Hypertriglyceridemic VLDL Decreases Plasminogen Binding to Endothelial Cells and Surface-Localized Fibrinolysis†

Xin-Nong Li,*,‡ Jay C. Koons,‡ Raymond L. Benza,‡ J. Michael Parks,‡ Vivek K. Varma,‡ William A. Bradley,§ Sandra H. Gianturco,§ Kenneth B. Taylor,|| J. Robert Grammer,‡ Edlue M. Tabengwa,‡ and Francois M. Booyse‡

Department of Medicine, Divisions of Cardiovascular Disease and Geriatric Medicine/Gerontology and Department of Biochemistry, University of Alabama at Birmingham, Birmingham, Alabama 35294-2170

Received August 25, 1995; Revised Manuscript Received February 27, 1996⊗

ABSTRACT: The effect of normo (NTG)- and hypertriglyceridemic (HTG)-VLDL on cultured human umbilical vein endothelial cell (HUVEC) surface-localized fibrinolysis was examined following preincubation with NTG-, HTG-VLDL, LDL (1−20 μg/mL) or buffer (control). Ligand binding assays, using ^{125}I-labeled tcu-PA, t-PA, or Glu-plasminogen (Glu-Pmg) were carried out in the absence/presence of lipoproteins. Scatchard analyses showed that HTG-VLDL decreased the B_{max} for ^{125}I-labeled Glu-Pmg ligand binding ~35% [(2.11 ± 0.39)−(1.40 ± 0.32) × 10^6 sites/cell, $p < 0.05$] and increased the $K_{d,app}$ ~5-fold (0.32 ± 0.03 to 1.74 ± 0.08 μM, $p < 0.01$), while NTG-VLDL, LDL, and buffer had no effect. ^{125}I-labeled PA ligand binding was unaffected by these lipoproteins. Receptor-bound PA activation of cell-bound ^{125}I-labeled Glu-Pmg was measured by quantitation of either the M_r 20 kDa light- or M_r 60 kDa heavy-chain of ^{125}I-labeled plasmin, following SDS−PAGE. Kinetic analysis of these data (HTG-VLDL vs controls) indicated that HTG-VLDL decreased the V_{max} of tcu-PA- and t-PA-mediated activation of plasminogen ~2.7-fold (0.317 ± 0.023 vs 0.869 ± 0.068 nM s^{-1}, $p < 0.01$) and ~2.9-fold (0.391 ± 0.098 vs 1.152 ± 0.265 nM s^{-1}, $p < 0.01$), respectively. Increasing concentrations of the HTG-VLDL increased $1/V_{max}$, yielding a series of parallel plots, typical for uncompetitive inhibition with a K_i for inhibition of ~10 μg/mL. The combined ligand binding and kinetic data best fit an uncompetitive inhibition model in which the binding of the large HTG-VLDL particle to the EC surface may directly affect Glu-Pmg binding and activation, thus contributing to early fibrin deposition and the increased thrombotic risk associated with HTG.

Normal hemostasis and vascular patency are maintained by a balance between coagulation and fibrinolysis. Endothelial cells (ECs) play an important role in this process by producing coagulation proteins that promote clot formation, fibrinolytic proteins that facilitate clot lysis, and through the localization of these proteins to the EC surface via specific receptors or binding proteins (Li et al., 1995; Haddock et al., 1991). Specific receptors or binding proteins for two-chain urokinase-type plasminogen activator (tcu-PA) (Haddock et al., 1991; Barnathan et al., 1990; Hajjar & Hamel, 1990), tissue-type plasminogen activator (t-PA) (Hajjar, 1991; Beebe et al., 1990; Ramakrishnan et al., 1990), and plasminogen (Pmg) (Plow et al., 1986; Manchanda & Schwartz, 1991) have been identified on endothelial and other cell types. The urokinase receptor (u-PAR) has been characterized most extensively (Haddock et al., 1991; Barnathan et al., 1990) and along with the receptors or binding proteins for t-PA, PA inhibitor (PAI-1), and Pmg serves to localize and facilitate the multicomponent interaction between these fibrinolytic proteins on the cell surface. Through the orchestration of these complex interactions, the overall catalytic efficiency of plasmin generation (fibrinolytic activity) is carefully regulated on the endothelial surface.

Several studies have suggested that an interrelationship may exist between impaired blood fibrinolytic activity, early fibrin deposition, and atherogenesis (Hamsten et al., 1985; Wiman et al., 1995; Nilsson et al., 1985). Individuals with atherosclerosis often have markedly impaired blood fibrinolytic activity. Alteration in plasma fibrinolytic capacity also occurs in pathological conditions associated with thrombotic episodes (Nordoy & Goodnight, 1990; Sherman et al., 1986; DeWood et al., 1980; Rentrop et al., 1981; Masuda et al., 1992; Sakamoto et al., 1992). Strong mechanistic links have been established between certain lipoproteins and the risk for developing atherosclerosis, including lipoprotein(a) [Lp(a)] (Miles et al., 1989; Oshima et al., 1991; Williams et al., 1993; Boomsma et al., 1993; Hajjar et al., 1989; Etingin et al., 1991; Frade et al., 1991), oxidized LDL (Latron et al., 1991; Kugiyama et al., 1993), acetylated LDL (Tremoli et al., 1993), and hypertriglyceridemic very low density lipoprotein (HTG-VLDL) (Stiko-Rahm et al., 1990), which have all been shown to alter fibrinolytic protein levels and activity. Lp(a) has been shown to competitively inhibit the binding of Pmg to the EC surface, decrease plasmin generation (Miles et al., 1989; Hajjar et al., 1989), and increase the expression of PAI-1 in cultured human umbilical vein ECs (HUVEC)

* Address of corresponding author: Xin-Nong Li, M.D., Division of Cardiovascular Disease, Department of Medicine, University of Alabama at Birmingham, 809 BBRB, 845 19th Street South, Birmingham, AL 35294-2170. Tel: (205) 934-4296. FAX: (205) 975-7121.

† This work was supported in part by National Institutes of Health Grant HL-52791. Drs. Li, Koons, and Benza were supported in part by the Institutional National Research Service Award T32 HL-07703 during the course of this study. Dr. Benza was a recipient of an American Heart Association Fellowship Award, Alabama Affiliate for 1994−1995.

‡ Division of Cardiovascular Disease.

§ Division of Geriatric Medicine/Gerontology.

|| Department of Biochemistry.

⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1996.

S0006-2960(95)02032-0 CCC: $12.00

(Etingin et al., 1991). Secretion of PAI-1 from cultured ECs can be induced by oxidized LDL, acetylated LDL, and VLDL. Secretion of t-PA from cultured HUVECs can be inhibited by LDL and Lp(a) (Levin et al., 1994). Previous studies have demonstrated that HTG-VLDL (S_f > 60) in sharp contrast to normal VLDL (NTG-VLDL) (S_f > 60) bind with high affinity to LDL receptors (Gianturco et al., 1978, 1980, 1982, 1983; Bradley et al., 1984) and to distinct receptors on monocytes and macrophages (Gianturco et al., 1988, 1994). These studies describe the direct effects of HTG-VLDL, as compared to NTG-VLDL, on the binding of t-PA, tcu-PA, and Pmg ligands to cultured HUVECs and the net expression of surface-localized fibrinolytic activity.

EXPERIMENTAL PROCEDURES

Materials. t-PA was obtained from American Diagnostica Inc.; human Glu-Pmg was from Enzyme Research Products, Inc.; collagenase (type I, CLS) was from Boehringer Mannheim Biochemicals; fetal bovine serum (FBS), Rehatuin FS, was from Intergen Corp.; heparin (porcine intestinal mucosa), phenylmethylsulfonyl fluoride (PMSF), and bovine serum albumin (BSA) were from Sigma Chemical Co.; frozen bovine hypothalamuses were from Pel-Freeze Inc.; Medium 199 was from GIBCO; Aprotinin (Trasylol) was from Mobay Corp.; $Na^{125}I$ (specific activity, 14.0 mCi/μg) was from Amersham Corp.; Iodo-Beads were from Pierce Chemical Co.; Sephadex G-25 column (PD-10) was from Pharmacia; acetylated LDL labeled with 1,1′-dioctadecyl-1,3,3,3′,3′-tetramethyliodocarbocyanine perchlorate (DiI-Ac-LDL) was from Biomedical Technologies, Inc.; D-Phe-Pro-Arg chloromethyl ketone (PPACK) was from Calbiochem Corp. tcu-PA was a generous gift from The Green Cross Corporation, Tokyo, Japan. Lipemic plasma was obtained from American Red Cross (Birmingham, AL).

