Unkeless, J. C. (1979) *J. Exp. Med. 150*, 580.
Unkeless, J. C., Healey, G. A. (1983) *J. Immunol. Methods 56*, 1.
Unkeless, J. C., Scigliano, E., & Freedman, V. H. (1988) *Annu. Rev. Immunol. 6*, 251.
Watts, T. H., Brian, A. A., Kappler, J. W., Marrack, P., & McConnell, H. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7564.
Watts, T. H., Gaub, H. E., & McConnell, H. M. (1986) *Nature 320*, 179.
Weinshank, R. L., Luster, A. D., & Ravetch, J. V. (1988) *J. Exp. Med. 167*, 1909.
Weis, R. M., Balakrishnan, K., Smith, B. A., & McConnell, H. M. (1982) *J. Biol. Chem. 257*, 6440.
Wright, L. L., Palmer, A. G., & Thompson, N. L. (1988) *Biophys. J. 54*, 463.
Young, J. D.-E., Unkeless, J. C., Kaback, H. R., & Cohn, Z. A. (1983a) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1636.
Young, J. D.-E., Unkeless, J. C., Young, T. M., Mauro, A., & Cohn, Z. A. (1983b) *Nature 306*, 186.

Tissue-Type Plasminogen Activator Binds to and Is Inhibited by Surface-Bound Lipoprotein(a) and Low-Density Lipoprotein†

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ABSTRACT: Elevated levels of lipoprotein(a) [Lp(a)] are associated with an increased risk of atherothrombotic disease, but the mechanism(s) by which Lp(a) potentiates atherogenesis is unknown. The extensive homology of apolipoprotein(a) [apo(a)] to plasminogen has led us and others to postulate that Lp(a) may impair fibrinolysis. We have previously shown that Lp(a) inhibits fibrin stimulation of plasminogen activation by tissue-type plasminogen activator (t-PA); however, we and other investigators have been unable to demonstrate direct inhibition of t-PA by Lp(a) in solution. We now report that t-PA binds reversibly and saturably to surface-bound Lp(a) and to low-density lipoprotein (LDL) and that as a result of this binding activation of plasminogen by t-PA is inhibited. The catalytic efficiency (k_{cat}/K_m) of t-PA when bound to polystyrene surface-bound fibrinogen increased 2.9-fold compared to t-PA bound to control wells. When bound to surface-bound Lp(a), however, the catalytic efficiency of t-PA was reduced 9.5-fold compared to t-PA bound to control wells; likewise, by binding to surface-bound LDL, the catalytic efficiency of t-PA was reduced 16-fold compared to the control. Studies with defined monoclonal antibodies suggest that major determinants of t-PA binding are its active site, the LDL receptor binding domain of apolipoprotein B-100 (apoB-100), and apo(a). These data suggest a unique mechanism by which Lp(a) and LDL incorporated in an atheroma can inhibit endogenous fibrinolysis and thereby contribute to the genesis of atherothrombotic disease.

The presence of both thrombus and atheroma in the atherosclerotic vessel wall has been recognized for many years (Haust et al., 1964, 1965), but the molecular and cellular mechanisms by which thrombotic and atherogenic processes interact remain poorly defined. One mechanism by which atherogenic determinants may influence thrombotic events occurring in atheromata involves a unique, highly atherogenic lipoprotein, lipoprotein(a) [Lp(a)].[1] First identified by Berg (1963), Lp(a) is comprised of low-density lipoprotein (LDL) covalently linked through a disulfide bridge from apoB-100 to one or two molecules of a unique apoprotein, apo(a). Lp(a) has been localized to the intima of atherosclerotic lesions (Rath et al., 1989), and elevated levels of Lp(a) (greater than approximately 30 mg/dL) are associated with a significantly increased risk of coronary artery disease (Dahlen et al., 1986; Kostner et al., 1981; Murai et al., 1986; Rhoads et al., 1986). The cDNA of apo(a) has recently been cloned and sequenced and demonstrates a remarkable homology to human plasminogen: it contains a serine protease domain that is 94% homologous with that of plasminogen, one copy of a kringle-5-like region, and 37 copies of kringle-4-like domains (McLean et al., 1988). Despite the structural homology to plasminogen, apo(a) does not generate plasmin-like activity when exposed to plasminogen activators owing to a critical amino acid substitution at the analogous activation site. However, recent work by several groups including our own has supported the view that Lp(a) influences fibrinolysis through its kringle domains. Lp(a) has been shown to inhibit plasminogen binding

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[1] Abbreviations: Lp(a), lipoprotein(a); apo(a), apolipoprotein(a); LDL, low-density lipoprotein; HDL, high-density lipoprotein; apoB-100, apolipoprotein B-100; t-PA, tissue-type plasminogen activator; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone hydrochloride; TBS, 10 mM Tris/0.15 M NaCl, pH 7.4; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; ANOVA, analysis of variance.

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to plasminogen receptors on endothelial (Hajjar et al., 1989) and mononuclear cells (Miles et al., 1989). Lp(a) has also been found to bind to fibrin and to compete with plasminogen and tissue-type plasminogen activator (t-PA) for fibrin binding (Loscalzo et al., 1990; Edelberg et al., 1990). As a consequence of these interactions, Lp(a) attenuates clot lysis induced by t-PA in an in vitro plasma system. Lp(a), however, has not previously been found to bind directly to t-PA or to inhibit t-PA activity in the absence of fibrin. In this report, we demonstrate that t-PA binds reversibly and saturably to surface-bound Lp(a) and LDL and that, as a result of this binding, activation of plasminogen by t-PA is attenuated. These data suggest a unique mechanism by which Lp(a) and LDL incorporated in an atheroma can inhibit endogenous fibrinolysis and thereby contribute to the genesis of atherothrombotic disease.

