Apolipoprotein E Mediates Uptake of $S_r\ 100-400$ Hypertriglyceridemic Very Low Density Lipoproteins by the Low Density Lipoprotein Receptor Pathway in Normal Human Fibroblasts*

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Our previous studies showed that very low density lipoproteins, $S_r = 100-400$, from hypertriglyceridemic subjects (HTG-VLDLr), but not VLDLr, from normolipidemic subjects, suppress the activity of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase in cultured normal human fibroblasts as a result of uptake and degradation via the low density lipoprotein (LDL) receptor. Uptake of HTG-VLDLr is reduced when apo-E is partially degraded by VLDL-associated protease(s) into two major fragments of $M_r = 12,000$ (E-12) and $M_r = 22,000$ (E-22). E-22 and E-12 are also produced by digestion of isolated purified apo-E with thrombin and are the NH₂- and COOH-terminal fragments, respectively.

We now report that treatment of HTG-VLDLr with thrombin at $37\ ^\circ C$ for 2 h abolishes or severely reduces its ability to compete with specific uptake and degradation of $^{125}$I-labeled LDL or to suppress HMG-CoA reductase (50-100% inactivation). Thrombin treatment of LDLr, however, has no effect on receptor-mediated reductase suppression. Radioimmunoassay for apo-B detected no change in immunoreactivity in either HTG-VLDLr or LDLr after thrombin treatment, indicating that thrombin inactivation of HTG-VLDLr may not be due to an effect on apo-B. Radioimmunoassay for apo-E of thrombin-inactivated HTG-VLDLr suggested a conformational reorganization of apo-E after thrombin digestion (enhanced immunoreactivity relative to native HTG-VLDLr). In addition, approximately 1 mol of apo-E/mol of VLDLr was lost when thrombin-inactivated HTG-VLDLr were reisolated by flotation. An immunochromatographic identification technique (western blot transfer) revealed that thrombin degrades some but not all the apo-E of HTG-VLDLr into E-22 and E-12.

Addition of total apo-E to thrombin-inactivated HTG-VLDLr (~1 mol/mol of VLDLr) restored its ability to suppress reductase. Addition of E-12 failed to restore activity whereas, on a molar basis, E-22 was 50% less effective than intact apo-E in restoring activity. We conclude that intact apo-E of an appropriate surface orientation (1 mol/mol of VLDLr), is necessary for LDL receptor recognition and uptake of HTG-VLDLr by fibroblasts. We hypothesize that the transfer of apo-E into triglyceride-rich lipoproteins in hypertriglyceridemia is a potential mechanism by which atherosclerosis is initiated or exacerbated due to the deleterious consequences of receptor-mediated uptake of these particles by cells in the arterial wall.

We have found that VLDLr isolated from the plasma of subjects with hypertriglyceridemia are functionally abnormal. HTG-VLDLr, but not normolipidemic VLDLr, $S_r = 400-600$, suppress the activity of HMG-CoA reductase in cultured human fibroblasts (1, 2) as a result of uptake via the classic LDL receptor-mediated pathway (3, 4). We have made two observations in vitro which, if similar phenomena occur in vivo, might represent potential cellular mechanisms by which atherosclerosis might be initiated or exacerbated in hypertriglyceridemia. First, low levels of HTG-VLDLr $S_r = 100-400$ (VLDLr), decrease the viability of cultured vascular endothelial cells (5). Second, uptake of hypertriglyceridemic VLDLr, but not normal VLDLr, via a distinct (non-LDL) receptor-mediated pathway produces triglyceride engorgement in unstimulated murine peritoneal macrophages; this produces a cell morphology histologically similar to foam cells (6). Since endothelial cell damage may be an initial event in atherogenesis (7) and arterial foam cells probably are derived from macrophages (8, 9), the deleterious metabolic consequences of uptake of HTG-VLDLr by these two cell types may be a primary factor in atherogenesis in hypertriglyceridemia.

