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Research Article

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Homocysteine-induced Modulation of Tissue Plasminogen Activator Binding to Its Endothelial Cell Membrane Receptor

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Abstract

Endothelial cells impart thromboresistance to the blood vessel wall. As modulators of fibrinolytic activity, these cells synthesize and secrete tissue plasminogen activator (t-PA) as well as its physiologic inhibitor, plasminogen activator inhibitor-1. In addition, endothelial cells support membrane-associated assembly of plasminogen and tissue plasminogen activator. Recently, an M_r ~ 40,000 protein expressed on endothelial cells has been shown to interact noncompetitively through disparate mechanisms with both t-PA and plasminogen, suggesting trimolecular assembly of enzyme, substrate, and receptor (Hajjar, K. A. 1991. *J. Biol. Chem.* 266:21962–21970). In the present study, treatment of cultured endothelial cells with DL-homocysteine was specifically associated with a selective reduction in cellular binding sites for t-PA. This 65% decrease in binding was associated with a 60% decrease in cell-associated t-PA activity. No change in affinity for t-PA or plasminogen or in the maximal number of binding sites for plasminogen was observed. Matrix-associated t-PA binding sites were not affected. These data suggest a new mechanism whereby homocysteine may perturb endothelial cell function, thus promoting a prothrombotic state at the surface of the blood vessel wall. (*J. Clin. Invest.* 1993. 91:2873–2879.) Key words: endothelial cells • homocysteine • homocystinuria • tissue plasminogen activator • thromboregulation

Introduction

Homocysteine is a normal metabolite which accumulates intracellularly and in plasma in certain inborn errors of transsulfuration. In homozygous cystathionine-β-synthase deficiency, the commonest form of homocystinuria, accumulation of homocysteine is accompanied by developmental delay, osteoporosis, and ectopia lentis (1). In addition, as many as 50% of patients with this disorder experience a major thromboembolic episode by 30 yr of age, and vascular occlusion is a primary factor in 66% of all fatal events (2). Recent evidence suggests, furthermore, that heterozygous homocysteinemia represents a significant independent risk factor for premature atherosclerotic vascular disease involving peripheral, cerebral, and coronary arteries (3–7). Although the mechanism by which homocysteine may promote atherothrombotic disease is not established, several recent reports have demonstrated a potential role for homocysteine in endothelial cell dysfunction in vitro. Reported alterations in the thromboregulatory properties of endothelial cells upon exposure to homocysteine (HC)[1] include induction of a protease activator of coagulation Factor V (8), induction of an inhibitor of protein C activation (9), aberrant secretion of thrombomodulin (10), and inhibition of thrombomodulin cofactor activity (11).

Endothelial cells line the vasculature and function to maintain the fluidity of blood (12). Plasmin generation in vitro is supported by cell surface assembly of its circulating precursor, plasminogen, and its endothelial cell–derived activator, tissue plasminogen activator (t-PA) (13). Recent evidence demonstrates that both the plasmin-forming enzyme, t-PA (K_d 10–20 nM), and its substrate, plasminogen (K_d 120–300 nM), interact noncompetitively with distinct domains associated with a common cell surface binding protein (t-PA/plasminogen receptor) synthesized by the endothelial cell (14). In a purified system, and on the surface of cultured cells, this M_r 40,000 protein enhances the catalytic activity of t-PA (14–16) and protects it from its physiologic inhibitor, plasminogen activator inhibitor-1 (PAI-1) (14). Upon binding to the endothelial cell, amino-terminal glutamic acid plasminogen is partially converted to a higher affinity, cleaved derivative (amino-terminal lysine plasminogen; Lys-PLG) through the action of a serine protease possessing plasmin-like specificity (17). Lys-PLG is activated by t-PA and urokinase approximately 10 to 20 times more efficiently than Glu-PLG (18, 19), thus providing for catalytic amplification at the cell surface.

Interactions of plasminogen and tissue plasminogen activator with endothelial cells appear to be independently regulated. Plasminogen binding to the endothelial cell or to its purified M_r 40,000 receptor appears to involve "kringle"-associated lysine binding sites since it can be blocked by basic amino acids such as ϵ-aminocaproic acid, tranexamic acid, lysine, and arginine (14, 20). t-PA binding, on the other hand, is relatively insensitive to these agents, indicating that lysine binding sites are not required for the interaction (14, 15). Binding of Lys-PLG to endothelial cells, furthermore, is also inhibited in the presence of molar excesses of lipoprotein(a) (21–23), an atherogenesis-

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1. *Abbreviations used in this paper:* AFC-56, 2-Gly-Gly-Arg-aminofluoromethylcoumarin; B_{max}, maximal binding capacity; EC, endothelial cells; HC, homocysteine; Lys-PLG, lysine plasminogen; PAI-1, plasminogen activator inhibitor-1; t-PA, tissue plasminogen activator.

associated low density lipoprotein whose apoprotein(a) contains multiple, tandemly repeated units of plasminogen-like kringle IV (24, 25). These structures appear to allow Lp(a) to compete with Lys-PLG, but not t-PA, for binding to the M_r 40,000 receptor (14). Factors that regulate t-PA binding to the t-PA/plasminogen receptor have been heretofore undefined. In the present study, exposure of endothelial cells to homocysteine was associated with a selective reduction in membrane binding sites for t-PA, and a concomitant reduction in cell surface t-PA activity, while plasminogen binding remained unperturbed.