Materials and Methods

Materials. t-PA was kindly provided by Genentech, Inc., South San Francisco, CA. S-2251 was purchased from Kabi Vitrum, Stockholm, Sweden. ϵ-Aminocaproic acid, Tween-20, bovine serum albumin (BSA), hydrogen peroxide, 3,3′-diaminobenzidine, bovine serum albumin, cathepsin D, and Trizma base were obtained from Sigma Chemical Co., St. Louis, MO. Soluble calf skin collagen was purchased from Worthington Biochemical Corp., Freehold, NJ. Human fibrinogen (plasminogen-free and von Willebrand factor-free) and monoclonal anti-kringle-4 antibody were obtained from Enzyme Research Laboratories, South Bend, IN. Lysine–Sepharose, Sephadex G-25, and benzamidine–Sepharose 2B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Porcine sodium heparin was obtained from Elkins-Sinn, Inc., Cherry Hill, NJ. D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone hydrochloride (PPACK) was purchased from Calbiochem, La Jolla, CA. Immobilon-P transfer membrane (PVDF) was obtained from the Millipore Corporation, Bedford, MA. Iodo-beads were purchased from Pierce Chemical Co., Rockford, IL. Polyclonal anti-t-PA antibodies bound to polystyrene microtiter wells were obtained from Corvas Biopharmaceuticals, La Jolla, CA. $Na^{125}I$, streptavidin-biotinylated horseradish peroxidase complex, and biotinylated ovine anti-murine IgG were purchased from Amersham, Inc., Arlington Heights, IL. Monoclonal antibodies to t-PA (1C8, 2G6, 62E8-3B6, 7D4B7) were kindly provided by Dr. Desire Collen. Monoclonal antibodies to apoB-100 (MB47, MB24, MB1, MB45, MB2, MB43, MB16) were generously provided by Dr. Linda Curtiss. All other chemicals were reagent grade or better.

Isolation of LDL and Lp(a). LDL and HDL were prepared from the plasma of fasting normolipidemic volunteers by sequential ultracentrifugation as previously described (Havel et al., 1955). SDS–polyacrylamide gradient gel electrophoresis was performed to ensure the purity of LDL preparation. Apo(a)-free LDL was prepared as previously described (Fless et al., 1986).

Lp(a) was prepared from blood drawn into sterile bottles that were immersed in wet ice and contained a final concentration of 0.15% EDTA, 0.01% NaN_3, and 0.4 μM soybean trypsin inhibitor. Plasma was separated immediately by low-speed centrifugation at 4 °C and diisopropyl fluorophosphate was added to a final concentration of 1 mM to minimize proteolysis. Total lipoproteins were then prepared by adjusting the plasma density to 1.21 g/mL with solid NaBr and centrifuging the sample in a 60 Ti rotor at 59000 rpm for 20 h at 15 °C. Lp(a) was isolated from the total lipoprotein fraction with use of a combination of rate zonal and density gradient ultracentrifugation as previously described (Fless et al., 1986). Lp(a) preparations were checked for purity by SDS–polyacrylamide gradient gel electrophoresis. When necessary, further purification was conducted by FPLC ion-exchange chromatography using a Mono-Q column (Pharmacia) (Armstrong et al., 1985). Lp(a) or LDL was eluted with a 20-min gradient from 0 to 0.5 M NaCl superimposed on 0.01 M Tris buffer, pH 7.4, at a flow rate of 1 mL/min at 8 °C. Lp(a) eluted at 0.41 M NaCl and LDL at 0.29 M NaCl. Purity of isolated Lp(a) and LDL was again checked electrophoretically.

The Lp(a) preparation used in the experiments presented here has a molecular weight of 914000. The molecular weight of apoB-100 is 514000.

Plasminogen Preparation. Glu-plasminogen was purified from fresh frozen plasma thawed at 37 °C using a modification of the method of Deutsch and Mertz (1970). Plasma was passed over a lysine–Sepharose column, and the column was washed with 0.3 M sodium phosphate, pH 7.4/3 mM EDTA. Plasminogen was eluted from the column with 0.2 M ϵ-aminocaproic acid/3 mM EDTA, pH 7.4. Contaminant plasmin was removed by passing the eluted volume over benzamidine–Sepharose 2B. The plasminogen obtained was subsequently dialyzed before use against 10 mM sodium phosphate, pH 7.4/0.15 M NaCl.

Preparation of Antisera to LDL and Lp(a). Antisera to FPLC-purified preparations of LDL, Lp(a), and reduced apo(a) were prepared in the goat (anti-LDL) and rabbit [anti-Lp(a) and anti-apo(a)] as described previously (Fless et al., 1989).

Radioiodination. t-PA was radioiodinated using Iodo-beads. Two Iodo-beads and 1.0 mCi $Na^{125}I$ were added to 1 mL of 10 mg/mL t-PA. The incubation was allowed to proceed for 30 min with gentle rocking, after which the solution was removed from the Iodo-beads to stop the iodination reaction and passed over a Sephadex G-25 column that had been preequilibrated first with one column volume of 10 mM Tris, pH 7.4/0.15 M NaCl (TBS)/1% BSA followed by two column volumes of TBS/0.1 M L-arginine, pH 8.0. Fifteen 0.5-mL fractions were collected and assayed for total and 100% trichloroacetic acid precipitable radioactivity. Peak fractions (routinely 6–9) routinely contained more than 96% precipitable radioactivity (using trichloroacetic acid) and had a specific activity of 90–120 cpm/ng of protein. t-PA was radioiodinated to this relatively low specific activity in order to minimize oxidative damage to t-PA (Vaughan et al., 1989). We have previously shown that its fibrin binding properties are unaffected by iodination (Vaughan et al., 1989). Fibrinogen was radioiodinated as previously described (Loscalzo et al., 1987).

Lp(a) and apo(a)-free LDL were iodinated by the iodine monochloride method of McFarlane (McFarlane, 1958) with modification (Loscalzo et al., 1987; Shepard et al., 1976). Radioiodinated Lp(a) was 97% precipitable with trichloroacetic acid and had a specific activity of 600–900 cpm/ng. Radioiodination did not affect fibrin binding properties (measured competitively), nor did it affect inhibition of fibrin enhancement of plasminogen activation by t-PA.

