibco BRL) containing 10 % (v/v) fetal calf serum (FCS; HyClone), penicillin (100 units/ml) and streptomycin (100 µg/ml) (Gibco BRL).

Pulse-chase analysis and immunoprecipitation

HepG2 cells were plated on 24-well plates. Culture medium was changed three times a week. Pulse-chase analysis of apoB, albumin and apoA-I was performed as detailed previously [8,24] on cells that had reached approx. 75 % confluence. Pharmacological manipulations before and during pulse-chase periods are indicated in the Figure legends. In brief, the cells were labelled for 60 min in methionine-free medium containing [^{35}S]methionine (100 µCi/ml). The chase was initiated by replacing the labelling medium with RPMI 1640 medium containing 10 % (v/v) FCS and unlabelled methionine (15 g/l). At the times indicated, the culture medium was harvested and the cells were lysed and immunoprecipitated with polyclonal antibodies against apoB, followed by polyclonal antibodies against apoA-I and anti-albumin as described previously [17,24]. Immunoprecipitates were released by boiling for 5 min in SDS/PAGE buffer in the presence of 5 % (v/v) 2-mercaptoethanol and analysed by SDS/PAGE [6 % (w/v) or 4–12 % (w/v) gradient gel for apoB; 4–20 % (w/v) gradient gel for apoA-I and albumin]. Gels were dried and an autoradiographic image was captured by a phosphor storage system (Cyclone[®]; Packard) and analysed with OptiQuant Image Analysis Software. Data are expressed as digital light units per hour.

Immunoblot analysis

Western blot analysis was performed on the culture medium or cell lysate as described previously [17,24]. The lipoproteins in the culture medium were concentrated with fumed silica [25], then washed with cold PBS and eluted by boiling for 5 min in SDS/PAGE buffer containing 5 % (v/v) 2-mercaptoethanol. The cells were washed with cold PBS and lysed in 2 % (w/v) sodium cholate in Hepes-buffered saline [50 mM Hepes/200 mM NaCl (pH 7.4)] containing 1 mM PMSF, 0.1 mM *N*-acetyl-L-leuciny-L-leucine-L-norleucine, 5 mM *N*-ethylmaleimide and complete protease inhibitors (Boehringer Mannheim). Insoluble material in the cell lysate was removed by centrifugation at 10000 *g* for 10 min. ApoB or albumin were immunoprecipitated with the respective antibodies as described above. The proteins were separated by SDS/PAGE, transferred overnight to nitrocellulose membranes, probed with monoclonal anti-ubiquitin or anti-apoB (1D1) or anti-albumin antibodies that had been peroxidase-conjugated with an EZ-Link[™] Plus Activated Peroxidase kit (Pierce) and detected by enhanced chemiluminescence (ECL[®] kit; Amersham). The relative intensities of the immunoblot bands were quantified with an AlphaImager[™] 2000 Documentation and Analysis system (Alpha Innotech Corporation).

Sucrose-gradient ultracentrifugation of lipoproteins

The lipoproteins present in the medium were separated by sucrose-gradient ultracentrifugation by the method of Borén et al. [26] as described previously [24]. Each gradient was formed by layering the following solutions from the bottom of the tube:

2 ml of 47 % (w/v) sucrose, 2 ml of 25 % (w/v) sucrose, 5 ml of sample in 12.5 % (w/v) sucrose, and 3 ml PBS. The gradients were centrifuged in a Beckman SW40 rotor at 35000 rev./min (217900 *g*) at 4 °C for 65 h. Gradients were unloaded from the top of the tube in 12 fractions. Lipoproteins in each fraction were concentrated with silica and washed with cold PBS; apolipoproteins were eluted into SDS/PAGE buffer and separated by SDS/PAGE [6 % (w/v) gel] as detailed above. ApoB was detected by immunoblot analysis with a peroxidase-conjugated monoclonal antibody against human apoB (1D1).

Statistics

Data are presented as means ± S.E.M. and were analysed by using Jandel Scientific Software SigmaStat 2.0. We used a *t* test to compare data from two groups and a one-way analysis of variance to evaluate the presence of significant differences between more than two groups, followed by post-hoc comparisons of the group means by the method of Tukey (SigmaStat 2.0).

