Vol. 139, No. 1, 1986
August 29, 1986

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 333-340

# APOLIPOPROTEIN E-MEDIATED BINDING OF HYPERTRIGLYCERIDEMIC VERY LOW DENSITY LIPOPROTEINS TO ISOLATED LOW DENSITY LIPOPROTEIN RECEPTORS DETECTED BY LIGAND BLOTTING

Spencer A. Brown, David P. Via, Antonio M. Gotto, Jr.,
William A. Bradley* and Sandra H. Gianturco*

Department of Medicine
Baylor College of Medicine and The Methodist Hospital
Houston, Texas

Received July 21, 1986

SUMMARY: HTG-VLDL$_1$, like LDL, bind with high affinity to electrophoretically transferred, isolated LDL receptors partially purified from bovine adrenal glands. Ligand blotting techniques show that binding is calcium dependent; little or no binding of LDL or HTG-VLDL$_1$ is observed in the presence of 10 mM EDTA. HTG-VLDL$_1$ does not bind in the presence of 7 mM suramin, an inhibitor of LDL binding to the LDL receptor. Pretreatment of LDL with either thrombin or trypsin does not affect apoB-mediated LDL binding to the LDL receptor. ApoE-mediated binding of HTG-VLDL$_1$ to the blotted LDL receptor is abolished or greatly decreased by thrombin treatment of HTG-VLDL$_1$; trypsin treatment of HTG-VLDL$_1$ abolishes binding. Reincorporation of apoE into trypsinized HTG-VLDL$_1$ restores binding. These studies demonstrate unequivocally that HTG-VLDL$_1$ bind to the LDL receptor, that the binding of HTG-VLDL$_1$ to the isolated LDL receptor is mediated through the thrombin-accessible apoE, and that HTG-VLDL$_1$ which bind via potentially dissociable apoE rather than non-tranferable apoB can be used for ligand blotting.


Certain forms of hypertriglyceridemia are associated with an increased risk of premature atherosclerosis, abnormal catabolism of large very low density lipoproteins (VLDL), and the accumulation of lipid-filled foam cells throughout the body (1). The abnormal LDL receptor binding, uptake, and degradation of VLDL $S_f$ 100-400 from hypertriglyceridemic subjects (HTG-VLDL$_1$) observed in cultured cells may account for the metabolic abnormalities; normal VLDL $S_f$ 100-400 do not bind to LDL receptors (2). Studies using cultured human skin fibroblasts indicate that VLDL $S_f$ 100-400 from hypertriglyceridemic patients bind to the low density lipoprotein receptor via a specific conformation of apolipopro-

*Address correspondence to Drs. Gianturco and Bradley at The Methodist Hospital, 6565 Fannin, M.S. A-601, Houston, Texas, 77030.

0006-291X/86 $1.50

tein E (apoE) accessible to thrombin cleavage (3,4). To date, binding of HTG-$VLDL_1$ to isolated LDL receptors has not been directly demonstrated.

Ligand blotting techniques are used to directly visualize electrophoretically isolated, functional LDL receptors (5,6) and acetyl LDL receptors (7). In this report we directly demonstrate by ligand blotting that 1) HTG-$VLDL_1$ bind to isolated LDL receptors, 2) apoE, not apoB, mediates this binding, and 3) lipoproteins that bind via the potentially dissociable apoE rather than the non-transferrable apoB as in LDL or acetyl-LDL can be used for ligand blotting.

## MATERIALS AND METHODS

Materials: We obtained leupeptin from Boehringer Mannheim; suramin from FBA Pharmaceuticals; nitrocellulose BA85 paper (0.45 µm pore size) from Schleicher and Schuell; prestained molecular weight standards from Bethesda Research Laboratories; bovine adrenal glands from a local slaughter-house; CNBr-activated Sepharose 4B from Pharmacia; PPACK and rabbit antiserum to human apolipoprotein B from Calbiochem-Behringer (512301); and peroxidase conjugated IgG fraction goat anti-rabbit IgG antibody from Cappel Laboratories (3212-0081). Other materials were obtained as described (3,4).

Lipoproteins: Plasma was obtained from fasting subjects with normal lipid values for isolation of normal LDL and LPDS or from subjects with types 2b, 4, and 5 lipoprotein profiles for HTG-VLDL. The diagnoses of subjects were based on commonly used criteria (1). Lipoproteins were isolated by standard techniques (8,9) from fresh plasma, as previously described (2-4,10,11). Total protein contents of the lipoproteins were obtained by a modified Lowry procedure (12,13).