Binding Assays. The binding of t-PA to Lp(a), LDL, and fibrinogen (FGN) was measured with the use of polystyrene microtiter wells [flat-bottom, high binding 96-well EIA plates, Catalog No. 3590 (Costar, Cambridge, MA) or 12-well strips (Dynatech, Chantilly, VA)]. Wells were first coated with 100 μL of equimolar concentrations of Lp(a) (0.04 μg/μL), LDL (0.02 μg/μL), or fibrinogen (0.08 μg/μL) in TBS, pH 7.4, and allowed to incubate for 2 h at 37 °C. The unbound proteins

were removed by draining the plate and washing three times with TBS/2% BSA. The remaining binding sites in the wells were blocked by incubating the plates with 320 μL/well TBS/2% BSA for 2 h at 37 °C. After blocking, the plates were blotted dry, sealed with parafilm, and stored at 4 °C until use (up to 10 days). To determine the amount of Lp(a), LDL, or FGN bound, the coating procedure was performed with radioiodinated Lp(a), LDL, or fibrinogen, respectively. Polystyrene wells bound 161 ng of LDL, 493 ng of Lp(a), and 2060 ng of FGN.

After the plates were equilibrated to room temperature, 100 μL of varying concentrations of ^{125}I-t-PA (150–1500 nM) in 0.1 M $NaHCO_3$/0.5 M NaCl containing 2% BSA and 0.1% Tween-20, pH 8.1, were added to the wells. This buffer was chosen because it minimized the nonspecific interaction between t-PA and control BSA-coated wells; in addition, we have previously shown that this buffer is necessary to prevent the nonspecific interaction between rabbit IgG, used in competition experiments described below, and human LDL or Lp(a) (Shepard et al., 1976). The microtiter plates or strips were incubated for 1.5 h at 37 °C, since time course experiments showed that binding reached equilibrium by 60 min. Subsequently, the plates were blotted dry and washed three times, one min/wash, with TBS/1% BSA, and then were washed three more times with TBS, pH 8.0, prior to the enzymatic assays described below. Radioactivity in the individual wells was then counted to measure total binding. Nonspecific binding was determined by the addition of a 20-fold excess of unlabeled t-PA at each concentration. Nonspecific binding accounted for approximately 35% of the total binding observed for t-PA to LDL or Lp(a) and for 25% of the total binding observed for t-PA to fibrinogen.

Competitive binding assays were performed by incubating increasing concentrations of free LDL or Lp(a) in solution with a fixed concentration of ^{125}I-t-PA. The amount of radioiodinated t-PA specifically bound at any concentration of Lp(a) or LDL (B) was compared to that bound to surface-bound Lp(a) or LDL, respectively, in the absence of free Lp(a) or LDL (B_0) and plotted as a ratio (B/B_0) vs total LDL or Lp(a) added.

In order to characterize further the nature of the binding between t-PA and LDL or Lp(a), binding experiments were performed in the presence of several potential inhibitors such as 10 mM ε-aminocaproic acid, 100 μM PPACK, 1000 units/mL heparin, and polyclonal or monoclonal antibodies to apoB-100 and t-PA. In these inhibition experiments, polystyrene-bound LDL or Lp(a) or fluid-phase t-PA was preincubated with 100 μL of 0.1 M $NaHCO_3$/0.5 M NaCl/0.1% Tween-20, pH 8.1, containing the particular inhibitor of interest for 1.5 h at 37 °C. A series of seven monoclonal antibodies to apoB-100 were screened in this manner to identify an antibody or antibodies that could block binding. Similarly, ε-aminocaproic acid, PPACK, heparin, or monoclonal antibodies to the fibrin binding (1C8), active site (2G6), and uncharacterized (62E8-3B6, 7D4B7) domains of t-PA were preincubated with ^{125}I-t-PA for 1.5 h at 37 °C, and the solution was then added to the microtiter wells. Percent inhibition was determined as above from the ratio (B/B_0) of t-PA binding with and without the putative inhibitor or antibody ($=1 - B/B_0$). Importantly, when binding assays were conducted in the absence of Tween-20, no significant difference in specific binding was observed.

Ligand/Immunoblot Analyses. Purified Lp(a), LDL, glu-plasminogen, bovine serum albumin, and cathepsin D were electrophoresed on a 4–15% SDS–polyacrylamide (gradient) gel and then transferred to an Immobilon-P (PVDF) membrane. t-PA (100 nM) was incubated with the transfer membrane for 1 h, after which the blot was developed with a murine monoclonal anti-t-PA antibody (that did not interfere with the active site or the fibrin binding properties of the plasminogen activator) followed by a biotinylated ovine anti-murine antibody.

Enzymatic Activity Assays. t-PA activity when t-PA was bound to Lp(a)-, LDL-, or fibrinogen-coated wells was assayed by use of the native substrate glu-plasminogen and the plasmin-specific chromogenic substrate S-2251. Substrate hydrolysis was measured spectrophotometrically with a Dynatech MR5000 Card Reader at 37 °C in TBS, pH 8.0. Initial reaction velocity was determined from the slope of the plot of absorbance at 405 nm/time vs time, as described previously (Ranby, 1982). The initial reaction velocities were measured over a range of plasminogen concentrations (0.1–4.0 μM) with a fixed concentration of S-2251 (0.8 mM). Velocities were converted to k's (and V_{max} to k_{cat}) by measuring the amount of t-PA bound to LDL, Lp(a), and fibrinogen with use of ^{125}I-t-PA. The enzymatic activity of t-PA not bound to Lp(a) nor LDL (i.e., basal activity that is not stimulated or inhibited, or true control) was determined by binding t-PA to a capture anti-t-PA antibody (bound to wells) that neither stimulates nor inhibits activity. The binding of t-PA to a capture antibody was necessary to ensure against t-PA aggregate formation (which occurred when t-PA was directly bound to polystyrene), yielding unreliable kinetic measurements. Formal kinetic analysis was performed with use of double-reciprocal plots.