Methods

Materials. DL-homocysteine, L-cysteine, L-homocystine, L-cystine, glutathione, diisopropyl fluorophosphate, catalase, and superoxide dismutase were from Sigma Immunochemicals, St. Louis, MO. $Na_2{}^{51}CrO_4$ and $Na^{125}I$ were purchased from ICN Biomedicals Inc., Costa Mesa, CA. 2-Gly-Gly-Arg-aminofluoromethylcoumarin (AFC-56) was obtained from Enzyme Systems Products, Livermore, CA. Tissue plasminogen activator was provided by Genentech, Inc., South San Francisco, and Lys-plasminogen, by Immuno, Vienna, Austria. Carboxypeptidase B from porcine pancrease was purchased from Calbiochem Corp., La Jolla, CA. Rabbit anti-angiotensin-converting enzyme IgG was a gift from Dr. E. Erdos, University of Texas Southwestern Medical School, Dallas, TX. A mouse monoclonal anti-M_r40,000 IgG was prepared by immunization with purified receptor protein (14) as described (26).

Cell culture. Endothelial cells (passages 1–5), propagated as previously described (14, 16, 19), were seeded in 24-well cluster dishes and reached confluency within 2–3 d. On day 5 or 6, 50% of postculture medium was replaced by complete medium containing DL-homocysteine or related compounds.

Cell treatment with sulfur-containing compounds. Twice final concentrations of DL-homocysteine, L-cysteine, L-methionine, and glutathione were prepared in complete culture medium. L-homocystine and L-cystine were dissolved first in 1 N HCl, added dropwise to an equal volume of 0.5 M $NaHCO_3$, and then diluted into 9 vol complete culture medium to achieve twice final concentration. The resulting media were pH-adjusted to 7.4 as necessary with 0.5 M $NaHCO_3$, sterile filtered, and added to wells from which 50% of postculture medium had been removed. Endothelial cells (EC) were cultured further for up to 96 h.

Cell viability. Cell viability was assessed essentially as described by Rodgers and Kane (8). Confluent endothelial cells were loaded with $Na_2{}^{51}CrO_4$ (1 μCi/ml complete culture medium, 18 h), washed three times with Hepes-buffered saline (HBS) (11 mM Hepes, 137 mM NaCl, 4 mM KCl, 11 mM glucose, pH 7.4), and incubated with doses of homocysteine (HC) (0–10 mM) in complete medium (18 h). Cytotoxicity was calculated as $(A - B)/(C - B)$, where A represents ^{51}Cr cpm released in the test sample, B represents ^{51}Cr cpm released in the control sample, and C represents maximal ^{51}Cr released upon treatment of cell monolayers with Tris-buffered saline-1% Triton X-100 (21°C, 10 min). ^{51}Cr release in response to 0.1, 1.0, 2.5, 5.0, and 10.0 mM HC was 3.3±2.7, 0.5±0.4, 3.6±1.7, 6.0±2.8, and 43.5±7.6% (SE, $n = 3$) above the spontaneous release level (24.2±2.6%, [SE, $n = 3$] of maximal after 18-h incubation).

Cell counts. Cells were enumerated in a hemocytometer after detachment with 0.02% type I collagenase in Hepes-buffered saline containing 0.01% EDTA and 0.25% BSA at 37°C.

Radioligand binding studies. t-PA and Lys-PLG were radiolabeled as previously described (15, 20). Murine antireceptor IgG was labeled as described (20). At specified times, control or HC-treated monolayers were equilibrated to 4°C, washed twice with ice cold IB(5) (HBS containing 3 mM $CaCl_2$, 1 mM $MgCl_2$, and BSA 5 mg/ml), once with IB(5)/10 mM ϵ-aminocaproic acid, and twice more with IB(5). ^{125}I-t-PA or ^{125}I-Lys-PLG were added, and cells incubated (4°C, 30 min) before sampling free radioactivity. Cell monolayers were washed five times rapidly with IB(5), and then solubilized (58°C, 60 min) in 1% SDS, 0.5 M NaOH, 0.01 M EDTA, before sampling bound radioactivity.

Ligand blotting. Ligand blotting was carried out as previously described (14, 16). Blots were imaged on a UMAX MaxColor UC630 and analyzed using the NIH Image 1.41 program.

Membrane preparation. Endothelial cell plasma membrane-enriched fractions were prepared as previously described (16).

