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# Angiopoietin-like protein 4/8 complex-mediated plasmin generation leads to cleavage of the complex and restoration of LPL activity

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**Triglyceride (TG) metabolism is highly regulated by angiopoietin-like protein (ANGPTL) family members [Y. Q. Chen *et al.*, *J. Lipid Res.* 61, 1203–1220 (2020)]. During feeding, ANGPTL8 forms complexes with the fibrinogen-like domain-containing protein ANGPTL4 in adipose tissue to decrease ANGPTL3/8- and ANGPTL4-mediated lipoprotein lipase (LPL)-inhibitory activity and promote TG hydrolysis and fatty acid (FA) uptake. The ANGPTL4/8 complex, however, tightly binds LPL and partially inhibits it in vitro. To try to reconcile the in vivo and in vitro data on ANGPTL4/8, we aimed to find novel binding partners of ANGPTL4/8. To that end, we performed pulldown experiments and found that ANGPTL4/8 bound both tissue plasminogen activator (tPA) and plasminogen, the precursor of the fibrinolytic enzyme plasmin. Remarkably, ANGPTL4/8 enhanced tPA activation of plasminogen to generate plasmin in a manner like that observed with fibrin, while minimal plasmin generation was observed with ANGPTL4 alone. The addition of tPA and plasminogen to LPL-bound ANGPTL4/8 caused rapid, complete ANGPTL4/8 cleavage and increased LPL activity. Restoration of LPL activity in the presence of ANGPTL4/8 was also achieved with plasmin but was blocked when catalytically inactive plasminogen (S760A) was added to tPA or when plasminogen activator inhibitor-1 was added to tPA + plasminogen, indicating that conversion of plasminogen to plasmin was essential. Together, these results suggest that LPL-bound ANGPTL4/8 mimics fibrin to recruit tPA and plasminogen to generate plasmin, which then cleaves ANGPTL4/8, enabling LPL activity to be increased. Our observations thus reveal a unique link between the ANGPTL4/8 complex and plasmin generation.**

ANGPTL | lipoprotein lipase | plasmin | plasminogen | triglycerides

## Significance

Lipoprotein lipase (LPL) must be active in adipose tissue postprandially for efficient triglyceride (TG) hydrolysis and fatty acid (FA) uptake into adipocytes. For this to occur, angiopoietin-like protein (ANGPTL) 8 forms a complex with ANGPTL4 to decrease the ability of ANGPTL4 to inhibit LPL. The ANGPTL4/8 complex, however, tightly binds and inhibits LPL in vitro, leaving LPL in a partially active state. We now show that ANGPTL4/8 also mimics fibrin, not only in its ANGPTL4 fibrinogen-like-domain structure but also by functional similarity. ANGPTL4/8 binds both tissue plasminogen activator (tPA) and plasminogen to generate plasmin, which cleaves LPL-bound ANGPTL4/8 to increase LPL activity. These observations, therefore, reveal a unique connection between the ANGPTL4/8 complex and plasmin generation.

The complex mechanisms governing triglyceride (TG) metabolism and lipoprotein lipase (LPL) activity in adipose tissue have begun to be elucidated, although many critical details are incompletely understood (1–6). LPL is the enzyme that lies at the center of TG metabolism in adipose tissue as well as oxidative tissues (7, 8). Its activity must be carefully regulated in the fat so that during fasting, LPL activity is low, allowing TG to reach the skeletal muscle where it can be used for energy. During feeding, however, LPL must be more active in adipose tissue so that TG in TG-rich lipoproteins can be hydrolyzed and the resulting fatty acids (FAs) can be taken up into adipocytes for storage.

Several events must occur for this to happen. LPL secreted by adipocytes into the subendothelial space must bind glycosylphosphatidylinositol high-density lipoprotein binding protein 1 (GPIHBP1) on the abluminal surface of capillary endothelium (9, 10). While this is occurring, LPL must be protected from irreversible inhibition by the potent LPL inhibitor angiopoietin-like protein (ANGPTL) 4, a fibrinogen-like domain-containing protein secreted by adipocytes into the subendothelial space (11–16). GPIHBP1 must then translocate LPL across endothelial cells so that LPL can ultimately be positioned on capillary luminal surfaces, where it can hydrolyze TG in TG-rich lipoproteins (17, 18).

Adipose tissue expression of ANGPTL4 is high in the fasting state to prevent LPL from reaching luminal surfaces of adipose capillary endothelium (19). During feeding, this needs to be reversed, and postprandial increases in ANGPTL8 in adipocytes caused by insulin and glucose insulinotropic peptide result in the formation of increased localized ANGPTL4/8 complexes (1, 2, 20). When ANGPTL4 is not associated with ANGPTL8 (during fasting), it is a potent, irreversible LPL inhibitor (11–16). Upon the formation of the ANGPTL4/8 complex during feeding, however, the LPL-inhibitory activity of ANGPTL4 is markedly reduced (1, 2).

ANGPTL8 thus decreases ANGPTL4-mediated inhibition of LPL in the adipose tissue during the postprandial state. However, a key missing detail relates to the fact that the ANGPTL4/8 complex itself inhibits LPL in vitro, albeit less potently than does

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ANGPTL4 (1, 2). In addition, ANGPTL4/8 binds LPL with almost no detectable off rate, suggesting that it cannot merely dissociate from LPL once LPL is positioned on capillary luminal surfaces (1, 2). This creates a potential paradox, since LPL should be more active in the fat after feeding, and LPL activity is partially inhibited while LPL is bound to ANGPTL4/8.