Protein Determination. Protein concentrations were determined by the methods of Lowry and colleagues (1951) and Bradford (1976).

Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). The Phast System (Pharmacia) was employed, and the gels were stained with Coomassie brilliant blue. Molecular weight standards (Bio-Rad, Richmond, CA) were used, and apparent molecular weights were estimated by interpolation.

Statistical Analysis. Initial reaction velocities were obtained from the slope of the plot A_{405}/time vs time with use of a linear regression analysis. Determination of statistical significance was carried out by nonpaired t-test and two-way analysis of variance (ANOVA).

Results

Binding of t-PA to Lp(a) and LDL. t-PA bound directly to Lp(a), apo(a)-free LDL, and fibrinogen when the latter were surface-bound to polystyrene wells (Figure 1). Binding was saturable, reversible, and specific. Analysis by Scatchard analysis using curve-fitting methods indicated that this binding likely involves more than one class of sites. At saturation, 0.20 mol of t-PA bound per mole of Lp(a) with an estimated apparent K_D of 750 nM, 0.42 mol of t-PA bound per mole of apo(a)-free LDL with an estimated apparent K_D of 750 nM, and 0.04 mol of t-PA bound per mole of fibrinogen with an estimated apparent K_D of 650 nM. No specific binding of t-PA was observed when HDL was surface-bound to polystyrene wells.

Competitive binding assays showed that fluid-phase Lp(a) and fluid-phase apo(a)-free LDL can effectively and equivalently compete with surface-bound apo(a)-free LDL (Figure 2a) and surface-bound Lp(a) (Figue 2b) for t-PA, displacing 70–80% of total bound t-PA at the maximal concentrations examined. In all cases, the IC_{50} (3–10 nM) for the fluid-phase lipoprotein was significantly less than the corresponding estimated K_D given above.

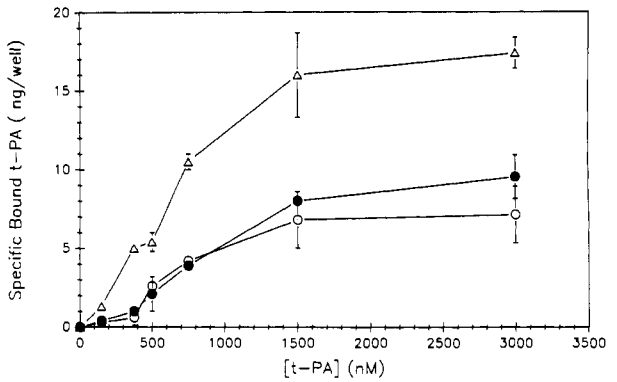

FIGURE 1: Specific binding of t-PA to surface-bound Lp(a), LDL, and fibrinogen. The binding of ^{125}I-t-PA to Lp(a)-, LDL-, and fibrinogen-coated polystyrene microtiter wells was performed as described under Materials and Methods. Each point represents the results of three experiments each performed in duplicate, plotted as mean ± standard deviation (○, Lp(a)-coated wells; ●, LDL-coated wells; △, fibrinogen-coated wells).

Table I: Inhibition of t-PA Binding to Surface-Bound Lp(a) and LDL[a]

	% inhibition	
	Lp(a)	LDL
monoclonal anti-t-PA Abs		
2G6	83 ± 3	70 ± 6
1C8	0	0
62E8-3	0	0
7D4B7	0	0
monoclonal anti-apoB-100 Abs		
MB47	49 ± 12	57 ± 9
MB24	0	52 ± 16
MB1	0	52 ± 5
MB45	0	0
MB2	0	0
MB43	0	0
MB16	0	0
PPACK (100 μM)	70 ± 3	66 ± 6
ε-aminocaproic acid (10 mM)	66 ± 14	43 ± 12
heparin (1000 units/mL)	0	0

[a] Percent specific inhibition is equal to $(1 - B/B_0)$, with each value given as the mean ± standard deviation of three experiments performed in duplicate.

To characterize the domain(s) of t-PA important for its binding to Lp(a) and LDL, monoclonal antibodies to the fibrin binding site (1C8), active site (2G6), and uncharacterized (62E8–3B6, 7D4B7) epitopes of t-PA were used in an attempt to inhibit binding. The active site monoclonal antibody 2G6 inhibited the binding of t-PA to Lp(a) by 83% and to LDL by 70%, suggesting that the active site domain of t-PA is in part responsible for its binding to these two lipoproteins (Table I). All other antibodies were ineffective inhibitors of binding, including 1C8, which is directed against the fibrin binding of domain of t-PA.

Because preliminary studies with a polyclonal antibody to apoB-100 suggested that this apoprotein was important for the binding of t-PA to both Lp(a) and LDL, we examined this interaction with specific monoclonal antibodies against apoB-100. A panel of seven monoclonal antibodies to apoB-100 were screened for their ability to block the binding of t-PA to Lp(a) or apo(a)-free LDL. Immuno-dot blots revealed that all seven antibodies bound to Lp(a) and apo(a)-free LDL (data not shown).

Three of the seven monoclonals (MB47, MB24, and MB1) inhibited the binding of t-PA to LDL by approximately 50–60%, while only the first of these (MB47) inhibited binding to Lp(a) (by 49%).