Plasminogen activator activity studies. Confluent endothelial cells were treated with complete medium with or without 5 mM homocysteine, and incubated for 24 h. After equilibration to 4°C, cells were washed three times with ice cold IB(2) (Hepes-buffered saline containing 3 mM $CaCl_2$, 1 mM $MgCl_2$, and BSA 2 mg/ml), and then incubated at 4°C with IB(2) containing 50 nM t-PA (30 min). Cells were washed twice rapidly, and exposed to the fluorogenic substrate AFC-56 (140 μM). Relative fluorescence units were monitored at 15-min-intervals (excitation 400 nm, emission 505 nm, slit widths 2 nm, range 3, 650-10S fluorescence spectrophotometer; Perkin-Elmer Corp., Norwalk, CT) in quadruplicate samples of cells with or without added t-PA and with or without pretreatment with DL-homocysteine. Incremental t-PA activity was estimated as relative fluorescence units/min which was linear (correlation coefficient 0.99) for the first 60 min of incubation. Wells were monitored for cell dropout which was < 5%.

Results

Preincubation of human umbilical vein endothelial cells with DL-HC (0.1–5.0 mM) had no significant effect on overall cell number or viability. ^{51}Cr release in response to 0.1 to 5.0 mM HC differed from control by 0.5 to 6.0%. When HC was increased to 10 mM or greater, however, ^{51}Cr release rose significantly to 44% above control. Wells treated for 18 h with 5 mM HC contained 168,000±16,400 cells/well, while control wells contained 176,000±15,400 cells/well (SE, $n = 3$). Endothelial cells exposed to concentrations of HC ranging from 2.5 to 7.5 mM, exhibited a spindle-shaped, whorled pattern compared with the typical cobblestone appearance of control cell monolayers (Fig. 1).

Endothelial cells, preincubated for 18 h with HC, showed no significant impairment of ^{125}I-Lys-PLG binding (Fig. 2 *A*). The same cells, however, exhibited a dose-related reduction in binding of ^{125}I-t-PA. The maximal effect on t-PA binding was observed at 1–7.5 mM HC and represented a 50–65% decrease in total specific binding. Treatment of HC-exposed cells with a polyclonal antireceptor antibody which blocks 65–70% of t-PA binding to control cells (16) resulted in further reduction of residual specific ^{125}I-t-PA binding to ~ 2% of the untreated control. These results suggested that a single class of immunologically related receptors promoted binding of t-PA to the cell surface. Nonspecific binding, defined as that observed in the presence of a 50-fold excess of unlabeled t-PA, was not significantly changed for any given input dose of HC.

Addition of catalase, superoxide dismutase, or both at the time of treatment with HC, had no effect on the observed decrement in specific t-PA binding, ruling out toxicity related to generation of oxidizing species (Fig. 2 *B*) (27). Pretreatment of t-PA with HC (1 mM, 1 h) did not impair its binding to endothelial cells. When the purified receptor protein (14) was applied to polystyrene wells and then treated with HC (1 mM, 37°C, 1 h), furthermore, there was no change observed in binding of either ^{125}I-t-PA (50 nM) or ^{125}I-Lys-PLG (50 nM) (145