RESULTS

Tunicamycin inhibits the biogenesis of apoB-100 in HepG2 cells

We first examined the effect of tunicamycin on the biogenesis of apoB-100. HepG2 cells were incubated in the presence or absence of tunicamycin (5 µg/ml) for 2 or 4 h. They were then labelled with [^{35}S]methionine for 60 min before a 2 h chase was performed by the addition of excess unlabelled methionine, during which time the tunicamycin was either present or absent. After the 2 h chase, approx. one-third of the labelled apoB-100 remained, irrespective of the presence or absence of the antibiotic ($P < 0.001$; Figure 1A). However, it is clear that the presence of tunicamycin substantially decreased the amount of intracellular radiolabelled apoB-100 during the 1 h labelling period. A 2 h pretreatment with tunicamycin produced an approx. 25 % ($P < 0.05$), and a 4 h pretreatment an approx. 80 % ($P < 0.05$), decrease in the amount of [^{35}S]methionine-labelled apoB-100 [compare tunicamycin-treated groups with control (Figure 1A, middle panel, filled columns)]. The relative amounts of apoB-100 secreted [approx. one-third of the remainder (Figure 1A, lower panel)] were essentially unaffected, indicating that poorly glycosylated or unglycosylated apoB-100 was secreted with an efficiency similar to that of the fully glycosylated protein. This observation is similar to that previously reported for the relative lack of effect of tunicamycin on apoB secretion [27,28].

As a control we examined the effect of treatment with tunicamycin on apoA-I and albumin, two non-glycosylated secretory proteins. Exposure of the cells to tunicamycin for 2 h had no effect on the amount of intracellular radiolabelled apoA-I; a 4 h exposure produced a small but significant effect [$P < 0.05$; compare tunicamycin-treated groups with control (Figure 1B, filled columns)]. The presence of tunicamycin substantially decreased the amount of the intracellular radiolabelled albumin after a 1 h labelling period [$P < 0.05$; compare tunicamycin-treated groups with control (Figure 1C, filled columns)]. Similar mild to moderate inhibitory effects of tunicamycin on albumin biosynthesis had been noted previously in avian hepatocytes [27]. There was little or no effect of tunicamycin on the relative amounts of apoA-I or albumin secreted by the HepG2 cells [compare open columns with corresponding filled columns in Figures 1(B) and 1(C)]. Both proteins were largely secreted after a 2 h chase.

Figure 1 Effect of tunicamycin on apoB biogenesis: pulse-chase analysis

HepG2 cells were pretreated for 2 or 4 h with tunicamycin (tuni) (5 μ g/ml) in 1 ml per well of culture medium containing 10% (v/v) FCS. Control cells received vehicle only. After pretreatment, cells were labelled with [³⁵S]methionine for 1 h, then chased with excess unlabelled methionine for 0 or 2 h in the presence or absence of tunicamycin as indicated. After the chase, the medium was collected and the cells were lysed. ApoB and then apoA-I and albumin were immunoprecipitated with anti-apoB followed by anti-(apoA-I) and anti-albumin antibodies and quantified as described in the Experimental section. (A) ApoB; (B) apoA-I; (C) albumin. Results represent three separate determinations and are expressed as means $\pm$ S.E.M. The absence of discernible S.E.M. bars indicates that they fell within the width of the line drawing. Abbreviation: DLU, digital light units. In (A) the bottom panel represents relative expression, taking as 100% the 1 h labelled apoB-100 at 0 h chase.

Figure 2 Effect of tunicamycin and lactacystin on apoB biogenesis: pulse-chase analysis

HepG2 cells were pretreated for 4 h with tunicamycin (5 μ g/ml) in 1 ml per well of culture medium containing 10% (v/v) FCS. Control cells received vehicle only. After pretreatment, the cells were labelled with [³⁵S]methionine for 1 h and chased with excess unlabelled methionine for 0 or 2 h in the presence or absence of tunicamycin (5 μ g/ml) or lactacystin (20 μ M). After the chase, the medium was collected and the cells were lysed. ApoB and then apoA-I and albumin were immunoprecipitated with anti-apoB followed by anti-(apoA-I) and anti-albumin antibodies and quantified as described in the Experimental section. (A) ApoB; (B) apoA-I; (C) albumin. Results represent three separate determinations and are expressed as means $\pm$ S.E.M. The absence of discernible S.E.M. bars indicates that they fell within the width of the line drawing. Abbreviation: DLU, digital light units. In (A) the top panel represents relative expression, taking as 100% the 1 h-labelled apoB-100 at 0 h chase.