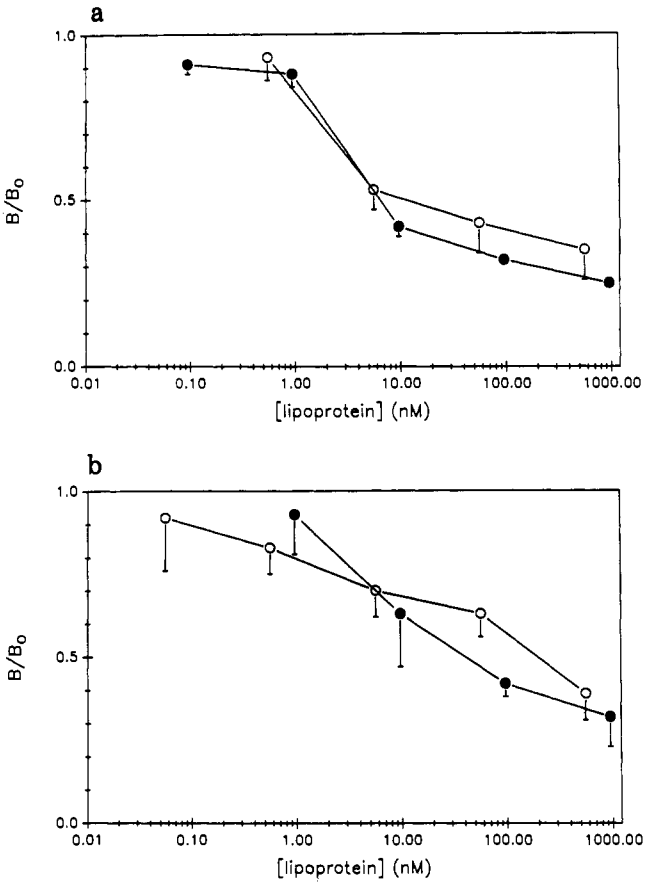

FIGURE 2: Inhibition of t-PA binding to Lp(a) and LDL. Competitive binding assays were performed by incubating increasing concentrations of fluid-phase Lp(a) or apo(a)-free LDL with a fixed concentration of ^{125}I-t-PA. The amount of radioiodinated ligand bound at any concentration of fluid-phase Lp(a) or LDL was compared to that bound to surface-bound Lp(a) or surface-bound LDL, respectively, in the absence of fluid-phase Lp(a) or LDL and plotted as a ratio (B/B_0) vs the concentration of lipoprotein added: (a) LDL-coated wells, (b) Lp(a)-coated wells. Each point represents three experiments performed in duplicate, plotted as mean ± standard deviation (○, fluid-phase Lp(a); ●, fluid-phase LDL).

The data in Table I also show the results of the binding of t-PA to Lp(a) or apo(a)-free LDL in the presence of various inhibitors of binding. PPACK (100 μM), a covalent serine protease active site blocker, inhibited t-PA binding to Lp(a) and LDL by 70 and 66%, respectively, confirming the results of the experiments using the active site monoclonal, 2G6. Interestingly, high concentrations of ε-aminocaproic acid (10 mM) also inhibited t-PA binding to Lp(a) and LDL by 66 and 43%, respectively, while a monoclonal directed against plasminogen kringle-4 [and, therefore, against the kringle-4-like domains of Lp(a)] had no effect on t-PA binding to Lp(a) and LDL. Thus, lysine binding domains in t-PA may play a role in its interaction with these surface-bound lipoproteins. Heparin had no effect on the binding of t-PA to either lipoprotein.

As one final measure of the direct interaction between t-PA and Lp(a) and LDL, we performed the ligand/immunoblotting studies shown in Figure 3. One microgram each of purified Lp(a), LDL, glu-plasminogen, bovine serum albumin, and cathepsin D were subjected to gradient gel electrophoresis and transferred to PVDF membranes, and the membranes were incubated with 100 nM t-PA. After incubation the blots were developed with a murine anti-t-PA antibody followed by a biotinylated ovine anti-murine antibody as described under Materials and Methods. As shown in the figure, under these rather stringent conditions t-PA formed a complex with

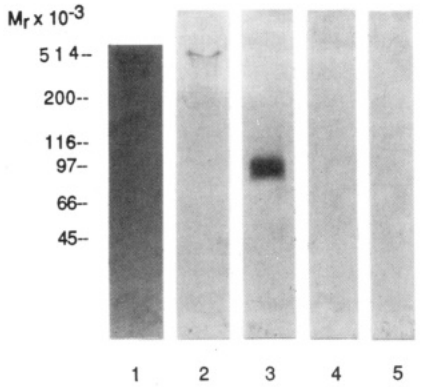

FIGURE 3: Binding of t-PA to Lp(a) and LDL apoproteins by immunoblot analysis. One microgram each of purified Lp(a) (lane 1), LDL (lane 2), glu-plasminogen (lane 3), bovine serum albumin (lane 4), and cathepsin D (lane 5) were subjected to gradient gel electrophoresis (4–15%), transferred to a PVDF membrane and then incubated with 100 nM t-PA. The blot was subsequently developed with a murine anti-t-PA antibody followed by a biotinylated ovine anti-murine antibody, as described under Materials and Methods. Molecular weight markers are indicated along the left side of the figure.

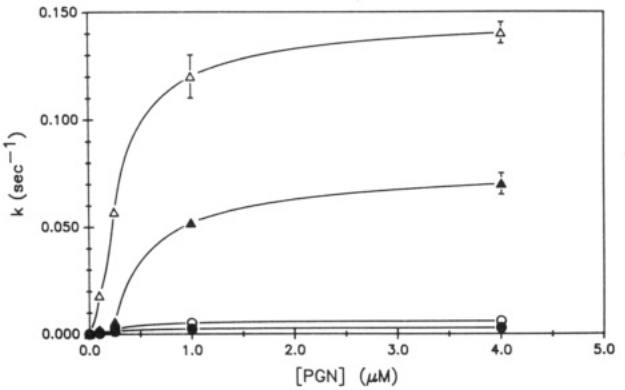

FIGURE 4: Effect of t-PA binding to Lp(a) and LDL on plasminogen activator activity of t-PA. The effect of t-PA binding to Lp(a), apo(a)-free LDL, capture anti-t-PA antibody, and fibrinogen on t-PA activation of the physiologic substrate glu-plasminogen was examined. Initial reaction velocities were calculated from the slope of A_{405}/time vs time and converted to k's by measuring the amount of t-PA bound to each coated well. Each point represents three experiments performed in triplicate plotted as mean ± standard deviation (○, Lp(a)–t-PA; ●, LDL–t-PA; △, FGN–t-PA; ▲, t-PA). ANOVA: $p <$ 0.005 for all pairs except Lp(a)-t-PA and LDL-t-PA.

apoB-100 (514-kDa band, lanes 1 and 2, and apo(a) 420-kDa and 400-kDa bands, lane 1). Binding to glu-plasminogen (lane 3) served as a positive control, while absence of detectable complex formation with bovine serum albumin (lane 4) or cathepsin D (lane 5) served as negative controls.