Methods. *Cell Culture*. HUVECs were obtained from freshly discarded umbilical cords by mild collagenase treatment (Li et al., 1995; Haddock et al., 1991), seeded onto human fibronectin-coated growth surfaces, and grown to confluency in complete culture medium consisting of M199, 0.025 M HEPES buffer, pH 7.4, 0.002 M fresh L-glutamine, 100 units of penicillin/mL, 100 μg of streptomycin/mL, 10% heat-deactivated FBC, 90 μg of heparin/mL, and 50 μg of unpurified endothelial cell growth factor/mL, as we have described previously (Li et al., 1995; Haddock et al., 1991). Cultures were re-fed every 48 h with complete culture medium, maintained in a 95% air−5% CO_2 humidified atmosphere, and serially subcultured using 0.25% trypsin. All experiments were carried out with post-confluent (2−3 days after reaching their stable confluency density), pooled (4−6 cords), first- or second-passage cultured HUVECs to minimize individual vessel variability.

Ligand binding assays for t-PA and tcu-PA were carried out with post-confluent cultures grown in 24-well plastic plates (2 cm^2/well). Glu-Pmg ligand binding and fibrinolytic activity assays were carried out with post-confluent cultures grown in 96-well plastic plates (0.33 cm^2/well). Cells were counted using phase microscopy and a 1.0 × 1.0 mm counting reticle.

Cultured cells were characterized as ECs by their content of immunologically identifiable von Willebrand factor antigen (immunofluorescence staining), uptake of the fluorescent probe, DiI-Ac-LDL (Voyta et al., 1984), and their typical monolayer "cobblestone" tight packing growth morphology (Voyta et al., 1984; Booyse et al., 1975, 1977, 1981, 1984, 1988; Jaffe et al., 1973; Thronton et al., 1983).

Isolation and Characterization of Lipoproteins. Plasma was obtained from fasting subjects with normal lipid values for isolation of NTG-VLDL, LDL, and HDL or from fasting patients with types 4 and 5 lipoprotein profiles for HTG-VLDL, after informed consent was obtained. The diagnoses were based on commonly used criteria (Fredickson et al., 1978) as previously described (Gianturco et al., 1978, 1980, 1982, 1983, 1988, 1994; Bradley et al., 1984). In some cases, HTG-VLDL were isolated from lipemic plasma obtained from the Red Cross. Lipoproteins for cell studies were isolated (Gianturco & Bradley, 1986) from fresh plasma containing 1 mM EDTA, 1 mM NaN_3, 10 μM PMSF, 10 μM PPACK, and 50 KIU of Aprotinin/mL. NTG- and HTG-VLDL were subfractionated through a discontinuous NaCl gradient from *d* 1.063 to 1.006 g/mL by Lindgren's cumulative flotation methods (Lindgren et al., 1972), as previously detailed (Gianturco & Bradley, 1986). VLDL used were S_f 100−400 and S_f 60−100. Characteristics of VLDL subclasses so isolated are detailed elsewhere (Gianturco et al., 1980, 1982, 1983, 1988, 1994; Bradley et al., 1984; Gianturco & Bradley, 1986). Control lipoproteins used in these studies included LDL and HDL. LDL were isolated at *d* 1.03−1.05 g/mL and washed at each density. HDL were isolated at *d* 1.125−1.21. LDL and HDL were dialyzed extensively to remove KBr and sterilized by filtration. HTG-VLDL, but not NTG-VLDL, used in these studies were shown to bind with high affinity to partially-purified bovine adrenal LDL receptors (Gianturco et al., 1983; Bradley et al., 1984) and to the monocyte-macrophage receptor for triglyceride-rich lipoproteins (Gianturco et al., 1988, 1994) in ligand blots. Total protein contents of the lipoproteins were obtained by a modified Lowry method (Lowry et al., 1951; Helenius & Simons, 1971) and triglyceride contents, using a kit (Boehringer-Mannheim) as previously described (Gianturco et al., 1994). All lipoproteins are also routinely analyzed by Western blotting to identify their apoprotein contents and to insure the absence of apo (a) in these preparations.

Iodination of tcu-PA, t-PA, and Glu-Pmg Ligands. Purified high molecular weight M_r 54 kDa tcu-PA (20 μg) or t-PA (50 μg) or purified Glu-Pmg (100 μg) in 0.2 mL of Dulbecco's PBS (DPBS) were iodinated with 200−300 μCi of $Na^{125}I$ by the Iodo-Bead method (Markwell, 1982). The reaction was terminated by removal of the Iodo-Beads from the sample, and the free iodine removed by gel-filtration chromatography using a Sephadex G-25 column. Specific activities of the ^{125}I-labeled proteins were determined at (1.5−2.2) × 10^6 cpm/μg for PAs and Glu-Pmg.

Binding of ^{125}I-Labeled Ligands to Cultured HUVECs. The binding of each of the ligands to live cultured HUVECs was carried out essentially as we have described previously for the binding of ^{125}I-labeled u-PA forms to cultured endothelial types (Li et al., 1995; Haddock et al., 1991). Confluent cultured HUVECs, in 24-well plates [2 cm^2/well, (1.6−2) × 10^5 cells] for PAs or 96-well plates [0.33 cm^2/well, (2.5−3.5) × 10^4 cells] for Pmg were washed three times with warm (37 °C) DPBS containing 1% bovine serum albumin (buffer A). Washed cultures, containing 250 μL/2 cm^2 well or 50 μL/0.33 cm^2 well of buffer A, were equilibrated at 4 °C for 20 min and then preincubated with

either buffer A (control) or buffer A containing NTG-VLDL or HTG-VLDL (20 μg/mL each) at 4 °C for 1 h prior to the addition of the specific ligand to be examined. In some experiments, additional controls included preincubation with either LDL or HDL. Varying concentrations of each of the ^{125}I-labeled binding ligands, tcu-PA (0.1−4 nM), or t-PA (15−180 nM) (Li et al., 1995; Haddock et al., 1991), or Glu-Pmg (0.1−2 μM), were then added directly to individual wells (in duplicate) in the presence/absence (buffer A control) of each of the different lipoproteins and incubated further at 4 °C for 30 min, with continuous gentle shaking. To determine the nonspecific binding of each ^{125}I-labeled PA ligand, a 50-fold molar excess of unlabeled ligand was added simultaneously with the ^{125}I-labeled ligand to parallel wells. In the case of ^{125}I-labeled Glu-Pmg, nonspecific ligand binding was determined by the simultaneous addition of 10 mM tranexamic acid to parallel wells. After incubation, the supernatants were removed from individual wells, individual wells were washed five times with buffer A to remove unbound ^{125}I-labeled ligand, and the cell monolayer in each well was solubilized with 1% SDS (w/v), 0.5 M NaOH, 0.01 M EDTA (Hoylaerts et al., 1982). The radioactivity content of each supernatant and corresponding cell lysate was determined in a γ counter. Specific binding was calculated by subtracting the nonspecific cell-associated radioactivity from its respective total cell-associated radioactivity. The specific binding data were analyzed using Munson's LIGAND program for estimating ligand binding parameters from a one-site model system (Munson & Rodbard, 1980). The dissociation constant (K_d) and the number of binding sites/cell (B_{max}) for ^{125}I-labeled tcu-PA, t-PA, or Glu-Pmg ligand binding to live cultured HUVECs were estimated in the presence of buffer (control), NTG-, or HTG-VLDL.

Experiments were also carried out to determine whether HTG-VLDL may affect ^{125}I-labeled Glu-Pmg ligand binding in a dose-dependent manner. Washed cultured HUVECs were preincubated with varying concentrations (0−20 μg/mL) of HTG-VLDL, followed by ^{125}-I-labeled Glu-Pmg ligand binding assays at each of the different HTG-VLDL concentrations, as described above.

