Unesterified Long Chain Fatty Acids Inhibit the Binding of Single Chain Urokinase to the Urokinase Receptor†

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ABSTRACT: The interaction of single chain urokinase with its receptor accelerates plasminogen activator activity on cell surfaces and induces intracellular signalling in several cell types. To date, no physiologic inhibitor of this binding has been identified. We report that the binding of scuPA to its cellular receptor is inhibited by long chain fatty acids such as oleic acid (C18,Δ9) at physiological plasma concentrations. Inhibition of single chain urokinase binding to human trophoblastic cells by long chain fatty acids was dose-dependent and saturable. Fifty percent of the binding was inhibited at an oleic acid concentration of 27 μM, while inhibition was maximal (75%) at 150 μM oleic acid. The inhibitory potency of oleic acid was unaffected by fatty acid free albumin or human plasma. Inhibition of single chain urokinase binding by free fatty acid analogues was critically dependent on chain length ($>C_{14}$ required for inhibition) and was proportional to the extent of unsaturation. Only the fraction of specific scuPA binding to trophoblasts that was dependent on uPAR was susceptible to inhibition by oleic acid, while binding of scuPA to vitronectin, thombospondin, and the α_2-macroglobulin receptor/low-density lipoprotein-related receptor was not. [^{3}H]Oleic acid bound specifically to recombinant soluble uPAR in a 1:1 molar ratio in the presence or absence of plasma and totally blocked its specific binding to a cell line expressing glycosyl phosphatidylinositol-linked single chain urokinase. These results indicate that oleic acid and other unsaturated long chain free fatty acids may serve as physiologic regulators of proteolytic events and intracellular signalling that depend upon the interaction of urokinase with its receptor.

Migration of cells through tissue barriers is a critical step in the formation of tumor metastases, organogenesis, neural development, and other physiologic and pathophysiologic processes (Dano et al., 1985). Migration into tissue is facilitated by enzymes such as plasmin which degrade noncollagenous portions of the basement membrane, activate pro-metalloproteases, and release growth factors from sites where they are normally sequestered (Naldini et al., 1992; Lyons et al., 1990). Plasmin is formed from plasminogen by limited proteolytic cleavage by plasminogen activators such as urokinase (uPA).[1] uPA binds with high affinity to a single 55 kDa glycoprotein which is attached to cell membranes by a glycosylphosphatidylinositol (GPI) anchor, while plasminogen binds to multiple low-affinity sites (Miles et al., 1989, 1991). Binding of single chain urokinase (scuPA) to its receptor (uPAR) promotes its enzymatic activity (Higazi et al., 1995), increases the local concentration of the reactants (Ellis et al., 1989, 1991), and may provide relative protection from plasma protease inhibitors (Ellis et al., 1990; Hall et al., 1991; Higazi et al., 1996). Therefore, regulation of the interaction between uPA and its receptor represents an important step in the control of diverse plasmin-mediated processes.

The factors that contain such plasmin-mediated processes have not been completely identified. The relative protection of cell-associated urokinase and plasmin from their plasma inhibitors makes the existence of additional substances plausible. Among the potential inhibitory substances in plasma are the free fatty acids which we have previously reported inhibit neutrophil migration (Higazi & Barghouti, 1994) and modulate urokinase-dependent plasminogen activator activity (Higazi et al., 1992, 1994). However, the capacity of these free fatty acids to modulate cell-associated PA binding is unknown. In the present study, we report that oleic acid (OA), the most abundant free fatty acid in normal plasma (Altman & Katz, 1977), and other long chain fatty acids inhibit the binding of scuPA to the urokinase receptor.

EXPERIMENTAL PROCEDURES

Materials. scuPA and ATF were the gifts of Drs. Andrew Mazar and Jack Henkin (Abbott Laboratories, Abbott Park,

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[1] Abbreviations: uPA, urokinase-type plasminogen activator; scuPA, single chain urokinase; uPAR, urokinase receptor; suPAR, recombinant soluble urokinase receptor; OA, oleic acid; Vn, vitronectin; TSP, thrombospondin; GPI, glycosylphosphatidylinositol; α_2MR/LRP, α_2-macroglobulin receptor/low-density lipoprotein-related receptor; rRAP, recombinant 39 kDa α_2-macroglobulin receptor-associated protein; PI-PLC, phosphatidylinositol-specific phospholipase C; DAF, decay-accelerating factor; PBS, phosphate-buffered saline (pH 7.4).