The distinctive features of VLDLr from patients with Type 4 and Type 5 hyperlipoproteinemia which result in enhanced interactions with cells have not yet been identified. We have found no consistent differences in major lipid or apolipoprotein components between the normal and the Type 4 or Type 5 VLDLr, that could account for their differences in reactivity with the LDL receptor of fibroblasts or with the distinct receptor of macrophages (4, 6). Potentially, either apo-E, apo-B, or both apolipoproteins of HTG-VLDLr might serve as recognition sites for the LDL receptor of fibroblasts. One or more apolipoproteins in hypertriglyceridemic VLDLr appears to be involved in binding, since modification of the arginyl residues of HTG-VLDLr with 1,2-cyclohexanedione abolished their ability to suppress HMG-CoA reductase activity in cultured human fibroblasts.

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1 The abbreviations used are: VLDL, very low density lipoprotein; HTG-VLDL, hypertriglyceridemic very low density lipoproteins; VLDLr, $S_r = 100-400$ small triglyceride-rich lipoproteins, 3-hydroxy-3-methylglutaryl CoA; LDLr, low density lipoproteins; apo, apolipoprotein; E-12, $M_r = 12,000$ COOH-terminal fragment of apo-E; E-22, $M_r = 22,000$ NH₂-terminal fragment of apo-E; RIA, radioimmunoassay.
via the LDL-receptor pathway (2). Several lines of evidence suggest that apo-E mediates binding of HTG-VLDL to the LDL receptor. First, apo-E has a higher affinity for the LDL-receptor than does apo-B (10). We have found that hypertriglyceridemic VLDL bind to the LDL receptor with a higher affinity than LDL (4) as is the case with apo-E-HDL (10), suggesting binding via apo-E. Second, the addition of apo-E to normal, nonselective VLDL results in a particle which can suppress HMG-CoA reductase activity in normal fibroblasts as effectively as LDL (4). Third, apo-E in VLDL is degraded by a protease(s) which co-isolates with \( S_1 = 60-400 \) VLDL purified from the \( d < 1.006 \) fraction. When added to fresh plasma prior to VLDL isolation and purification, the serine protease inhibitor phenylmethylsulfonyl fluoride protects against apo-E degradation and the concomitant loss of the ability of HTG-VLDL to suppress HMG-CoA reductase (11). The breakdown of apo-E seen in purified VLDL subclasses by plasma-derived protease(s) can be mimicked by treating purified apo-E with thrombin, a plasma protease which potentially could associate and/or co-isolate with VLDL. Two major proteolytic fragments of apo-E are produced by thrombin treatment of apo-E and apparently by VLDL protease(s) and associated proteolysis \( M_r = 22,000 \) (E-22) and \( M_r = 12,000 \) (E-12), the NH\( _2 \)- and COOH-terminal fragments, respectively (11).

These observations raise the question whether thrombin itself can degrade apo-E in intact HTG-VLDL and thereby affect uptake of HTG-VLDL via the LDL receptor. We now report that thrombin hydrolyzes a portion of apo-E in HTG-VLDL into the two major fragments, E-12 and E-22, that are produced by thrombin cleavage of isolated apo-E. Although thrombin degrades only a portion of the apo-E in HTG-VLDL, it abolishes or significantly reduces uptake of HTG-VLDL by cultured normal human fibroblasts. Since receptor recognition is restored when purified intact apo-E is added back to thrombin-inactivated HTG-VLDL, we conclude that apo-E is the primary determinant in HTG-VLDL for binding to the LDL receptor (12).

**Materials and Methods**

**Cells**—Monolayer cultures of normal human newborn foreskin fibroblasts were grown and maintained as described previously (1, 2). For experiments, approximately \( 5 \times 10^5 \) cells (third to tenth passage) were seeded into dishes (60 mm x 15 mm) with 5 ml of complete medium containing 10% fetal calf serum. When the cells were approximately 75% confluent (2–3 days), the cells were washed with saline and the medium replaced with 2 ml of medium containing 5% human lipoprotein-deficient serum (13) for 24 h to deplete the LDL pathway. Indicated quantities of lipoproteins were added to duplicate dishes and incubated at \( 37 \) °C for 16 h. HMG-CoA reductase activity, determined by a modification (1, 2) of published methods (14, 15), was used as the intracellular endpoint for assessing receptor-mediated uptake (4).