Because ANGPTL4/8 binds almost irreversibly to LPL, ANGPTL4/8 should remain bound to LPL on capillary luminal surfaces, where it could interact with circulating proteins (1, 2). In our current study, we, therefore, investigated what additional serum proteins LPL-bound ANGPTL4/8 might interact with to modulate LPL activity. We present evidence that LPL-bound ANGPTL4/8 functionally resembles fibrin and engages both tissue plasminogen activator (tPA) and plasminogen. Once this occurs, ANGPTL4/8 enables tPA-mediated activation of plasminogen almost as efficiently as does fibrin, leading to generation of plasmin, which then cleaves ANGPTL4/8 itself. This in turn leads to increased LPL activity. Together, these observations reveal a link between the ANGPTL4/8 complex and plasmin generation.

## Results

**Mass Spectrometry Shows that Plasminogen Specifically Binds ANGPTL4/8.** To identify serum proteins that bind ANGPTL4/8, serially diluted DYKDDDDK octapeptide (FLAG)-tagged ANGPTL4/8 (0.125 to 1.0 μg) was immobilized using an anti-FLAG antibody or an anti-ANGPTL4/8 complex-specific antibody. After incubation with human serum followed by washing, bound proteins were eluted, reduced, and alkylated prior to trypsin digestion. Tryptic peptides were analyzed using liquid chromatography tandem mass spectrometry (LC–MS/MS), and proteins were identified through searches of tandem mass spectrometry (MS/MS) data against a human protein database, with peak areas (AUC) extracted for each ion and analyzed.

Plasminogen was identified as the only ANGPTL4/8 concentration-dependent binding protein present when ANGPTL4/8 was captured using anti-FLAG or anti-ANGPTL4/8 antibodies (Fig. 1*A*). In samples with the highest amount of ANGPTL4/8 added, plasminogen sequence coverage was 76%. The mean $\log_2$area under the curve (AUC) for ions from plasminogen, ANGPTL4,

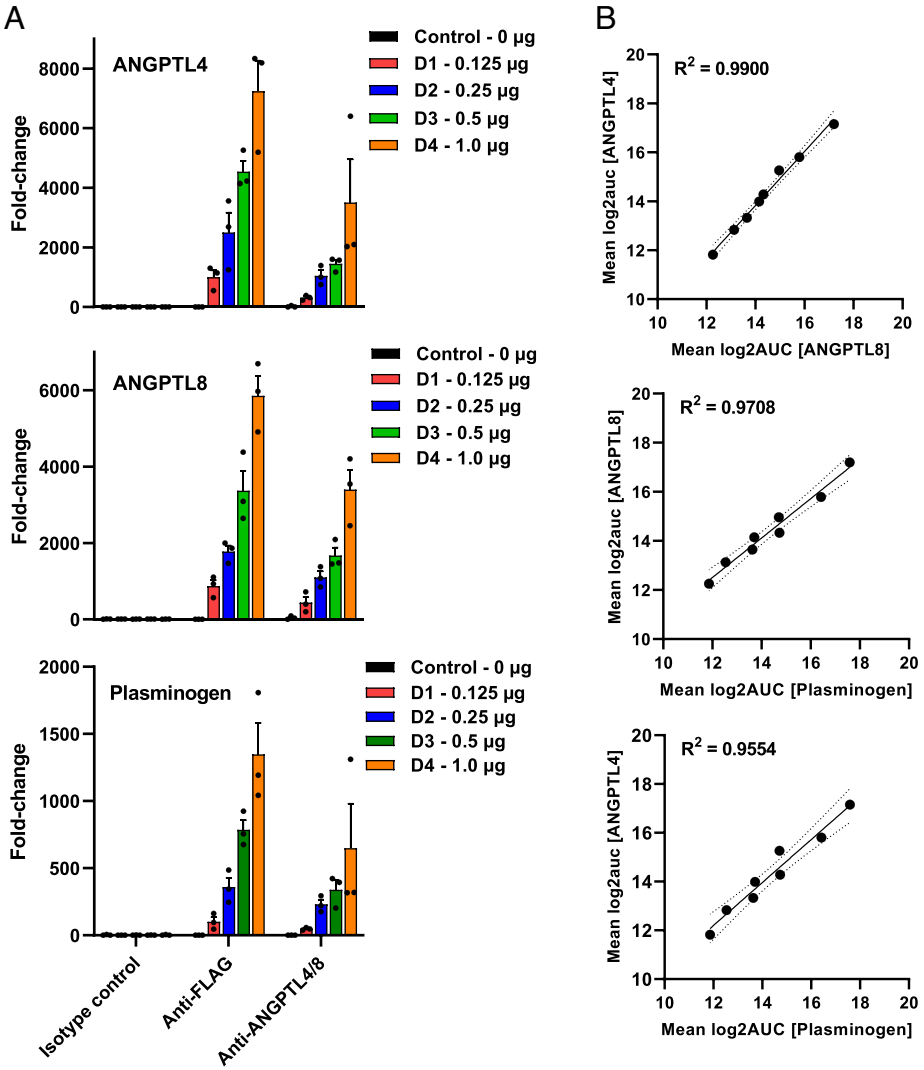


**Fig. 1.** Mass spectrometry demonstrates ANGPTL4/8 binds human serum plasminogen. Recombinant ANGPTL4/8 was serially diluted (D1 to D4: 0.125 to 1.0 μg, with 0 μg as a control), immobilized, and incubated with human serum. Interacting proteins were eluted and identified by mass spectrometry after enzymatic digestion. (*A*) Plots show fold-changes of the mean ion intensities of ANGPTL4, ANGPTL8, and plasminogen for each dilution compared to corresponding control ion intensities. Each condition was analyzed in triplicate. All *P* values were <0.001 for D1 to D4 with anti-FLAG and anti-ANGPTL4/8 antibodies versus the isotype-matched control antibody. (*B*) Graphs show correlations of the mean $\log_2$AUC ion intensities between plasminogen, ANGPTL4, and ANGPTL8 in samples with anti-FLAG and anti-ANGPTL4/8 antibodies. Dashed lines represent 95% CIs. Results are representative of two independent experiments.

and ANGPTL8 in anti-FLAG and anti-ANGPTL4/8 captured samples were combined and compared with each other (Fig. 1*B*). Excellent correlations were obtained for ANGPTL4 and ANGPTL8 in the serially diluted samples. Robust correlations were also observed for plasminogen versus ANGPTL4 and ANGPTL8, suggesting that plasminogen binding occurred directly through ANGPTL4/8 interaction. No plasminogen was detected in blank wells or wells containing an isotype-matched control antibody.