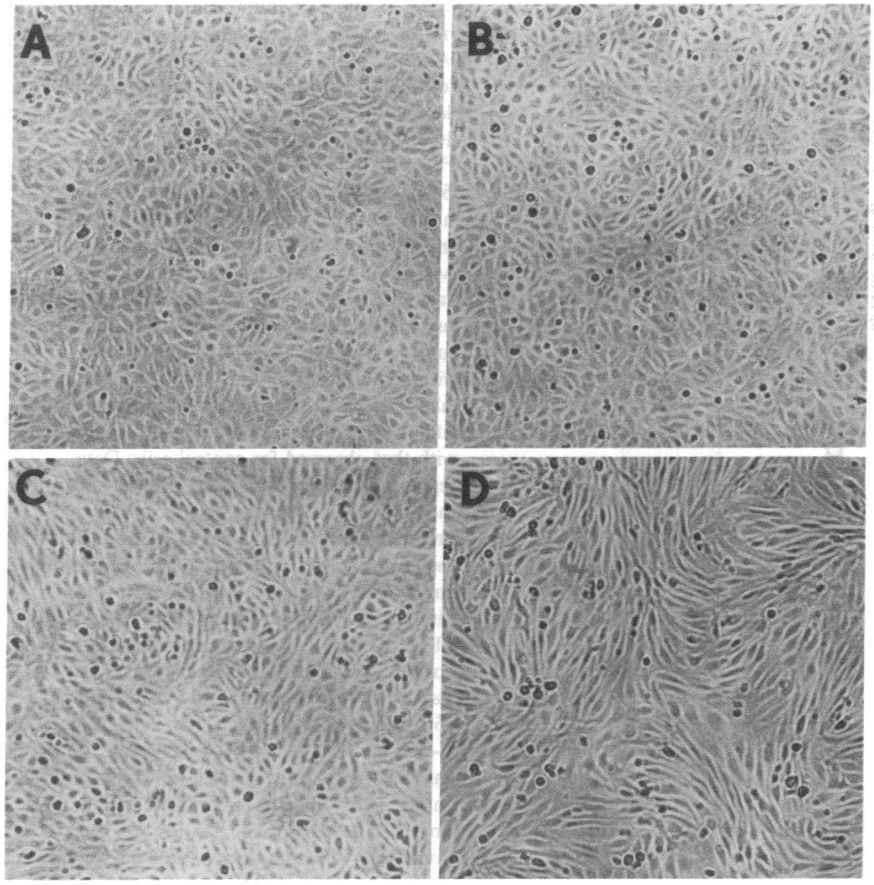

Figure 1. Effect of DL-homocysteine on endothelial cell morphology. Confluent EC, propagated as described under Methods, were preincubated for 18 h with HC at concentrations of (*A*) 0, (*B*) 2.5, (*C*) 5.0, or (*D*) 10.0 mM, and then photographed. ×150.

vs 147 fmol per well for t-PA and 39 vs 38 fmol per well for plasminogen). These data indicated that direct exposure of either receptor or ligand to HC did not impair its ability to engage in binding.

Inhibition of ^{125}I-t-PA binding was maximal after preincubations of 20–48 h (Fig. 2 *C*). Recovery of ^{125}I-t-PA binding capacity to within 15% of untreated cells was observed at 72–96 h, possibly reflecting expression of newly synthesized receptors. The observation of a 20-h lag period preceding the maximal HC effect suggested that one or more processing events might be required before an effect on t-PA binding could be appreciated at the cell surface. Among cells pretreated with HC analogues (L-homocystine, L-cysteine, L-cystine, L-methionine, or glutathione), binding of ^{125}I-t-PA did not differ by > 15% when compared with that of untreated cells (Fig. 2 *D*). This result suggested that modulation of ^{125}I-t-PA binding was specific to homocysteine, and not simply a consequence of excess reducing potential in the extracellular environment. In addition, the effect of HC on ^{125}I-t-PA binding was completely abrogated by coincubation of cells with excess L-cysteine, a competitive inhibitor of HC uptake by endothelial cells (Fig. 2 *E*) (28). Half-maximal restoration of ^{125}I-t-PA binding to HC-treated cells (5 mM) was observed upon coincubation with roughly equimolar doses of L-cysteine (~ 4.8 mM). Thus, cellular uptake of HC appeared to be necessary for modulation of ^{125}I-t-PA binding.

Previous studies have identified two discrete, saturable binding sites for t-PA associated with cultured EC monolayers (15, 29). These two classes can be resolved by Scatchard analysis of binding isotherms (15). A high affinity site (K_d 29 pM, maximal binding capacity [B_{max}] 3,700 sites per cell), representing the physiologic t-PA inhibitor, PAI-1, is located primarily in extracellular matrix (29, 30). A lower affinity site (K_d 18 nM, B_{max} 815,000 sites per cell), representing an M_r 40,000 membrane protein, preserves catalytic activity (14–16). To determine which binding site(s) might be targeted by HC, isotherms depicting the dose-dependent interaction of a range of concentrations (0.05–40 nM) of ^{125}I-t-PA with HC-treated or untreated confluent EC monolayers were developed (Fig. 3 *A*). At 25–40 nM input doses of ^{125}I-t-PA, cells pretreated with HC displayed significantly dampened binding capacity for the labeled ligand. Scatchard transformation of these data (Fig. 3 *A, inset*) yielded biphasic plots which differed only in their lower affinity components. While HC treatment had no significant effect upon the ligand affinity for either class of t-PA binding site, it was associated with a 65% decrease in B_{max} for the membrane site, and a nonsignificant increase in capacity for the matrix-associated site (Table I). On the other hand, neither K_d nor B_{max} values for the major ^{125}I-Lys-PLG binding site on control and HC-treated EC differed significantly (167±9 vs 156±5 nM and 752,000±39,000 vs 710,000±20,500 sites per cell, respectively [SE, n = 3]). Furthermore, both K_d and B_{max} values for binding of a monoclonal ^{125}I-antireceptor IgG to control and HC-treated EC were also essentially identical (365±5 vs 376±6 nM and 594,000±8,000 vs 588,000±14,000 sites per cell, respectively [SE, n = 3]). Similarly, EC reactivity to IgG directed against angiotensin-converting enzyme, an integral membrane protein, was not affected by HC. These data indicate that the effect of HC on ligand binding to the t-PA/

