

Apolipoprotein[a] secretion from hepatoma cells is regulated in a size-dependent manner by alterations in disulfide bond formation

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Abstract Apolipoprotein[a] (apo[a]) is a large disulfide linked glycoprotein synthesized by hepatocytes. We have examined the role of disulfide bond formation in the processing of apo[a] using human and rat hepatoma cells expressing apo[a] isoforms containing varying numbers of kringle 4 (K4) domains, following treatment with DTT. Hepatoma cells expressing 6- or 9-K4 isoforms revealed ~90% inhibition of apo[a] secretion following DTT treatment, although larger isoforms containing 13- or 17-K4 domains demonstrated continued secretion (up to 30% of control values), suggesting that a fraction of the larger isoforms is at least partially DTT resistant. Wash-out experiments demonstrated that these effects were completely reversible for all isoforms studied, with no enhanced degradation associated with prolonged intracellular retention. DTT treatment was associated with enhanced binding of apo[a] with the endoplasmic reticulum-associated chaperone proteins calnexin, calreticulin, and BiP, which was reversible upon DTT removal. The chemical chaperone 6-aminohexanoic acid, previously demonstrated by others to rescue defective apo[a] secretion associated with alterations in glycosylation, failed to alter the secretion of apo[a] following DTT treatment. The demonstration that DTT modulates apo[a] secretion in a manner influenced by both the type and number of K4 repeats extends understanding of the mechanisms that regulate its exit from the endoplasmic reticulum.—Nassir, F., Y. Xie, and N. O. Davidson. Apolipoprotein[a] secretion from hepatoma cells is regulated in a size-dependent manner by alterations in disulfide bond formation. *J. Lipid Res.* 2003. 44: 816–827.

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Apolipoprotein[a] (apo[a]) is a large and highly polymorphic glycoprotein whose interaction with apoB-100 results in the formation of an atherogenic lipoprotein particle referred to as lipoprotein[a] (Lp[a]) (1–4). Both

protein components of Lp[a] are synthesized by hepatocytes, although the production of apo[a] is confined to the liver of humans and certain primates while production of apoB-100 is universally observed in mammalian hepatocytes (5, 6). Elevated circulating levels of Lp[a] in humans is associated with increased risk for a number of atherosclerotic diseases, including coronary artery disease and stroke, and this association has driven continued interest in the mechanisms that regulate plasma levels of this enigmatic lipoprotein species (4, 5, 7).

Studies of the mechanisms underlying the remarkable genetic variability in Lp[a] levels in humans have demonstrated that differences largely reside in the production rate of this lipoprotein rather than changes in its catabolism (8–11). These differences in production rate appear to be reproducible within individuals and are not subject to significant modulation by diet or pharmacologic manipulations directed at lowering plasma cholesterol levels (12, 13). The most informative insight into the potential mechanisms regulating hepatic production of apo[a] derives from a series of studies demonstrating that variations at the *APOA* gene locus result in transcription of a variable number of copies of a repeating domain that resembles that of the kringle 4 (K4) found in plasminogen (14–20). Apo[a] contains varying numbers of these K4-like repeats and, in general, plasma levels of Lp[a] are inversely related to the number of these repeat domains (20, 21). Studies by White and colleagues have demonstrated that the most plausible mechanism for this relationship is that apo[a] isoforms with larger numbers of K4 repeats undergo a size-dependent maturation process, with retention and folding in the endoplasmic reticulum representing an important and possibly dominant restriction point in the processing, folding, and secretion process (11, 22).

Much effort has focused on understanding the post-translational control of apo[a] secretion from hepato-

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cytes, and a consensus of studies indicates that the efficiency of apo[a] secretion is linked to its processing and exit rate from the endoplasmic reticulum, with endoplasmic reticulum-associated degradation assuming a crucial role (23). These findings are consistent with earlier studies demonstrating that alterations in the glycosylation of apo[a] play a major role in its secretion efficiency and modulate its interaction with resident chaperone proteins (23, 24). In particular, inhibitors of N-linked glycosylation greatly reduced (23) or eliminated apo[a] secretion (24), suggesting that alterations in folding or abnormal processing may play a major role in determining the efficiency of apo[a] secretion. This prediction is consistent with the observation that apo[a] is extensively glycosylated (23–28% of the weight of the protein is carbohydrate) (25, 26).

In order to understand the contribution of other mechanisms to the folding and processing of apo[a], we evaluated the role of disulfide bond formation in regulating the secretion of apo[a] isoforms of different size. The strategy was predicated on the observation that apo[a] contains three internal disulfide bonds in each kringle (27) and employed an approach similar to that used in the analysis of disulfide bond formation in the regulation of apoB secretion from hepatocytes (28, 29). The findings demonstrate that DTT treatment alters folding and modulates apo[a] secretion in a manner that is fully reversible and not accompanied by alterations in intracellular degradation. The results further suggest that DTT treatment alters apo[a] processing in a manner that reflects both the number and type of K4 repeats.

MATERIALS AND METHODS

Materials

DMEM, Eagle's minimum essential medium (MEM), methionine- and cysteine-free DMEM, FBS, and DTT were all obtained from Life Technologies, Inc. (Gaithersburg, MD). Protein G-agarose was obtained from Boehringer Mannheim. Goat anti-apo[a] antiserum was purchased from Biodesign Int. (Kennebunk, ME). Rabbit anti-human α 1-antitrypsin was purchased from Calbiochem (La Jolla, CA). Sheep anti-rat albumin was obtained from ICN Immunologicals (Lisle, IL). Rabbit anticalnexin, anticalreticulin, and anti-Grp78 polyclonal antibodies were purchased from StressGen Biotechnologies Corp (Canada). [³⁵S]protein labeling mix (1,175 Ci/mmol) was purchased from NEN Life Science Products, Inc (Boston, MA). Reagents for gel electrophoresis were purchased from Invitrogen (Carlsbad, CA). Complete mini-protease inhibitor cocktail tablets were from Roche Diagnostics (Indianapolis, IN). All other chemicals and reagents were of the highest grade available and purchased from Sigma.

Cell culture

Stable clones of HepG2 and McA-RH7777 cells expressing a recombinant apo[a] containing 6 K4-like domains were generated as described previously (24, 30). McA-RH7777 cells were also stably transfected with apo[a] expression constructs encoding 9-, 13-, or 17-K4 repeats (24, 30). The 13-K4 apo[a] construct was provided by H-J. Müller (20). These expression constructs are annotated (Figs. 1A, 3A) according to the nomenclature proposed by Morrisett and colleagues for K4 repeats 1–10 (31). HepG2 cells were maintained in minimum essential medium

supplemented with 10% FBS, 2 mM L-glutamine, 1 mM pyruvate, 0.1 mM nonessential amino acids, 100 mg/ml streptomycin, and 100 U/ml penicillin. McA-RH7777 cells were grown in DMEM containing 4 mM L-glutamine and 10% FBS.