Effect of t-PA Binding to Lp(a) and LDL on t-PA Plasminogen Activator Activity. The effect of t-PA binding to Lp(a) and apo(a)-free LDL on t-PA activation of glu-plasminogen was examined (Figure 4). In these experiments, t-PA bound to Lp(a)-coated, LDL-coated, capture anti-t-PA antibody (basal, control) coated, or fibrinogen-coated wells was incubated with a range of plasminogen concentrations (0.1–4.0 μM) and 0.8 mM S-2251 in TBS, pH 8.0. Initial reaction velocities were calculated from the slope of A_{405}/time vs time and converted to k's by measuring the amount of t-PA bound to LDL, Lp(a), capture anti-t-PA antibody, or fibrinogen. When bound to fibrinogen-coated wells, the catalytic efficiency of t-PA (k_{cat}/K_m) increased by 2.94-fold compared with basal (control) t-PA activity, whereas, when bound to Lp(a)- or LDL-coated wells, the catalytic efficiency decreased by 9.4- and 16.0-fold, respectively, compared with basal t-PA activity with t-PA. Compared with t-PA bound to fibrinogen-coated wells, t-PA binding to Lp(a)-coated wells and LDL-coated wells reduced the catalytic efficiency 27.6- and 47.0-fold, respectively (Table II).

Table II: Kinetic Constants for t-PA Bound to Surface-Bound Proteins and Lipoproteins

surface-bound (lipo)protein	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})
fibrinogen	0.14	0.30	0.47
anti-t-PA Ab	0.070	0.45	0.16
Lp(a)	0.0061	0.35	0.017
LDL	0.0030	0.30	0.010

Discussion

We have demonstrated that t-PA binds reversibly and saturably to surface-bound Lp(a) and LDL and that as a result of this binding the activation of plasminogen by t-PA is inhibited. These are unique observations because (1) Lp(a) has not previously been found to bind directly to t-PA or to inhibit t-PA in the absence of fibrin and (2) there are no prior reports of direct interaction between LDL and t-PA.

LDL and Lp(a) accumulate in the vessel wall (Rath et al., 1989; Cushing et al., 1989), and in this location interact with plasma proteins of the clotting and fibrinolytic systems. Surface binding of these lipoproteins provides an opportunity to investigate in vitro the determinants of the vessel wall/fluid-phase interaction. Earlier observations suggest that surface binding of these lipoproteins alters their conformation. Zawadzki and colleagues (1988) showed that monoclonal antibodies raised against apoB-100 react variably depending on whether or not LDL is surface-bound or free in solution and whether or not LDL is linked to apo(a); some epitopes in apoB-100 are, therefore, inaccessible or structurally modified by the conformation adopted by surface binding of LDL and its association with apo(a). Such surface binding dependent changes in tertiary structure may be important for the functional effects of these lipoproteins in the microenvironment of the vessel wall/blood interface to be optimally manifest.

The estimated apparent K_D of t-PA for surface-bound fibrinogen in our binding assay was 650 nM. Several groups have reported that t-PA binds to fibrin with a K_D of approximately 150 nM; however, this lower K_D is dependent on the presence of plasminogen. In the absence of plasminogen, the K_D for t-PA binding to fibrin has been reported to be as high as 600 nM (Ranby, 1982; Liu & Wallen, 1984) in the range observed in this report for surface-bound fibrinogen. The K_D of t-PA for surface-bound Lp(a) or LDL was 750 nM. However, competitive binding assays showed that fluid-phase Lp(a) and fluid-phase LDL effectively and equivalently competed with surface-bound Lp(a) and surface-bound LDL for t-PA with an IC_{50} ranging from 3 to 10 nM. Interestingly, we and others have been unable to demonstrate any inhibition of t-PA activity by Lp(a) or apo(a)-free LDL in solution. The data reported here showing (1) that surface-bound Lp(a) and LDL impair t-PA activity in the absence of fibrin and (2) that fluid-phase Lp(a) and LDL compete for t-PA binding to their surface-bound lipoprotein counterparts with IC_{50}'s significantly lower than the estimated apparent K_D's suggest that t-PA binding to Lp(a) and LDL in solution does not impair active site access to substrate or alter the catalytic mechanism. Thus, these data suggest that the interaction of t-PA with Lp(a) and LDL occurs both in solution and on surfaces, but through significantly different domains. That Lp(a) and LDL do not alter t-PA activity in the fluid phase but do so on a surface is consistent with the greater fibrinolytic relevance of surface-bound t-PA as compared to fluid-phase t-PA.