For uptake and degradation studies, LDL and reisolated LDL were iodinated (16), desalted by gel filtration over a P-10 column and extensive dialysis, and filtered (0.45-μm Millex) immediately prior to use. The cells were preincubated in lipoprotein-deficient medium for 36 h prior to the uptake studies. Duplicate dishes of cells and empty dishes were incubated with labeled lipoproteins alone and in the presence of indicated quantities of unlabeled lipoprotein at \( 37 \) °C for 4 h. Total cell-associated activity (representing both surface-bound and internalized) was determined after the cells were washed extensively with chilled buffer containing 2 ml of albumin/ml (17). The amount of non-iodide, non-lipid, trichloroacetic acid-soluble radioactivity in the medium was used as a measure of iodinated lipoprotein degradation (17). All values were corrected for nonspecific uptake and degradation by subtracting the amount bound and degraded in the no-cell control dishes.

**Lipoproteins**—Plasma was obtained from fasting subjects with normal lipid values for isolation of LDL and lipoprotein-deficient serum or from subjects with Types 4 and 2b lipoprotein profiles for HTG-VLDL. The diagnoses were based on commonly used criteria (18). Lipoproteins for cell studies were isolated (19) and the VLDL fraction (20) by standard procedures as previously detailed (1, 2) from fresh plasma containing 1 mM EDTA, 1 mM Na\( _3 \)S, and 10 μM phenylmethylsulfonyl fluoride (Sigma). Total protein contents of the lipoproteins were obtained either by a modified Lowry (21, 22) or by amino acid analysis.

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receptor-mediated uptake of HTG-VLDL1, we incubated HTG-VLDL1 (starting VLDL) with bovine thrombin (200–400 units thrombin/mg of VLDL protein) in 0.15 M NaCl, 20 mM Tris, pH 7.4, 10 mM CaCl2 (buffer) or with buffer alone (control). After incubation at 37°C for 2 h, aliquots of the thrombin-treated and control VLDL were then recentrifuged through a discontinuous salt gradient (d 1.05–1.06 g/ml) to isolate the Sf 100–400 VLDL (20). The lipoproteins were then tested for their effects on HMG-CoA reductase activity in normal fibroblasts as a sensitive indicator of receptor-mediated uptake (4). One such experiment is shown in Fig. 1. Compared to starting HTG-VLDL1, HTG-VLDL1 treated with thrombin were about 60% less effective in suppressing reductase activity, indicating loss of receptor-recognition factors. By contrast, the suppressive effects of control HTG-VLDL1 (incubated at 37°C for 2 h) were indistinguishable from starting VLDL in reductase suppression. When these control 37°C-incubated VLDL1 were resiolated by flotation, there was little to no decrease in suppressive ability (<10%).

In seven experiments, bovine thrombin (200 units/mg) decreased the ability of HTG-VLDL1 to suppress reductase by 50–100%. Purified human α-thrombin at 50 units/mg of VLDL protein inactivated HTG-VLDL1 to a similar extent.

Since thrombin can stimulate cell division in cultured cells (32), it is conceivable that thrombin alone could elevate reductase activities by stimulating cell growth. Cellular requirements for cholesterol and other critical products derived from mevalonate are increased when cells are actively growing. Such an effect might account for the higher reductase activities of fibroblasts exposed to thrombin-treated HTG-VLDL1 relative to control VLDL. Therefore, as additional controls, starting HTG-VLDL1 and thrombin were mixed at 4°C immediately prior to their incubation with the cells. HTG-VLDL1 plus thrombin were identical with starting HTG-VLDL1 in reductase suppression, indicating that thrombin itself does not elevate HMG-CoA reductase activity by stimulating cell growth and, therefore, cholesterol requirements.

Likewise, thrombin added to the medium without other lipoproteins or with LDL did not change HMG-CoA reductase specific activities.

Conceivably, TI HTG-VLDL1 may bind normally to the LDL receptor, but uptake necessary for suppression of reductase activity may be impaired. To test this possibility, we compared the abilities of HTG-VLDL1, resolated TI HTG-VLDL1, and LDL to compete with the specific uptake and degradation of 125I-labeled LDL. Consistent with the relative effects of these lipoproteins on reductase activity, LDL, was the most efficient competitor, followed by HTG-VLDL1. Reisolated TI HTG-VLDL1 failed to compete with the uptake of iodinated LDL or to effectively compete with the degradation of LDL (Fig. 2). This shows that TI HTG-VLDL1, like normal VLDL1, do not bind specifically to the LDL receptor.