Pulse-chase studies

Transfected HepG2 and McA-RH7777 cells were grown to 90% confluence in T-25 flasks. Prior to each experiment, the cells were washed twice with PBS, preincubated for 1 h in methionine- and cysteine-free DMEM without serum, pulse-labeled in the same medium containing 250 μ Ci/ml [³⁵S]protein labeling mix, and chased in complete medium containing 3 mM cysteine and 10 mM methionine for the times indicated in the figure legends. There was no preincubation step with DTT. Pulse and chase media were supplemented where indicated with 2 mM DTT, which was freshly prepared prior to each experiment. Where indicated, washout experiments were performed using three brief washes in PBS. At the indicated times following the pulse chase, cells were quickly chilled to 4°C and treated with 100 mM iodoacetamide (IAA) in PBS for 10 min to alkylate intracellular sulfhydryl groups (29). Media were collected and a cocktail of protease inhibitors was added. The cells were washed three times with ice-cold PBS and subsequently lysed in cold lysis buffer (M-Per, Pierce Chemicals, Chicago, IL, containing 0.1% SDS) along with protease inhibitors. Cell lysates and media were clarified by centrifugation at 10,000 rpm at 4°C for 5 min to remove cellular debris, and immunoprecipitations were conducted as described below.

Single protein and sequential immunoprecipitations

Immunoprecipitations were conducted as described (24, 30). For single immunoprecipitations, aliquots were immunoprecipitated with saturating quantities of anti-apo[a], anti-albumin, or anti- ∞ 1 antitrypsin antisera. For immunoprecipitation of culture medium, 5 \times IP buffer was added to a final concentration of 150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.4), 1% Triton X-100, 1 mg/ml BSA, 20 mM methionine, and 0.02% sodium azide. Antiserum was added to cell lysates and medium samples and incubated overnight at 4°C. After overnight incubation at 4°C, protein G-agarose beads were added and the incubation continued for another 2–3 h at 4°C. The final pellet was washed three times in wash buffer [50 mM Tris (pH 7.4), 0.65 M NaCl, 10 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS] two times in water. The beads were then boiled for 10 min in SDS sample buffer [4% SDS, 20% glycerol, 0.001% bromophenol blue, 125 mM Tris (pH 6.8), and 100 mM DTT]. After centrifugation, the supernatant was analyzed by SDS-PAGE and fluorography. Quantitation was conducted using a PhosphorImager (SI, Molecular Dynamics, Sunnyvale, CA) and the ImageQuant software.

Analysis of chaperone protein binding to apo[a]

The binding of calnexin, calreticulin, and BiP to the precursor form of apo[a] was determined as described by Bonen et al. (24). Transfected McA-RH7777 cells expressing the 13-K4 apo[a] isoform were grown to 90% confluence in T-25 flasks. The cells were preincubated for 1 h in methionine- and cysteine-free DMEM without serum, and pulse-labeled in the same medium containing 250 μ Ci/ml [³⁵S]protein labeling mix. One set of cells was chased in complete medium containing 3 mM cysteine, 10 mM methionine, and 2 mM DTT for up to 3 h. Another set of cells was chased in the presence of 2 mM DTT for 3 h, after which DTT was washed out by three brief washes in PBS and the cells were rechased in media without DTT for the indicated times in the figure legends. At the end of each chase, cells were quickly chilled to 4°C and treated with 100 mM IAA in PBS for 10 min (29). The cells were washed three times with ice-cold PBS

and then lysed in nondenaturing buffer [0.1 M NaCl, 25 mM Tris (pH 7.5) 1% Triton-X-100, 5 mM EDTA] and protease inhibitor cocktail as above (Roche). For BiP immunoprecipitation, cells were alkylated in situ followed by the addition of 10 U/ml aprotinin and incubation for 60 min at 4°C (to enzymatically deplete ATP). Cell lysates were first immunoprecipitated in lysis buffer with a rabbit polyclonal antibody to calnexin, calreticulin, or BiP. The immune complex from the first immunoprecipitation was captured using protein A agarose and washed three times in Tris saline (TS) wash buffer [10 mM Tris (pH 7.4), 150 mM NaCl]. The protein A agarose pellet was then boiled for 5 min in TS buffer containing 1% SDS. After centrifugation, the supernatant was diluted with 1 ml of IP buffer and used for recapture-immunoprecipitation with goat anti-human apo[a] antiserum. All immunoprecipitates were analyzed by SDS-PAGE and fluorography.

RESULTS

DTT treatment eliminates secretion of apo[a] from HepG2 cells

Previous studies have demonstrated that alterations in N-glycosylation result in retention of apo[a] within the endoplasmic reticulum and greatly decrease or eliminate secretion of different isoforms (23, 24). In order to address

the role of disulfide bond formation as a mechanism to regulate folding and processing of apo[a], HepG2 cells expressing a 6-K4 isoform were subjected to pulse-chase analysis in the presence of 2 mM DTT, using the study design described by Lodish and colleagues and Shelness and colleagues (29, 32). The 6-K4 isoform (**Fig. 1A**) has been previously demonstrated to undergo efficient processing and secretion by hepatoma cells and was selected for initial evaluation (24, 30). As shown in Fig. 1B, DTT treatment eliminated secretion of apo[a] into the media and led to accumulation of the precursor form within cell lysates. Recovery of apo[a] averaged 90% in control cells and 96% in DTT treated cells (means of three experiments), suggesting that the intracellular accumulation of apo[a] following DTT treatment was not accompanied by increased degradation. Secretion of albumin was also significantly decreased (**Fig. 1C**) in parallel with intracellular accumulation, as previously described (28, 29).

The effects of DTT treatment on apo[a] secretion are reversible

A subsequent series of experiments were conducted to determine whether the effects of DTT treatment on the