To determine whether the bound ^{125}I-labeled Glu-Pmg ligand could be displaced by NTG- or HTG-VLDL, cultured HUVECs containing prebound ^{125}I-labeled Glu-Pmg ligand were subsequently incubated with NTG- or HTG-VLDL, unlabeled Glu-Pmg, tranexamic acid, or buffer A. Cultured HUVECs containing total prebound ^{125}I-labeled Glu-Pmg ligand (see above) were washed four times with buffer A to remove unbound ^{125}I-labeled Glu-Pmg ligand and then incubated with fresh buffer A, containing NTG- or HTG-VLDL (20 μg/mL) or a 20-fold molar excess of Glu-Pmg or 10 mM tranexamic acid. After various incubation times (0, 5, 10, 15, and 20 min) at 4 °C the cell-associated radioactivity was determined in each individual washed and solubilized cell monolayer, as described above.

Analysis of Surface-Localized Fibrinolytic Activity in Cultured HUVECs. The direct effect of NTG- and HTG-VLDL on the net expression of cultured HUVEC surface-localized fibrinolytic activity was measured in the absence/presence of NTG- or HTG-VLDL. Surface-localized fibrinolytic activity was measured, using live confluent cultured HUVECs grown in 96-well plates, by the direct conversion of HUVEC-bound single-chain ^{125}I-labeled Glu-Pmg (Mussoni et al., 1984) by receptor-bound PAs, followed by quantitation of either ^{125}I-labeled plasmin M_r 20 kDa light- or M_r 60 kDa heavy-chain formation by phosphorimaging autoradiography. Confluent cultured HUVECs (in triplicate) were washed three times with 0.01 M HEPES, 0.1 M sodium acetate, pH 7.4, containing 1% BSA (buffer B), equilibrated with buffer B (50 μL/well) at 4 °C for 10 min and then preincubated in the absence (buffer B only) or presence of NTG- or HTG-VLDL (20 μg/mL in buffer B) at 4 °C for 1 h. PA (tcu-PA, 2 nM final or t-PA, 200 nM final) was then added to each well, cultures incubated at 4 °C for 30 min and washed five times with cold buffer B (4 °C) containing 100 KIU of Aprotinin/mL. Varying concentrations of ^{125}I-labeled Glu-Pmg (0.25−2.0 μM) in buffer B containing 1000 KIU of Aprotinin/mL (40 μL) were added to each well and incubated at 4 °C for 30 min. Culture plates were placed in a 37 °C water bath to initiate the receptor-bound PA-mediated conversion of ^{125}I-labeled Glu-Pmg to ^{125}I-labeled plasmin. Reactions were stopped at various times (0−16 min) by the rapid addition of 40 μL of hot (56 °C) solubilizing buffer (4% SDS, 10% glycerol, 0.2 M Tris-HCl, pH 6.8). The total solubilized contents in each well, containing generated ^{125}I-labeled plasmin were removed and reduced by the addition of 5% mercaptoethanol and boiling for 5 min. Reduced samples were analyzed by SDS−PAGE, and the radioactivity content of either the ^{125}I-labeled plasmin light- or heavy-chains was determined by quantitative phosphorimaging autoradiography analyses, as described below.

To determine the K_i for inhibition of HTG-VLDL, different concentrations of HTG-VLDL (0, 10, 20, and 30 μg/mL) were added to individual wells and incubated for 1 h at 4 °C. PA (tcu-PA, 2 nM final) and varying concentrations of ^{125}I-labeled-Glu-Pmg were added, and the receptor-bound tcu-PA-mediated conversion of ^{125}I-labeled Glu-Pmg to ^{125}I-labeled plasmin was analyzed, as described above.

To determine the contribution of HUVEC surface-associated vs solution phase activation of Pmg to the measured kinetic rate, experiments were carried out in wells without cells or with cultured HUVECs in absence/presence of tranexamic acid (10 mM final), as described above. For experiments without cells, buffer B containing varying concentrations of ^{125}I-labeled Glu-Pmg (40 μL/well) was placed in empty wells at 4 °C. PA (tcu-PA, 0.25 nM final) was added to each well, and then the plate was placed in a 37 °C water bath to initiate the tcu-PA-mediated conversion of ^{125}I-labeled Glu-Pmg to ^{125}I-labeled plasmin. The amount of tcu-PA added to the wells was calculated to be equal to the amount of tcu-PA bound to cultured HUVECs, as we have described previously (Haddock et al., 1991; Li et al., 1995). After incubation for 10 min, the reactions were stopped by the rapid addition of solubilizing buffer and the amount of ^{125}I-labeled plasmin generated was analyzed as described above.

SDS−PAGE and Quantitative Phosphorimaging Autoradiography. Reduced samples containing ^{125}I-labeled Glu-Pmg and ^{125}I-labeled plasmin were analyzed by SDS−PAGE, using a 1.5 × 82 × 74 mm polyacrylamide slab gel consisting of an upper 4% stacking gel and a lower 5%−12.5% gradient running gel, according to Laemmli (1970). Following electrophoresis, gels were dried and exposed in phosphorimaging cassettes for 10−12 h. The amount of remaining ^{125}I-labeled Glu-Pmg and newly generated ^{125}I-labeled M_r 20 kDa plasmin light-chain in each individual

gel lane was quantitated by measuring the radioactivity content in each band by phosphorimaging autoradiography, using a Molecular Dynamics Series 425F PhosphorImager in combination with ImageQuant software (Molecular Dynamics). The radioactivity content in each band was then converted to a plasmin concentration by comparing the radioactivity content of each individual band with the radioactivity content of ^{125}I-labeled M_r 20 kDa plasmin light-chain derived from a known amount of fully activated ^{125}I-labeled Glu-Pmg. A ^{125}I-labeled plasmin standard was obtained by complete activation of ^{125}I-labeled Gul-Pmg (1.0 μg) in buffer B containing 1000 KIU of Aprotinin/mL (minus BSA) by incubation with tcu-PA (2 IU/mL) at 37 °C for 1 h (Li et al., 1995).

Data Analysis. The data for analyses of activation rates of EC-bound ^{125}I-labeled Glu-Pmg, in the presence of NTG- or HTG-VLDL, were fitted to several model equations, 1−3, for competitive, uncompetitive, and noncompetitive inhibition, where V_i and P_B are the initial velocity of Pmg activation and bound Pmg, respectively, V_{max} and K_m are the maximum velocity and the Michaelis−Menten constant, respectively, I is the concentration of lipoprotein, and K_i is the inhibition constant

$$V_i = V_{max}P_B/P_B + K_m(1 + I/K_i) \quad (1)$$

$$V_i = V_{max}P_B/P_B(1 + I/K_i) + K_m \quad (2)$$

$$V_i = V_{max}P_B/(P_B + K_m)(1 + I/K_i) \quad (3)$$

Curve fitting was done with a program (MINSQ program, Micromath Inc.) (Taley, 1990) for nonlinear least-squares optimization. Model selection is based on a minimum value of the sum of the squared deviations (SDS).

RESULTS

Effects of Lipoproteins on the Binding of ^{125}I-Labeled PAs and Glu-Pmg Ligands to Cultured HUVECs. Studies were initially carried out to determine whether varying concentrations (1−20 μg/mL) of each of the isolated lipoproteins of interest (NTG-, HTG-VLDL, LDL, and HDL) affected HUVEC monolayer tight-packing density or morphology under the experimental conditions and time course of these ligand binding studies. No apparent deleterious effects were observed at any concentration of each individual lipoprotein examined in these studies. Since 20 μg/mL of each individual lipoprotein did not appear to affect cultured HUVECs and represented the higher range of VLDL in plasma of normal individuals and was above the saturation level for the LDL receptor (Gianturco et al., 1983; Bradley et al., 1984) and the triglyceride-rich lipoprotein receptor on the human macrophages (Gianturco et al., 1988), most of the studies described here were carried out only at this saturating level of lipoprotein receptors.