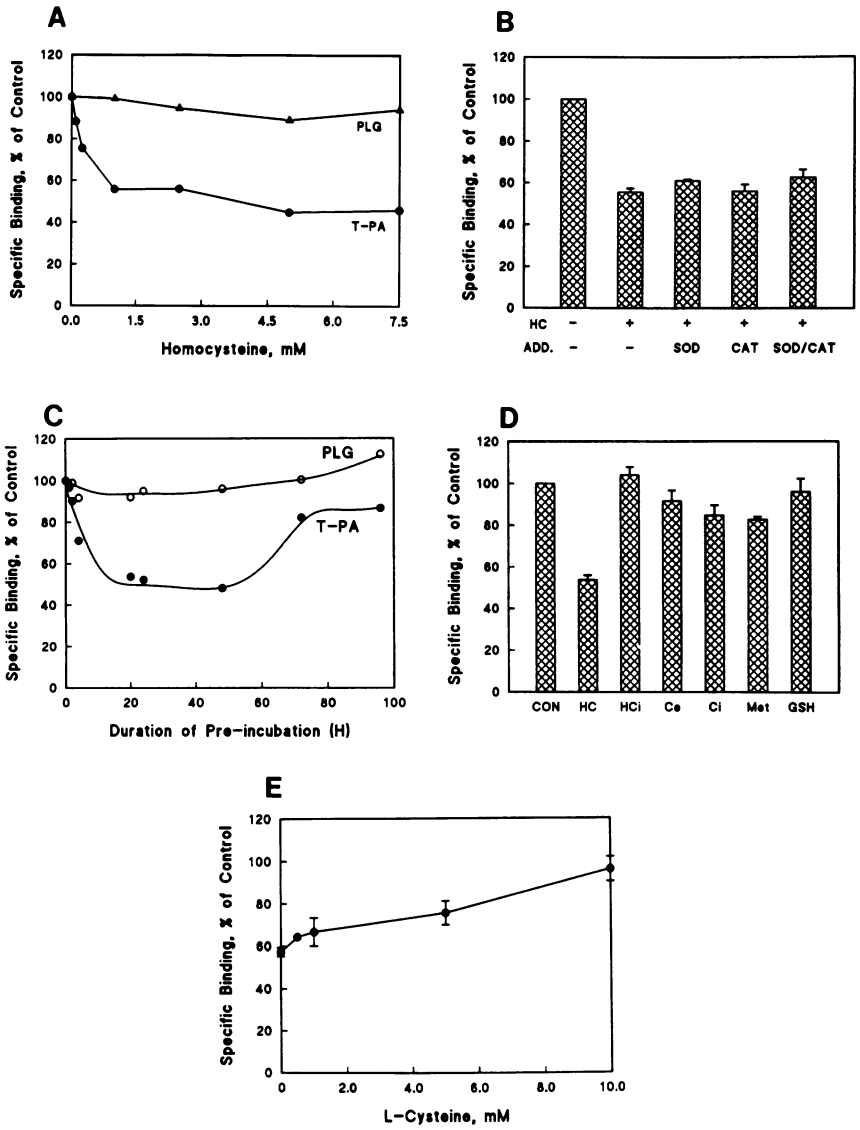

Figure 2. Effect of endothelial cell treatment with DL-homocysteine on binding of ^{125}I-t-PA and ^{125}I-Lys-PLG. (*A*) Dose-response. Confluent EC were preincubated with complete culture medium supplemented with HC (0.1–7.5 mM, 18 h). Specific binding of ^{125}I-t-PA (47 nM) (15) and ^{125}I-Lys-PLG (50 nM) (20) was defined as that inhibited in the presence of 50-fold molar excess of unlabeled ligand. Each point represents the average of triplicate determinations. (*B*) Lack of EC protection by superoxide dismutase and catalase. Confluent EC were preincubated with HC (5 mM, 18 h) with or without additions of SOD (200 μg/well), catalase (CAT, 200 μg/ml), or both, and specific binding of ^{125}I-t-PA (55 nM) compared with that observed for control cells. Data represent mean±SE for four separate experiments. (*C*) Time course. Confluent EC were preincubated with HC (5 mM) or control medium for 3–96 h. Data represent mean values of triplicate determinations for specific binding of ^{125}I-t-PA (25 nM) and ^{125}I-Lys-PLG (50 nM) expressed as a percentage of control. (*D*) Effect of HC analogues. Confluent EC were preincubated (18 h) with HC (5 mM), L-homocystine (HCi, 2.5 mM), L-cysteine (Ce, 5 mM), L-cystine (Ci, 2.5 mM), L-methionine (Met, 5 mM), or glutathione (GSH, 5 mM) in complete medium, and specific binding of ^{125}I-t-PA (55 nM) compared with that of cells pretreated with normal medium (CON). L-homocystine and L-cystine were tested at 2.5 mM due to their limited solubility. Data represent mean±SE of three separate experiments. (*E*) Preservation of ^{125}I-t-PA binding by coincubation with L-cysteine. Confluent EC were preincubated with control or HC-containing medium (5 mM) supplemented with L-cysteine (0–10 mM). Specific binding of ^{125}I-t-PA (50 nM) was determined for duplicate samples.

plasminogen receptor selectively involves its t-PA binding domain.