Figure 3 Effect of tunicamycin on the time course of apoB synthesis

HepG2 cells were pretreated for 3 h with tunicamycin (5 $\mu\text{g/ml}$) in 1 ml per well of culture medium containing 10% (v/v) FCS. Lactacystin was then added to some wells; other wells received an equal volume of vehicle for a further 0.5 h of incubation. Medium was changed to 0.5 ml of methionine-free medium containing [^{35}S]methionine for 5, 10, 30 and 60 min with or without tunicamycin or lactacystin as indicated. After labelling, the cells, together with medium, were mixed with 0.5 ml of lysis buffer and lysed overnight. The lysate was cleared of cell debris by centrifugation and the supernatant was immunoprecipitated with anti-apoB followed by anti-(apoA-I) and anti-albumin antibodies. The proteins in the immunoprecipitated lysates were separated by SDS/PAGE. The positions of molecular mass markers are indicated at the left for apoB and right for albumin and apoA-I.

Effect of lactacystin and tunicamycin on apoB-100 biogenesis in HepG2 cells

To examine whether the effect of tunicamycin on apoB biogenesis was mediated by the proteasome pathway, we studied whether lactacystin, a proteasome-specific inhibitor, influenced the tunicamycin effect. HepG2 cells were pretreated with tunicamycin (5 $\mu\text{g/ml}$) for 4 h. They were then analysed by pulse-chase analysis as described above, except that lactacystin was present in some of the cultures. In agreement with previous observations [17], the presence of lactacystin (20 μM) almost doubled the amount of intracellular radiolabelled apoB-100 after 1 h of

labelling [$P < 0.001$; Figure 2A (compare group 3 with group 1, filled columns)]. This can be explained by the fact that lactacystin inhibits the co-translational as well as the post-translational degradation of apoB-100 [10,12,17]. In contrast, lactacystin did not increase the amount of intracellular radiolabelled apoA-I or albumin after a 1 h labelling [Figures 2B and 2C (compare group 3 with group 1, filled columns)], indicating that there is insignificant proteasome-mediated degradation of these non-glycoproteins. As in the experiment shown in Figure 1, tunicamycin substantially decreased the amount of intracellular radiolabelled apoB-100 after a 1 h labelling period [$P < 0.001$; Figure 2A (compare group 2 with group 1, middle panel, filled columns)].

Figure 4 Time course of the effect of tunicamycin on intracellular and secreted apoB

HepG2 cells were cultured in the presence or absence of tunicamycin (5 μ g/ml) for 2, 4, 7, 16, 20 and 25 h. At various times, the medium was collected and the lipoproteins in the medium were concentrated with silica. The cells were lysed; the lysate was cleared of debris by spinning and the supernatant (1500 μ g of protein of each sample) was immunoprecipitated with goat anti-apoB antibody followed by rabbit anti-albumin antibody. The proteins in the concentrated medium and in the immunoprecipitated cell lysates were separated by SDS/PAGE, transferred to nitrocellulose and probed with peroxidase-conjugated antibodies as described in the Experimental section. (A) Cellular apoB; (B) secreted apoB; (C) cellular albumin. The positions of molecular mass markers are indicated at the left.

The presence of lactacystin attenuated this effect of tunicamycin [$P < 0.001$; Figure 2A (compare group 4 with group 2, middle panel, filled columns)].

After a 2 h chase, the amount of apoB-100 that remained (cellular plus secreted) in lactacystin-treated (group 3) was approx. 4-fold higher than in the control (group 1) [$P < 0.001$; Figure 2A (middle panel, hatched column plus open column)]. However, lactacystin had no effect on the relative amount (i.e. the percentage) of apoB-100 that remained (cellular plus secreted), whether tunicamycin was present or not [Figure 2A (compare group 4 with group 3, top panel)]. Furthermore, of the remaining 35 S-apoB-100, approximately one-quarter to one-third was recovered from the medium after the 2 h chase, irrespective of the presence or absence of tunicamycin.

Figure 5 Dose-dependent effect of tunicamycin on apoB secretion

HepG2 cells were treated for 20 h with various amounts of tunicamycin (0, 1.25, 2.5, 5, 10, 20 and 40 μ g/ml; lanes 1–7) or with vehicle (DMSO) (2, 4 and 8 μ l/ml; lanes 5'–7'). The medium was then collected and concentrated with silica. The cells were lysed. The cell lysate was cleared of debris by centrifugation and the supernatant (1500 μ g of protein of each sample) was immunoprecipitated with goat anti-apoB antibody followed by rabbit anti-albumin antibody. The proteins in the concentrated medium and in the immunoprecipitated cell lysates were separated by SDS/PAGE, transferred to nitrocellulose and probed with peroxidase-conjugated antibodies as described in the Experimental section. (A) Cellular apoB; (B) secreted apoB; (C) cellular albumin. The positions of molecular mass markers are indicated at the left.