Binding of ^{125}I-Labeled tcu-PA, t-PA, and Glu-Pmg Ligands to Cultured HUVECs in the Absence of Lipoproteins. Specific binding of ^{125}I-labeled tcu-PA, t-PA, and Glu-Pmg ligands to confluent live cultured HUVECs was time- and concentration-dependent and reached saturation at about 2−3 nM, 150−180 nM, and 2−3 μM, respectively. Typically, nonspecific ligand binding represented about 25%, 10%, and 10% of the total ^{125}-I-labeled ligand binding for tcu-PA, t-PA, and Glu-Pmg, respectively. Scatchard analysis of each of these specific ligand binding data showed no significant statistical improvement in the fit of a two-site model over that described by a one-site model (F-test, $p > 0.5$). Estimations of the K_d and B_{max} values using a one-site model for each of these binding ligands are summarized in Table 1.

Table 1: Binding of ^{125}I-Labeled Ligands to Cultured HUVECs in the Absence/Presence of Lipoproteins

ligand	treatment[a]	K_d (nM)[b,c]	B_{max} ($\times 10^6$ sites/cell)[b,c]
tcu-PA	buffer A	0.83 ± 0.28	0.154 ± 0.042
	NTG-VLDL	0.75 ± 0.22	0.163 ± 0.035
	HTG-VLDL	0.79 ± 0.18	0.171 ± 0.038
t-PA	buffer A	61.74 ± 6.85	1.39 ± 0.28
	NTG-VLDL	59.65 ± 8.35	1.34 ± 0.15
	HTG-VLDL	72.95 ± 3.39	1.42 ± 0.21
Glu-Pmg	buffer A	320 ± 30	2.11 ± 0.50
	NTG-VLDL	350 ± 150	2.01 ± 0.71
	HTG-VLDL	1740 ± 80[d]	1.40 ± 0.41[e]

[a] Pre-incubated with buffer A, NTG-VLDL (20 μg/mL), or HTG-VLDL (20 μg/mL). [b] Values for binding constants, K_d (association constant), and B_{max} (sites/cell) were estimated from Scatchard analysis using the LIGAND program. [c] Mean ± SD (three separate experiments, each carried out in duplicate). [d] $p < 0.01$ and [e] $p < 0.05$, as compared to the buffer-incubated control.

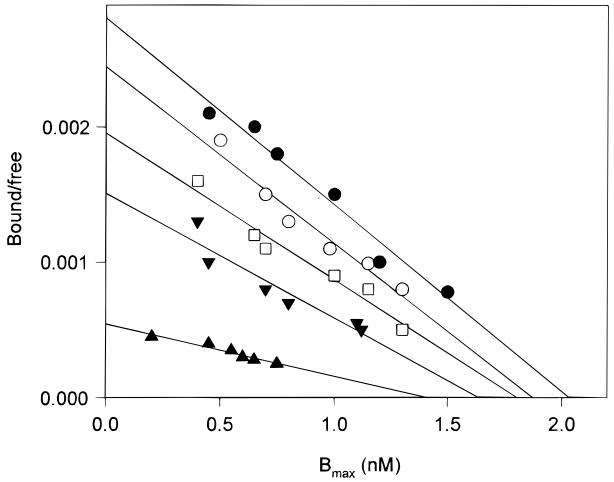

FIGURE 1: Scatchard analysis of ^{125}I-labeled Glu-Pmg ligand binding to cultured HUVECs in the presence of various concentration of HTG-VLDL. Confluent HUVEC cultures were treated with various concentration of HTG-VLDL [0 (●), 5 (○), 10 (□), 15 (▽), 20 (△) μg/mL] at 4 °C for 60 min. ^{125}I-labeled Glu-Pmg ligand binding was carried out, and the specific binding data were analyzed using Munson's LIGAND program for estimating ligand parameters for a one-model system as described in Experimental Procedures.

Binding of ^{125}I-Labeled PAs and Glu-Pmg Ligands to Cultured HUVECs in the Presence of Lipoproteins. To determine whether NTG- and HTG-VLDL or control LDL and HDL may affect the specific binding of ^{125}I-labeled PAs and Glu-Pmg ligands to cultured HUVECs, ^{125}I-labeled ligands were either added simultaneously with or after preincubation (1 h) with the various lipoproteins. The simultaneous addition of or preincubation with NTG- or HTG-VLDL did not affect the K_d and B_{max} values for ^{125}I-labeled tcu-PA and t-PA ligand binding to cultured HUVECs as compared to buffer-incubated controls. In contrast, preincubation of cultured HUVECs with varying concentrations of HTG-VLDL (0, 5, 10, 15, 20 μg/mL) showed a concentration-dependent decrease in the binding of ^{125}I-labeled Glu-Pmg ligand (Figure 1). Only a small decrease was observed at 5 μg/mL with the maximal decrease

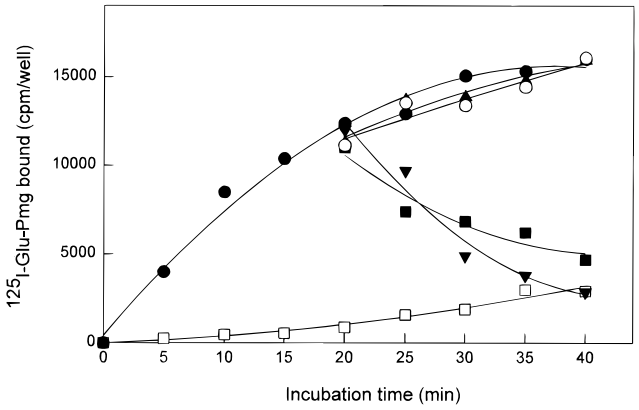

FIGURE 2: Reversibility of ^{125}I-labeled Glu-Pmg ligand binding to cultured HUVECs. Confluent HUVEC cultures were incubated 20 min at 4 °C with saturating levels of ^{125}I-labeled Glu-Pmg (2 μM). Following saturation, parallel wells were washed four times and then incubated at 4 °C with each of the following unlabeled ligands: HTG-VLDL (▲, 20 μg/mL); NTG-VLDL (○, 20 μg/mL); Glu-Pmg (■, 50-fold molar excess); tranexamic acid (▼, 10 mM). At 5-min intervals the contents of each well were solubilized for quantitation. Total (●) and nonspecific bound (box) ^{125}I-labeled Glu-Pmg ligand were determined as detailed under Experimental Procedures.

observed at 20 μg/mL, as evidenced by a ~5-fold increase in the $K_{d,app}$ and a ~30% decrease in the B_{max} values, as compared to buffer-incubated controls (Table 1). Preincubation of cultured HUVECs with NTG-VLDL had no effect on ^{125}I-labeled Glu-Pmg ligand binding. The effects of NTG- and HTG-VLDL on ^{125}I-labeled PAs and Glu-Pmg binding to cultured HUVECs are summarized in Table 1. Control LDL and HDL had no demonstrable effects on ^{125}I-labeled PAs or Glu-Pmg ligand binding to cultured HUVECs (data not shown). These effects of NTG- and HTG-VLDL on ^{125}I-labeled PAs and Glu-Pmg ligand binding to cultured HUVECs, were reproducible and observed in five separate independent experiments using lipoproteins isolated from different individuals.

Reversibility of ^{125}I-Labeled Glu-Pmg Ligand Binding to Cultured HUVECs. The capacity for displacement of specifically prebound ^{125}I-labeled Glu-Pmg from cultured HUVECs by lipoproteins and various ligands is shown in Figure 2. At the highest concentrations of NTG- and HTG-VLDL examined (20 μg/mL), neither NTG- nor HTG-VLDL was capable of displacing the specifically prebound ^{125}I-labeled Glu-Pmg after 20 min incubation at 4 °C (Figure 2). In contrast, a 20-fold molar excess of unlabeled Glu-Pmg and tranexamic acid (10 mM) showed a time-dependent displacement of the specifically prebound ^{125}I-labeled Glu-Pmg, achieving a maximum displacement of ~92% and ~100%, respectively, after 20 min of incubation at 4 °C (Figure 2). In these experiments, nonspecific binding accounte for 10%−17% of the total ^{125}I-labeled Glu-Pmg ligand binding data.