The ligand binding capacity of the M_r 40,000 receptor, extracted from membrane preparations from control cells and cells pretreated with HC, was also evaluated by ligand blotting (Fig. 3 *B*). Although the M_r 40,000 receptor from normal cells bound t-PA efficiently, as previously reported (14, 16), the same protein from HC-treated cells showed a 65% loss of receptor interaction with t-PA by image analysis of ligand blots. Urokinase, a negative control (14), did not bind to the M_r 40,000 protein in either case. Binding of both Lys-PLG and apo(a) to the t-PA/plasminogen receptor remained intact upon pretreatment of endothelial cells with HC. These data demonstrate that t-PA and plasminogen interact with EC through disparate mechanisms, only the former of which is sensitive to HC.

Active site blockade of t-PA with diisopropylfluorophosphate had no effect on the observed decrease in specific binding to EC (Fig. 3 *C*). This result provided further evidence that HC targeted a binding site other than PAI-1, a protein which lacks cysteine residues (31). Interestingly, a previous preliminary report suggests that HC has no effect on PAI-1 secretion while significantly hindering cellular processing of the integral membrane protein, thrombomodulin (32).

Our previous studies have demonstrated that the endothelial cell membrane receptor for t-PA preserves its catalytic activity (14–16), whereas PAI-1 rapidly inactivates this protease (31, 32). To further distinguish between PAI-1 and the EC membrane receptor, HC-treated endothelial cell monolayers were tested for the ability to support t-PA activity. Upon exposure of t-PA to HC-treated EC, hydrolysis of the fluorogenic t-PA substrate AFC-56 was reduced to 39% of that associated with control cells (0.00443±0.00127 vs 0.01140±0.00114 relative fluorescence units/min, mean±SE, n = 5). This finding reflected a significant loss of t-PA catalytic activity at the surface of HC-treated cells. This result could not be explained by enhanced binding of t-PA to PAI-1 since binding of active site blocked t-PA was unchanged by HC (Fig. 3 *C*). The observed decrease in cell associated plasminogen activating activity, thus, further localized the HC effect to the membrane-associated t-PA binding site.