Surface-bound Lp(a) inhibits t-PA in a mechanistic manner that is distinctly different from that noted in solution (Loscalzo et al., 1990; Edelberg et al., 1990) in prior studies. In these studies, Lp(a) was found to bind to soluble fibrin and to compete with plasminogen and t-PA binding for soluble fibrin; as a result of this interaction in solution, Lp(a) inhibits the fibrin stimulation of plasminogen activation by t-PA with a K_i ranging from 15 to 22 nM (Loscalzo et al., 1990; Edelberg et al., 1990). Solution-phase Lp(a) or LDL had no direct inhibitory effects on t-PA in the absence of fibrin. By binding Lp(a) to polystyrene wells, Lp(a) directly inhibits t-PA activity in the absence of fibrin(ogen). One possible mechanism for this inhibition is suggested by the inhibitory binding experiments with anti-t-PA monoclonal antibodies. The active site of t-PA is largely responsible for its binding to Lp(a)—namely, the active site monoclonal antibody 2G6 inhibits specific binding by 83%, and PPACK does so by 70%. Ligand/immunoblotting experiments suggest that t-PA binds to apo(a) as well as to apoB-100 (vide infra). By binding to Lp(a), the active site of t-PA is, thus, unavailable for interaction with substrate plasminogen. The catalytic efficiency of t-PA therefore decreases and does so largely by a decrease in k_{cat}. In addition to active site dependent binding, the data with ϵ-aminocaproic acid suggest that lysine-dependent binding is also important for the interaction of t-PA with Lp(a), although inhibition occurred only at very high concentrations of the lysine analogue (10 mM) and may, therefore, be of less pathophysiologic relevance. Importantly, the lack of effect of an anti-kringle-4 antibody on this interaction and the equipotent inhibition of t-PA binding to LDL by 2G6 and PPACK suggest that it is not through the kringle-4-like domains of apo(a) that t-PA and Lp(a) interact when Lp(a) is bound to a surface.

Additional information about the mechanism of interaction of t-PA with Lp(a) is apparent from the binding and kinetic experiments with LDL. t-PA binds to surface-bound apo(a)-free LDL, and fluid-phase LDL is able to compete for t-PA binding to surface-bound Lp(a) or LDL. As a result of this binding, the activity of t-PA is inhibited. The active site of t-PA is also responsible for approximately 70% of this specific binding, and experiments with monoclonal antibodies to apoB-100 suggest that at least one important region in LDL for t-PA binding is blocked by the monoclonal MB47. MB47 has been found to block LDL receptor binding and binds to an apoB-100 proteolytic fragment spanning amino acids 3441–3586 (Hoover et al., 1988; Young et al., 1986; Knott et al., 1986). Within this domain (Petersen et al., 1990) are two candidate sequences (GAV and GSV) with neutral amino acids substituted in the second position for Arg561 [located at the activator site (GRV) of plasminogen] that may serve as a binding site for the active site of t-PA. Importantly, preliminary studies show that t-PA does not cleave Lp(a) or LDL (data not shown), indicating that if t-PA does bind through its active site to one of these two candidate sequences in apoB-100, it does so by forming a dead-end complex.

Detailed analysis of the kinetics of activation of plasminogen by t-PA and the role of fibrin have been reported previously by several groups (Holyaerts et al., 1982, Binder & Spragg, 1980). Binder and Spragg found that in the presence of fibrin the K_m remained unaltered but the V_{max} increased 20-fold with glu-plasminogen as substrate. Hoylaerts and colleagues, in contrast, found that the K_m decreased and the V_{max} increased in the presence of fibrin, resulting in a 50–600-fold increase in the catalytic efficiency of t-PA. Ranby (1982) has shown previously that the degree of stimulation of t-PA activity is less with fibrinogen than that with fibrin. In the presence of fibrinogen, Ranby found that the activity of t-PA was stimulated 1.5–9-fold over a range of fibrinogen concentrations from 0.01 to 10 μM. Similar to Binder and Spragg, we found a predominant effect on V_{max} when t-PA is bound to surface-bound fibrinogen compared to surface-bound Lp(a) or LDL, resulting in an approximate 3-fold increase in the catalytic efficiency of t-PA when bound to fibrinogen. The binding of t-PA to surface-bound Lp(a) or LDL leads to a reduction in the V_{max} with a relatively unaltered K_m and is consistent with the interpretation that the active site of t-PA is involved in the binding mechanism.

The presence of both thrombus and atheroma in the atherosclerotic vessel wall has been recognized for many years, but the molecular and cellular mechanisms by which thrombotic and atherogenic processes interact remain poorly defined. Recent work by several groups has demonstrated interactions between lipoproteins and the clotting/fibrinolytic systems. The data presented here suggest a unique mechanism by which the two lipoproteins, Lp(a) and LDL, can impair fibrinolysis. The physiologic relevance of these observations awaits additional experiments in more complex systems.

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REFERENCES

Armstrong, V. W., Walli, A. K., & Seidel, D. (1985) *J. Lipid Res. 26*, 1314–1323.

Berg, K. (1963) *Acta Pathol. Microbiol. Scand. 59*, 369–382.

Binder, B. R., & Spragg, J. (1980) in *Protides of the Biological Fluids* (Peters, H., Ed.) Vol. 28, pp 391–394, Pergamon, Oxford.

Bradford, M. (1976) *Anal. Biochem. 72*, 248.

Cushing, G. L., Gaubatz, J. W., Nava, M. L., Burdick, B. J., Bocan, T. M. A., Guyton, J. R., Weilboecher, D., DeBakey, M. E., Lawrie, G. M., & Morrisett, J. D. (1989) *Arteriosclerosis 9*, 593–603.

Dahlen, G. H., Guyton, J. R., Attar, M., Farmer, J. A., Kautz, J. A., & Gotto, A. M. (1986) *Circulation 74*, 758–765.

Deutsch, D. G., & Mertz, E. T. (1970) *Science 170*, 1095–1096.

Edelberg, J. M., Gonzalez-Gronow, M., & Pizzo, V. (1990) *Thromb. Res. 57*, 155–162.

Fless, G. M., ZumMallen, M., & Scanu, A. M. (1986) *J. Biol. Chem. 261*, 8712–8718.

Fless, G. M., Snyder, M. L., & Scanu, A. M. (1989) *J. Lipid Res. 30*, 651–662.

Hajjar, K. A., Gavish, D., Breslow, J. L., & Nachman, R. L. (1989) *Nature 339*, 303–305.

Haust, M. D., Wylie, J. C., & More, R. H. (1964) *Am. J. Pathol. 44*, 255–267.