Effects of Lipoproteins on Activation of Cultured HUVEC-Bound ^{125}I-Labeled Glu-Pmg Mediated by Receptor-Bound PA. Verification that the described assay for quantitation of fibrinolytic activity reflected primarily the activation of HUVEC surface-associated Pmg rather than unbound Pmg was obtained by analyses of HUVEC-bound ^{125}I-labeled Glu-Pmg activation by receptor-bound tcu-PA, in the absence/presence of tranexamic acid as well as in solution. The direct activation of HUVEC-bound ^{125}I-labeled Glu-Pmg by receptor-bound tcu-PA obeyed Michaelis−Menten kinetics, as determined by Lineweaver−Burke plot analyses (summarized in Table 2). Activation of HUVEC-bound ^{125}I-labeled Glu-Pmg by receptor-bound tcu-PA demonstrated that the k_{cat}/K_m was decreased ~25-fold in the presence of tranexamic acid. The solution activation of ^{125}I-labeled Glu-Pmg by tcu-PA further demonstrated that the k_{cat}/K_m was similar to that observed in the activation of unbound Pmg (presence of tranexamic acid) (Table 2). These combined activation results on HUVEC-bound and -unbound ^{125}I-labeled Glu-Pmg indicated that the measured kinetic rate data reflected primarily the activation of HUVEC surface-bound Pmg.

To determine whether the decrease in ^{125}I-labeled Glu-Pmg ligand binding to cultured HUVECs by lipoproteins was additionally associated with a concomitant decrease in HUVEC surface-localized fibrinolytic activity, the kinetics of HUVEC-bound ^{125}I-labeled Glu-Pmg activation by receptor-bound PAs were examined in the absence/presence of lipoproteins. Quantitation of the formation of either ^{125}I-labeled M_r 20 kDa light- or M_r 60 kDa heavy-chain plasmin indicated that the direct activation of HUVEC-bound ^{125}I-labeled Glu-Pmg by receptor-bound PA obeyed Michaelis−Menten kinetics both in the absence and presence of lipoproteins, as shown in the Lineweaver−Burke plot (Figure 3). As required for an uncompetitive inhibition model, the

Table 2: Kinetic Constants for the Activation of Cell-Bound ^{125}I-Labeled Glu-Pmg by Receptor-Bound PA in Cultured HUVECs in the Absence or Presence of Lipoproteins[a]

treatment[b]	K_m (μM)	V_{max} (nM s^{-1})	k_{cat} (s^{-1})	k_{cat}/k_m (μM^{-1} s^{-1})
tcu-PA[c]				
buffer A	1.06 ± 0.35	0.869 ± 0.068	3.22 ± 0.26	3.038 ± 0.246
NTG-VLDL	0.69 ± 0.42[d]	0.543 ± 0.070[e]	2.01 ± 0.26[e]	2.945 ± 0.378
HTG-VLDL	0.52 ± 0.19[f]	0.317 ± 0.023[e]	1.17 ± 0.09[e]	2.249 ± 0.173
tranexamic acid	30.4 ± 2.3[e]	0.093 ± 0.007[e]	0.37 ± 0.03[e]	0.014 ± 0.001[e]
solution phase	28.8 ± 3.5[e]	0.134 ± 0.023[e]	0.54 ± 0.14[e]	0.018 ± 0.007[e]
t-PA[c]				
buffer A	2.19 ± 0.61	1.152 ± 0.265	0.0192 ± 0.0044	0.0088 ± 0.0020
NTG-VLDL	2.87 ± 0.60[d]	1.127 ± 0.147[d]	0.0188 ± 0.0020[d]	0.0066 ± 0.0008
HTG-VLDL	1.22 ± 0.43[f]	0.391 ± 0.098[e]	0.0065 ± 0.0016[e]	0.0053 ± 0.0013

[a] Values for each of the kinetic constants, K_m and V_{max} were determined from the Lineweaver−Burke plot analyses of plasmin generation rates vs Glu-Pmg concentration in the absence/presence of lipoproteins, as shown in Figure 3. The values for k_{cat} were calculated from their respective V_{max} values for buffer A and NTG- and HTG-VLDL at a receptor-bound tcu-PA concentration of 0.27 nM or t-PA concentration of 60 nM. All values represent the mean ± SD of three individual experiments, each carried out in triplicate. [b] Pre-incubated with buffer A, NTG-VLDL (20 μg/mL), or HTG-VLDL (20 μg/mL), see Experimental Procedures. [c] Plasmin generation was mediated by tcu-PA or t-PA, see Experimental Procedures. [d] $p > 0.05$, [e] $p < 0.01$, and [f] $p < 0.05$, as compared to the buffer-incubated control.

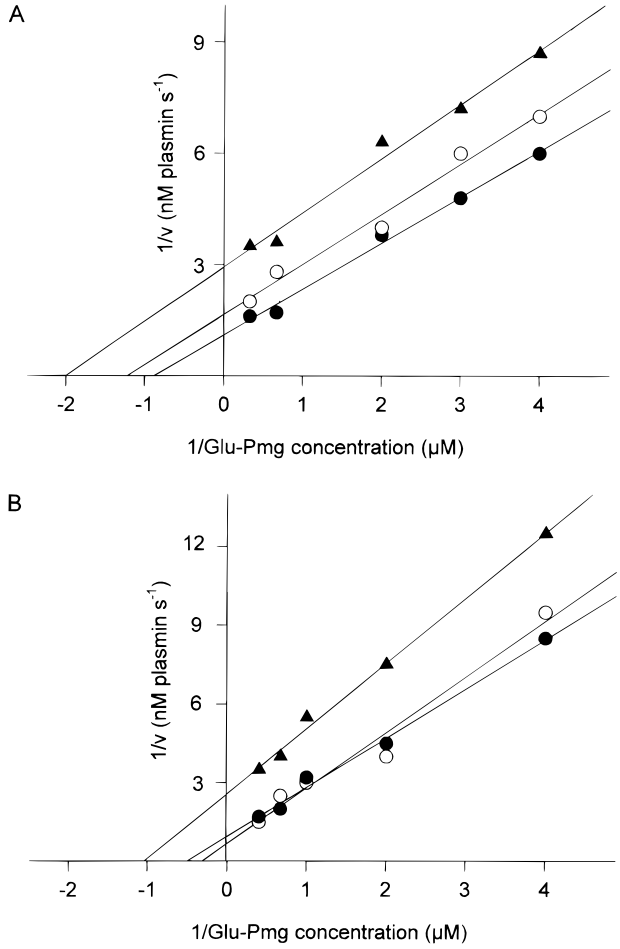

FIGURE 3: Kinetic analysis of HUVEC-bound ^{125}I-labeled Glu-Pmg activation in the presence of lipoproteins. ^{125}I-labeled Glu-Pmg were bound to cultured HUVECs and activated to plasmin in the absence (●) or presence of NTG- (○) or HTG-VLDL (▲); ^{125}I-labeled plasmin light chain were quantitated and plotted in Lineweaver−Burke plots as detailed under Experimental Procedures. (A) Pmg activation by tcu-PA. (B) Pmg activation by t-PA.