Modification of lipoproteins by proteases: LDL from normal subjects and HTG-$VLDL_1$ from hypertriglyceridemic subjects were incubated with trypsin or with thrombin in buffer or with buffer alone (control) and reisolated before use (3,4). The lipoproteins were then tested for their effects on HMG-CoA reductase activity in normal fibroblasts as a sensitive intracellular indicator of the receptor-mediated uptake of LDL (2,10,11). Apoproteins B and E were characterized by SDS/PAGE (14) and Western blotting (15), as described (3,4). To show that apoE can restore binding to trypsinized HTG-$VLDL_1$, the reisolated, trypsinized HTG-$VLDL_1$ were incubated with purified human apoE as described (16).

Receptor preparations by chromatography on DEAE cellulose: The LDL receptor from bovine adrenal glands was partially purified per Schneider et al. (6). The fractions containing LDL receptor activity were combined and stored at -145°C.

Antibodies: Rabbit antiserum to human apolipoprotein B was purified by affinity chromatography using an LDL-Sepharose column.

Ligand blotting: One-dimensional electrophoresis was performed on 6% polyacrylamide slab gels (14 x 8.5 x 0.15 cm) containing 0.1% SDS (v/v) using the buffer system of Laemmli (14). Solubilized membrane samples (25-400 µg of protein) in 0.5% SDS and 10% glycerol were electrophoresed at 4°C at 25 mA/gel for 4-5 hr and transferred to nitrocellulose paper at 4°C and 30 V for 16-18 hr. Nitrocellulose papers were then incubated in blocking buffer (50 mM Tris-HCl, 2 mM $CaCl_2$, 5% bovine serum albumin and 90 mM NaCl at pH 8.0) for 60 min at 37°C and then incubated in 0.5% BSA-buffer containing the indicated amount of lipoprotein for 6 hr

at room temperature. Where indicated, $CaCl_2$ was omitted and 10 mM EDTA was added to the buffer. After lipoprotein binding, the strips were washed three times with blocking buffer in a 15 min period.

Visualization of the LDL receptor: The strips were incubated with affinity-purified rabbit anti-human apoB IgG (10-25 µg protein/ml) in buffer without albumin for 90 min at room temperature, washed three times with buffer and incubated for 60 min with goat anti-rabbit IgG coupled to horseradish peroxidase. The enzymatic reaction was initiated in 0.01M Tris-HCl, pH 7.4, containing 0.0025% O-Dianisidine and 0.01% hydrogen peroxide. The reaction mixture was shielded from light and color-development was terminated by quenching in distilled water.

## RESULTS

**Visualization of LDL receptors with HTG-$VLDL_1$:** HTG-$VLDL_1$, like LDL, bind to LDL receptors partially purified from the bovine adrenal cortex (Figure 1). Maximal intensities were obtained with HTG-$VLDL_1$ at one-half the concentration of LDL (11 nM vs. 22 nM, respectively (Figures 1-3), indicating that HTG-$VLDL_1$ bind with high affinity to the LDL receptor. The receptor has an apparent $M_r$ of approximately 130,000. An additional minor band is seen at $M_r$ ~120,000 with both LDL and HTG-$VLDL_1$. This minor band in receptor preparations from bovine adrenal cortex has been reported previously (5,17) and may be either a proteo-