As shown above in Figures 1(B) and 1(C), the presence of tunicamycin also decreased the amount of intracellular radio-labelled apoA-I and albumin [$P < 0.001$; Figures 2B and 2C (compare group 2 with group 1, filled columns)]. The inhibitory effect of tunicamycin on these non-glycoproteins was not affected by the addition of lactacystin [Figures 2B and 2C (compare group 4 with group 2)], which suggests that the effect of tunicamycin on apoA-I and albumin was not mediated by the

Figure 6 Effect of tunicamycin on ubiquitination of apoB

HepG2 cells were cultured in 162 cm² flasks until they had reached approx. 75% confluence. The cells were then incubated with or without tunicamycin (5 µg/ml) in 10 ml of culture medium containing 10% (v/v) FCS for 19 h. During the last 2 h of incubation, lactacystin (10 µM) was added as indicated. The culture medium was then collected; lipoproteins in the medium (1 ml) were concentrated with silica (asterisk). The cells were lysed in 5 ml lysis buffer. The supernatant was cleared of cell debris by centrifugation; an aliquot (1 ml) of the supernatant was immunoprecipitated with goat anti-human apoB antibody (Ab). The lipoproteins in the concentrated medium and in the anti-apoB immunoprecipitates from the cell lysates were separated by SDS/PAGE and blotted with peroxidase-conjugated anti-ubiquitin antibody [(A), left panel]. To probe for apoB, the filter was stripped and re-blotted with anti-apoB–peroxidase conjugate [(A), right panel]. (B) The anti-apoB immunoprecipitates from the lactacystin-treated cell lysates were subjected to SDS/PAGE and immunoblotted for ubiquitin and for apoB. Various amounts of the sample were loaded on the gel in this experiment. The ubiquitin conjugates (the entire lane from the top of the gel to approx. 46 kDa) and the apoB-100 band were quantified as described in the Experimental section. The ratios of the ubiquitin–apoB conjugate over apoB-100 were calculated and plotted in (C) as arbitrary units relative to control, which was set at 1.0. It should be noted that tunicamycin markedly decreased the intracellular mass of apoB-100 [compare lanes 3' and 1' and lanes 4' and 2' in (A)]. However, the intensity of apoB ubiquitinate conjugates in the cells treated with tunicamycin plus lactacystin was roughly similar to that in the cells treated with lactacystin only [compare lanes 3 and 1 in (A)], whereas the intracellular mass of apoB-100 in the cells treated with lactacystin plus tunicamycin was approx. one-quarter of that in the cells treated with lactacystin only [compare lanes 3' and lane 1' in (A)]. Therefore the tunicamycin-treated samples had a much higher ubiquitin content per weight basis of apoB in the sample. This is clearly evident in (B), as calculated in (C). The positions of molecular mass markers are indicated at the left.

proteasome. In both cases, most of the newly synthesized apoA-I and albumin were secreted both in the presence and the absence of lactacystin or tunicamycin after a 2 h chase.

Effect of tunicamycin and lactacystin on the time course of biogenesis of apoB polypeptides

We examined in some detail the effects of tunicamycin and lactacystin on the time-dependent accumulation of [³⁵S]-methionine-labelled growing apoB polypeptide chains. HepG2 cells were pretreated with tunicamycin for 3 h; lactacystin was then added to some of the cells and the preincubation continued for a further 0.5 h, at which time [³⁵S]methionine was added to

the cultures for 5, 10, 30 or 60 min in the presence or absence of tunicamycin and lactacystin as indicated (Figure 3). The inhibitory effect of lactacystin has a lag period [29]; we therefore added this drug 30 min before labelling, to ensure that the inhibitor was present in its active form during the radiolabelling. As shown in Figure 3 (left panels), lactacystin enhanced the total amount of [³⁵S]methionine-labelled apoB, an observation consistent with the previously established fact that apoB undergoes co-translational (as well as post-translational) degradation by the proteasome pathway [10,12,17]. The presence of tunicamycin resulted in a substantial decrease in the apparent incorporation of [³⁵S]methionine into apoB. This was evident even at 5 min and was observed throughout the 60 min labelling period. The effect

seemed to be much smaller or absent in apoB polypeptides that were shorter than approx. 220 kDa. This inhibitory effect of tunicamycin was largely, but not completely, reversed by the addition of lactacystin, an observation consistent with the participation of both proteasomal and non-proteasomal pathways in the effect of tunicamycin. In this experiment, lactacystin had essentially no effect on the incorporation of [35 S]methionine into apoA-I or albumin. The effect of tunicamycin on these non-glycoproteins was minimal and unaffected by the presence or absence of lactacystin (Figure 3, right panels).