**Western Blotting Demonstrates That Plasminogen and tPA Specifically Bind ANGPTL4/8.** Additional sets of samples were prepared like those used for the mass spectrometry study but with more control proteins included. These included FLAG-tagged ANGPTL3/8, polyhistidine (His)-tagged ANGPTL3, and His-tagged ANGPTL4, in addition to the FLAG-tagged ANGPTL4/8. The two FLAG-tagged complexes were captured using anti-FLAG antibody, and the two His-tagged proteins were captured using anti-His antibody. After incubation with human serum, washing, and acid elution, bound proteins were separated electrophoretically and transferred to the polyvinylidene fluoride (PVDF) membrane, which was probed with an antiplasminogen antibody. As shown in Fig. 2*A*, plasminogen was detected only in samples in which ANGPTL4/8 was present, thus confirming its specific binding to ANGPTL4/8.

To test whether tPA also bound ANGPTL4/8, samples were prepared like those used for the plasminogen Western blotting study. Because circulating tPA is largely inaccessible since it is bound to endogenous inhibitors (21), we incubated the ANGPTL proteins and complexes with recombinant tPA in PBS. After washing and acid elution, bound proteins were separated electrophoretically and transferred to the PVDF membrane, which was probed with an anti-tPA antibody. As Fig. 2*B* shows, tPA was detected only in the sample containing ANGPTL4/8, thus demonstrating that tPA, like plasminogen, specifically bound ANGPTL4/8.

To address the possibility that pull-downs of His-tagged ANGPTL3 and ANGPTL4 and FLAG-tagged ANGPTL4/8 and ANGPTL3/8 were affected by binding the proteins by their respective FLAG or His tags, we repeated the experiments shown above using an anti-ANGPTL3 polyclonal antibody (for ANGPTL3 and ANGPTL3/8) and an anti-ANGPTL4 polyclonal antibody (for ANGPTL4 and ANGPTL4/8). The results from these additional experiments confirmed that only ANGPTL4/8 bound human serum plasminogen (*SI Appendix*, Fig. S1*A*). For tPA, only ANGPTL4/8 demonstrated marked binding, with very slight binding for ANGPTL4, ANGPTL3, and ANGPTL3/8; this binding was not much more than that observed in the absence of any ANGPTL proteins (*SI Appendix*, Fig. S1*B*).

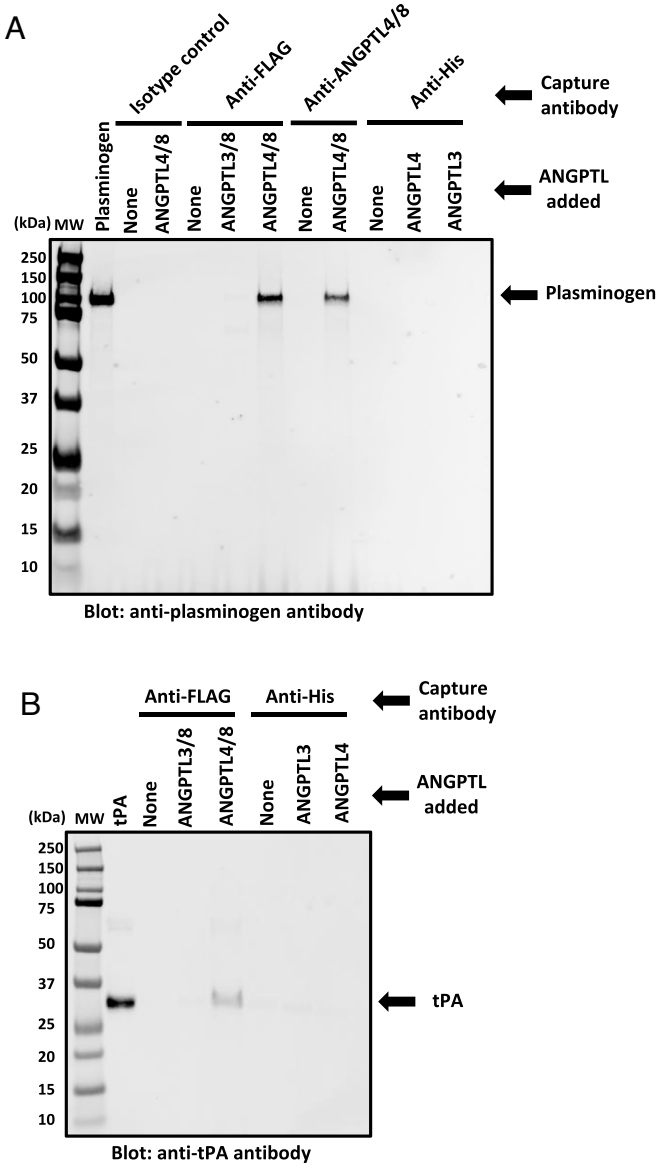


**Fig. 2.** Western blotting confirms ANGPTL4/8 binds plasminogen and tPA. (*A*) Immobilized FLAG-tagged ANGPTL4/8, FLAG-tagged ANGPTL3/8, His-tagged ANGPTL4, or His-tagged ANGPTL3 were incubated with human serum. Bound proteins were eluted, separated electrophoretically, and transferred to PVDF membrane probed with rabbit polyclonal anti-plasminogen primary antibody, with Alexa 680-labeled anti-rabbit antibody used for detection. Results are representative of two independent experiments. (*B*) The above experiment was repeated except immobilized proteins were incubated with phosphate buffered saline (PBS) containing tPA. Bound proteins were eluted, separated electrophoretically, and transferred to PVDF membrane probed with mouse monoclonal anti-tPA primary antibody, with Alexa 680-labeled anti-mouse antibody used for detection. Results are representative of two independent experiments.