In the same experiment illustrated in Fig. 2, 125I-labeled resolated TI HTG-VLDL1 (125I-labeled TI HTG-VLDL1) were also tested for uptake and degradation by the fibroblasts to determine whether these in vitro inactivated VLDL1, like normal VLDL1, bind to the cells in a nonspecific manner (4). Both 125I-LDL and 125I-labeled TI HTG-VLDL1 were tested at a final concentration of 5 μg of protein/ml of medium. After 4 h at 37°C, 205 ng of protein/dish of 125I-LDL were cell-associated (surface bound plus internalized) and 387 ng/dish were degraded. By contrast, essentially no 125I-labeled TI HTG-VLDL1 were degraded (1.2 ng/dish), although 19.2 ng/dish were associated with the cells. This cell-associated radioactivity probably represents surface bound, primarily, and little to no internalized TI HTG-VLDL1, since there was no significant lipoprotein degradation or reductase suppression with the TI HTG-VLDL1. Like the nonspecific adsorption of normal 125I-labeled VLDL1 to fibroblasts and to plastic dishes (4), this low level, nonspecific adsorption of 125I-labeled TI HTG-VLDL1 was diminished in the presence of unlabeled VLDL1 and, to a lesser extent, by LDL. This is consistent with the competitive adsorption of VLDL peptide (apo-C and apo-E) to plastic and glass (33). The presence of unlabeled LDL (HTG-VLDL1) in the medium caused an apparent increase in the degradation of 125I-labeled TI HTG-VLDL1 (from 1.2 ng/dish to 6 ng/dish (4 ng/dish)) at unlabeled lipoprotein concent...
trations of 5 to 50 μg of protein/ml). We have previously reported this phenomenon in degradation studies with low levels of 125I-labeled HTG-VLDL and 1–50 μg/ml of unlabelled LDL (4). The apparent increase in degradation of labeled VLDL in the presence of unlabelled LDL or HTG-VLDL, is probably due to the transfer of labeled soluble (non-apo-B) peptides from iodinated VLDL to unlabelled lipoproteins, which are then taken up by the LDL receptor pathway and degraded. The presence of unlabelled TI HTG-VLDL, however, competed only with the nonspecific binding of 125I-labeled TI HTG-VLDL, but had no effect on its degradation. We conclude that TI HTG-VLDL are not specifically bound, internalized, nor degraded by the LDL receptor-mediated pathway.

These experiments further emphasize the importance of measuring an intracellular end point, such as reductase suppression or acyl:cholesterol acyl transferase activation, in uptake studies. This point was originally made by Dana et al. (34) for iodinated LDL. We found that it is critical in dealing with labeled VLDL, since 1) VLDL have a high affinity for plastic and 2) since labeled soluble peptides can dissociate from the VLDL and transfer to originally unlabeled lipoproteins, and thereby complicate interpretation of binding and degradation studies.

In additional studies, the binding and low level degradation of 125I-labeled TI HTG-VLDL, was linear over the range of 1–30 μg of protein/ml, whereas high affinity binding and degradation of 125I-HTG-VLDL, saturated at about 25 μg of protein/ml (4). We conclude that TI HTG-VLDL, like normal LDL (4), adsorb to cells and dishes nonspecifically rather than to the LDL receptor.

Thrombin Does Not Reduce Receptor-mediated Uptake of LDL—If the thrombin inactivation of HTG-VLDL, were due to an effect on apo-B, one might expect that thrombin digestion of LDL would also cause diminished receptor-mediated uptake of LDL. LDL incubated with bovine thrombin at 200–400 units/mg of LDL protein for 2 h, however, were as effective as native LDL in suppressing HMG-CoA reductase activity (Fig. 3). Likewise, incubation of LDL with purified human thrombin (200 units/mg) also failed to affect the ability of LDL to suppress HMG-CoA reductase, indicating that thrombin digestion does not diminish receptor binding and uptake mediated by LDL, which consists primarily of apo-B-100 (35).

The lack of effect of thrombin on LDL is not altogether surprising, however, since digestion of LDL with trypsin or plasmin also failed to diminish its receptor-mediated uptake and degradation (36). Consistent with these observations, we too detected no decrease in receptor-mediated reductase suppression by LDL after incubation with trypsin. Trypsin-digested HTG-VLDL, however, were ineffective in suppressing reductase activity. These observations lend strength to the working hypothesis that thrombin inactivation of HTG-VLDL, is due not to an effect on apo-B but to an effect on apo-E.