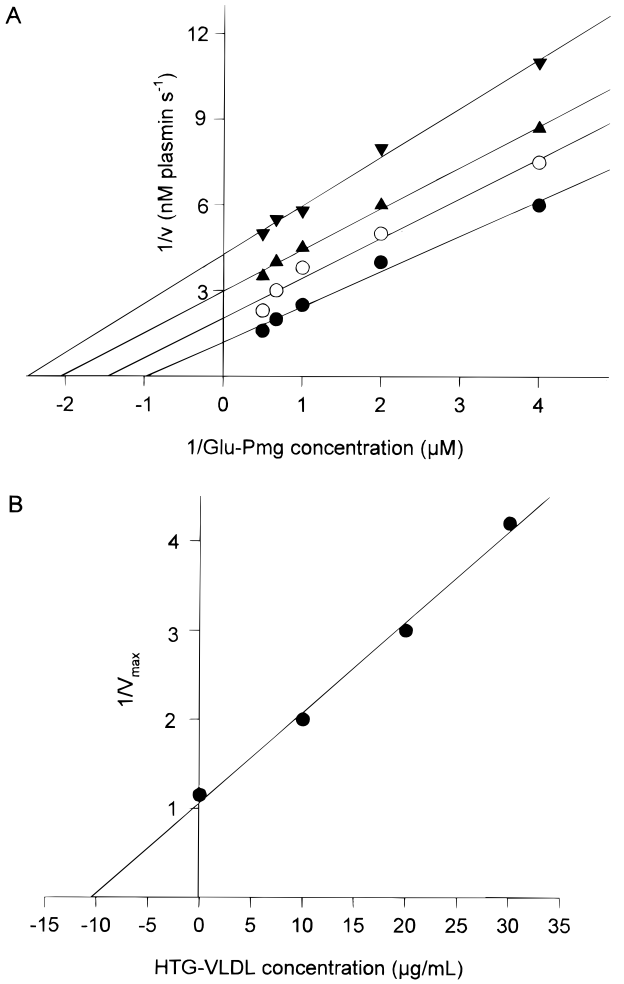

FIGURE 4: Kinetic analysis of HUVEC-bound ^{125}I-labeled Glu-Pmg activation in the presence of different concentrations of HTG-VLDL. ^{125}I-labeled Glu-Pmg were bound to cultured HUVECs and activated to plasmin in the presence of different concentrations of HTG-VLDL (●, 0; ○, 10; ▲, 20, and ▼, 30 μg/mL). ^{125}I-labeled plasmin light-chain was quantitated and plotted in Lineweaver−Burke plots (A) and replots of $1/V_{max}$ vs [HTG-VLDL] (B) as detailed under Experimental Procedures.

K_m as well as V_{max} for the receptor-bound PA-mediated activation of HUVEC-bound ^{125}I-labeled Glu-Pmg activation was decreased in the presence of HTG-VLDL (Table 2). The concentration-dependent inhibition of receptor-bound PA-mediated activation of HUVEC-bound ^{125}I-labeled Glu-Pmg activation by HTG-VLDL was further examined by Lineweaver−Burke plot analyses and showed a series of parallel plots typical for uncompetitive inhibition (Figure 4A). A replot of the $1/V_{max}$ vs HTG-VLDL concentration gave a K_i for inhibition of ~10 μg/mL (Figure 4B).

The data for the HUVEC-bound ^{125}I-labeled Glu-Pmg activation by receptor-bound PA in the presence of lipoproteins can also be fitted to mathematical equations describing competitive, noncompetitive, and uncompetitive inhibition models using the MINSQ program. However, correlation coefficients and sum of squares calculations obtained when these different models were each fitted with the HTG-VLDL inhibition of the ^{125}I-labeled Glu-Pmg activation data demonstrated the best fit for both noncompetitive and uncompetitive inhibition models, as summarized in Table 3. The combined use of the MINSQ and Lineweaver−Burke methods of analysis of the ^{125}I-labeled Glu-Pmg activation data has provided results that are consistent with HTG-VLDL acting as an uncompetitive inhibitor of surface-localized ^{125}I-labeled Glu-Pmg activation.

Table 3: Analysis of EC-Bound ^{125}I-Labeled Glu-Pmg Activation Rates in the Presence of Lipoproteins (20 μg/mL) Fitted to Model Equations Describing Competitive, Uncompetitive, and Noncompetitive Inhibition Models in which Curve Fitting Was Done Using the MINSQ Program[a]

	inhibition model					
	competitive		uncompetitive		noncompetitive	
inhibitor type	SDS[b]	corr[c]	SDS	corr	SDS	corr
NTG-VLDL	3.393	0.83	2.116	0.79	2.289	0.98
HTG-VLDL	2.760	0.72	0.989[d]	0.62	0.405[d]	0.85

[a] Model equations, eqs 1−3, for competitive, uncompetitive, and noncompetitive inhibition are described under Experimental Procedures. [b] SDS, sum of squared deviations. [c] Corr, correlation coefficients. [d] Model selection is based on a minimum value of SDS

DISCUSSION

These studies have demonstrated that HTG-VLDL, but not NTG-VLDL, decreased the B_{max} for ^{125}I-labeled Glu-Pmg ligand binding by ~25%−35% and increased the $K_{d,app}$ ~5-fold (from ~0.3 to 1.7 μM) to live cultured HUVECs (Table 1). Lipoproteins had no effects on ^{125}I-labeled tcu-PA and t-PA ligand binding. In addition, the decrease in ^{125}I-labeled

Glu-Pmg ligand binding was associated with a concomitant decrease in receptor-bound PA activation of EC-bound ^{125}I-labeled Glu-Pmg, resulting in decreased EC surface-localized fibrinolytic activity, as evidenced by the significant decrease in V_{max} in the presence of HTG-VLDL, as compared to buffer controls (Table 2).

Atherosclerosis and thrombosis represent two of the major pathophysiologic processes that underlie occlusive vascular diseases such as stroke, myocardial infarction (MI), and peripheral vascular disease. Well-established factors for coronary artery disease (CAD) and MI, such as hyperlipoproteinemia, HTG, Lp(a), diabetes, and obesity, have been associated with decreased fibrinolytic activity (Hamsten et al., 1985; Wiman et al., 1995; Nilsson et al., 1985; Sherman et al., 1986; DeWood et al., 1980; Rentrop et al., 1981; Masuda et al., 1992; Sakamoto et al., 1992). The specific effects of several different lipoproteins on EC-mediated fibrinolysis have been well documented. For example, Lp(a) will decrease cultured EC fibrinolytic activity by the competitive inhibition of Pmg binding, induction of PAI-1 antigen, and mRNA expression and by the inhibition of t-PA secretion (Miles et al., 1989; Oshima et al., 1991; Williams et al., 1993; Boomsma et al., 1993; Hajjar et al., 1989; Etingin et al., 1991; Frade et al., 1991). Oxidized LDL and acetylated LDL have been shown to induce the secretion of PAI-1 antigen in cultured HUVECs (Latron et al., 1991; Kugiyama et al., 1993; Tremoli et al., 1993). Previous studies in patients with CAD and in young survivors of MI have suggested that the decreased fibrinolytic activity associated with certain types of HTG may be correlated with elevated PAI-1 levels (Hamsten et al., 1985). Studies with cultured HUVECs have shown that HTG-VLDL is a more potent stimulus of PAI-1 antigen secretion than NTG-VLDL (Stiko-Rahm et al., 1990). This increased secretion of PAI-1 was dependent, at least in part, on the specific binding of large HTG-VLDL particles to the LDL receptor on ECs, since this effect was decreased by an antibody to the LDL receptor.

Scatchard analysis of specific ^{125}I-labeled Glu-Pmg ligand binding data, obtained in the absence/presence of lipoproteins, indicated that HTG-VLDL, but not NTG-VLDL, significantly increased the $K_{d,app}$ of the ^{125}I-labeled Glu-Pmg ligand for its HUVEC binding site and simultaneously also interfered with ^{125}I-labeled Glu-Pmg ligand binding to the HUVEC surface, as evidenced by the decrease in the B_{max} for ^{125}I-labeled Glu-Pmg ligand binding (Table 1). However, HTG-VLDL (20 μg/mL) did not displace prebound ^{125}I-labeled Glu-Pmg from the HUVEC surface (Figure 1), which suggests that HTG-VLDL does not inhibit Pmg binding to the HUVEC surface by a competitive type inhibition mechanism. The mechanism by which HTG-VLDL inhibits Pmg binding to the HUVEC surface is not clear at present time but appears to be different from that proposed for Lp(a) (Miles et al., 1989; Hajjar et al., 1989). Lp(a) shares extensive structural homology with Pmg and is presumed to competitively inhibit the binding of Pmg to the EC surface via its apolipoprotein(a) Pmg "kringle" repeats. Inhibition of ^{125}I-labeled Glu-Pmg binding to HUVEC by Lp(a) and unlabeled Pmg was comparable, indicating that Lp(a) can occupy the same binding sites for Pmg on the cell surface (Miles et al., 1989). In HTG but not normal subjects, VLDL $S_f > 60$ contain additional apoE, which mediates its binding to the LDL receptor (Gianturco et al., 1978, 1980, 1982, 1983; Bradley et al., 1984). An apoE-independent receptor distinct from the LDL receptor that binds HTG-VLDL, but not NTG-VLDL or LDL, with high affinity has also been identified on the surface of macrophages, human monocytes, and ECs (Gianturco et al., 1988, 1994). Furthermore, similar analyses of the specific ^{125}I-labeled PA ligand binding data, obtained in the absence/presence of lipoproteins, indicated that these lipoproteins had no additional effect on the binding of either tcu-PA or t-PA to cultured HUVECs (Table 1). The HTG-VLDL-induced decrease in ^{125}I-labeled Glu-Pmg but not ^{125}I-labeled t-PA ligand binding is consistent with the suggestion that Pmg and t-PA may bind to different sites of the same cellular binding protein, annexin II (Hajjar, 1995). Pmg appears to bind to a specific site on annexin II, following the "activation" of annexin II through cleavage by an unknown serine protease which generates a new C-terminal lysine residue. The amino acid homocysteine appears to disable the t-PA-binding domain of annexin II, but has no effect on Pmg binding to annexin II. Lp(a) directly inhibits binding of Pmg, but not t-PA, to annexin II, providing further evidence for the presence of discrete ligand binding domains for Pmg and t-PA (Hajjar, 1995). These combined ligand binding and displacement results strongly favor an inhibition model in which subsequent ^{125}I-labeled Glu-Pmg ligand binding may be interfered with by the presence of the large receptor-bound HTG-VLDL particle, either through occupancy of significant space on the cell surface and/or by affecting the "activation" of annexin II.