Finally, purified receptor protein was treated with carboxy-

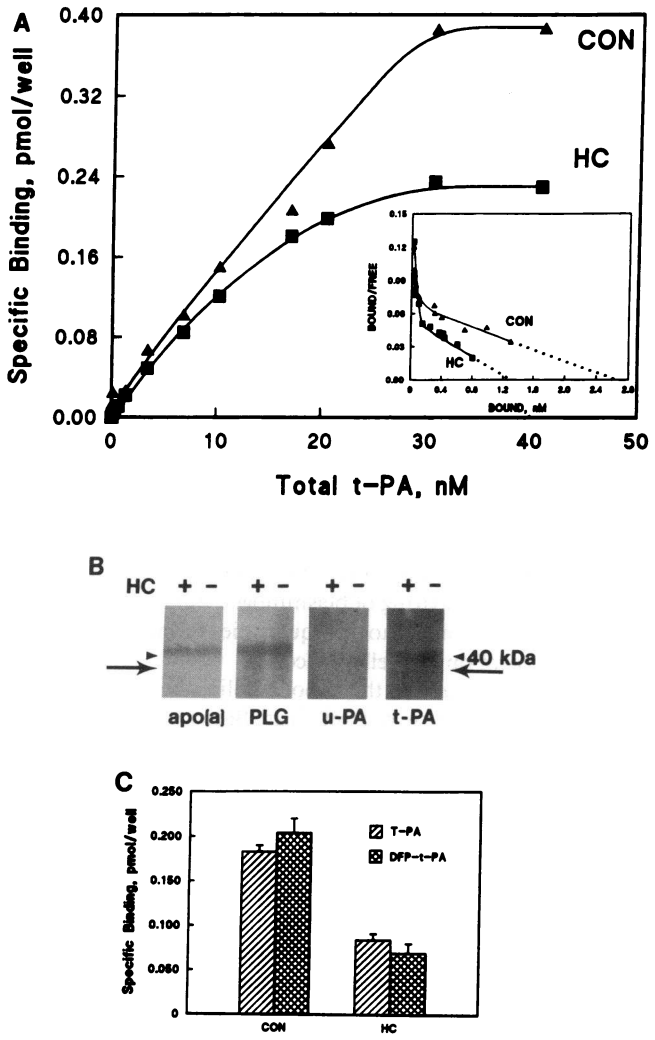

Figure 3. Effect of DL-homocysteine on ^{125}I-t-PA binding to its endothelial cell membrane receptor. (*A*) Binding isotherms. Confluent EC, preincubated with HC (5 mM, 18 h) or complete medium, were washed and exposed to a range of concentrations of ^{125}I-t-PA (sp act 433,000 cpm/pmol). Specific binding was quantified as described (14). (*Inset*) Scatchard analysis. Data depicted in *A* were linearly transformed using the Ligand program (39). (*B*) Ligand blot. Detergent extracts of plasma membrane fractions from EC treated with or without HC (1 mM, 18 h) were prepared, resolved by SDS-PAGE, and blotted with t-PA (10 μg/ml), u-PA (10 μg/ml), Lys-PLG (1.0 μg/ml), or apolipoprotein(a) (1.0 μg/ml) as previously described (14, 16). The arrows indicate the position of a 39-kD molecular mass marker. (*C*) Active-site blocked ^{125}I-t-PA. t-PA (147 μM) was treated twice with diisopropylfluorophosphate (DFP, 250 mM) at 24-h intervals (4°C), radiolabeled, and evaluated at an input dose of 15 nM for binding to control and HC-treated EC. Efficiency of active site blockade (99.2%) was estimated in a fluorogenic assay of plasmin generation (14).

peptidase B to remove COOH-terminal lysine residues (Fig. 4). Specific binding of ^{125}I-Lys-PLG to carboxypeptidase B-modified protein was reduced in a dose-dependent fashion, and was completely eliminated at 250 U/ml (Fig. 4 *A*). ^{125}I-t-PA binding, on the other hand was only minimally affected by digestion of receptor with carboxypeptidase B, as only a 14% reduction in binding was observed at 250 U/ml of CPB (Fig. 4 *B*). These data indicated that while receptor binding of plasminogen is COOH-terminal lysine dependent, t-PA binding uses a different mechanism consistent with its selective sensitivity to HC.

Table I. Selective Effect of Homocysteine on the Endothelial Cell Membrane Receptor for t-PA

Site	Condition	K_d	B_{max}
		nM	*sites/cell*
Matrix	Control	0.037±.005	8,800±3,300
(PAI-1)	HC	0.078±.042	15,800±8,400
Membrane	Control	12±4	621,000±165,000
(40 kD)	HC	7±3	217,000±65,000

Binding affinity (K_d) and capacity (B_{max}) were estimated by Scatchard analysis of isotherm pairs depicting binding of ^{125}I-t-PA (0.05–40 nM) to control and HC-treated (5 mM, 18 h) endothelial cells. Values for high affinity (matrix – PAI-1) and low affinity (membrane – 40 kD) binding sites represent means±SE for three separate experiments.

Discussion

The present study demonstrates for the first time that t-PA binding to cultured endothelial cells can be modulated by homocysteine uptake in a dose- and time-dependent fashion. The maximal effect on t-PA binding was achieved at HC concentrations of 1 mM or greater and after a 20–48-h preincubation (Fig. 2, *A* and *C*). This effect was not related to generation of oxidizing species such as superoxide radicals or hydrogen peroxide (Fig. 2 *B*), and was relatively specific for HC since related analogues such as cysteine, methionine, glutathione, homocystine, or cystine did not affect binding by > 15% (Fig. 2 *D*). That cellular entry of HC was involved in modulation of t-PA binding is supported by the shape change observed in HC-treated endothelial cells suggesting cytoskeletal rearrangement (Fig. 1), the time delay of several hours needed to achieve the full HC effect (Fig. 2 *C*), blockade of the HC effect by a competitive inhibitor of its endothelial cell uptake (Fig. 2 *E*), and the lack of effect on binding when the purified receptor was treated with HC.

Several lines of evidence indicate that homocysteine targeted the lower affinity membrane receptor for t-PA rather than its high affinity, matrix-associated binding site (PAI-1). Scatchard analyses revealed a selective reduction in membrane binding sites available to t-PA with no change in B_{max} for PAI-1 binding sites (Fig. 3 *A*; Table I). Ligand blots of EC membrane extracts revealed 65% impairment of t-PA binding to the M_r 40,000 receptor (Fig. 3 *B*). In addition, HC treatment of EC was associated with a commensurate loss of t-PA catalytic activity at the cell surface. Finally, covalent blockade of the active site of t-PA did not alter the susceptibility of binding to HC, indicating that HC affected a binding compartment which was active site independent (Fig. 3 *C*).

Fasting plasma levels of homocysteine in patients affected with homocystinuria range from 0.03 to 0.24 mM (33–35). In the present report, as in other published studies using normal EC (8–11), relatively high doses of HC (≥ 1 mM) were required to achieve a maximal effect on cell function. Indeed, EC from normal individuals are known to be relatively resistant to