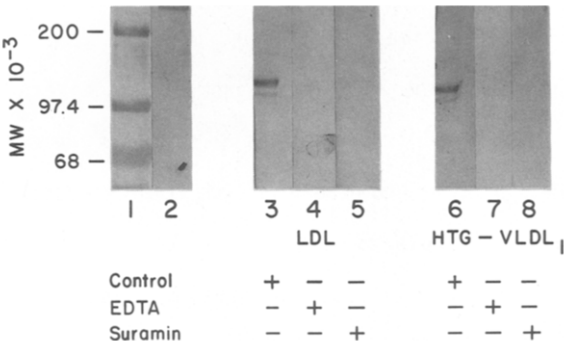


FIGURE 1: Ligand blotting demonstrates that HTG-$VLDL_1$ bind to LDL receptors and that this binding is inhibited by EDTA and suramin. The DEAE-cellulose fraction of bovine adrenal cortex was prepared as described in Methods. Aliquots (60 µg of protein/lane) were electrophoresed in 6% slab gels containing 0.1% SDS. The proteins were transferred electrophoretically to nitrocellulose paper, blocked, and incubated with lipoproteins and inhibitors as indicated. Lane 1, prestained molecular weight standards (myosin ~200K, phosphorylase B ~94.7K, and albumin ~68K); Lane 2, apoB of LDL; Lanes 3, 4, and 5 were incubated with LDL (10 µg protein/ml; 21.5 pmole/ml) alone (Lane 3), with 10 mM EDTA (Lane 4), or with 7 mM suramin (Lane 5). Lanes 6, 7, and 8 were incubated with HTG-$VLDL_1$ (20 µg protein/ml; 11.3 pmole/ml) alone (Lane 6), with 10 mM EDTA (Lane 7), or with 7 mM suramin (Lane 8). The LDL receptor was visualized as described in METHODS.

lytic fragment of the mature receptor or an immature LDL receptor which retains the ability to bind LDL. In the presence of 10 mM EDTA or 7 mM suramin, no binding of LDL to either form of the LDL receptor is observed (1). Likewise, EDTA and suramin inhibited the binding of HTG-$VLDL_1$ (Figure 1, lanes 7, 8). HTG-$VLDL_1$ was also used to visualize LDL receptors from cultured human fibroblasts (data not shown).

These experiments demonstrate that native HTG-$VLDL_1$, bind to electrophoretically isolated and transferred LDL receptors with the binding characteristics of LDL.

**Thrombin and trypsin abolish binding of HTG-VLDL, but not LDL, to isolated LDL receptors:** To confirm that apoE, and not apoB, mediates binding of the HTG-$VLDL_1$ to the LDL receptor as studies with cultured human fibroblasts indicate, HTG-$VLDL_1$ were pretreated with either thrombin or trypsin prior to ligand blotting (3,4). Our previous studies in intact fibroblasts have shown that thrombin treatment abolishes or greatly diminishes the ability of HTG-$VLDL_1$ to bind to the LDL receptor by cleaving a specific conformation of the total apoE. Trypsin treatment of HTG-$VLDL_1$ hydrolyzes all apoE and abolishes apoE-mediated binding (3,4). Thrombin-inactivated HTG-$VLDL_1$ did not bind to the isolated LDL receptor (Figure 2, lane 7). Five thrombin-inactivated preparations from three different hypertriglyceridemic subjects demonstrated no or greatly diminished binding to the blotted LDL receptor, identical to studies in intact fibroblasts (3,4). Likewise, trypsin treatment abolished the binding of HTG-$VLDL_1$ (Figure 2, lane 8) but not of LDL (Figure 2, lane 5) to the isolated receptor, again as previously reported for intact fibroblasts (3,4). Apoprotein analysis of the samples used in these studies by SDS/PAGE and immunochemical blotting techniques demonstrated that some but not all of the apoE and some of the apoB-100 was cleaved by thrombin as previously described (3,4). ApoE was not detected after trypsin treatment and apoB was degraded into species of $M_r$ < 100,000 in these experiments and as previously reported

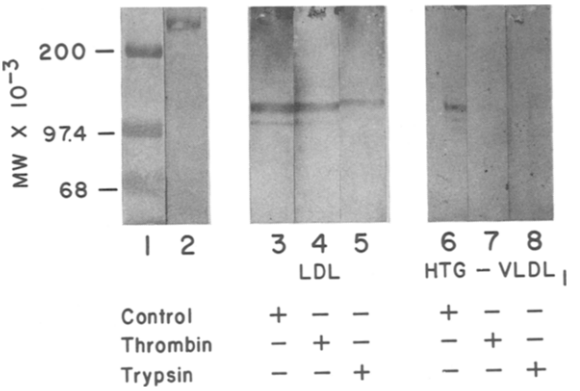


Figure 2: Protease treatment of HTG-VLDL$_1$, but not of LDL, abolishes interaction with partially purified LDL receptors. DEAE-cellulose fractions from bovine adrenal cortex (125 µg protein/lane) were electrophoresed and transferred to nitrocellulose paper, blocked, and incubated with the indicated lipoproteins. Lane 1, prestained molecular weight standards (myosin ~200K, phosphorylase B ~94.7K, and albumin ~68K); Lane 2, apoB of LDL. The remainder of the strips were incubated with LDL (10 µg protein/ml), Lane 3; LDL (10 µg protein/ml) modified with thrombin, Lane 4; LDL (10 µg protein/ml) modified with trypsin, Lane 5; HTG-VLDL$_1$ (20 µg protein/ml), Lane 6; HTG-VLDL$_1$ (20 µg protein/ml) modified with thrombin, Lane 7; HTG-VLDL$_1$ (20 µg protein/ml) modified with trypsin, Lane 8. Modifications of LDL and HTG-VLDL$_1$ and visualization of the LDL receptors are described in the METHODS section.