Haust, M. D., Wylie, J. C., & More, R. H. (1965) *Exp. Mol. Pathol. 4*, 206–217.

Havel, R. M., Eder, H. A., & Bradgon, J. H. (1955) *J. Clin. Invest. 34*, 1345–1353.

Holyaerts, M., Rijkin, D. C., Lijnen, H. R., & Collen, D. (1982) *J. Biol. Chem. 257*, 2912–2919.

Hoover, G. A., McCormick, S., & Kalant, N. (1988) *Arteriosclerosis 8*, 525–534.

Knott, T. J., Pease, R. J., Powell, L. M., Wallis, S. C., Rall, S. C., Innerarity, T. L., Blackhart, B., Taylor, W. H., Marcel, Y., Milner, R., Johnson, D., Fuller, M., Luis, A.

J., McCarthy, B. J., Mahley, R. W., Levy-Wilson, B., & Scott, J. (1986) *Nature 323*, 734–738.
Kostner, G. M., Avogaro, P., Cazzolato, G., Marth, E., Bittolo-Bon, G., & Quinci, G. B. (1981) *Atherosclerosis 38*, 51–61.
Laemmli, U. K. (1970) *Nature 227*, 680–682.
Liu, C. Y., & Wallen, P. (1984) *Circulation 70 (Suppl. II)*, 365.
Loscalzo, J., Freedman, J., Rudd, M. A., & Vaughan, D. E. (1987) *Arteriosclerosis 7*, 450–455.
Loscalzo, J., Weinfeld, M., Fless, G. M., & Scanu, A. M. (1990) *Arteriosclerosis 10*, 240–245.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, L. (1951) *J. Biol. Chem. 193*, 265–275.
McFarlane, A. S. (1958) *Nature 182*, 58.
McLean, J. W., Tomlinson, J. E., Kuang, W. J., Eaton, D. L., Chen, E. Y., Fless, G. M., Scanu, A. M., & Lawn, R. M. (1988) *Nature 330*, 132–137.
Miles, L. A., Fless, G. M., Levin, E. G., Scanu, A. M., & Plow, E. F. (1989) *Nature 339*, 301–302.
Murai, A., Miyahara, T., & Fujimoto, N. (1986) *Atherosclerosis 59*, 199–204.
Petersen, T. E., Martzen, M. R., Ichinose, A., & Davie, E. W. (1990) *J. Biol. Chem. 265*, 6104–6111.
Ranby, M. (1982) *Biochim. Biophys. Acta 704*, 461–469.
Rath, M., Neindorf, A., Tjark, R., Dietel, M., Krebber, H., & Beisiegel, U. (1989) *Atherosclerosis 9*, 579–592.
Rhoads, G. G., Dahlen, G., Berg, K., Morton, N. E., & Dannenberg, A. L. (1986) *J. Am. Med. Assoc. 256*, 2540–2544.
Shepard, J., Bradford, D. K., & Morgan, H. G. (1976) *Clin. Chim. Acta 66*, 97–109.
Vaughan, D. E., Mendelsohn, M. E., Declerck, P. J., van Houtte, E., Collen, D. E., & Loscalzo, J. (1989) *J. Biol. Chem. 264*, 15869–15874.
Young, S. G., Witztum, J. L., Casal, D. C., Curtiss, L. K., & Bernstein, S. (1986) *Arteriosclerosis 6*, 178–188.
Zawadzki, Z., Terce, F., Seman, L. J., Thealis, R. T., Breckenridge, W. C., Milne, R. W., & Marcel, Y. L. (1988) *Biochemistry 27*, 8474–8481.

Comparison of Operator-Specific and Nonspecific DNA Binding of the λ *cI* Repressor: [KCl] and pH Effects†

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ABSTRACT: The effects of proton and KCl activity on the nonspecific λ *cI* repressor–DNA interactions and on the site-specific repressor–O_R interactions were compared, in order to assess their roles in site specificity. The repressor–O_R interactions were studied by using DNase I footprint tiration. The Gibbs free energy changes for binding and for cooperativity were determined between 25 and 300 mM KCl, from individual-site isotherms for the binding of repressor to O_R and to reduced-valency mutants. The proton-linked effects on repressor–O_R interactions have been published [Senear, D. F., & Ackers, G. K. (1990) *Biochemistry 29*, 6568–6577; Senear, D. F., & Bolen, D. W. (1991) *Methods Enzymol.* (in press)]. Nonspecific binding was studied by using a nitrocellulose filter binding assay, which proved advantageous in this case, due to the relatively weak nonspecific binding, and precipitation of repressor–DNA complexes. Filter binding provided measurements at low binding density where precipitation did not occur. The data provide estimates of the Gibbs free energy changes for nonspecific, intrinsic binding, but not for cooperativity. The KCl concentration dependencies of the intrinsic binding constants indicate that ion release plays similar roles in distinguishing between the operators and in discriminating operator from nonoperator DNA. Binding to DNA is accompanied by net proton absorption. Near neutral pH, proton linkages to operator and nonoperator binding are the same. Differences at acid and at basic pH implicate the same ionizable repressor groups in distinguishing between the operators and in discriminating operator from nonoperator DNA. The results indicate similar overall modes of operator and nonoperator binding of repressor, but implicate indirect effects of DNA sequence as important contributors to sequence recognition.

The key steps in the regulation of developmental and metabolic pathways often occur at the level of initiation of transcription. In both prokaryotes and eukaryotes, this regulation involves DNA sequence dependent interactions between regulatory proteins and DNA. Due to enormous effort expended in recent years, a few general principals of sequence-specific recognition of DNA by regulatory proteins have emerged. However, a complete picture remains elusive. While it was felt, at one time, that specificity must arise strictly from contacts to functional groups in the major groove of the DNA, it is now recognized that interactions with the backbone are also important to specificity, due to sequence-dependent variations in DNA conformation and susceptibiity to protein-induced deformation of DNA [see Steitz (1990) and

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