**ANGPTL4/8 and tPA Synergistically Activate Plasminogen to Convert It to Plasmin.** Plasminogen can be converted to plasmin by tPA, its principal endogenous activator in blood, and the canonical substrate for plasmin is fibrin (22). We, therefore, used a kinetic plasmin activity assay to examine how ANGPTL4/8 affects tPA-mediated plasminogen activation. As Fig. 3*A* shows, no plasmin activity was detected for samples with plasminogen alone, and only minimal activity was detected in samples containing tPA + plasminogen or ANGPTL4 + tPA + plasminogen. Somewhat more activity was detected in samples containing ANGPTL3 + tPA + plasminogen or ANGPTL3/8 + tPA + plasminogen. However, when ANGPTL4/8, tPA, and plasminogen were combined, a marked increase in plasmin activity was observed that was only slightly less than that observed in the presence of fibrin + tPA + plasminogen. These results indicated that ANGPTL4/8 + tPA synergistically activated plasminogen almost to the same effect that fibrin + tPA did.

This synergistic effect observed for plasminogen activation with tPA and ANGPTL4/8 was further evaluated by comparing ANGPTL4/8 with fibrin in an assay in which the plasminogen concentration was varied. As shown in Fig. 3*B*, with increasing plasminogen concentrations, there was a modest increase in the plasmin activity observed with tPA, and the degree of plasmin activity generated was not increased by the combination of ANGPTL4 + tPA. In the presence of ANGPTL3 + tPA or ANGPTL3/8 + tPA, there was a moderate increase in plasmin activity. In the presence of ANGPTL4/8 + tPA, however, there was

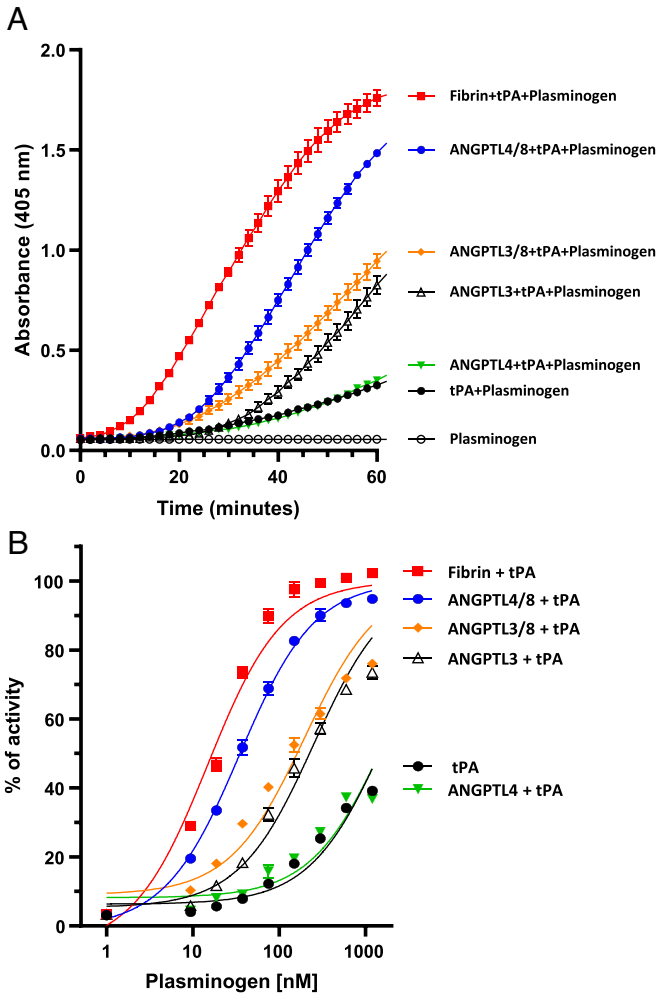


**Fig. 3.** ANGPTL4/8 and fibrin increase tPA-mediated plasmin generation. (*A*) Activation of plasminogen to plasmin was monitored using the chromogenic plasmin substrate D-Val-Leu-Lys-pNA. Plasminogen alone, tPA + plasminogen, ANGPTL4 + tPA + plasminogen, ANGPTL4/8 + tPA + plasminogen, ANGPTL3 + tPA + plasminogen, ANGPTL3/8 + tPA + plasminogen, or fibrin + tPA + plasminogen were added to wells containing chromogenic substrate. Samples were incubated at 37 °C for 60 min, and absorbance at 405 nm was recorded every 2 min. Results are representative of three independent experiments. (*B*) Increasing concentrations of plasminogen (0 to 1,200 nM) were incubated with tPA alone, ANGPTL4 + tPA, ANGPTL4/8 + tPA, ANGPTL3 + tPA, ANGPTL3/8 + tPA, or fibrin + tPA in buffer containing chromogenic plasmin substrate for 60 min at 37 °C. Wells containing only plasminogen and chromogenic substrate served controls. Maximal absorbance was normalized to 100% plasmin activity. Results are representative of three independent experiments.