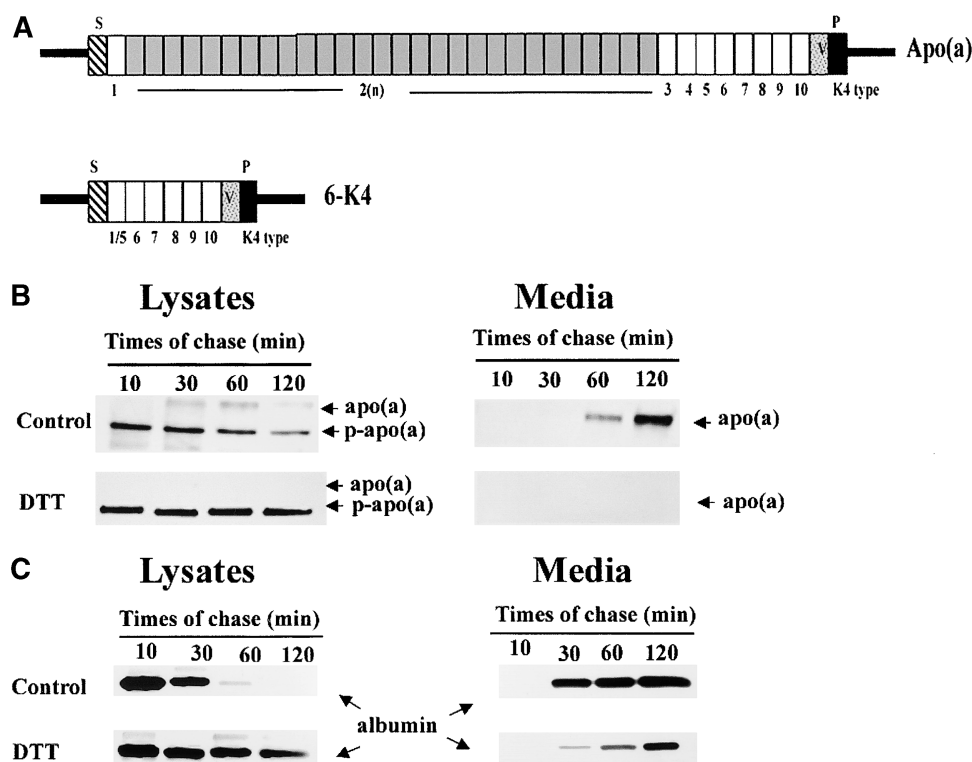


Fig. 1. DTT blocks the secretion of apo[a] from HepG2 cells. **A:** The 6-kringle 4 (K4) expression construct used to transfect HepG2 cells. This construct is aligned with the apolipoprotein[a] (apo[a]) cDNA, annotated according to the nomenclature of K4 repeats (K4 1–10) proposed by Morrisett et al. (31). The 6-K4 construct contains the 5' untranslated region, a signal sequence (S) followed by 6-K4 repeats, a single kringle 5 like-region (V), the protease domain (P), and a 3' untranslated region. The location of the disulfide bonds (three per K4 repeat) is as detailed by McLean et al. (27). **B:** HepG2 cells expressing the 6-K4 apo[a] isoform were pulse labeled for 10 min and chased for up to 120 min in the absence (control) or presence of 2 mM DTT (DTT). Cells were placed on ice and alkylated in situ with 100 mM iodoacetamide. Lysates and media were collected at the indicated times of chase, and apo[a] and albumin (**C**) were immunoprecipitated and analyzed by 4–12% SDS-PAGE under reducing conditions. Migration of the precursor, (p-apo[a]), and mature form (apo[a]) is indicated by the arrows. This is a representative of three replicate experiments.

kinetics of folding and secretion of apo[a] were reversible. HepG2 cells expressing the 6-K4 isoform were studied using a modified pulse-chase protocol described in detail in the legend to **Fig. 2**. Following a 10 min pulse and 2 h chase in the presence of DTT, cells were washed and chased for a further 2 h in the absence of DTT. The results (**Fig. 2A–C**) demonstrate that, following removal of DTT, apo[a] undergoes refolding and is secreted in a time-dependent manner quantitatively similar to that demonstrated in untreated cells.

Inhibition of disulfide bond formation: effects of apo[a] size

In order to examine the effects of DTT on the processing and secretion of different apo[a] isoforms, we turned to McA 7777 rat hepatoma cells as a model, since transfection of these cells with larger isoforms of apo[a] allowed us to select clones that consistently expressed the target protein at levels that permit ready detection. In our previous studies (24, 30) as well as in studies reported by others (20), stably transfected HepG2 cells exhibited low synthesis rates for the larger apo[a] isoforms, as evidenced by the extended labeling periods (several hours) required for apo[a] detection in those studies (20). Others have also commented on the lack of suitable models of human liver-derived cell lines expressing apo[a] (22). Accordingly, we turned to McA cells expressing apo[a] isoforms with 6-, 9-, 13-, or 17-K4 repeats (**Fig. 3A**), which were subject to pulse-chase analysis in the presence or absence of DTT. The results indicate that DTT inhibits secretion of all isoforms of apo[a], although a greater proportion of the larger isoforms (13- and 17-K4) was secreted in comparison to the 6- and 9-K4 isoforms (**Fig.**

3B). By way of control, the effects of DTT treatment appear confined to secretory proteins that contain disulfide linkage, since the secretion of α -1 antitrypsin, a protein with a single cysteine residue and no predicted disulfide bonds, was unimpaired by addition of DTT (**Fig. 3B**).

The results of multiple experiments in which pulse-chase analysis was conducted in the presence or absence of DTT in the pulse and/or chase media, indicate that inclusion of DTT in both the pulse and chase media reduces secretion of the 6-K4 isoform by $\sim 90\%$, findings consistent with the data from HepG2 cells presented earlier (**Fig. 3C**). By contrast, secretion of apo[a] isoforms containing 13- or 17-K4 repeats was reduced to only $\sim 30\%$ of control values (**Fig. 3C**). In addition, the experiments demonstrated in **Fig. 3B** suggest that the effects of DTT are reversible for all apo[a] isoforms, since refolding and secretion resumed normally when McA cells were pulse labeled in the presence of DTT and chased in the absence of DTT (compare lanes where pulse and chase both contained DTT to lanes where the pulse contained DTT but the chase contained no DTT).

A final series of experiments was conducted to determine the effects of increasing concentrations of DTT on the secretion of the 13-K4 isoform from McA cells. These studies revealed that addition of higher concentrations of DTT (5–10 mM) progressively eliminated secretion of the 13-K4 apo[a] isoform (**Fig. 3D**). These findings suggest that the folding of apo[a] isoforms containing the K4 type 2 domain (13-K4 and 17-K4) requires higher concentrations of DTT to disrupt disulfide bond formation and eliminate secretion than the truncated isoforms (6-K4 and 9-K4).

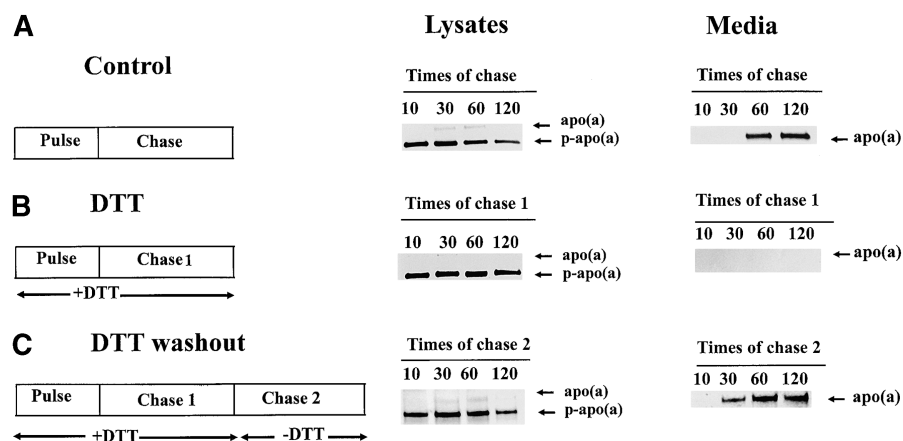


Fig. 2. Refolding and secretion of the 6-K4 apo[a] isoform in HepG2 cells after DTT removal. On the left (A–C) is indicated the experimental protocol employed for the three different studies. HepG2 cells expressing the 6-K4 apo[a] isoform were pulse labeled for 10 min and chased for up to 120 min in the absence of DTT (A; Control). Alternatively, cells were studied in the presence of 2 mM DTT (B) or pulse labeled and chased in the presence of DTT (chase 1), after which DTT was washed out by three brief washes in PBS and the cells re-chased for 10–120 min in media without DTT (chase 2) (C). Apo[a] in the cell lysates and media from these cells were immunoprecipitated and analyzed by 4–12% SDS-PAGE under reducing conditions. Migration of p-apo[a] and apo[a] is indicated by the arrows. This is a representative of three replicate experiments.