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IL) (Mazar et al., 1995). Recombinant soluble uPA receptor (suPAR) was prepared and isolated as described (Crowley et al., 1993; Mizukami et al., 1994). scuPA, ATF, and suPAR were radiolabeled with ^{125}I as previously described (Barnathan et al., 1990; Higazi et al., 1995). Monoclonal antibodies to the uPA receptor and to the catalytic subunit of two-chain urokinase as well as plasminogen were provided by American Diagnostica, Inc. (Greenwich, CT). Anti-thrombospondin antibody was the kind gift of Dr. Jack Lawler (Adams & Lawler, 1994). Unlabeled free fatty acids and [^{3}H]oleic acid were obtained from Sigma and Amersham, respectively. Rabbit polyclonal antibody to urea-purified vitronectin and rabbit polyclonal antibody to fibronectin were obtained from Calbiochem (La Jolla, CA) and Becton Dickinson Labware (Bedford, MA), respectively. Phosphatidylinositol-specific phospholipase C (PI-PLC) was from Boehringer Mannheim. Radioimmunoprecipitation experiments described below were performed in a buffer (RIPA) composed of 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.5% deoxycholate sodium salt, 0.2% SDS, 100 mM NaCl, and 1.0 mM EDTA. PD-10 columns (Sephadex-G-25 prepacked) were obtained from Pharmacia. Stock solutions of fatty acids (24 mM) in ethanol were diluted in PBS as described (Higazi et al., 1992, 1994). Controls with the vehicle alone indicated that the diluted ethanol had no effect on binding.

Cells. Cultures of human umbilical vein endothelial cells (HUVECs) were prepared and characterized as previously described (Cines et al., 1984). LM-TK$^-$ fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). First trimester human placental trophoblastic cells were obtained from transabdominal chorionic villous samplings, maintained in culture and characterized as previously described (Diss et al., 1992; Kniss et al., 1994). By the fifth passage, a uniform population of epithelioid cells was obtained which express an extensive number of microvilli on their apical surface characteristic of syncytiotrophoblasts, synthesize both estradiol and progesterone, and stain positive for both α and β human choriogonadotrophic hormone and cytokeratin-peptide 8 but not for vimentin and von Willebrand factor.

Construction of a CHO Cell Line Expressing GPI-Anchored scuPA. A plasmid was constructed encoding GPI-anchored urokinase (Lee et al., 1992) under the control of cytomegalovirus immediate early promoter−enhancer sequences (Invitrogen catalog). Specifically, a 1.5 kb *Hin*dIII fragment containing 95 bp of 5′-untranslated DNA, the entire scuPA coding sequence, and 82 bp of 3′-untranslated DNA was excised from pULSCUPA (Nelles et al., 1987) and ligated into *Hin*dIII-digested pcDNA2 (Boshart et al., 1985). The resultant plasmid was digested with *Bam*HI, removing a 143 bp fragment containing 43 bp of 3′-scuPA coding sequence, 82 bp of 3′-untranslated sequence from pULSCUPA, and 18 bp of polylinker sequences of pcDNA3. This 143 bp *Bam*HI fragment was replaced with a 174 bp *Bam*HI fragment from pLUK+ASN (Lee et al., 1992), regenerating the 3′-scuPA coding sequence, replacing the scuPA stop codon with the carboxy terminal 38 codons of decay-accelerating factor (DAF), and regenerating the 3′-untranslated sequences from pULSCUPA. This plasmid, encoding for GPI-anchored scuPA, was termed pSCUPA/GPI.c. The nucleotide sequence and proper orientation of the 174 bp *Bam*HI fragment in pSCUPA/GPI.c were confirmed by dideoxy-sequence analysis (Sequenase, U.S. Biochemical Corp.). The synthesis of enzymatically active scuPA, attached to the surface of eukaryotic cells by a carboxy terminal GPI anchor, has been established previously (Lee et al., 1992, 1994).

A stably transfected cell line was then established using calcium phosphate precipitation (Chen & Okayama, 1987). Briefly, CHO cells were transfected during their exponential growth phase with purified (Qiagen, Chatsworth, CA) pSCUPA/GPI.c. Transfected cells were incubated for 24 h at 37 °C in an atmosphere containing 2% CO_2, washed, and incubated for another 36 h in complete Iscove's modified Dulbeco's medium containing penicillin−streptomycin, 200 mM L-glutamine, 50 mg/mL gentamicin, hypoxanthine, and thymidine, supplemented with 10% heat-inactivated fetal bovine serum at 37 °C under humidified conditions in an atmosphere containing 5% CO_2. The cells were split 1:10 and selected for G418 (400 μg/mL) resistance the following day. Resistant colonies were isolated and maintained in the same complete medium containing 250 μg/mL G418 after an additional 3 week incubation. Medium from the clone was assayed for scuPA expression determined by ELISA (American Diagnostica, Inc.). Expression of scuPA on the cell surface was determined by measuring the specific binding of [^{125}I]suPAR (14 pg/10^6 cells). Control CHO cells bound no labeled suPAR. Preincubation of the cell line with 1 u/mL PI-PLC for 1 h at 37 °C inhibited the specific binding of [^{125}I]suPAR >80%, confirming GPI linkage of the cell surface-associated scuPA.