(4). In control studies not shown, the binding of modified HTG-VLDL$_1$ to the LDL receptor in cultured fibroblasts was abolished or drastically diminished, as reported (3,4). Under the same conditions, thrombin and trypsin treatment of LDL cleaved apoB, as in HTG-VLDL, but the protease-treated LDL still bound to the blotted bovine adrenal LDL receptor (Figure 2, lanes 4,5; Figure 3, lane 2) as reported in studies with human fibroblasts (3,4).

**Reincorporation of intact apoE into trypsin-inactivated HTG-VLDL$_1$ restores binding to isolated LDL receptors:** Reincorporation of apoE into thrombin-inactivated HTG-VLDL$_1$ (3) or into trypsin-inactivated HTG-VLDL$_1$ (16) restores full binding to cultured human fibroblasts. Likewise, reincorporation of apoE into trypsinized HTG-VLDL$_1$ restores its binding to the isolated, transferred LDL receptor (Figure 3, lane 6).

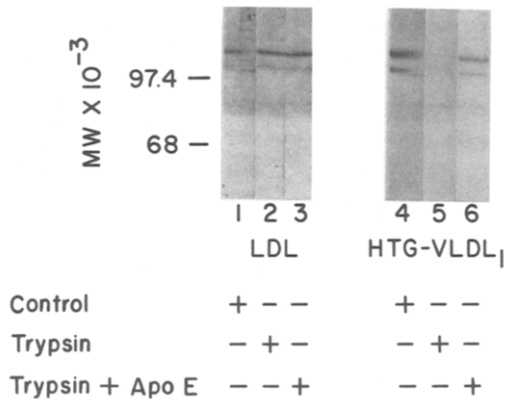


Figure 3: ApoE restores ability of trypsinized HTG-VLDL$_1$ to bind to the partially purified LDL receptor. DEAE-cellulose purified LDL receptors from bovine adrenals (125 µg protein/lane) were electrophoresed, transferred to nitrocellulose paper, and incubated as follows: LDL (10 µg protein/ml), Lane 1; LDL (10 µg protein/ml) modified with trypsin, Lane 2; LDL (10 µg protein/ml) modified with trypsin and incubated with apoE, Lane 3; HTG-VLDL$_1$ (20 µg protein/ml), Lane 4; HTG-VLDL$_1$ (20 µg protein/ml) modified with trypsin, Lane 5; HTG-VLDL$_1$ (20 µg protein/ml) modified with trypsin and incubated with apoE, Lane 6. Ligand detection was as described in METHODS.

## DISCUSSION

Ligand blotting directly demonstrates that HTG-VLDL$_1$ bind to LDL receptors and that this binding absolutely requires apoE in HTG-VLDL$_1$ as a binding determinant for the LDL receptor. These data from studies using isolated LDL receptors directly support conclusions from our earlier studies with intact fibroblasts (3,4) as well as the reports by others (18,19) which confirmed, through the use of monoclonal antibodies, that apoE and not apoB is the primary binding determinant of large hypertriglyceridemic VLDL, $S_f > 60$.

This report also represents the first unequivocal demonstation that apoE-mediated receptor binding, as opposed to apoB-mediated binding, can be detected by ligand blotting techniques. Previously, human β-VLDL ( d < 1.006 g/ml) from patients with type 3 hyperlipoproteinemia were used to visualize the LDL receptor (17). However, the β-VLDL isolated from the d < 1.006 fraction of plasma are heterogenous (20) and contain particles that can bind to the LDL receptor via the non-transferable apoB rather than apoE (3,4,18). Thus, the observed reaction could have been

mediated by a subpopulation of smaller particles that bind via apoB. Kroon et al. detected LDL receptor binding with rabbit β-VLDL (d < 1.006 g/ml), a mixed population of cholesteryl ester-rich particles from cholesterol-fed animals (21). Again, these experiments did not distinguish between receptor binding of the large lipoproteins (which bind via apoE) and the small lipoproteins (which bind via apoB) of the respective heterogeneous lipoprotein preparations (3,4,18). Our studies demonstrate that a more homogeneous subpopulation of large HTG-VLDL, $S_f$ 100-400, known to bind via apoE and not apoB (3,4,16,18), can be used for ligand blotting.