Effect of tunicamycin on steady-state levels of apoB-100 in HepG2 cells

To assess the effects of tunicamycin on the steady-state level of apoB-100 present in HepG2 cells and on that secreted into the culture medium, we performed an immunoblot analysis of cell lysate (Figure 4A) and medium (Figure 4B) after the cells had been exposed to tunicamycin (5 μ g/ml) for various periods from 2 to 25 h. It is evident that tunicamycin decreased the amount of cellular apoB after a 7 h treatment and detectably decreased that in the culture medium after 16 h. It had a minimal effect on the cellular albumin level throughout the 25 h incubation (Figure 4C).

To examine the tunicamycin effects on these proteins further, we treated HepG2 cells with increasing amounts of tunicamycin (0, 1.25, 2.5, 5, 10, 20 and 40 μ g/ml; Figure 5, lanes 1–7) or vehicle (DMSO) alone (2, 4 and 8 μ l; Figure 5, lanes 5'–7') for 20 h. Cellular apoB-100 (Figure 5A) and apoB-100 in the medium (Figure 5B) were analysed by immunoblotting. It is evident that tunicamycin decreased, in a dose-dependent manner, the amount of apoB inside the cell as well as in the medium; vehicle alone was completely without effect. Consistent with the results shown in Figure 4 is our observation that the inhibitory effect of tunicamycin was much stronger on cellular apoB than on secreted apoB (compare Figures 5A and 5B). The differential sensitivity of cellular and secreted apoB, coupled with the experiments shown in Figures 1 and 2, suggests that the effect on the amount of apoB secreted was a result of impaired apoB production in the cell instead of a direct effect of tunicamycin on apoB secretion. The effect of tunicamycin on cellular albumin was minimal under these conditions (Figure 5C).

Tunicamycin promotes the ubiquitination of apoB in HepG2 cells

Multi-ubiquitination is a major mechanism for targeting proteins to the proteasomal pathway. We examined whether tunicamycin enhances the ubiquitination of apoB in HepG2 cells. Cells were preincubated in the presence or absence of lactacystin and/or tunicamycin (see the legend to Figure 6 for details). Cellular immunoreactive apoB was precipitated by a monospecific goat antiserum against human apoB and the immunoprecipitates were fractionated by SDS/PAGE. These were immunoblotted with monoclonal anti-apoB or anti-ubiquitin antibody. As shown in Figure 6(A), apoB-ubiquitin conjugates were readily detected in the cells treated with lactacystin alone and in those treated with lactacystin plus tunicamycin (Figure 6A, lanes 1 and 3); they were barely detectable in the absence of lactacystin (lanes 2 and 4). Although the intensities of the cellular apoB-ubiquitin conjugates in the cells treated with lactacystin plus tunicamycin were roughly similar to those in cells treated with lactacystin only (Figure 6A; compare lanes 3 and 1), the intracellular mass of apoB-100 in the cells treated with lactacystin plus tunicamycin was approx. one-quarter of that in cells treated with lactacystin only (Figure 6A; compare lanes 3' and 1'). Therefore the tunicamycin-treated samples had a much higher ubiquitin content

Figure 7 Effect of tunicamycin on the density distribution of apoB secreted into the medium

HepG2 cells were cultured in 162 cm² flasks until they had reached approx. 75% confluence. The cells were then incubated with or without tunicamycin (5 μ g/ml) in culture medium containing 10% (v/v) FCS for 19 h, as indicated. During the last 2 h of incubation, lactacystin (10 μ M) was added to some cultures, after which the culture medium was collected and the lipoproteins in the medium were separated by sucrose-gradient ultracentrifugation. The gradients were unloaded from the top of the tube into 12 fractions. Lipoproteins in each fraction were concentrated with silica; the apolipoproteins were separated by SDS/PAGE [6% (w/v) gel]. ApoB was detected by immunoblot analysis with a monoclonal antibody (1D1) against human apoB.

per unit mass of apoB-100. In the sample that was treated with tunicamycin only, the intracellular apoB was barely detectable (Figure 6A; lane 4').