Binding Experiments. Cells were grown to confluency in 48-well Falcon multiwell culture dishes (Becton Dickinson, Lincoln Park, NJ) (approximately 5×10^4 cells/well). The cells were prechilled to 10 °C for 10 min and washed twice in phosphate-buffered saline (PBS, pH 7.4). Each ^{125}I-labeled ligand was added at the indicated concentrations for 90 min at 10 °C in the presence or absence of a 50-fold molar excess of the respective unlabeled ligand or in the presence of the indicated concentrations of oleic acid or other free fatty acids. Unbound ligand was removed by washing the cells four times with prechilled PBS. Cell-associated ligand was released by adding glycine buffer (pH 3) for 7 min. Nonspecific binding was defined as the cell-associated radioactivity not inhibited by excess unlabeled ligand. Specific binding was defined as the difference between total and nonspecific binding. Specific binding reached a plateau by 90 min under these conditions. The inhibitory effect of each free fatty acid was defined as the decrease in specific binding.

In some experiments, [^{125}I]scuPA (1 nM) was added to PBS containing 1% fatty acid free albumin (Sigma) or pooled citrated plasma (0.38% final concentration) collected from 32 healthy volunteers instead of PBS, and the specific binding to trophoblasts was determined in the presence and absence of added OA (150 μM) as described above. Another series of experiments was performed to determine whether the inhibition of scuPA binding by long chain fatty acids described below was due to an effect on scuPA itself, on its receptor, or on both molecules. To determine if OA directly inhibits the capacity of scuPA to bind to uPAR, [^{125}I]scuPA (200 nM) was preincubated with or without OA (200 μM) for 15 min. The samples were diluted 200-fold to give a final concentration of OA and ligand of 1 μM and 1 nM, respectively, and the specific binding to trophoblasts was

measured as above. To determine if OA directly inhibits the capacity of the uPA receptor to bind scuPA, trophoblasts were preincubated with or without OA (150 μM) for 15 min, washed four times with PBS to remove unbound OA, and the specific binding of [^{125}I]scuPA was determined. To determine whether the effect of OA was on suPAR or on any other component of the cell membrane, 200 nM [^{125}I]-suPAR was preincubated with 200 μM OA as above, the complex was diluted 1:200, and the specific binding to CHO cells expressing GPI-anchored scuPA was determined.

In other experiments, cells were preincubated for 1 h at 4 °C (LM-TK$^-$ cells) or at 10 °C (trophoblasts) with anti-vitronectin (Vn) antibody (1:10 dilution), anti-fibronectin antibody (20 μg/mL), anti-thrombospondin (20 μg/mL), with normal rabbit IgG, with 200 nM 39 kDa α_2-macroglobulin receptor/low-density related protein receptor (α_2MR/LRP)-associated protein (rRAP), or with buffer. The preincubation solutions were removed, and [^{125}I]scuPA was added in the presence of the same concentrations of inhibitors. The experiments involving rRAP were performed in PBS/1% bovine serum albumin supplemented with 2 mM $CaCl_2$. Binding was measured as described above. In other experiments, HUVECs were preincubated with 1.0 unit/mL PI-PLC for 60 min at 37 °C and/or with a monoclonal anti-uPAR antibody prior to addition of [^{125}I]scuPA.

Immunoprecipitation and Gel Filtration. scuPA or suPAR (100 nM each) was incubated with 50 μM [^{3}H]OA in the presence or absence of a 6-fold molar excess of unlabeled OA in RIPA buffer (final reaction volume of 30 μL) containing the specific antibody or normal rabbit serum as a control. In other experiments, 100 μM [^{3}H]OA was added to plasma and 1.4 nM suPAR was added for 10 min prior to immunoprecipitation. The amount of anti-urokinase, anti-urokinase receptor, and anti-plasminogen antibody needed to immunoprecipitate equal amounts of each protein and to provide optimal specificity compared with control IgG was determined in preliminary experiments using ^{125}I-labeled proteins. The mixture was incubated for 18 h at 4 °C with constant agitation. Staph protein A agarose (GIBCO) (30 μL) was added for an additional 4 h under the same conditions, after which 750 μL of RIPA buffer was added and the mixture was centrifuged at 5000 rpm for 5 min. The precipitate was washed four times in 750 μL of RIPA buffer, and the radioactivity in the final pellet was measured. In other experiments, scuPA or suPAR (200 nM) was incubated for 15 min with [^{3}H]OA (50 μM) in the presence and absence of a 6-fold molar excess of unlabeled OA. The sample was loaded on PD-10 columns, and the OD_{280} and the radioactivity in the various fall-through fractions were measured. In other experiments, 0.5 nmol of suPAR was incubated with 10 nmol of [^{3}H]OA (specific activity of 6.2×10^4 cpm/nmol) in a total volume of 200 μL of PBS for 15 min in the presence or absence of a 6-fold molar excess of unlabeled OA. The sample was loaded onto a G-25 column (Pharmacia), and the OD_{280} and the radioactivity in each fraction were determined. To determine whether OA might cause the suPAR to self-associate, suPAR (2.5 μM) was incubated in the absence and presence of 600 μM OA. The sample was loaded onto a G-75 column (Pharmacia), and the OD_{280} in each fraction was determined.