**ACKNOWLEDGMENTS:** This work was supported in part by a Specialized Center of Research (SCOR) in Arteriosclerosis Grant HL-27341; a Grant-in-Aid from the American Heart Association (SHG); and HL-34111 (DPV). SHG is an Established Investigator of the AHA; SAB was supported by a Cardiovascular Science Multi-Disciplinary Training Grant during a portion of this work. We thank Flora Brown, Shiah-Lian Hwang, Alice Lin, and Jill Young for technical assistance. We thank Dr. John W. Fenton for human α-thrombin, Drs. John Guyton and Jeff Probstfield for plasma from hypertriglyceridemic patients, and Virginia McGuffin and Rosetta Ray for editorial assistance and manuscript preparation.

## REFERENCES

1. Fredrickson, D.S., Goldstein, J.L., and Brown, M.S. (1978) In: The Metabolic Basis of Inherited Diseases (4th edition). J.G. Stanbury, M.F. Wyngaarden, and D.S. Fredrickson, editors. McGraw-Hill Publications, New York, NY. 604-655.
2. Gianturco, S.H., Brown, F.B., Gotto, A.M., Jr., and Bradley, W.A. (1982) J. Lipid Res. 23: 984-993.
3. Gianturco, S.H., Gotto, A.M., Jr., Hwang, S.C., Karlin, J.B., Lin, A.H.Y., Prasad, S.C., and Bradley, W.A. (1983) J. Biol. Chem. 258: 4526-4533.
4. Bradley, W.A., Hwang, S.-L. C., Karlin, J.B., Lin, A.H.Y., Prasad, S.C., Gotto, A.M., Jr., and Gianturco, S.H. (1984) J. Biol. Chem. 259: 14728-14735.
5. Daniel, T.O., Schneider, W.J., Goldstein, J.L., and Brown, M.S.. (1983) J. Biol. Chem. 258: 4606-4611.
6. Schneider, W.J., Beisiegel, U., Goldstein, J.L., and Brown, M.S. (1982) J. Biol. Chem. 257: 2664-2673.
7. Via, D.P., Dresel, H.A., Cheng, S.-L., and Gotto, A.M., Jr. (1985) J. Biol. Chem. 260: 7379-7386.
8. Havel, R.J., Eder, H.A., and Bragdon, J.H. (1955) J. Clin. Invest. 34: 1345-1353.
9. Lindgren, F.T., Jenson, L.C., and Hatch, F.T. (1972) In: Blood Lipids and Lipoproteins. G.J. Nelson, editor. Wiley-Interscience, New York, NY. 181-274.
10. Gianturco, S.H., Packard, C.J., Shephard, J., Smith, L.C., Catapano, A.L., Sybers, H.D., and Gotto, A.M., Jr. (1980) Lipids 15: 456-463.
11. Gianturco, S.H., and Bradley, W.A. (1986) In: Methods in Enzymology 129: 319-344.
12. Lowry, O. H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193: 265-275.

13. Helenius, A., and Simons, K. (1971) Biochemistry 10: 2542-2547.
14. Laemmli, U.K. (1970) Nature (Lond.) 227: 680-685.
15. Twobin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci., USA 76: 4350-4354.
16. Bradley, W.A., and Gianturco, .S.H. (1986) J. Lipid Res. 27: 40-48.
17. Wade, D.P., Knight, B.L., and Soutar, A.K. (1985) Biochem J. 229: 785-790.
18. Krul, E.S., Tikkanen, M.J., Cole, T.G., Davie, J.M., and Schonfeld, G. (1985) J. Clin. Invest. 75: 361-369.
19. Hui, D.Y., Innerarity, T.L., and Mahley, R.W. (1984) J. Biol. Chem. 259: 860-869.
20. Gianturco, S.H., Gotto, A.M., Jr., Jackson, R.L., Patsch, J.R., Sybers, H.D., Taunton, O.D., Yeshurun, D.L., and Smith, L.C. (1978) J. Clin. Invest. 61: 320-328.
21. Kroon, P.A., Thompson, G.M., and Chao, Y. (1984) Biochem. J. 233: 329-335.