a dramatic increase in plasmin activity, which was almost comparable to the combination of fibrin + tPA. The calculated half-maximal effective concentration ($EC_{50}$) values for ANGPTL4 + tPA, ANGPTL4/8 + tPA, and fibrin + tPA were 1,734, 34.8, and 14.9 nM, respectively, representing 116-fold and 50-fold increases in plasmin activity for fibrin + tPA and ANGPTL4/8 + tPA, respectively, versus ANGPTL4 + tPA. These results indicated that ANGPTL4/8 behaved functionally like fibrin. Subsequent experiments described below in *SI Appendix*, Figs. S3–S5 confirmed that only ANGPTL4/8 (and not ANGPTL4, ANGPTL3, or ANGPTL3/8) was cleaved by plasmin and that only ANGPTL4/8-mediated LPL inhibition was reversed by tPA + plasminogen.

**ANGPTL4/8 Is Cleaved by Plasmin.** Because fibrin binds tPA and plasminogen to generate plasmin that cleaves fibrin itself (23), we tested whether ANGPTL4/8 binding of tPA + plasminogen to generate plasmin would result in ANGPTL4/8 cleavage. We examined culture media obtained from LPL-stable expressing cells preincubated with recombinant ANGPTL4/8 and then exposed to tPA + plasminogen for various periods of time. After the reaction was stopped, proteins were separated electrophoretically and transferred to separate PVDF membranes probed with anti-ANGPTL4, anti-ANGPTL8, and anti-LPL antibodies. When ANGPTL4/8 alone was added to the cells, no ANGPTL4 or ANGPTL8 degradation was detected. However, when tPA and plasminogen were added to the cells incubated with ANGPTL4/8, almost all ANGPTL4 and ANGPTL8 in the ANGPTL4/8 complex were degraded within 30 min (Fig. 4 *A* and *B*).

In contrast, the band representing intact LPL remained much more constant, although there appeared to be some decrease in the amount of full-length LPL at 30 min (Fig. 4*C*). There was also some variability in the presence of a lower band (of unknown identity) that did not appear to correlate in a manner related to the intensity of the intact LPL band. Each of these observations was noted both times the experiment was performed (*SI Appendix*, Fig. S2). The slight decrease in the intact LPL band at 30 min suggested that there could be some effect of plasmin on LPL, although results from subsequent LPL activity experiments (described below) did not show any reduction in LPL enzymatic activity.

To verify the cleavage pattern of ANGPTL4/8 and confirm the specificity of the ANGPTL4/8 observations, we performed several additional experiments. We first examined the cleavage pattern of ANGPTL4/8 in assay buffer when tPA and plasminogen were added in the absence of LPL-expressing cells. As *SI Appendix*, Fig. S3 shows, ANGPTL4/8 again manifested a similar cleavage pattern compared to the LPL cell-based assays, indicating that ANGPTL4/8 not bound to LPL was also degraded by tPA + plasminogen. Interestingly as this figure shows, all cleaved forms of ANGPTL4 protein tended to decrease with time, suggesting that while plasmin may have an initial preferred cleavage site in ANGPTL4 in the ANGPTL4/8 complex, it may also cleave ANGPTL4 at other sites as well when given enough time.

We also examined the cleavage patterns of ANGPTL4, ANGPTL3, or ANGPTL3/8, and as *SI Appendix*, Fig. S4 demonstrates, no cleavage of ANGPTL4, ANGPTL3, or ANGPTL3/8 was observed when these were added to LPL-stable expressing cells in the presence of tPA + plasminogen. Together, these results suggested that the preferred substrate for the plasmin generated by the combination of ANGPTL4/8 + tPA + plasminogen was ANGPTL4/8 itself.

**The Addition of tPA + Plasminogen Reverses ANGPTL4/8-Mediated LPL Inhibition.** Based on the above results, we hypothesized that the purpose for LPL-bound ANGPTL4/8-mediated plasminogen activation by tPA might be to cleave ANGPTL4/8 so that the ANGPTL4/8 complex would no longer be able to bind LPL and inhibit its activity. To assess this possibility, we performed LPL cell-based experiments in which the levels of ANGPTL4/8, ANGPTL4, ANGPTL3, and ANGPTL3/8 were steadily increased in the presence or absence of tPA + plasminogen. As *SI Appendix*, Fig. S5 shows, LPL activity was completely restored by the addition of tPA + plasminogen across the entire range of ANGPTL4/8 concentrations tested, while there was no effect observed with ANGPTL4, ANGPTL3, or ANGPTL3/8. We next used kinetic assays with LPL stable-expressing cells to investigate the ability of tPA + plasminogen to selectively reverse ANGPTL4/8-mediated LPL inhibition. As Fig. 5 *A*–*C* demonstrates, the addition of tPA + plasminogen had no ability to reverse LPL inhibition by ANGPTL3, ANGPTL3/8, or ANGPTL4. When the same experiments were performed after preincubation with ANGPTL4/8, however, the addition of tPA + plasminogen fully restored LPL activity (Fig. 5*D*).