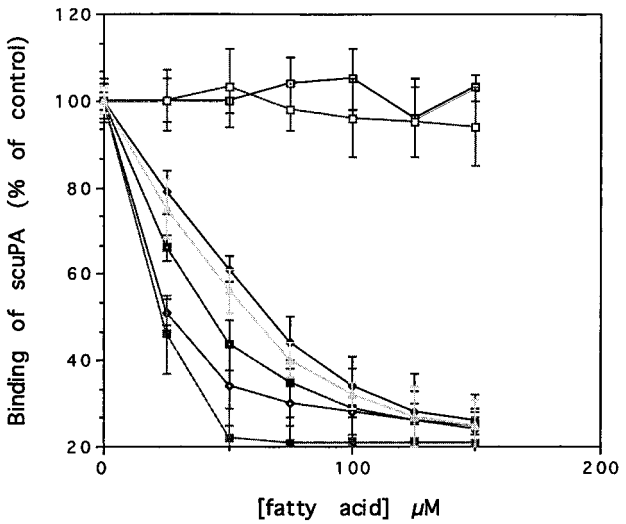

FIGURE 1: Effect of free fatty acids on the binding of scuPA to human trophoblasts. ^{125}I-labeled scuPA (1.5 nM) was incubated with a human trophoblastic cell line in the presence or absence of a 50-fold molar excess of unlabeled scuPA or with the indicated concentrations of various free fatty acids for 90 min at 10 °C, and the specific binding was determined. The data in this and all subsequent figures are expressed as the percent of specific binding in the absence and presence of increasing concentrations of free fatty acid. Specific binding in the absence of free fatty acids was 2.1 pM/10^6 cells. The mean ± SD of three experiments is shown. The inhibitory effect of each free fatty acid was defined as the decrease in specific binding: octanoic acid (⊡), myristic acid (□), palmitic acid (♦), stearic acid (▲), oleic acid (solid square with dot in center), linoleic acid (◊), and linolenic acid (■).

RESULTS

Binding of [^{125}I]scuPA to a trophoblast cell line was inhibited by *cis*-Δ^9-oleic acid (C_{18}) (OA) in a dose-dependent, saturable manner (Figure 1).

Binding of scuPA was inhibited 50% at an OA concentration of 27 μM and 75 ± 4% at an OA concentration of 150 μM. However, higher concentrations of OA had no additional effect. These concentrations of OA are both within the range of OA found in normal human serum (Altman & Katz, 1977). Indeed, almost identical results (75 ± 8% inhibition, mean ± SD, $n = 3$) were obtained when the binding of [^{125}I]scuPA was measured in the presence of OA (150 μM) added to a buffer containing 1% fatty acid free albumin or to undiluted normal plasma.

The inhibitory effect of free fatty acids on scuPA binding was not limited to OA, which has 18 carbons and one double bond in the cis configuration between C_9 and C_{10}. Other long chain fatty acids were able to inhibit scuPA binding as well, albeit with variable potency (Figure 1). To identify some of the important structural features in this process, we examined the inhibitory capacity of several analogues of OA. We first examined the contribution of the double bond. Stearic acid, which has the same chain length as OA (C_{18}) but which lacks the double bond, was a less potent inhibitor of scuPA binding (50% inhibition at a concentration of 42 μM). In contrast, the inhibitory effect was enhanced by increasing the extent of unsaturation through the addition of double bonds in the cis configuration (Figure 1). For example, the 2- and 3-*cis*-OA analogues linoleic acid (*cis,cis*-Δ^9,Δ^{12}-octadecatrienoic acid) and linolenic acid (*all-cis*-$\Delta^9,\Delta^{12},\Delta^{15}$-octadecatrienoic acid) inhibited scuPA binding more efficiently than OA. Moreover, linolenic acid inhibited