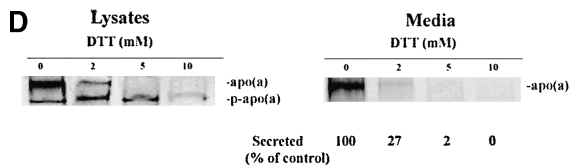
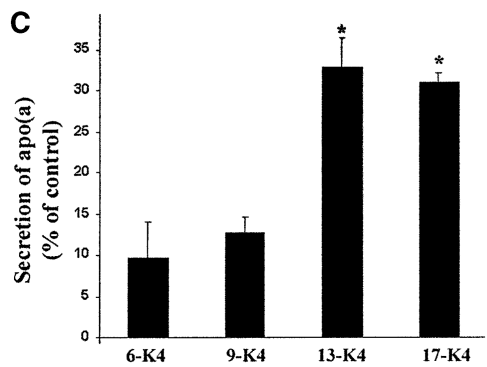
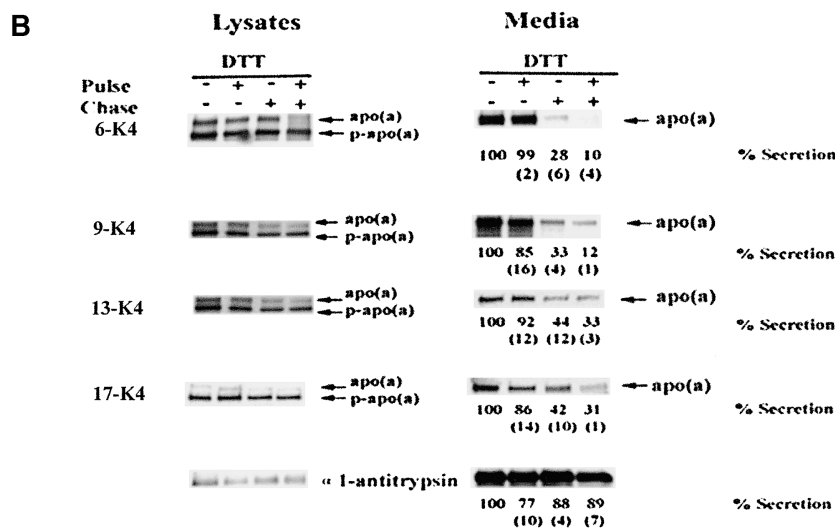
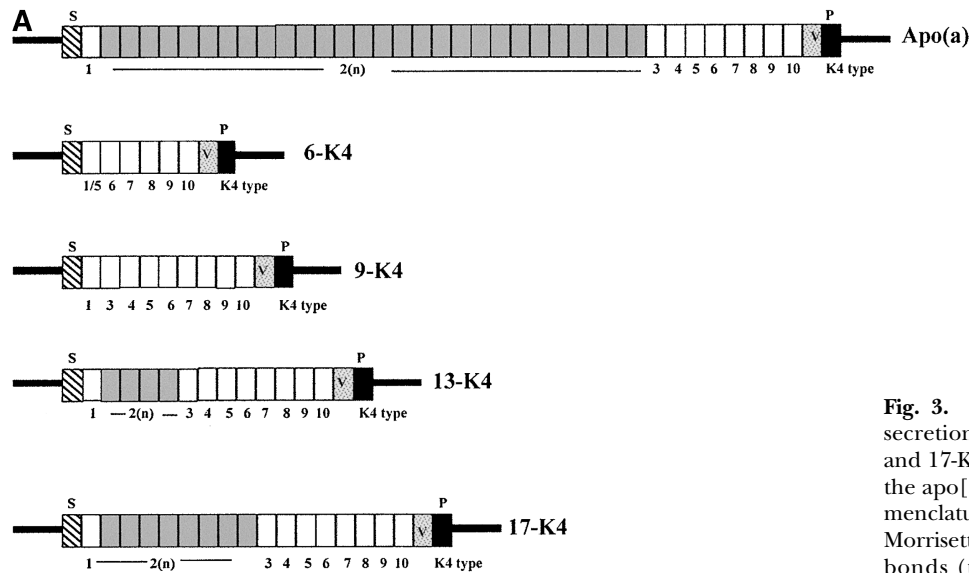


Fig. 3. Size dependent effects of DTT on apo[a] secretion in McA-RH7777 cells. **A:** The 6-, 9- 13-, and 17-K4 expression constructs are aligned with the apo[a] cDNA annotated according to the nomenclature of K4 repeats (K-4 1–10) proposed by Morrisett et al. (31). The location of the disulfide bonds (three per K4 repeat) is as detailed by McLean et al. (27). **B:** McA-RH7777 cells expressing 6-, 9-, 13-, and 17-K4 apo[a] isoforms were pulse labeled for 30 min in the absence (–) or presence (+) of DTT and chased for 4 h with (+) or without (–) 2 mM DTT. Apo[a] and α 1-antitrypsin were immunoprecipitated from cell lysates and media and analyzed by 4–12% SDS-PAGE under reducing conditions. Migration of p-apo[a] and the mature form of apo[a] is indicated by the arrows. This is a representative of three replicate experiments. The amount of apo[a] recovered in the media after the pulse and chase in the presence of 2 mM DTT was quantitated by Phosphor-Imager analysis and expressed as a percentage of the amount secreted in control cells. Data are presented as mean \pm SEM ($n = 3$ observations). **C:** Graph representing the size-dependent effect of DTT on apo[a] isoform secretion. Asterisks indicate statistically significant differences from either 6- or 9-K4 apo[a] isoform secretion ($P < 0.05$). **D:** McA-RH7777 expressing the 13-K4 apo[a] isoform were pulse labeled for 30 min and chased for 3 h in the presence of increasing concentrations of DTT. Apo[a] was immunoprecipitated from cell lysates and analyzed on SDS-PAGE under reducing conditions. This is a representative of duplicate experiments.

Kinetics of refolding and secretion of apo[a]: effect of apo[a] size

Studies detailed above suggest that the effects of DTT are reversible. In order to examine the kinetics of refolding, studies were undertaken in McA cells expressing different apo[a] isoforms in which pulse and chase studies were conducted in the presence of DTT, followed by a washout and a further chase in the absence of DTT. The results demonstrate that, as in HepG2 cells, apo[a] isoforms containing 6-, 13-, or 17-K4 repeats undergo complete refolding with resumption of secretion following the removal of DTT (Fig. 4A). In addition, these studies demonstrate that the time to maximal secretion of the larger isoforms is longer than that observed for the shorter isoforms (120 min for the 6-K4 vs. 180 min for the 13-K4 vs. ~240 min for the 17-K4 species) (Fig. 4A). The time course for apo[a] secretion following DTT removal is again similar to that routinely observed in cells unexposed to DTT (24, 30) (Fig. 4B, C), reinforcing the suggestion that the effects of DTT on apo[a] secretion are completely reversible for all isoforms.

As a further measure of the size dependence of apo[a] folding kinetics, lysates from McA cells expressing either a 6-K4 or 13-K4 isoform were examined after pulse-chase analysis in the absence of DTT. Immunoprecipitated apo[a] was resolved by SDS-PAGE either with or without reduction in order to compare the appearance of the folding intermediates with respect to time of chase. As illustrated in Fig. 5A, apo[a] expressed from the 6-K4 isoform rapidly (within 30 min) achieved its fully folded state. By

contrast, nonreducing SDS-PAGE of immunoprecipitated apo[a] expressed from the 13-K4 isoform (Fig. 5B) revealed subtle but reproducible changes in migration detectable until 90 min of chase. These findings, together with the data presented in Fig. 4, strongly suggest that the folding and refolding kinetics of apo[a] exhibits size dependence that in turn reflects the number of K4 repeats.