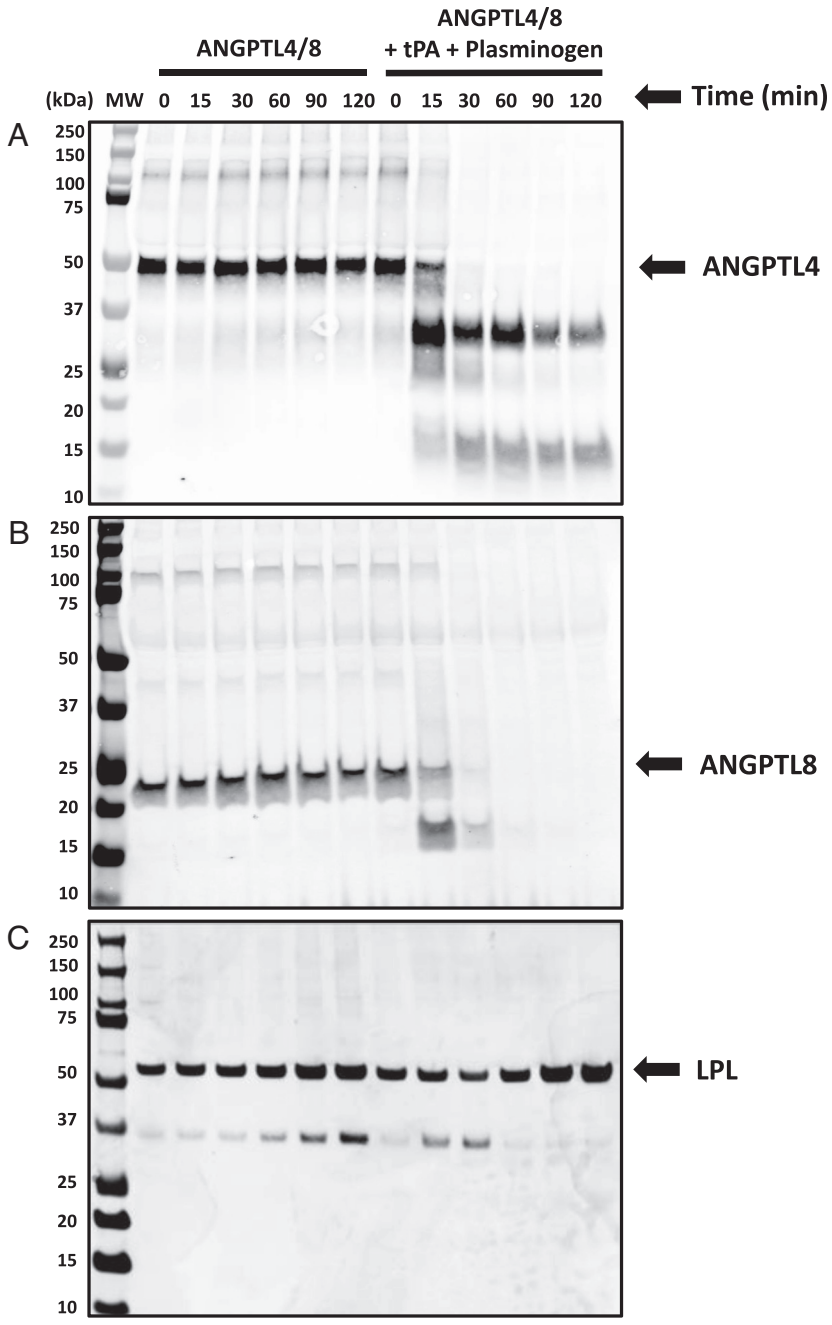


**Fig. 4.** LPL-bound ANGPTL4/8 is rapidly degraded by tPA + plasminogen. Recombinant ANGPTL4/8 was incubated with LPL stable-expressing cells for 1 h at 37 °C. The incubation was continued for 0 to 120 min at 37 °C in the absence or presence of tPA + plasminogen. Aliquots were removed at times indicted, and reactions stopped for ANGPTL4/8 and LPL analyses. Samples were separated on different sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to separate PVDF membranes. All results are representative of two independent experiments. (*A*) The membrane was probed with biotinylated anti-ANGPTL4 antibodies, followed by Alexa 680-labeled streptavidin. (*B*) The membrane was probed with biotinylated anti-ANGPTL8 antibodies, followed by Alexa 680-labeled streptavidin. (*C*) The membrane was probed with goat anti-LPL antibody, followed by Alexa 680-labeled anti-goat antibody.

Because in vivo, LPL would be expected to be bound to GPIHBP1 on capillary luminal surfaces, we performed additional experiments using recombinant GPIHBP1–LPL complex. As *SI Appendix*, Fig. S6 *A* and *B* demonstrates, the addition of increasing amounts of GPIHBP1–LPL did not block the cleavage of ANGPTL4/8 observed with the addition of tPA + plasminogen. Furthermore, the addition of tPA + plasminogen to GPIHBP1–LPL in the presence of ANGPTL4/8 also resulted in the restoration of LPL enzymatic activity (*SI Appendix*, Fig. S6*C*).

**Plasminogen Activation Is Required for Reversal of ANGPTL4/8-Mediated LPL Inhibition.** We next evaluated the effects of tPA alone, plasminogen alone, tPA + plasminogen, plasmin, plasminogen activator inhibitor-1 (PAI-1) alone, tPA + plasminogen + PAI-1, catalytically inactive plasminogen(S760A), and tPA + plasminogen (S760A) on LPL activity in the absence or presence of recombinant ANGPTL4/8 (Fig. 6*A*). The addition of either tPA alone or plasminogen alone had no effect, while addition of plasmin completely restored LPL activity following preincubation with ANGPTL4/8, like the full restoration of LPL activity observed with tPA + plasminogen. The effect of tPA + plasminogen was completely blocked by PAI-1, indicating that tPA-mediated activation of plasminogen to plasmin was required for restoration of LPL activity. To confirm this concept, we tested catalytically inactive plasminogen (S760A) alone and in combination with tPA. Plasminogen (S760A) alone had no effect, and the combination