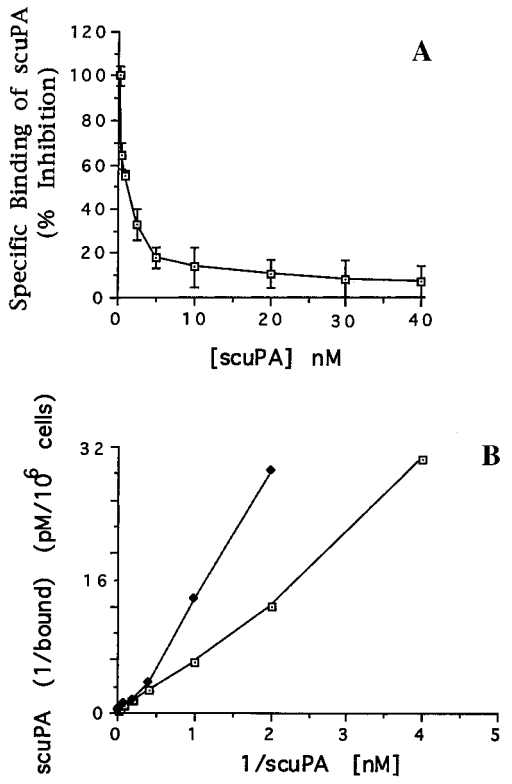

FIGURE 2: scuPA and oleic acid compete for binding to trophoblasts. (A) A trophoblastic cell line was incubated with 27 μM OA and the indicated concentrations of [^{125}I]scuPA for 90 min at 10 °C, and the cell-associated radioactivity was determined. The data are expressed as percent inhibition of specific binding in the absence of OA. The mean ± SD of three experiments is shown. (B) The data from one of the three competition experiments described in panel A was analyzed using a double reciprocal plot. Binding of [^{125}I]scuPA in the presence (♦) and absence (⊡) of 27 μM OA.

the binding of scuPA 50% at a concentration of 17.6 μM. We then examined the contribution of the remaining portion of the fatty acid molecule by comparing the inhibitory capacity of free fatty acids of different chain lengths (Figure 1). Palmitic acid (C_{16}) was almost comparable to stearic acid (50% inhibition at 45 μM), while myristic (C_{14}) and octanoic acid (C_8) had little or no inhibitory effect at any concentration tested.

The inhibitory effect of OA (27 μM) was overcome by increasing the concentration of scuPA, suggesting a competitive pattern of inhibition (Figure 2A). Competition was evident at physiologic concentrations of each reactant (1−2 nM scuPA and 27 μM OA). Of interest, 27 μM OA totally inhibited the binding of scuPA when its concentration was lowered to 0.25 nM (Figure 2A). Analysis of this data using a reciprocal plot (Figure 2B) indicated that the binding of scuPA is nonlinear in the presence and absence of OA; the nonlinearity is more apparent in the presence of OA. One explanation for this finding is that scuPA binds to more than one class of receptors on the trophoblasts, the one with the highest affinity being more sensitive to inhibition by long chain fatty acids than the other (Figure 2B). This explanation would also be in accord with the total inhibition of scuPA binding at low concentrations seen in Figure 2A and the plateau of inhibition seen in Figure 1. To clarify the nature of the putative "fatty acid-sensitive" and "fatty acid-resistant" sites, we examined the binding of scuPA to LM-TK$^-$ fibroblasts, which we have previously reported bind scuPA although they lack GPI-anchored urokinase receptors (Li et al., 1994). Binding of scuPA to LM-TK$^-$ cells was inhibited by a combination of rRAP (which inhibits binding of scuPA to α_2MR/LRP) and antibodies to vitronectin and thrombospondin, while the cells were preincubated with an anti-fibronectin antibody (Figure 3A) or with PI-PLC, which releases GPI-anchored uPAR from the cell surface with no effect (not shown). Thus, scuPA binds to specific sites on LM-TK$^-$ cells other than uPAR. As seen in Figure 3A, specific binding of scuPA to LM-TK$^-$ cells was totally resistant to OA.

Taken together, the data in Figures 1, 2B, and 3A suggest that long chain fatty acids inhibit the high-affinity interaction of scuPA with uPAR expressed on the trophoblast cell line but not the binding of scuPA to other lower-affinity sites that might be expressed on these cells. We tested this hypothesis in several ways. First, we examined the effect of OA on the binding of scuPA to HUVECs. Approximately 90% of scuPA binding was uPAR-dependent as it was inhibited by preincubating HUVECs with PI-PLC or with a monoclonal anti-uPAR antibody (not shown). As shown in Figure 3B, the binding of scuPA to HUVECs was almost totally inhibited by 150 μM OA. Second, we studied the binding of ^{125}I-labeled ATF to trophoblasts as an additional marker of binding to uPAR. OA inhibited the total binding of 1 nM [^{125}I]ATF by >80% (not shown). Third, we studied the effect of OA on the binding of ^{125}I-labeled soluble uPA receptor to a stably transformed CHO cell line expressing GPI-anchored scuPA (Figure 4). Again, OA inhibited suPAR binding to cell-associated scuPA >95%. Fourth, we characterized the fatty acid-resistant scuPA binding sites on the trophoblastic cells using the same antibodies described in Figure 3A. The results shown in Figure 5 demonstrate that trophoblastic cells indeed express fatty acid-resistant binding sites since part of the binding of scuPA can be inhibited by rRAP and by an antibody to vitronectin, while anti-TSP had no measurable effect in this cell type.