DTT sensitivity of apo[a] secretion from McA cells is dependent on the timing of its addition in relation to apo[a] synthesis

McA cells expressing a 6-K4 or 13-K4 isoform of apo[a] were pulse labeled for 10 or 30 min, respectively, and chased for 4 h with DTT addition at the indicated times of chase (Fig. 6, top panel). The results of this experiment indicate that secretion of apo[a] is decreased following DTT addition at times up to 30 min for the 6-K4 isoform (Fig. 6A) (40% control values prior to 30 min); however, secretion of the 13-K4 isoform remained sensitive to DTT treatment up to 90 min into the chase (Fig. 6B) (35% control values prior to 90 min). Addition of DTT to the chase after longer intervals resulted in progressively less inhibition (compare the effects at 30 min to >60 min for the 6-K4 and 60 min to >120 min for the 13-K4) (Fig. 6). These data, coupled with the findings presented in Fig. 5 concerning the differences in folding kinetics of the 6-K4 and 13-K4 apo[a] isoforms suggest that there is a finite interval (distinct for each isoform) during which disulfide bond formation may be altered by the inclusion of DTT. Beyond this period, however, the effects of DTT be-

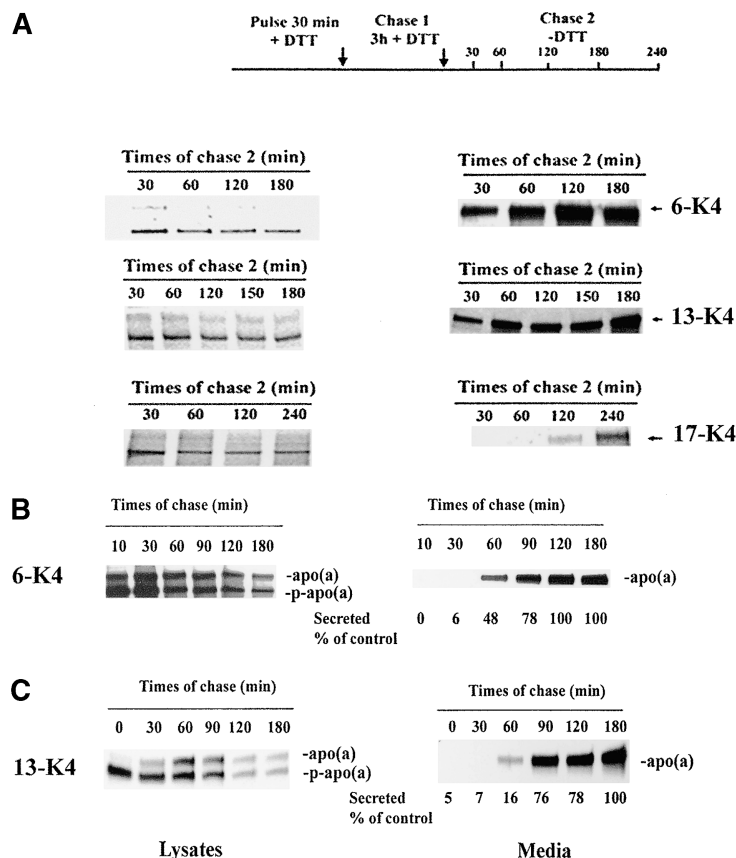


Fig. 4. Size dependent secretion of apo[a] in transfected McA-RH7777 cells after DTT removal. A: McA-RH7777 cells expressing a 6-, 13-, or 17-K4 apo[a] isoform were pulse labeled for 30 min and chased for 3 h in the presence of 2 mM DTT. The DTT was then washed out by three brief washes with PBS and the cells rechased (chase 2) for the indicated times in the absence of DTT. Apo[a] was immunoprecipitated from the lysates (left panel) and media (right panel) and analyzed by SDS-PAGE under reducing conditions. This is a representative of three replicate experiments. B: McA-RH7777 cells expressing the 6-K4 (B) and the 13-K4 (C) were pulse labeled for 10 min or 30 min, respectively, and chased for the indicated times in the absence of DTT. Apo[a] immunoprecipitates from lysates and media were analyzed by SDS-PAGE under reducing conditions.

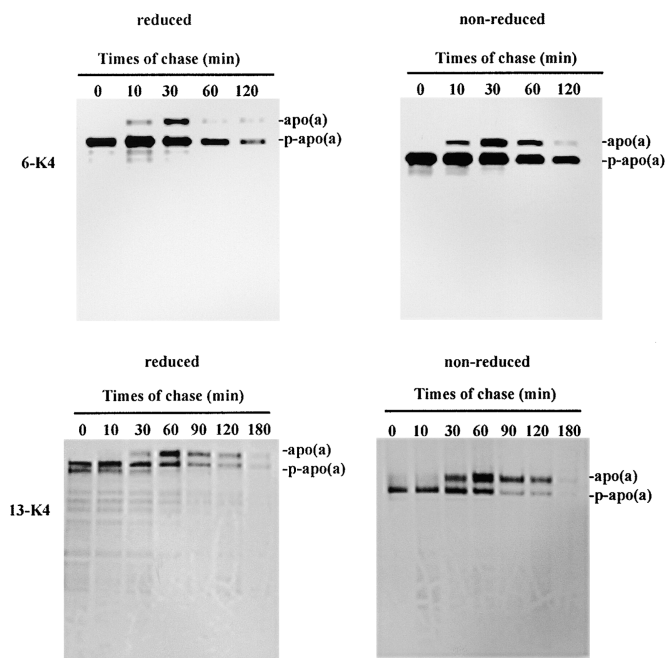


Fig. 5. Folding and secretion of newly synthesized apo[a] in the absence of DTT. McA-RH7777 cells expressing the 6-K4 (A) or the 13-K4 (B) were pulse labeled for 10 (6-K4) or 30 min (13-K4) and chased for the indicated times in the absence of DTT. Apo[a] was immunoprecipitated from lysates and analyzed by 4–15% SDS-PAGE under either reducing or nonreducing conditions. Migration of p-apo[a] and mature form of apo[a] is indicated. This is a representative of duplicate experiments.

come diminished, suggesting that disulfide bond formation is complete.

An alternative and complementary explanation for these findings relates to the secretion kinetics of apo[a] illustrated in Fig. 4B and C. At 60 min of chase, 48% of the 6-K4 isoform destined for secretion has been recovered in the medium (Fig. 4B), compared with 16% for the 13-K4 isoform. However, in the case of the 6-K4 isoform, adding DTT to cells at 60 min of chase reveals negligible effects on apo[a] secretion (84% control, Fig. 6A), suggesting that folding of this isoform is largely complete. On the other hand, DTT addition to cells expressing the 13-K4 isoform at 90 min of chase reduced apo[a] secretion to 57% of control levels (Fig. 6B), despite the observation in Fig. 4C that 76% of the material destined for secretion had been recovered in the medium at this time point in untreated cells. These findings point to intrinsic differences in processing and folding as the likely mechanism for DTT inhibition of secretion rather than the existence of distinct pools of apo[a] with distinct secretion potential.