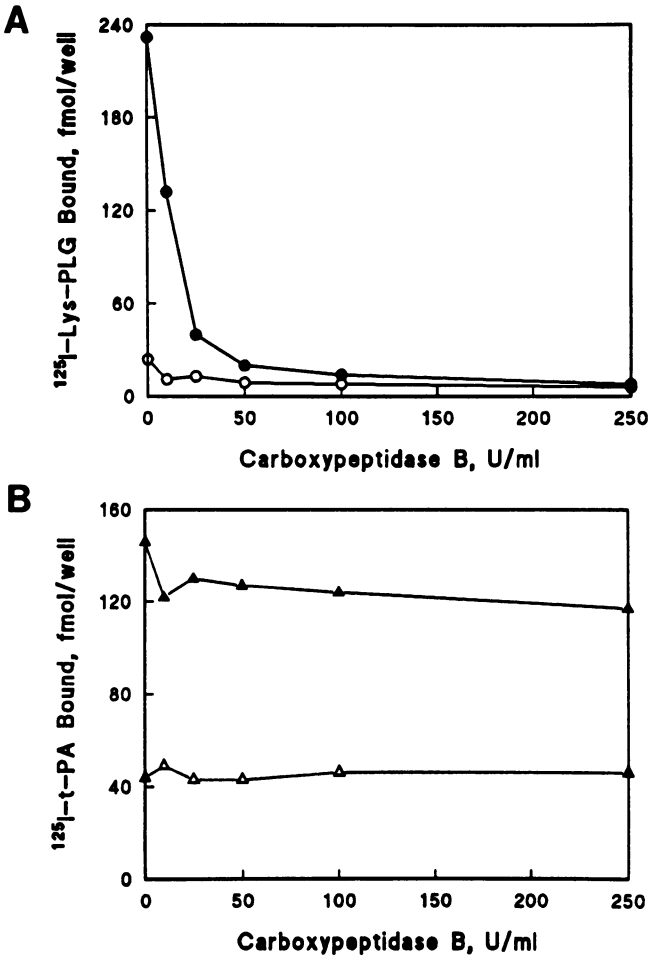

Figure 4. Effect of carboxypeptidase B on ^{125}I-Lys-PLG and ^{125}I-t-PA binding to purified receptor protein. Solid phase binding studies were carried out as previously described (14). Wells coated with purified receptor protein (10 μg/ml) were washed three times, treated with carboxypeptidase B (0–250 U/ml, 30 min, 37°C), and washed again three times. Radiolabeled ligands were added (120 min, 37°C), and bound and unbound radioactivity estimated (14). Total binding (*solid symbols*) and nonspecific binding (*open symbols*), defined as that remaining in the presence of a 50-fold molar excess of unlabeled ligand, were determined. (*A*) ^{125}I-Lys-PLG (150 nM; 186,000 cpm/pmol). (*B*) ^{125}I-t-PA (35 nM; 259,000 cpm/pmol).

the effects of HC when compared to EC from individuals heterozygous for homocystinuria (36). The high threshold noted for normal EC in vitro most likely reflects the need to transport externally presented HC to the intracellular space. In the normal cell, furthermore, the cystathionine β-synthase pathway, which catalyzes the condensation of HC and serine to form the thioether cystathionine, would need to be overcome. This may explain why, in our system, preincubation with HC resulted in a less than complete loss of t-PA binding and activity at the cell surface, and why we observed recovery of binding at 72–96 h. Recovery may also be due to inactivation of HC secondary to formation of mixed disulfides with serum proteins (5).

The mechanism by which HC selectively disables the t-PA binding domain of its membrane receptor is currently under investigation. Although EC are known to maintain homocysteine in the reduced state possibly due to high intracellular concentrations of glutathione (9), there is, at present, no clear evidence that HC becomes incorporated into cellular proteins during message translation (1). In recent in vitro studies, however, protein disulfide isomerase, an enzyme residing in the lumen of the endoplasmic reticulum has been shown to mediate disulfide-dependent protein folding (37). Cysteine-containing peptides act as strong competitive inhibitors of this activity (38). Interference with protein disulfide isomerase-mediated protein folding by HC might result in immunologically cross-reactive receptor protein that is functionally deficient. SDS gel electrophoresis experiments indicate that the M_r 40,000 membrane receptor for t-PA and plasminogen contains at least one intrachain disulfide bond (15). Thus, if interaction with t-PA requires a correctly folded binding domain, HC-induced disulfide mismatch might result in fewer functional binding sites for this ligand. Plasminogen binding, on the other hand, which seems to require only carboxy-terminal lysine residues (Fig. 4), would be resistant to disulfide rearrangement. This mechanism would explain the selective effect of HC on t-PA binding with sparing of plasminogen binding, and is supported by the finding of normal quantities of immunoreactive receptor protein on the cell surface.

The precise cause of thromboembolic disease in patients with homocystinuria is not yet clear. Based upon infusion of homocysteine in an animal model, Harker et al. suggested that vascular occlusion might arise secondary to a direct toxic effect on vascular endothelium with subsequent platelet consumption (33). Two later studies, however, found no evidence of altered platelet kinetics in homocystinuric patients and suggested that mechanisms other than platelet adhesion to the subendothelial surface might be operative clinically (34, 35). More recent in vitro studies have focused on homocysteine-associated alterations in the thromboregulatory properties of the endothelial cell which were not accompanied by cell lysis (8–11). Our results are most consistent with the latter hypothesis, but the extent to which endothelial cell dysfunction may contribute to vascular occlusion will await clinical study.

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