Fifth, we performed a series of experiments to identify the site of action of long chain fatty acids directly, i.e. to determine whether they inhibit the capacity of scuPA to bind to uPAR, the capacity of uPAR to bind scuPA, or both. First, we examined whether OA bound to both molecules. ^{3}H-labeled OA was incubated with scuPA or suPAR (or with human IgG as a non-kringle-containing protein control) in the presence and absence of a 6-fold molar excess of unlabeled OA, and the complexes were immunoprecipitated by the addition of antibodies to scuPA or to uPAR or with an irrelevant rabbit IgG. [^{3}H]OA bound specifically to scuPA and suPAR but not to IgG (Figure 6). More [^{3}H]OA bound to suPAR than to scuPA, raising the possibility that OA exerts its inhibitory effect primarily upon uPAR. Almost identical results were obtained when incorporation of labeled OA was examined by gel filtration (not shown). To quantify these results, suPAR was preincubated with a 60-fold molar excess of [^{3}H]OA or with [^{3}H]OA and excess unlabeled OA, the protein isolated by gel filtration, and the specific binding to suPAR determined. As shown in Figure 7, 76% of the total binding of [^{3}H]OA to suPAR was specific. Approximately 1 mol of [^{3}H]OA was incorporated specifically per mole of suPAR under these conditions. OA did not cause suPAR to self-associate. The migration of suPAR was unaffected by the presence of OA even when the latter was added at concentrations as high as 600 μM. Further, the peak containing [^{3}H]OA and suPAR was clearly distinguish-

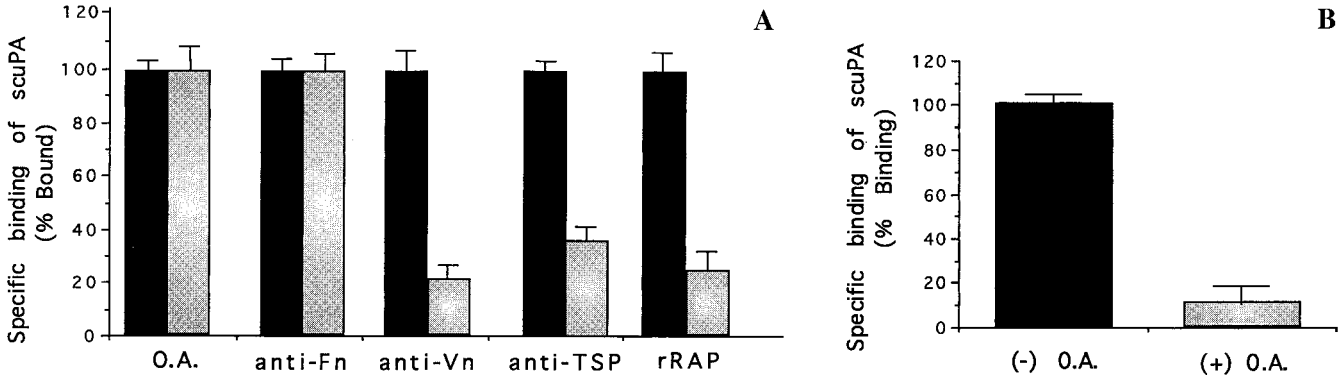

FIGURE 3: (A) Oleic acid does not inhibit the binding of scuPA to LM-TK$^-$ cells. The binding of [^{125}I]scuPA (2 nM) to LM-TK$^-$ cells was measured in the presence and absence of anti-vitronectin antibody (anti-Vn), anti-thrombospondin (TSP), anti-fibronectin (FN), recombinant rRAP (rRAP), or 200 μM OA. Data are expressed relative to the specific binding of scuPA in the absence of inhibitor. The mean ± SD of three experiments is shown. (B) Oleic acid inhibits the binding of scuPA to HUVEC. The binding of [^{125}I]scuPA (1 nM) to HUVEC was measured in the presence and absence of 150 μM OA. Data are expressed relative to the specific binding of scuPA in the absence of inhibitor. The mean ± SD of three experiments is shown.

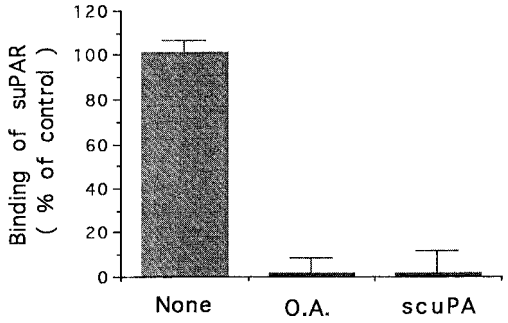

FIGURE 4: Oleic acid inhibits the binding of soluble uPA receptor to cell-associated scuPA. The binding of ^{125}I-soluble uPA receptor (1 nM) to a CHO cell line expressing GPI-anchored scuPA was measured in the presence and absence of a 50-fold molar excess of unlabeled scuPA or 150 μM OA. Data are expressed relative to the binding of labeled suPAR in the absence of inhibitor. The mean ± SD of three experiments is shown.