To quantify the relative degrees of ubiquitination of the cellular apoB under these conditions, we blotted various amounts of the anti-apoB immunoprecipitate from the cells treated with lactacystin only and compared them with a sample containing a fixed amount of anti-apoB immunoprecipitate from the cells treated with lactacystin plus tunicamycin (Figure 6B). Comparison of samples that had approximately similar amounts of apoB-100 revealed that the apoB in tunicamycin-treated cells displayed an approx. 400% increase in ubiquitin content (Figure 6C). In contrast, tunicamycin did not induce albumin ubiquitination under these conditions (results not shown).

Effect of tunicamycin on density distribution of apoB-100 secreted by HepG2 cells

ApoB-100 is secreted by HepG2 cells in association with lipids. We examined whether the density distribution of the apoB-100-associated lipoproteins was changed by treatment with tunicamycin. We compared the density profiles of apoB-containing lipoprotein particles in the medium of cells cultured in the presence and in the absence of tunicamycin or lactacystin. As shown in Figure 7, we detected no significant difference between the density distributions of apoB under the different culture conditions. Inhibition of apoB glycosylation therefore did not affect its differential association with lipoproteins secreted by HepG2 cells.

DISCUSSION

Tunicamycin inhibits the formation of *N*-acetylglucosamine-containing lipid intermediates involved in the generation of the *N*-linked oligosaccharide chains [23]. There are 19 potential

N-linked glycosylation sites in apoB-100, of which only 16 are actually glycosylated [18]. The effect of inhibition of N-linked glycosylation on protein conformation and secretion varies with the protein [30]. Here we have shown that the inhibition of N-glycosylation in apoB-100 interferes with the production of this protein in HepG2 cells.

In HepG2 cells approx. 30–75% of the newly synthesized apoB-100 is degraded within a 2–3 h period before the balance of the protein is secreted [4,5,8,24]. We [8] and others [9–11] have shown previously that the intracellular degradation of apoB in HepG2 cells is mediated mainly by the ubiquitin–proteasome pathway. It occurs both co-translationally [12,17] and post-translationally [17,31]. Here we have shown that the co-translational degradation of apoB-100 was enhanced further when N-linked glycosylation of the protein was blocked by treatment with tunicamycin. Interestingly, some mild to moderate inhibition of albumin and apoA-I production was also evident. For these non-glycoproteins the mechanism is clearly different because, whereas lactacystin partly reversed the tunicamycin-induced co-translational degradation of apoB-100, it had no effect on albumin or apoA-I. Inhibition of non-glycoprotein synthesis by treatment with tunicamycin had been reported previously [27]. It might be related to the effect of the antibiotic on the proper glycosylation of protein factors involved in protein translation. We note that lactacystin only partly reversed the effects of tunicamycin on apoB, indicating that both proteasome-mediated and non-proteasome-mediated pathways are involved in the tunicamycin-enhanced co-translational degradation of apoB-100. The involvement of the proteasome pathway was further supported by the stimulation of the multi-ubiquitination of apoB in the presence of tunicamycin. The role of the non-proteasomal pathway is unclear but is probably related at least partly to the same process that inhibits the production of albumin and apoA-I, possibly by interference with protein factors involved in translation.

Despite its enhanced co-translational degradation in the presence of tunicamycin, the under-glycosylated apoB-100 seemed to be secreted with relatively normal efficiency. The smaller amount of apoB-100 secreted in tunicamycin-treated cells is caused mainly by a decrease in the amount of protein produced in the cell. Approximately the same proportion of the newly synthesized apoB-100 was secreted from the HepG2 cells as from untreated cells under these conditions. Furthermore, the secreted apoB-100 was associated with lipoproteins of roughly the same buoyant densities as those produced by control cells. Thus inhibition of N-linked glycosylation in apoB-100 seems not to interfere with the assembly of apoB-100 into lipoproteins.

Why are more of the under-glycosylated apoB molecules degraded? Normally, a substantial proportion of apoB is degraded intracellularly. The most popular hypothesis is that, being such a large and complex molecule, apoB-100 is prone to misfolding in HepG2 cells. The intracellular degradation of apoB is thought to be a mechanism by which the cell rids itself of misfolded apoB molecules. This interpretation is supported by the observation that a supply of extra lipids to HepG2 cells down-regulates the intracellular degradation of apoB [32], presumably by increasing the proportion of molecules that are properly folded. It is tempting to speculate that inhibition of N-glycosylation promotes misfolding of apoB, predisposing it to removal by the cell. If this interpretation is correct, we are intrigued by the ability of the remaining under-glycosylated apoB-100 molecules to be secreted with similar efficiency and to associate normally with the secreted lipoprotein particles.