Lineweaver−Burke plot analyses of the HUVEC-bound ^{125}I-labeled Glu-Pmg activation data, in the presence of prebound lipoproteins, indicated that the HTG-VLDL-mediated inhibition of HUVEC-bound ^{125}I-labeled Glu-Pmg activation was consistent with an uncompetitive inhibition model. The inhibitor, HTG-VLDL, decreased both the V_{max} and K_m, as required in uncompetitive inhibition in which the large receptor-bound HTG-VLDL particle may additionally also affect the rate of cell-bound Pmg·PA complex formation, subsequent efficiency of Pmg activation, and hence the net expression of surface-localized EC fibrinolysis. Further support of this conclusion was obtained by the fact that increasing concentrations of the HTG-VLDL increased $1/V_{max}$, yielding a series of parallel plots (Figure 4A), typical for uncompetitive inhibition with a K_i for inhibition of $\sim$10 μg/mL (Figure 4B). In addition, when the inhibition effects of HTG-VLDL on these activation data were separately curve-fitted to model equations for competitive, uncompetitive, and noncompetitive inhibition (MINSQ program), the minimum value of the sum of the squared deviations favored either the uncompetitive or noncompetitive inhibition models (Table 3). Noncompetitive inhibitions do not usually decrease the K_m of a reaction, but since a significant decrease in K_m ($p < 0.05$) was, in fact, observed in the presence of HTG-VLDL, as determined by Lineweaver−Burke plot analyses, we have interpreted these kinetic results to further suggest and favor an uncompetitive rather than a noncompetitive model of inhibition. Finally, if the decrease in activation of HUVEC-bound ^{125}I-labeled Glu-Pmg by the HTG-VLDL inhibitor is best described by an uncompetitive type mechanism, then this conclusion will also be consistent with and accommodate the decrease in ^{125}I-labeled Glu-Pmg ligand binding observed in the presence of HTG-VLDL. A plausible explanation for how uncompetitive inhibition can

explain these results may be that the binding and receptor proteins for Pmg, PAs, and HTG-VLDL may be architecturally arranged in such a manner that the HTG-VLDL receptor is juxtaposed to the Pmg rather than the t-PA binding site on annexin II. The presence of a large receptor-bound HTG-VLDL particle may then simultaneously and specifically interfere with the juxtaposed Pmg binding site, resulting both in the observed decreased Pmg binding as well as decreased V_{max} and K_m due to the interference of Pmg·PA complex, typical of an uncompetitive inhibitor. A typical uncompetitive inhibitor, in this case HTG-VLDL, does not usually exert its inhibitory effect by directly binding the free enzyme, as evidenced by the lack of inhibition of ^{125}I-labeled PA binding described in these experiments. In these studies we have now described an additional mechanism by which HTG-VLDL may directly affect the net expression of HUVEC surface-localized fibrinolytic activity by decreasing the interaction of ^{125}I-labeled Glu-Pmg ligand with the surface of cultured HUVECs.

Alternatively the binding of HTG-VLDL to the EC surface may cause a conformational change in HTG-VLDL, resulting in the binding of Pmg, as has been described for the binding of Pmg to Lp(a), following binding to fibrin, which has been attributed to a required conformational change in Lp(a) (Liu et al., 1994). Since lipoproteins have been shown to bind a variety of proteins, such as prothrombin and factor X (Bradley et al., 1986; Bradley & Gianturco, 1988), it is conceivable that the apparent decrease in ^{125}I-labeled Glu-Pmg ligand binding to ECs may be due to preferentially binding to pre-bound HTG-VLDL and is therefore not available to interact with the cell surface. Studies are in progress in this laboratory to examine this possibility.

These studies have provided additional evidence that HTG-VLDL can directly impair the net expression of EC surface-localized fibrinolytic activity by an uncompetitive type mechanism. These results demonstrate that prebound HTG-VLDL, but not NTG-VLDL, is capable of inhibiting the catalytic efficiency of HUVEC-bound ^{125}I-labeled Glu-Pmg activation by EC-bound PAs. If the observed *in vitro* decrease in EC surface-localized plasmin generation in the presence of receptor-bound HTG-VLDL can be extrapolated to *in vivo* conditions, then these studies would further provide a partial mechanistic explanation for the increased thrombotic risk often associated with certain types of HTG. Although the HTG-VLDL-induced decrease in catalytic efficiency appeared to be small, this low level of HTG-VLDL-induced perturbation of the fibrinolytic system may be more commensurate with the level of fibrinolytic dysfunction associated with HTG. In HTG, fibrinolytic dysfunction must occur gradually and represent a long-term chronic rather than a short-term acute consequence of HTG-VLDL perturbation since thrombotic complications usually occur at a later age. If HTG-VLDL were to induce a rapid and significant decrease in fibrinolytic activity, *in vivo*, then consequences arising from the resultant extensive thrombotic complications would be considerably more extensive than commonly associated with HTG. It may, therefore, be more reasonable to assume that HTG-VLDL-induced effects on fibrinolysis, *in vivo*, are, in fact, similarly quite small as we have demonstrated in these *in vitro* experiments. The results presented here further emphasize the potential pathogenic role that chronic exposure to high HTG-VLDL levels may have in the early initiation of thrombosis, in the early and progressive stages of atherosclerosis, and in influencing acute ischemic events such as unstable angina and MI.

ACKNOWLEDGMENT

We thank the labor and delivery staff of AMI Brookwood Medical Center in Birmingham, AL, for providing the umbilical cords used for the isolation of HUVECs used in these studies.

REFERENCES

Barnathan, E. S., Kuo, A., Kariko, K., Rosenfeld, L., Murray, S. C., Behrendt, N., Ronne, E., Weiner, D., Henkin, J., & Cines, D. B. (1990) *Blood 76*, 1795.

Beebe, D. P., Wood, L. L., & Moos, M. (1990) *Thromb. Res. 59*, 339.

Boomsma, D. I., Kaptein, A., Kempen, H. J. M., Gevers Leuven, J. A., & Princen, H. M. G. (1993) *Atherosclerosis 99*, 23.

Booyse, F. M., Sedlak, B. J., & Rafelson, M. E., Jr. (1975) *Thromb. Diasth. Haemorrh. 34*, 825.

Booyse, F. M., Quarfoot, A. J., Bell, S., Fass, D. N., Lewis, J. C., Mann, K. G., & Bowie, D. J. W. (1977) *Proc. Natl. Acta. Sci. U.S.A. 74*, 5702.

Booyse, F. M., Quarfoot, A. J., Chediak, J., Stemerman, M. B., & Maciag, T. (1981) *Blood 58*, 788.

Booyse, F. M., Osikowicz, G., Feder, S., & Scheinbuks, J. (1984) *J. Biol. Chem. 259*, 7198.

Booyse, F. M., Scheinbuks, J., Lin, P., Traylor, M., & Bruce, R. (1988) *J. Biol. Chem. 263*, 15129.