Effects of Thrombin on Apo-B and Apo-E Immunoreactivities—We have used an apo-B and two apo-E radioimmunoassays to detect potential changes in apo-E and/or apo-B in the thrombin-treated VLDL and LDL samples. To preserve possibly subtle conformational differences in apo-E or apo-B of the native versus the thrombin-treated lipoproteins, these samples were not exposed to denaturing agents prior to radioimmunoassay.

The apo-B immunoreactivities of all the HTG-VLDL, preparations were parallel to the normolipidemic LDL standard. There was no significant change in the apo-B immunoreactivity after treatment of HTG-VLDL, with thrombin or after incubation in buffer at 37°C either with or without recentrifugation. Likewise, and consistent with the lack of effect on reductase suppression, incubation of LDL with thrombin at 37°C for 2 h did not change its apo-B immunoreactivity. The apo-B content of the HTG-VLDL, preparations averaged 27 ± 3.3 per cent of total protein.

In both apo-E RIAs, the apo-E immunoreactivity of starting HTG-VLDL, was not parallel to that of the apo-E standard (Fig. 4, A and B). The apo-E immunoreactivities of the TI HTG-VLDL, changed relative to the native HTG-VLDL, suggesting conformational reorganization of the apo-E (Fig. 4, A and B). The apo-E immunoreactivity of the thrombin-treated HTG-VLDL, measured with the R-I1 antisera (Fig. 4B), was parallel to the standard and showed an apparent increase in the apo-E immunoreactivity from an average of 3.6 ± 1.9% (HTG-VLDL,) to 6.1 ± 0.5% (TI HTG-VLDL,) of total protein (Fig. 4B). The apo-E immunoreactivity of the recentrifuged TI HTG-VLDL, was not parallel to that of the TI HTG-VLDL, but was parallel to that of the starting HTG-VLDL, (Fig. 4, A and B). Relative to the starting HTG-VLDL, the R-II antisera detected a consistent decrease in this recentrifuged TI HTG-VLDL, (4.9 ± 3.2 to 3.7 ± 2.1%) suggesting that thrombin treatment followed by centrifugation causes a loss of apo-E (approximately 1 mol of apo-E/mol of VLDL,) from the HTG-VLDL, and that the remaining apo-E on the TI HTG-VLDL, is again in a similar conformation to that on the starting HTG-VLDL. By contrast, there was no change in apo-E (or apo-B) immunoreactivities of recentrifuged control HTG-VLDL.
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FIG. 4. Displacement of ¹²⁵I apo-E from anti-apo-E antibodies: two radioimmunoassays for apo-E in HTG-VLDL. These assays were performed as described under "Materials and Methods." Each point is the average of duplicate determinations. A, antisera R-11; B, antisera R-34. O—O, isolated apo-E; □—□, E-22, the M₉ = 22,000 fragment isolated after digestion of isolated apo-E with human α-thrombin (50 units/mg of protein); □—□, HTG-VLDL. HTG-VLDL incubated with bovine thrombin (200 units/mg of protein) without (A-A) and with (A—A) recentrifugation.

Thus, in all experiments, the cells recognized a drastic loss of receptor sites after thrombin digestion of HTG-VLDL, but not of LDL. Thrombin treatment caused no change in apo-B immunoreactivity of either LDL or HTG-VLDL. That the radioimmunoassay detected enhanced apo-E immunoreactivity of HTG-VLDL after thrombin treatment suggests that, although receptor recognition was essentially abolished, more immunoreactive sites of apo-E were accessible after thrombin digestion. That this was more apparent with the R-34 antisera, with which E-22 was more reactive (Fig. 4B), suggests that the E-22 fragment may have been released by thrombin digestion.