In conclusion, we found that the inhibition of N-glycosylation of apoB-100 in HepG2 cells promotes its co-translational degradation

by proteasomal and non-proteasomal pathways. The absence of any significant effect of tunicamycin on the secretion and the lipoprotein association of apoB-100 is noteworthy. These observations provide insight into the biogenesis, structure–conformation and functional aspects of apoB-100, a central building block of atherogenic plasma lipoproteins.

This work was supported by a grant (HL-16512) from the National Institutes of Health (to L. C.) and a grant (9960083Y) from the American Heart Association, Texas Affiliate (to W. L.). W. L. is an American Liver Foundation Clarence A. Kruse Memorial Liver Scholar.

REFERENCES

- Borchardt, R. A. and Davis, R. A. (1987) Intrahepatic assembly of very low density lipoproteins. Rate of transport out of the endoplasmic reticulum determines rate of secretion. *J. Biol. Chem.* **262**, 16394–16402
- Sato, R., Imanaka, T., Takatsuki, A. and Takano, T. (1990) Degradation of newly synthesized apolipoprotein B-100 in a pre-Golgi compartment. *J. Biol. Chem.* **265**, 11880–11884
- Yeung, S. J. and Chan, L. (1998) Hepatic apolipoprotein B biogenesis: an update. *Trends Cardiovasc. Med.* **8**, 6–12
- Ginsberg, H. N. (1995) Synthesis and secretion of apolipoprotein B from cultured liver cells. *Curr. Opin. Lipidol.* **6**, 275–280
- Yao, Z., Tran, K. and McLeod, R. S. (1997) Intracellular degradation of newly synthesized apolipoprotein B. *J. Lipid Res.* **38**, 1937–1953
- Sparks, J. D. and Sparks, C. E. (1990) Insulin modulation of hepatic synthesis and secretion of apolipoprotein B by rat hepatocytes. *J. Biol. Chem.* **265**, 8854–8862
- Twisk, J., Gillian-Daniel, D. L., Tebon, A., Wang, L., Barrett, P. H. R. and Attie, A. D. (2000) The role of the LDL receptor in apolipoprotein B secretion. *J. Clin. Invest.* **105**, 521–532
- Yeung, S. J., Chen, S.-H. and Chan, L. (1996) Ubiquitin–proteasome pathway mediates intracellular degradation of apolipoprotein B. *Biochemistry* **35**, 13843–13848
- Fisher, E. A., Zhou, M., Mitchell, D. M., Wu, X., Omura, S., Wang, H., Goldberg, A. L. and Ginsberg, H. N. (1997) The degradation of apolipoprotein B100 is mediated by the ubiquitin–proteasome pathway and involves heat shock protein 70. *J. Biol. Chem.* **272**, 20427–20434
- Benoist, F. and Grand-Perret, T. (1997) Co-translational degradation of apolipoprotein B100 by the proteasome is prevented by microsomal triglyceride transfer protein. *J. Biol. Chem.* **272**, 20435–20442
- Chen, Y., Le Cahérec, F. and Chuck, S. L. (1998) Calnexin and other factors that alter translocation affect the rapid binding of ubiquitin to apoB in the Sec61 complex. *J. Biol. Chem.* **273**, 11887–11894
- Zhou, M., Fisher, E. A. and Ginsberg, H. N. (1998) Regulated co-translational ubiquitination of apolipoprotein B100. A new paradigm for proteasomal degradation of a secretory protein. *J. Biol. Chem.* **273**, 24649–24653
- Mitchel, D. M., Zhou, M., Pariyath, R., Wang, H., Aitchison, J. D., Ginsberg, H. N. and Fisher, E. A. (1998) Apoprotein B100 has a prolonged interaction with the translocon during which its lipidation and translocation change from dependence on the microsomal triglyceride transfer protein to independence. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 14733–14738
- Liang, J.-S., Wu, X., Jiang, H., Zhou, M., Yang, H., Angkeow, P., Huang, L.-S., Sturley, S. L. and Ginsberg, H. (1998) Translocation efficiency, susceptibility to proteasomal degradation, and lipid responsiveness of apolipoprotein B are determined by the presence of β sheet domains. *J. Biol. Chem.* **273**, 35216–35221
- Cavallo, D., McLeod, R. S., Rudy, D., Aiton, A., Yao, Z. and Adeli, K. (1998) Intracellular translocation and stability of apolipoprotein B are inversely proportional to the length of the nascent polypeptide. *J. Biol. Chem.* **273**, 33397–33405
- Du, E. Z., Fleming, J. F., Wang, S.-L., Spitsen, G. M. and Davis, R. A. (1999) Translocation-arrested apolipoprotein B evades proteasome degradation via a sterol-sensitive block in ubiquitin conjugation. *J. Biol. Chem.* **274**, 1856–1862
- Liao, W., Yeung, S.-C. J. and Chan, L. (1998) Proteasome-mediated degradation of apolipoprotein B targets both nascent peptides cotranslationally before translocation and full-length apolipoprotein B after translocation into the endoplasmic reticulum. *J. Biol. Chem.* **273**, 27225–27230
- Chan, L. (1992) Apolipoprotein B, the major protein component of triglyceride-rich and low density lipoproteins. *J. Biol. Chem.* **267**, 25621–25624
- Segrest, J. P., Jones, M. K., Mishra, V. K., Anantharamaiah, G. M. and Garber, D. W. (1994) ApoB-100 has a pentapartite structure composed of three amphipathic α -helical domains alternating with two amphipathic β -strand domains. *Arterioscler. Thromb.* **14**, 1674–1685