Bradley, W. A., & Gianturco, S. H. (1988) *Semin. Thromb. Hemostasis 14*, 253.

Bradley, W. A., Hwang, S. C., Karlin, J. B., Lin, A., & Prasad, S. C. (1984) *J. Biol. Chem. 259*, 14728.

Bradley, W. A., Song, J. N., & Gianturco, S. H. (1986) *Ann. N.Y. Acad. Sci. 485*, 159.

DeWood, M. A., Spores, J., Notske, R., Mouser, L. T., Burroughs, R., Golden, M. S., & Lang, H. T. (1980) *N. Engl. J. Med. 303*, 897.

Etingin, O. R., Hajjar, D. P., Hajjar, K. A., Harpel, P. C., & Nachman, R. L. (1991) *J. Biol. Chem. 268*, 2459.

Frade, L. J. G., Alvarez, J. J., Rayo, I., Torrado, M. C., Lasuncion, M. A., Avello, A. G., Hernandez, A., & Marin, E. (1991) *Thromb. Res. 63*, 407.

Fredickson, D. S., Goldstein, J. L., & Brown, M. S. (1978) in *The Metabolic Basis of Inherited Diseases* (Stanbury, J. G., Wyngaarden, M. F., & Fredickson, D. S., Eds.) pp 604−655, McGraw-Hill, New York.

Gianturco, S. H., & Bradley, W. A. (1986) in *Methods in Enzymology* (Albers, J. J. & Segrest, J. P. Eds.) pp 319−344, Academic Press, New York.

Gianturco, S. H., Gotto, A. M., Jackson, R. L., Patsch, J. R., Sybers, H. D., Taunton, O. D., Yesurun, D. L., & Smith, L. C. (1978) *J. Clin. Invest. 61*, 320.

Gianturco, S. H., Parkard, C. J., Shepherd, J. B., Smith, L. C., Catapano, A. L., Sybers, H. D., & Gotto, A. M. (1980) *Lipids 5*, 456.

Gianturco, S. H., Brown, F. P., Gotto, A. M., & Bradley, W. A. (1982) *J. Lipid Res. 23*, 984.

Gianturco, S. H., Gotto, A. M., Hwang, S. C., Karlin, J. B., & Lin, A. (1983) *J. Biol. Chem. 258*, 4526.

Gianturco, S. H., Lin, A., Hwang, S. C., Young, J., Brown, S. A., Via, D. P., & Bradley, W. A. (1988) *J. Clin. Invest. 82*, 1633.

Gianturco, S. H., Ramprasad, M. P., Lin, A., Song, R., & Bradley, W. A. (1994) *J. Lipid Res. 35*, 1674.

Haddock, R. C., Baker, C. D., Grammer, J. R., Parks, J. M., Speidel, M. T., Spell, M. L., & Booyse, F. M. (1991) *J. Biol. Chem. 266*, 21466.

Hajjar, K. A. (1991) *J. Biol. Chem. 266(32)*, 21962.

Hajjar, K. A. (1995) *Thromb. Haemostasis 74*, 294.

Hajjar, K. A., & Hamel, N. M. (1990) *J. Biol. Chem. 265(5)*, 2908.

Hajjar, K. A., Gavish, D., Breslow, J. L., & Nachman, R. L. (1989) *Nature 339*, 303.

Hamsten, A., Wiman, B., de Faire, V., & Blomback, M. (1985) *N. Engl. J. Med. 313*, 1557.

Helenius, A., & Simons, K. (1971) *Biochemistry 10*, 2547.
Hoylaerts, M., Rijken, D. C., Lijnen, H. R., & Collen, D. (1982) *J. Biol. Chem. 257*, 2912.
Jaffe, E. A., Nachman, R. L., Becker, C. G., & Minick, C. R. (1973) *J. Clin. Invest. 52*, 2745.
Kugiyama, K., Sakamoto, T., Misumi, I., Sugiyama, S., Ohgushi, M., Ogawa, H., Horiguchi, M., & Yasue, H. (1993) *Circ. Res. 73*, 335.
Laemmili, U. K. (1970) *Nature 227*, 680.
Latron, Y., Chautan, M., Anfosso, F., Alessi, M. C., Nalbone, G., Lafont, H., & Juhan-Vague, I. (1991) *Arterioscler. Thromb. 11*, 1821.
Levin, E. G., Miles, L. A., Fless, G. M., Scanu, A. M., Baynham, P., Curtiss, L. K., & Plow, E. F. (1994) *Arterioscler. Thromb. 14*, 438.
Li, X. N., Varma, V. K., Parks, J. M., Benza, R. L., Koons, J. C., Grammer, J. R., Grenett, H. E., Tabengwa, E. M., & Booyse, F. M. (1995) *Arterioscler. Thromb. Vasc. Biol. 15*, 410.
Lindgren, F. T., Jensen, L. C., & Hatch, F. T. (1972) in *Blood Lipids and Lipoproteins* (Nelson, G. J., Ed.) pp 181−274, Wiley Interscience, New York.
Liu, J. N., Harpel, P. C., & Gurewich, V. (1994) *Biochemistry 33*, 2554.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Manchanda, N., & Schwartz, B. S. (1991) *J. Biol. Chem. 266*, 14580.
Markwell, M. A. (1982) *Anal. Biochem. 125*, 427.
Masuda, T., Yasue, H., Ogawa, H., Misumi, I., Sakamoto, T., Okubo, H., Miyao, Y., & Kato, H. (1992) *Am. Heart J. 124*, 314.
Miles, L. A., Fless, G. M., Levin, E. G., Scanu, A. M., & Plow, E. F. (1989) *Nature 339*, 301.
Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220.
Mussoni, L., Lawrence, D., & Loskutoff, D. J. (1984) *Thromb. Res. 34*, 241.
Nilsson, I. M., Ljungerner, H., & Tengborn, L. (1985) *Br. Med. J. Clin. Res. 290* (6480), 1453.
Nordoy, A., & Goodnight, S. H. (1990) *Arteriosclerosis 10*, 149.
Oshima, S., Uchida, K., Uasu, T., Uno, K., Nonogi, H., & Hze, K. (1991) *Arterioscler. Thromb. 11*, 1772.
Plow, E. F., Freaney, D. E., Plescia, J., & Miles, L. A. (1986) *J. Cell Biol. 103* (6-1), 2411.
Ramakrishman, V., Sinicropi, D. V., Dere, R., Darbonne, W. C., Bechtol, K. B., & Baker, J. B. (1990) *J. Biol. Chem. 265*, 2755.
Rentrop, K. P., Blande, H., Kaiser, H., Karsch, H., Kostering, H., & Leitz, K. (1981) *Circulation 63*, 307.
Sakamoto, T., Yasue, H., Ogawa, H., Misumi, I., & Masuda, T. (1992) *Am. J. Cardiol.* 271.
Sherman, C. T., Litvack, F., Grundfest, W., Lee, M., Hickey, A., Chaux, A., Kass, R., Blanche, C., Matloff, J., Morgenstern, L., Ganz, W., Swan, H. J. C., & Forrester, J. (1986) *N. Engl. J. Med. 315*, 913.
Stiko-Rahm, A., Wiman, B., Hamsten, A., & Nilsson, J. (1990) *Arteriosclerosis 10*, 1067.
Talley, J. R. (1990) *Hosp. Pharm. 25*, 185.
Thronton, S. C., Mueller, S. N., & Levine, E. M. (1983) *Science 222*, 623.
Tremoli, E., Camera, M., Maderna, P., Sironi, L., Prati, L., Colli, S., Piovella, F., Bernini, F., Corsini, A., & Mussoni, L. (1993) *Arterioscler. Thromb. 13*, 338.
Voyta, J. C., Via, D. P., Butterfield, C. E., & Zetter, B. R. (1984) *J. Cell Biol. 99*, 2034.
Williams, J. K., Bellinger, D. A., Nichols, T. C., Griggs, T. R., Bumol, T. F., Fouts, R. L., & Clarkson, T. B. (1993) *Arterioscler. Thromb. 13*, 548.
Wiman, B., Ljungberg, B., Chmielewska, J., Urden, G., Blomback, M., & Johnsson, H. (1995) *J. Lab. Clin. Med. 105*, 265.

BI952032I