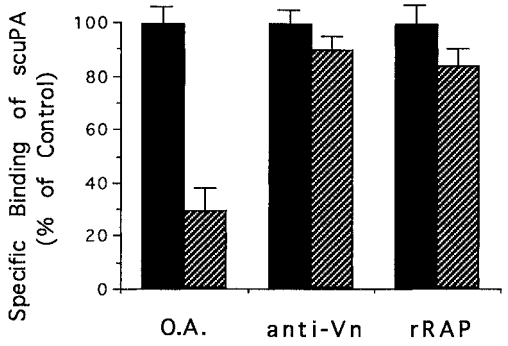

FIGURE 5: scuPA binds to several sites on trophoblasts. The binding of [^{125}I]scuPA (1.5 nM) to a trophoblast cell line was measured in the presence and absence of a 50-fold molar excess of unlabeled scuPA, 150 μM OA, anti-vitronectin antibody, or rRAP. Data are expressed relative to the specific binding of scuPA in the absence of inhibitor. The mean ± SD of three experiments is shown.

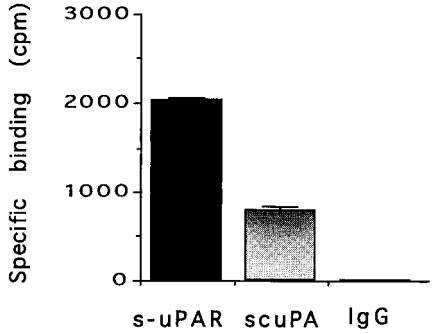

FIGURE 6: Immunoprecipitation of protein-bound [^{3}H]OA. Equal amounts of [^{3}H]OA were incubated with 100 nM of each of the following proteins, soluble uPA receptor (suPAR), scuPA, or normal rabbit IgG, in the presence and absence of a 6-fold molar excess of unlabeled OA and immunoprecipitated by specific antibody. The data shown represent the specific counts immunoprecipitated. The mean ± SD of two experiments performed in triplicate is shown.

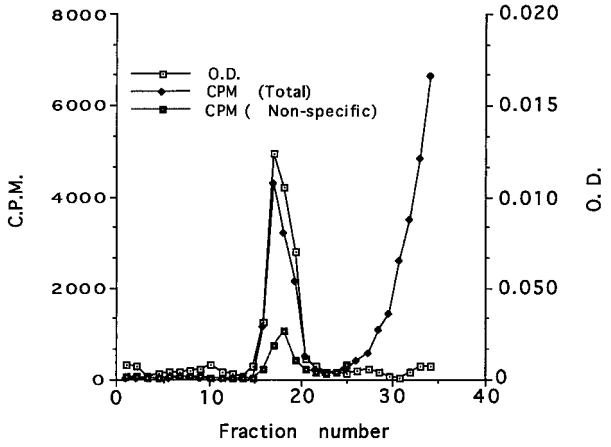

FIGURE 7: Specific binding of [^{3}H]OA to suPAR by gel filtration. suPAR (0.5 nmol) was incubated with [^{3}H]OA (30 nmol; specific activity of 2.2 × 10^4 cpm/nmol) for 15 min in the presence or absence of a 6-fold molar excess of unlabeled OA. The sample was loaded onto a G-25 column (Pharmacia), and the OD_{280} and the total radioactivity in each fraction were determined.

able from the migration of fibrinogen used as a marker of the void volumn. This result excludes the possibility of receptor aggregation by OA (not shown).

A second series of experiments was performed to determine whether the binding of OA to uPAR was responsible for the inhibition of scuPA binding. To examine the effect of OA on the uPA receptor itself, trophoblasts were preincubated with or without OA and washed to remove unbound OA, and the residual specific binding of scuPA was measured. In other experiments, [^{125}I]scuPA was preincubated with or without OA prior to measurement of its binding. As shown in Figure 8, preincubation of trophoblasts with OA inhibited the binding of [^{125}I]scuPA, while preincubation of [^{125}I]scuPA with OA did not, suggesting a direct effect on the receptor itself. To exclude the possibility that fatty acids inhibit urokinase binding indirectly through an

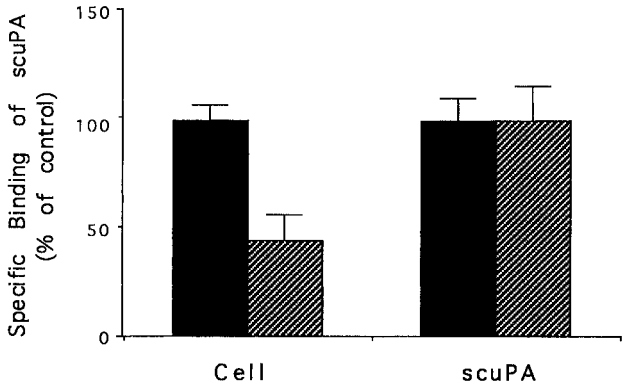

FIGURE 8: OA inhibits scuPA binding when preincubated with trophoblasts but not scuPA itself. Trophoblasts (Cell) were preincubated with or without OA (150 μM), and the specific binding of [^{125}I]scuPA was measured. The mean ± SD of two experiments performed in triplicate is shown. The data are expressed as the specific binding of scuPA to trophoblasts preincubated with OA (cross-hatched column) relative to the specific binding to cells not preincubated with OA (filled column). [^{125}I]scuPA (200 nM), with or without OA (200 μM), was preincubated for 15 min and diluted 200-fold, and the specific binding to trophoblasts was measured. The mean ± SD of three experiments performed in triplicate is shown. Data are expressed as binding of labeled scuPA preincubated with OA (cross-hatched column) relative to binding of scuPA not preincubated with OA (filled column).