Immunological Identification of Apo-E Fragments in Thrombin-treated HTG-VLDL.—To determine if thrombin degraded apo-E in HTG-VLDL, or merely changed its conformation, we visualized the effects of thrombin treatment on apo-E of HTG-VLDL using the western blot transfer technique (Fig. 5). Apo-E and apo-E treated with purified thrombin, to produce the E-12 and E-22 fragments, were used as standards (Fig. 5, lane 1 and 2, respectively). After thrombin digestion, much of the apo-E remains intact, but a portion of the apo-E of HTG-VLDL was hydrolyzed into, primarily, the E-22 and E-12 fragments (lane 4). Thus thrombin need degrade only a portion of apo-E in HTG-VLDL to produce profound loss of LDL receptor binding determinants. The sensitivity of this technique indicates that other apo-E fragments are also produced from both purified apo-E and HTG-VLDL digestions. It is obvious and striking that with recentrifugation of thrombin-inactivated HTG-VLDL, all of the E-22 fragments were lost from the VLDL; the smaller fragments remained associated with the VLDL. Based on this observation and on a Chou-Fasman analysis of the complete sequence of apo-E (37), the E-12 fragment appears to be a major lipid-binding region of apo-E. The western blots indicate that the
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Fig. 5. Immunological identification of apo-E fragments in thrombin-inactivated HTG-VLDL. HTG-VLDL were incubated with human a thrombin (50 units/mg) for 2 h at 37°C and tested for reductase suppression ± reconstitution as described in the legend to Fig. 1. The samples were extracted and electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) before transfer to nitrocellulose paper for immunological detection of apo-E and apo-E fragments using R-I1 antisera used in Fig. 4A. Lane 1, purified total apo-E; Lane 2, isolated apo-E incubated with thrombin (50 units/mg, 2 h at 37°C); Lane 3, HTG-VLDL (starting material); Lane 4, HTG-VLDL incubated with thrombin (50 units/mg, 2 h at 37°C; TI HTG-VLDL). Lane 5, TI HTG-VLDL, reisolated by flotation through a salt gradient.

quantitative loss of approximately 1 mol of apo-E/mol of TI HTG-VLDL upon recentrifugation indicated by RIA is more accurately designated as loss of E-22.

Addition of Apo-E to Thrombin-inactivated HTG-VLDL Restores LDL Receptor Recognition—We have demonstrated that specific cleavage of approximately 1 mol of apo-E in HTG-VLDL by VLDL-associated protease(s) (11) or by thrombin treatment occurs concomitantly with a loss of receptor-binding determinants. If apo-E degradation is indeed responsible for this loss of biological activity, then receptor recognition should be restored when purified intact apo-E is added back to thrombin-inactivated HTG-VLDL (TI HTG-VLDL). Therefore, we incubated recentrifuged TI HTG-VLDL with purified iodinated apo-E at 37°C for 2 h. The 125I-apo-E TI HTG-VLDL were nearly as effective in suppressing reductase as LDL (Fig. 6). A portion of the apo-E TI HTG-VLDL was reisolated by flotation (20) prior to testing for reductase suppression to equate 125I-apo-E content with reductase suppression. Reisolated apo-E TI HTG-VLDL contained only 2% of the total VLDL protein as 125I-apo-E (calculated from the specific activities) yet it was highly effective in reductase suppression (Fig. 6). This amount of apo-E is equivalent to approximately 1 mol of apo-E/mol of VLDL. That 1 mol of apo-E/mol of TI HTG-VLDL is so effective in restoring receptor uptake is compatible with our earlier observation that adding 1 mol of apo-E/mol of normal, nonsuppressive VLDL, conferred upon the VLDL the ability to suppress reductase as effectively as LDL (4).