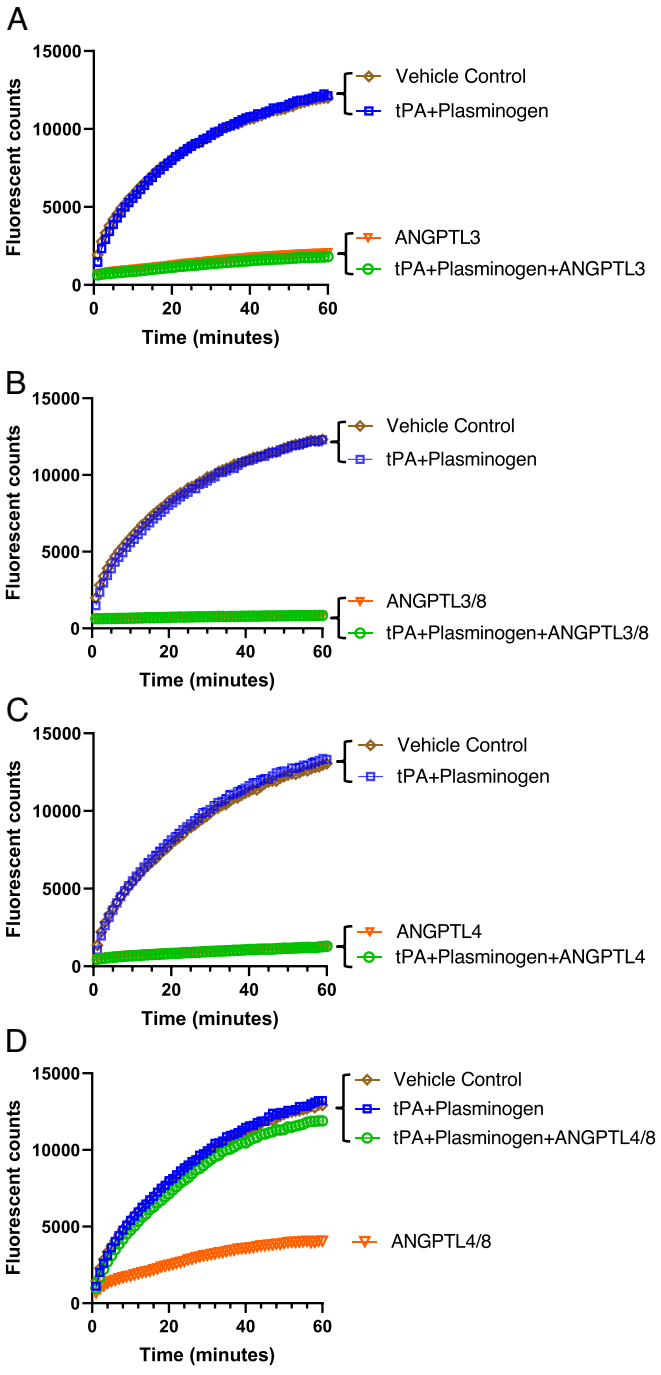


**Fig. 5.** The combination of tPA + plasminogen specifically reverses ANGPTL4/8-mediated LPL inhibition. The ability of 30 nM ANGPTL3 (*A*), ANGPTL3/8 (*B*), ANGPTL4 (*C*), or ANGPTL4/8 (*D*) to inhibit LPL activity in the absence or presence of tPA + plasminogen was assessed using LPL-stable expression cells with fluorescent lipase substrate. Fluorescence was monitored continuously every minute for 60 min. In each case, cells preincubated in the absence of ANGPTL proteins served as the vehicle control. All results are representative of at least two independent experiments.

of plasminogen (S760A) + tPA was unable to restore LPL activity in ANGPTL4/8 pretreated LPL expressing cells, indicating that activation of plasminogen to generate plasmin was required to reverse ANGPTL4/8-mediated LPL inhibition. To confirm these observations, we repeated the above experiments using very low-density lipoprotein (VLDL) substrate and quantitated the release of nonesterified fatty acids (NEFAs) as a measure of LPL activity. As Fig. 6*B* shows, almost identical results were obtained compared to those using the fluorometric substrate.