Chaperone protein interactions with apo[a] are altered in the presence of DTT

Previous studies have demonstrated that alterations in apo[a] processing as a result of modifications in N-linked carbohydrate addition are associated with changes in the kinetics of association of the precursor form with chaperone

one proteins, including calnexin, calreticulin, and BiP (23, 24). In order to understand the mechanisms underlying the alterations noted in secretion of apo[a] in the presence of DTT, studies were conducted using McA cells expressing a 13-K4 isoform in which the association of newly synthesized apo[a] with these different chaperone proteins was examined by sequential immunoprecipitation. Cells were pulsed for 30 min and chased for up to 3 h in the presence of DTT, following which a second chase was conducted for 3 h in the absence of DTT (Fig. 7, upper panel). The results of these studies demonstrate that apo[a] associates with calnexin, calreticulin, and BiP throughout the 3 h chase in the presence of DTT (Fig. 7A–C). Only the precursor form of apo[a] was demonstrated in these coimmunoprecipitation experiments, findings consistent with previously published observations (23, 24). Following washout of DTT, the association of apo[a] with all three chaperone proteins examined was found to diminish in conjunction with the appearance, as early as 30 min of the second chase, of apo[a] in the media (Fig. 7D).

Reversible inhibition of apo[a] secretion: differential effects of a chemical chaperone

Previous studies from White and colleagues have demonstrated that the lysine analog, 6-aminoheptanoic acid (6-AHA), increases the secretion of apo[a] from mouse hepatocytes and rescues the inhibition of apo[a] secretion produced by inhibition of N-glycosylation produced by castanospermine (33). The effects of this chemical chaperone were attributed by these authors to enhanced efficiency of exit of the incompletely processed glycoprotein from the endoplasmic reticulum (23, 33). In order to determine whether this chemical chaperone exerts a similar effect on the secretion efficiency of an incompletely or misfolded apo[a] following alterations in disulfide bond formation, McA cells expressing a 13-K4 isoform were studied by pulse chase methodology, as outlined in the upper panel of Fig. 8. Cells were radiolabeled for 30 min in the presence of the indicated agent and then chased for either 30 min or 4 h. This protocol permitted an evaluation of both intracellular apo[a] processing (30 min) and its secretion (4 h). Lysates and media were immunoprecipitated, the results demonstrating the following findings. First, as previously demonstrated in mouse hepatocytes expressing a human 17-K4 apo[a] transgene (33), 6-AHA increases apo[a] secretion into the media from McA cells expressing a 13-K4 apo[a] isoform (Fig. 8, compare lane 7 and 8). Second, treatment with castanospermine results in a decrease in apo[a] secretion (100% vs. 36%, compare lanes 7 and 10, Fig. 8), and this decrease was rescued by the addition of 6-AHA (110%, compare lanes 10 and 12, Fig. 8). These findings are in agreement with the data from White and colleagues who used hepatocytes from transgenic mice to examine apo[a] secretion in relation to alterations in glycosylation (33). Third, and in contrast to these latter findings, the inhibition of apo[a] secretion following DTT treatment was not rescued by 6-AHA treatment (39% vs. 46%, compare lanes 9 and 11,

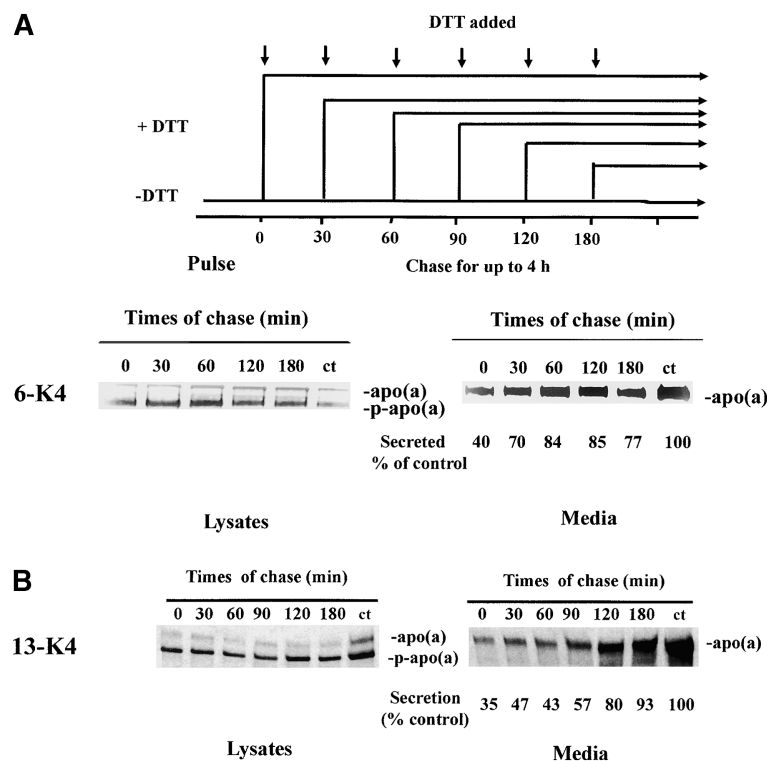


Fig. 6. Sensitivity of the 6-K4 and the 13-K4 apo[a] isoforms to posttranslational addition of DTT in McA-RH7777 cells. Top panel: McA-RH7777 cells expressing the 6-K4 (A) or the 13-K4 (B) apo[a] isoform were pulse labeled for 10 min or 30 min, respectively and chased for 0 min, 10 min, 30 min, 60 min, 120 min, and 180 min in control medium before the addition of 2 mM DTT. The chase was then continued up to 4 h. Control cells (ct) were examined in parallel without DTT addition. Apo[a] was immunoprecipitated from cell lysates and media and analyzed by 4–12% SDS under reducing conditions. Migration of p-apo[a] and mature form of apo[a] is indicated by the arrows. Secretion (percentage of control) for a single experiment is shown. This is a representative of two replicate experiments with similar findings.

Fig. 8). Taken together, the data suggest that 6-AHA treatment augments apo[a] secretion in the setting of altered glycosylation but that the same rescue mechanism, presumed to be related to augmented exit from the endoplasmic reticulum, does not permit abnormally folded apo[a] (following disruption of disulfide bonding) to be secreted in the same manner.

DISCUSSION

The results of these studies demonstrate that different isoforms of apo[a] demonstrate informative responses to treatment with DTT, findings that suggest potentially distinctive roles for different regions of the protein in initiating and maintaining its folding. The initial experiments were undertaken using an isoform of apo[a] that contains six of the K4 domains (K4, types 1.5–10) found in plasminogen, but none of the repeating type 2 domains that are the prime determinant of apo[a] size (21, 34). The 6-K4 apo[a] isoform has been previously characterized in HepG2 cells and demonstrates certain important characteristics of the naturally occurring isoforms, including its ability to associate with apoB-100, its requirement for glycosylation and other posttranslational processing events, and its extensive intracellular degradation when retained within the endoplasmic reticulum (24, 30, 34).