effect on another membrane component, we preincubated [^{125}I]suPAR with 200 μM OA, and the binding to CHO cells expressing GPI-anchored scuPA was determined. Specific binding of suPAR was completely inhibited under these conditions (not shown).

Lastly, we performed a series of experiments to consider whether long chain fatty acids such as OA would retain their ability to bind to suPAR under more physiologic conditions. [^{3}H]OA (100 μM) was added to undiluted human plasma, 1.4 nM suPAR was added 10 min later and the receptor immunoprecipitated with specific antibody. Under these conditions, [^{3}H]OA was again incorporated into the suPAR in a 1:1 molar ratio. These results are in accord with those cited above indicating that OA retains its potency to inhibit scuPA binding to suPAR in the presence of plasma.

DISCUSSION

The results of this study indicate that OA, the most abundant free fatty acid in plasma, and other long chain fatty acids inhibit the binding of scuPA to cultured trophoplasts, endothelial cells, and soluble uPA receptor. The inhibitory effect of long chain fatty acids is dose-dependent and saturable, consistent with involvement of a specific binding site(s). Inhibition of scuPA binding was not a general feature of free fatty acids. For example, although other long chain fatty acids also inhibited scuPA binding, short and middle chain length fatty acids did not. Further, the inhibitory capacity of long chain fatty acids was proportional to the number of unsaturated bounds present ($C_{18} < C_{18:9} < C_{18:9,12} < C_{18:9,12,15}$), again indicating that specific structural features are required for a free fatty acid to inhibit scuPA binding. This specificity differs from the structural features previously reported to modulate plasmin activity (Higazi et al., 1994).

OA specifically inhibited the binding of scuPA to its high-affinity cellular receptor but not binding to lower-affinity sites such as α_2MR/LRP, vitronectin, or thrombospondin. Thus, OA had essentially no effect on the binding of scuPA to LM-TK$^-$ cells which do not express uPAR, partially inhibited binding to the trophoblastic cells which express uPAR as well as the other two proteins, and almost completely blocked binding to a HUVEC, a cell on which >90% of the binding of scuPA is sensitive to PI-PLC and can be blocked by anti-uPAR antibody. The difference in the sensitivity of the various scuPA binding sites to the effect of OA explains why inhibition reached a plateau (Figure 1) and the kinetics of binding and inhibition were nonlinear (Figure 2B) when trophoblasts were studied. In less complex systems, it was evident that OA only inhibits the binding of scuPA to uPAR and not binding to its lower-affinity sites (Figures 3−5). This observation, combined with the capacity of suPAR to incorporate [^{3}H]OA (Figure 6), makes it likely that OA inhibits scuPA binding through a direct effect on the receptor itself. This conclusion is supported by the fact that preincubation of trophoblasts with OA inhibited the binding of scuPA while preincubation of the scuPA with OA did not (Figure 8) and that preincubation of suPAR with OA totally blocked its specific binding to cells expressing GPI-anchored scuPA. Thus, the capacity of OA to inhibit scuPA binding to uPAR in plasma at physiologic concentrations raises a question as to whether the fatty acid concentration and composition of the blood modulates plasminogen activator activity and other uPA-dependent processes in vivo.

The concentrations of OA employed in these studies are within the range found in normal human serum (Altman & Katz, 1977). The fact that oleic acid added initially to plasma becomes incorporated into suPAR is consistent with the known physiologic role of albumin in the transport of free fatty acids to high-affinity binding sites on other proteins and supports the physiologic relevance of the observation that OA inhibits scuPA binding to its cellular receptor in a plasma environment. Therefore, the present studies suggest that urokinase may be displaced from its cellular receptor into the fluid phase at plasma concentrations of OA attainable in vivo, thereby promoting the inhibitory capacity of plasminogen activator type 1 (Ellis et al., 1990; Hall et al., 1991), preventing the stimulatory effect of uPAR on scuPA activity (Higazi et al., 1995) and thus limiting plasmin formation. Further, these results may be relevant both to the reported protective effect of diets rich in oleic acid in both human and animal studies of metastatic tumors (Rose et al., 1986) and in the well-described role of urokinase and its receptor in mediating the invasiveness of tumor cells (Dano et al., 1985). Therefore, plasma and pericellular concentrations of OA and related free fatty acids may have multiple, previously unappreciated effects on the fibrinolytic system and other urokinase and plasmin-mediated processes.

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