To determine if E-2 or E-12 alone or together could restore uptake of TI HTG-VLDL as does intact apo-E (E-34), we incubated recentrifuged TI HTG-VLDL with E-34, E-22, E-12, and with both E-12 and E-22. The TI HTG-VLDL failed to suppress reductase, indicating abolishment of receptor recognition factors. RIA indicated that recentrifuged TI HTG-VLDL contain 2-3 mol of apo-E/mol of VLDL versus 3-4 mol of apo-E/mol of starting HTG-VLDL. As shown in Fig. 7, the E-12 fragment was ineffective in restoring activity. Only preparations containing E-22 showed any ability to mediate reductase suppression. This was expected, since residues implicated in the binding of apo-E to the LDL receptor are located in the E-22 fragment (residues 145 and 158) (38). Compared to preparations containing intact apo-E, however, the E-22-containing preparations were far less effective (Fig. 7). We reisolated the complexes by flotation in order to calculate per cent incorporation from specific activities. The E-34 TI HTG-VLDL complex contained 2.9 mol of apo-E incorporated/mol of TI HTG-VLDL (from E-34 specific activities) and gave 48% suppression at 4 to 15 µg of protein/ml of medium. The E-22 TI HTG-VLDL complex contained, on a molar basis, an identical amount of E-22/particle (2.9 mol of E-22 incorporated/mol of TI HTG-VLDL), yet this complex was 50% less effective than the E-34 TI HTG-VLDL complex in suppressing reductase activity (24% suppression at 4 to 15 µg of VLDL protein/ml). In the E-12 TI HTG-VLDL complex, 4.9 mol of E-12 was incorporated/mol of TI HTG-VLDL, consistent with its greater lipid-binding properties, yet it had no effect on the reductase activity. Although the E-22 fragment may contain residues critical for receptor binding (38), a domain within the E-12 fragment appears to be essential for efficient receptor binding of HTG-VLDL, either to anchor the molecule to the lipid surface and/or to maintain the appropriate apoprotein conformation for binding. We conclude that, for LDL receptor recognition, large VLDL (either E-34 or E-34 TI HTG-VLDL, HTG-VLDL, or normal VLDL) complexed to E-34 (4) must have bound to its surface at least one molecule of intact apo-E of the appropriate conformation.

Thrombin Inactivates and Apo-E Restores Receptor Recognition of HTG-VLDL—Fig. 8 summarizes our findings in schematic fashion. First, HTG-VLDL are potent reductase suppressors because of the high affinity of HTG-VLDL for

![Fig. 6. Addition of apo-E to reisolated TI HTG-VLDL restores ability to suppress HMG-CoA reductase in normal fibroblasts. HTG-VLDL were inactivated with thrombin (200 units/mg) and reisolated to remove thrombin and incomplete thrombin fragments, as described under "Materials and Methods" and the legend to Fig. 1, and incubated with 125I-apo-E (total apo-E from pooled normolipidemic plasma containing isoforms E5, E6, and E7) 200 µg of 125I-apo-E/mg of VLDL protein, at 37°C for 2 h and an additional 16 h at 4°C. A portion was reisolated by flotation through a salt gradient (0.105-0.106) before testing in the fibroblasts. Each data point is the average of duplicate determinations of HMG-CoA reductase activity in cells from duplicate dishes, which varied by 0-5%. ○○○○ normal LDL; ○○○○ reisolated TI HTG-VLDL; ○○○○ reisolated TI HTG-VLDL incubated with apo-E at 37°C for 2 h without (△△△△) and with recentrifugation (-----).]{http://www.jbc.org/}

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The LDL receptor (4). Second, incubation of HTG-VLDL₁ with thrombin greatly reduces or abolishes receptor-mediated suppression by the HTG-VLDL₁, suggesting that the receptor-binding protein(s) are disrupted by thrombin. Thrombin treatment produces no change in apo-B immunoreactivity of either LDL or HTG-VLDL₁, as measured by RIA using antisera of the VLDL₁ surface and free in solution. Third, when TI HTG-VLDL₁ is recentrifuged, there is little or no further loss of biological activity (reductase suppression); the western blot technique demonstrates that the E-22 but not the E-12 fragment is completely lost during recentrifugation. It is noteworthy, first, that not all of the apo-E in HTG-VLDL₁ was degraded by thrombin and, second, that the binding ability of HTG-VLDL₁ was lost prior to removal of the E-22 fragment by recentrifugation. Even though the E-22 fragment contains residues critical for receptor binding (38), our observations suggest that these regions are insufficient for efficient binding and that additional residues (in the E-12 region) are required to properly orient the E-22 domain for receptor interaction of large VLDL. Thus the LDL receptor is exquisitely sensitive to the integrity of apo-E in HTG-VLDL₁.

Finally, receptor recognition is restored when intact apo-E is added back to TI HTG-VLDL₁. Addition of E-12 failed to restore receptor recognition to the TI HTG-VLDL₁; E-22 alone or together with E-12 were ~50% less effective than intact apo-E. These observations suggest that the breakdown of apo-E in HTG-VLDL₁ caused by thrombin was indeed responsible for loss of receptor uptake. Addition of approximately 1 mol of apo-E/mol of TI HTG-VLDL₁ restored efficient reductase suppression.