This said, behavior of the 6-K4 isoform cannot be held as representative of the naturally occurring isoforms of apo[a], particularly in view of the absence of the repeating Type 2 domains that characterize these latter products. Nevertheless, one of the central aims of this study

was to examine aspects of the inverse relationship between apo[a] size and its secretion efficiency, particularly in view of the knowledge that each kringle domain contains three disulfide bonds. Accordingly, we considered it likely that interruption of folding with DTT would be informative with respect to the regulation of apo[a] secretion, particularly by providing comparisons between the truncated 6-K4 apo[a] isoform and the larger, naturally occurring isoforms.

The results suggest that secretion of the smaller isoforms containing 6- or 9-K4 repeats is virtually eliminated following DTT treatment, while there is preservation of secretion of the larger isoforms containing 13-K4 and 17-K4 species (Fig. 9). Features of the sensitivity to DTT appear to be shared between the smaller and larger isoforms. For example, both small and larger isoforms demonstrate complete reversibility of the effects of the reducing agent, and each demonstrates time dependence in attaining DTT resistance. These features, particularly the greater amount of time required for the larger isoforms to become DTT resistant (Fig. 6), are consistent with the a priori prediction that apo[a] isoforms containing larger numbers of K4 repeat domains would remain sensitive to the effects of DTT proportionately longer than isoforms with fewer K4 repeats. The finding that DTT sensitivity is maintained for at least 30–60 min or 120 min after the addition of radiolabel with the 6-K4 and 13-K4 isoform, respectively, supports the prediction that disulfide bond formation *in vivo* proceeds in a serial rather than parallel manner. This prediction is consistent with the possibility that the longer time required for translation of the larger isoforms in turn allows folding of the N terminus (con-

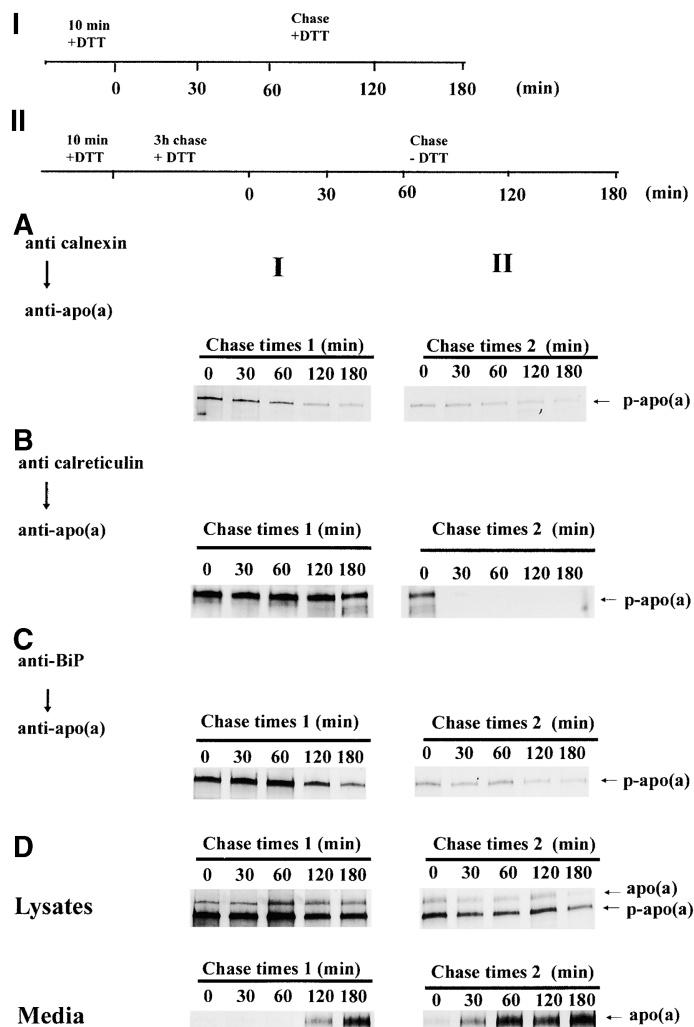


Fig. 7. Association of the 13-K4 apo[a] isoform with calnexin, calreticulin, and BiP in relation to DTT treatment. Upper panel: Two distinct protocols were employed. Protocol I (I): McA-RH7777 cells expressing a 13-K4 apo[a] isoform were pulse labeled for 30 min and chased for up to 180 min in the presence of 2 mM DTT. Protocol II (II): McA-RH7777 cells expressing a 13-K4 apo[a] isoform were pulse labeled for 30 min, chased for 3 h with DTT, following which DTT was washed out with PBS and the cells rechased without DTT for 0–180 min. At the indicated times in the relevant chase period, the cells were lysed under nonreducing conditions and cell lysates were subject to sequential immunoprecipitation, first with anticalnexin (A), anticalreticulin (B), or anti-BiP (C) polyclonal antibodies (Materials and Methods), followed by reimmunoprecipitation using anti-apo[a] as a capture antibody. Immunoprecipitates were then analyzed by 4–12% SDS-PAGE under reducing conditions. D: Total apo[a] was immunoprecipitated in cell lysates and media. The migration of p-apo[a] and apo[a] is shown. This is a representative of three independent experiments.

taining the type 2 repeats) into a more stable, less DTT-sensitive conformation. This said, the results of these experiments do not necessarily imply that disulfide bond formation in the larger isoforms takes proportionately longer to occur, since it is unknown whether this modification occurs co- or posttranslationally in McA cells, perhaps preferentially within one or more kringle domains.

Earlier studies from White and colleagues using baboon hepatocytes demonstrated that the bulk of disulfide bond formations occurred posttranslationally, with folding being completed 30–60 min after synthesis with a hierarchy of early folding intermediates that might represent incompletely processed forms (35). These authors found no differences in the patterns or kinetics of folding with isoforms of different size. Our results using nonreducing SDS-PAGE demonstrate size dependent kinetics of apo[a] folding. In addition, no higher molecular weight apo[a] complex was detected in our studies, in contrast to the findings of White and colleagues (35). We suspect that the differences between these findings may be explained by differences in the cell systems (i.e., rat hepatoma cells vs. baboon hepatocytes) used in these studies.

Another feature to emerge from these studies is that the addition of DTT led to a decrease in apo[a] secretion

that appeared disproportionately greater in the smaller isoforms studied. At the outset, at least two possible scenarios were predicted in this regard. One outcome predicted that the larger isoforms, by virtue of the proportionately greater numbers of disulfide bonds, would exhibit greater sensitivity to the disruption of folding accompanied by a graduated, incremental inhibition of secretion. This outcome would be similar to the effects noted with tunicamycin, which led to a decreased secretion of the 6-K4 isoform from McA cells but completely eliminated secretion of two larger isoforms containing 9-K4 and 17-K4 repeats (24). The alternative prediction was that folding of the larger isoforms occurs in a sequential manner such that certain conformations permit subsequent folding steps to occur. This latter prediction is consistent with the observations that the apparent resistance of a subset of intracellular apo[a] to the effects of DTT and the continued secretion, at ~30% control levels, of the resistant isoform. Differences in solvent accessibility with the larger isoform may account for the resistance, though the basis for such differences remains to be determined. It should be emphasized that while all the isoforms studied contain K4 types 5–10, only the larger isoforms (13-K4, 17-K4) contain iterations of the repeating