- 20 Swaminathan, N. and Aladjem, F. (1976) The monosaccharide composition and sequence of the carbohydrate moiety of human serum low density lipoproteins. *Biochemistry* **15**, 1516–1522
- 21 Imperiali, B. and O'Connor, S. E. (1999) Effect of N-linked glycosylation on glycopeptide and glycoprotein structure. *Curr. Opin. Chem. Biol.* **3**, 643–649
- 22 Grabenhorst, E., Schlenke, P., Pohl, S., Nimtz, M. and Conradt, H. S. (1999) Genetic engineering of recombinant glycoprotein and the glycosylation pathway in mammalian host cells. *Glycoconj. J.* **16**, 81–97
- 23 Elbein, A. D. (1987) Inhibitors of the biosynthesis and processing of N-linked oligosaccharide chains. *Annu. Rev. Biochem.* **56**, 497–534
- 24 Liao, W., Kobayashi, K. and Chan, L. (1999) Adenovirus-mediated overexpression of microsomal triglyceride transfer protein (MTP): mechanistic studies on the role of MTP in apolipoprotein B-100 biogenesis. *Biochemistry* **38**, 7532–7544
- 25 Vance, D., Weinstein, D. and Steinberg, D. (1984) Isolation and analysis of lipoproteins secreted by rat liver hepatocytes. *Biochim. Biophys. Acta* **792**, 39–47
- 26 Borén, J., Rustaeus, S. and Olofsson, S.-O. (1994) Studies on the assembly of apolipoprotein B-100- and B-48-containing very low density lipoproteins in McA-RH7777 cells. *J. Biol. Chem.* **269**, 25879–25888
- 27 Struck, D. K., Siuta, P. B., Lane, M. D. and Lennarz, W. J. (1978) Effect of tunicamycin on the secretion of serum proteins by primary cultures of rat and chick hepatocytes. *J. Biol. Chem.* **253**, 5332–5337
- 28 Patel, S. B. and Grundy, S. M. (1996) Interactions between microsomal triglyceride transfer protein and apolipoprotein B within the endoplasmic reticulum in a heterologous expression system. *J. Biol. Chem.* **271**, 18686–18694
- 29 Dick, L. R., Cruikshank, A. A., Destree, A. T., Grenier, L., McCormack, T. A., Melandri, F. D., Nunes, S. L., Palombella, V. J., Parent, L. A., Plamondon, L. and Stein, R. L. (1996) Mechanistic studies on the inactivation of the proteasome by lactacystin in cultured cells. *J. Biol. Chem.* **272**, 182–188
- 30 Bauer, H. C., Parent, J. B. and Olden, K. (1985) Role of carbohydrate in glycoprotein secretion by human hepatoma cells. *Biochem. Biophys. Res. Commun.* **128**, 368–375
- 31 Huang, X. F. and Shelness, G. S. (1999) Efficient glycosylation site utilization by intracellular apolipoprotein B: implications for proteasomal degradation. *J. Lipid Res.* **40**, 2212–2222
- 32 Dixon, J. L., Furukawa, S. and Ginsberg, H. N. (1991) Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from Hep G2 cells by inhibiting early intracellular degradation of apolipoprotein B. *J. Biol. Chem.* **266**, 5080–5086

Received 3 August 2000/25 October 2000; accepted 13 November 2000