Thus it appears that apo-E is necessary for receptor recognition by HTG-VLDL₁. Apo-B does not appear to be sufficient for receptor-mediated uptake of large (S₁ = 100–400) VLDL₁, since normal VLDL₁ contain similar levels of apo-B as do HTG-VLDL₁, and yet the normal VLDL do not bind to the LDL receptor (1, 2, 4). The requirements for receptor binding of large lipoproteins (VLDL₁) are, therefore, different from those of smaller lipoproteins (LDL₁, high density lipoproteins containing apo-E as the only apoprotein), where either apo-B or apo-E alone is sufficient for interaction with the LDL receptor.

The conformation/configuration of apo-E on the surface of VLDL appears to be critical for receptor recognition, since the total mass of apo-E in normal VLDL₁ is not significantly different from that of HTG-VLDL₁ (1, 2), although normal VLDL₁ fail to bind to the receptor (4). We hypothesize that

**Fig. 7.** Addition of E-12 or E-22 to TI HTG-VLDL₁ does not restore ability to suppress HMG-CoA reductase activity in normal fibroblasts. Cells were grown and preincubated as described in the legend to Fig. 1. Resolated TI HTG-VLDL₁ (600 μg of protein), prepared as described under "Materials and Methods" and the legend to Fig. 1, were incubated (2 h at 37 °C) with buffer or with 5 nmol total, of [³⁵S]-apo-E (E-34); with [³⁵S]-E-12 (12 nmol); [³⁵S]-E-22 (12 nmol); or with both [³⁵S]-E-12 and [³⁵S]-E-22 (6 nmol each). E-12 and E-22 were prepared from isolated apo-E as described in the legend to Fig. 4. Each data point is the average of duplicate determinations of reductase activity in duplicate dishes. ○——○ TI HTG-VLDL₁ incubated with buffer; ⊗——⊗ TI HTG-VLDL₁ incubated with E-34; △——△ TI HTG-VLDL₁ incubated with E-34; •——• TI HTG-VLDL₁ incubated with E-22; ≪——≪ TI HTG-VLDL₁ incubated with E-22; ●——● TI HTG-VLDL₁ incubated with E-12 and E-22; ⧫——⧫ normal LDL.

**Fig. 8.** Schematic summary of thrombin inactivation and apo-E restoration of HTG-VLDL activity.
Apo-E Mediates LDL-receptor Uptake of Sf 100-400 VLDL

newly transferred apo-E, either acting alone or in tandem with apo-B (or some other component), is required for interaction of large triglyceride-rich lipoproteins with the LDL receptor. In hypertriglyceridemia, large VLDL have a longer residence time in plasma than large VLDL in normolipidemic subjects. In hypertriglyceridemia, most apo-E is in VLDL, not high density lipoproteins (in vivo mass transfer of apo-E to VLDL) (39). The in vitro addition of apo-E to VLDL may be considered analogous to the transfer of apo-E into VLDL in hypertriglyceridemia. We postulate that HTG-VLDL, in contrast to normal VLDL, contain apo-E that has the appropriate surface conformation to mediate binding to the LDL receptor. This apo-E may be acquired by transfer in the plasma compartment. Normal VLDL may contain only apo-E that was originally packaged by the liver and, although the mass of apo-E is similar to that of HTG-VLDL, this “endogenous” apo-E may not be appropriately configured or exposed for interaction with the LDL receptor. It is also possible that with the mass shift of apo-E into VLDL in hypertriglyceridemia, a subpopulation is generated which contains an increased mass of apo-E and therefore increased statistical chance of containing the appropriate conformation for receptor recognition.

In hypertriglyceridemia, receptor-mediated uptake of triglyceride-rich particles may produce large amounts of intracellular fatty acids after lysosomal hydrolysis of the particle. Such an occurrence may be responsible for the endothelial cell toxicity induced by hypertriglyceridemic but not normal VLDL, that we have observed (5) and this may promote endothelial injury if it occurs in vivo. We also suggest that rapid receptor-mediated triglyceride accumulation induced by hypertriglyceridemia but not normal VLDL in macrophages (6) may trigger foam cell formation. Thus receptor-mediated uptake of HTG-VLDL may be an initiating or exacerbating event in atherosclerosis associated with hypertriglyceridemia. Knowledge of the binding determinants that are present in HTG-VLDL, but not expressed in normal VLDL, may provide insight into some causes of atherosclerosis at the cellular level.

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