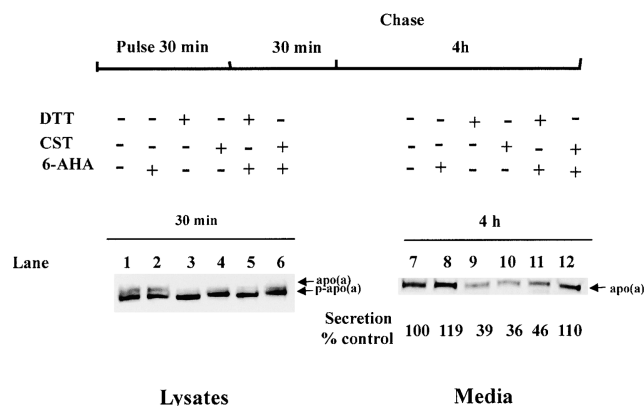


Fig. 8. Independent effects of 6-aminohexanoic acid on the rescue of apo[a] secretion following treatment of transfected McA-RH7777 cells with either castanospermine or DTT. Upper panel shows the overall experimental protocol used. McA-RH7777 cells expressing a 13-K4 apo[a] isoform were preincubated and pulse labeled for 30 min in the presence of the indicated agents. The cells were then chased for either 30 min (to permit analysis of intracellular apo[a]) or 4 h in the presence of the same agents (to evaluate apo[a] secretion). Apo[a] was immunoprecipitated from either cell lysates or media and analyzed by denaturing SDS-PAGE under reducing conditions. The lower panel shows the results from a representative series of lysates (left panel) and media (right panel). The migration of p-apo[a] and apo[a] is indicated by the arrows. Secretion (percentage of control) for a single experiment is shown. This is a representative of two replicate experiments with similar findings.

domain (K4-type) in the N terminus of the protein, raising the possibility that this domain is intrinsically more resistant to defects in disulfide bond disruption; however, it is equally possible, as alluded to above, that translation of the larger isoforms requires longer times than the shorter isoforms, which in turn permits domains in the N terminus (containing the Type 2 repeats) to fold into a more stable conformation. These features are summarized in the model depicted in Fig. 9.

An intriguing comparison may be drawn between the size-dependent effects of DTT on apo[a] secretion and the effects of this agent on the secretion of apoB. A series of studies by the laboratories of Shelness and colleagues and by Hersokovitz and colleagues has defined the sensitivity of N terminal-truncated apoB species to DTT, specifically in relation to their secretion competence and susceptibility to irreversible denaturation and degradation (28, 29, 36). In the amino terminal, ~21% of apoB-100 contains seven of the eight known disulfide bonds, and studies using truncated apoB species (B28 or 29) have demonstrated that DTT resistance is acquired extremely rapidly, within 1–5 min (28, 36). Shelness and colleagues also demonstrated continued secretion of apoB-100 from HepG2 cells exposed to DTT in both the pulse and chase, albeit at reduced levels (29). The results suggest an important role for the N-terminus of apoB-100 in attaining a rapid and ordered folding conformation that in turn permits a fraction of the full-length protein to become DTT resistant. The findings of the current study lead us to spec-

ulate that domains present in the larger isoforms studied (13-K4, 17-K4) may similarly facilitate the appearance of a DTT resistant population. This is consistent with our finding that higher concentrations of DTT (5–10 mM) maximize the inhibition of 13-K4 apo[a] secretion compared with 2 mM. In contrast with the studies in apoB, however, the current results show no evidence for a state of irreversible misfolding. Although the shorter apoB truncations studied appeared to undergo reversible misfolding with only small changes in degradation, apoB-100 becomes irreversibly misfolded following DTT treatment and is degraded (29). The current results indicate that all the isoforms studied, including the 17-K4 isoform that is larger than apoB-100, demonstrate completely reversible inhibition of folding and resumption of secretion to levels observed with untreated cells. Of particular note in this regard is the fact that there was no alteration in intracellular

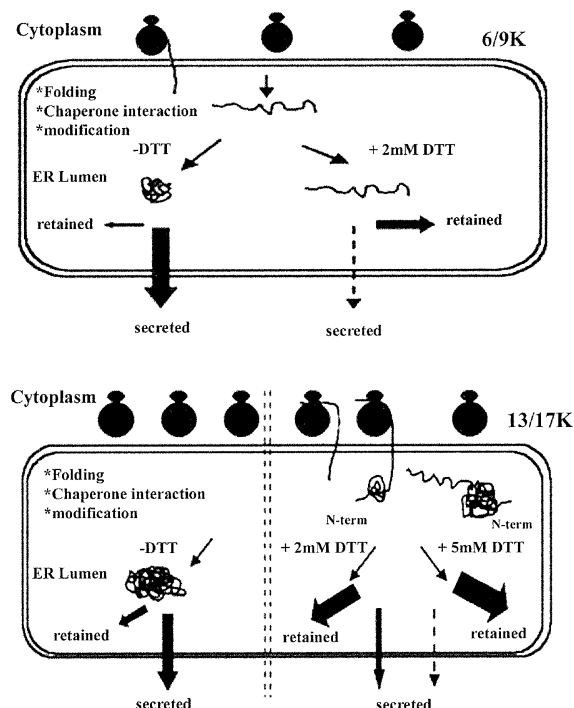



Fig. 9. Schematic model for apo[a] folding and secretion in the absence or presence of DTT. Upper panel (6- and 9-K4 isoforms): In the absence of DTT, apo[a] is translated and subject to folding, interaction with chaperone proteins, and other intracellular modifications. The majority of the newly synthesized apo[a] is secreted. Unfolded or incompletely processed apo[a] is retained in the endoplasmic reticulum (ER) and eventually degraded. In the presence of 2 mM DTT, the secretion of the 6- and 9-K4 apo[a] is completely inhibited, and unfolded apo[a] is retained in the ER. Lower panel (13- and 17-K4 isoforms): Translation of the larger isoforms of apo[a] containing the K4 type 2 repeat domains permits the protein to acquire a more stable conformation with intrinsically higher resistance to DTT. At low concentrations of DTT (2 mM), a larger proportion of these isoforms is secreted, though secretion is eliminated when cells are incubated with higher concentrations of DTT (5 mM). In all instances, the unfolded protein is retained in an intracellular compartment, presumably ER, and undergoes completely reversible renaturation and folding upon removal of DTT, without accompanying increases in intracellular degradation.

degradation associated with prolonged retention. Among the interpretations for the absence of degradation is the prolonged and enhanced binding by the three chaperone proteins studied in DTT treated cells. These latter findings are in agreement with the results of studies from White and colleagues who demonstrated that proteasomal degradation of apo[a] in baboon hepatocytes could be prevented under conditions that favor its interaction with calnexin (23, 35).

In this regard, the studies of White and colleagues further demonstrated that the chemical chaperone 6-AHA rescued the defect in apo[a] secretion associated with castanospermine treatment of murine hepatocytes (33). The current findings demonstrate that this agent does facilitate exit from the endoplasmic reticulum of misfolded apo[a] following DTT treatment, suggesting that informative distinctions may exist between misfolded proteins following different metabolic perturbations that could potentially be exploited to examine the pathways involved. Further study of the reversible misfolding of apo[a] species in liver cells may illuminate other aspects of its regulated delivery through the secretory pathway. These and other issues will be the focus of future reports. 

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