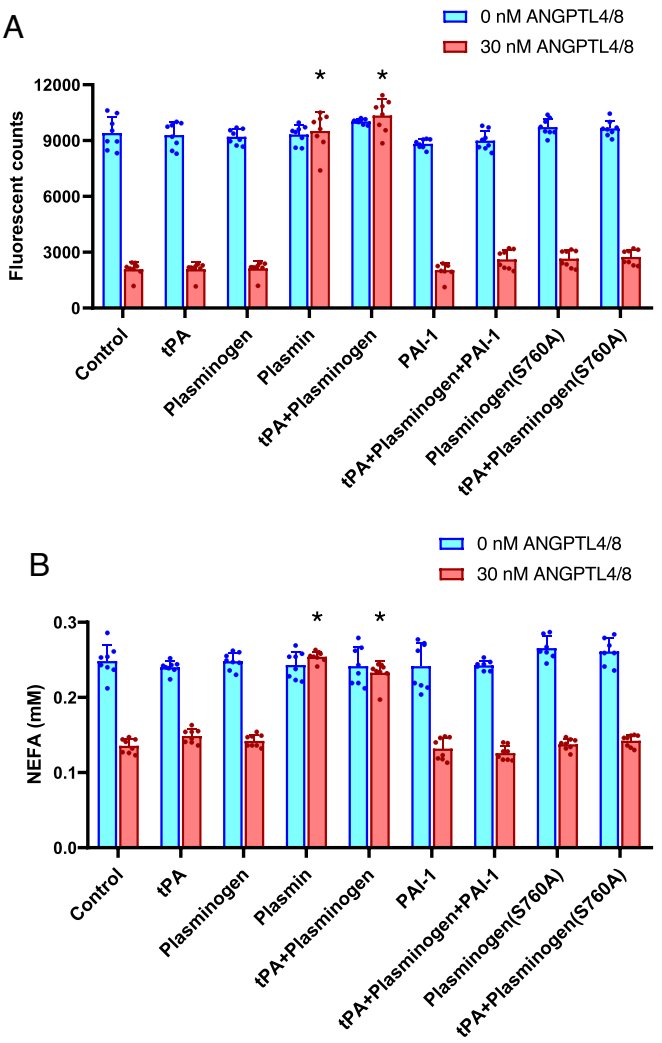


**Fig. 6.** Plasminogen activation is required for reversal of ANGPTL4/8 mediated LPL inhibition. The ability of recombinant ANGPTL4/8 to reduce LPL activity in the presence of plasminogen, tPA, tPA + plasminogen, plasmin, PAI-1, tPA + plasminogen + PAI-1, plasminogen (S760A), or tPA + plasminogen (S760A) was assessed using LPL-stable expression cells with fluorescent lipase substrate and VLDL substrate. LPL activity in the absence of ANGPTL4/8 served as the maximum activity reference. All results are shown as the mean ± SD (n = 8 from two independent experiments) (**P* < 0.0001 versus ANGPTL4/8 alone). (*A*) LPL activity with fluorescent lipase substrate. (*B*) LPL activity with VLDL substrate and NEFA measurement.

**The Main ANGPTL4 Fragment in Human Serum Is Consistent with Plasmin Cleavage of ANGPTL4/8.** In human serum, the main ANGPTL4 species has been shown through the pioneering work of the Kersten laboratory to be a C-terminal fragment (24, 25). To determine whether this ANGPTL4 fragment could come from plasmin cleavage of ANGPTL4/8, endogenous ANGPTL4 was immunoprecipitated from human serum using an anti-ANGPTL4 polyclonal antibody. Immunoprecipitated proteins were analyzed using Western blotting, along with material from recombinant ANGPTL4/8 digestions with tPA + plasminogen in assay buffer and LPL-stable expression cell culture media to which tPA + plasminogen were added. As Fig. 7*A* shows, a predominant 30 kD band was detected in human serum, and this band comigrated with the major ANGPTL4 degradation product generated in assay buffer or culture media when ANGPTL4/8 was combined with tPA and plasminogen.

To determine if the circulating ANGPTL4 fragment could be explained by ANGPTL4/8 plasmin cleavage, we excised gel sections corresponding to the ANGPTL4 bands and performed in-gel

digestion analyses using Asp-N and chymotrypsin. ANGPTL4 was deglycosylated using peptide:N-glycosidase F (PNGase F) to remove N177-glycans, which converts it to an aspartate residue, creating an Asp-N recognition site. The N-terminal ANGPTL4 peptides identified from Asp-N digestion of endogenous ANGPTL4 had sequences from 165 to 172 (LPEMAQPV) or 165 to 176 (LPEMAQPVDPAH). The MS/MS spectrum for the doubly charged 165 to 176 ion is shown in *SI Appendix*, Fig. S7*A*. The N-terminal ANGPTL4 peptides from chymotrypsin digestion of endogenous ANGPTL4 had sequences in the ranges 165 to 179 (LPEMAQPVDPAHNVS), 165 to 181 (LPEMAQPVDPAHNVSRL), and 165 to 182 (LPEMAQPVDPAHNVSRLH) with N177 deaminated by PNGase F. The MS/MS spectrum for the triply charged 165 to 181 ion is shown in *SI Appendix*, Fig. S7*C*. These results showed that the circulating ANGPTL4 fragment began around L165.

Similar analyses performed on plasmin-generated products of recombinant ANGPTL4/8 from assay buffer and LPL culture media showed the 30 kDa ANGPTL4 band from these ANGPTL4/8 digestions contained the same fragment starting at L165 in both the Asp-N and chymotrypsin digestions, as well as additional fragments starting with R162, K163, and R164. The MS/MS spectra for peptides 165 to 176 from Asp-N digestion and for peptides 165 to 181 from chymotrypsin digestion are shown in *SI Appendix*, Fig. S7 *B* and *D*, respectively. Both spectra matched their counterpart spectra from endogenous ANGPTL4, indicating that some of the circulating ANGPTL4 fragment may come from ANGPTL4/8 plasmin cleavage.

Because the plasmin cleavage site for ANGPTL4 contained amino acids arginine-arginine-lysine-arginine (RRKR) (161 to 164), which also make up a canonical furin cleavage site where furin and other proprotein convertases may cleave ANGPTL4 to generate fragments starting with R162 or L165 (24), these data indicated that the endogenous C-terminal ANGPTL4 fragment could also result from furin or related enzymes cleaving ANGPTL4/8 (or ANGPTL4). We, therefore, incubated recombinant ANGPTL4/8 with either tPA + plasminogen or furin. Afterward, proteins were analyzed by western blotting with anti-ANGPTL4 antibodies. As Fig. 7*B* shows, ANGPTL4 in the ANGPTL4/8 complex was completely degraded after 30 min with tPA + plasminogen. In contrast, furin cleavage of ANGPTL4 in the ANGPTL4/8 complex was markedly slower, with much smaller amounts of the 30 kD fragment formed, even after 300 min. We next compared the incubation of recombinant ANGPTL4/8 + furin versus recombinant ANGPTL4 + furin (Fig. 7*C*). While furin cleaved ANGPTL4 alone, there was minimal furin cleavage of ANGPTL4/8. Together, these data suggested that some of the human serum ANGPTL4 fragment may come from ANGPTL4/8 plasmin cleavage.

## Discussion

The major finding of our study is that the fibrinogen-like domain-containing ANGPTL4/8 complex behaves like fibrin by binding plasminogen and tPA to enable tPA to activate plasminogen and generate plasmin. The plasmin produced then cleaves

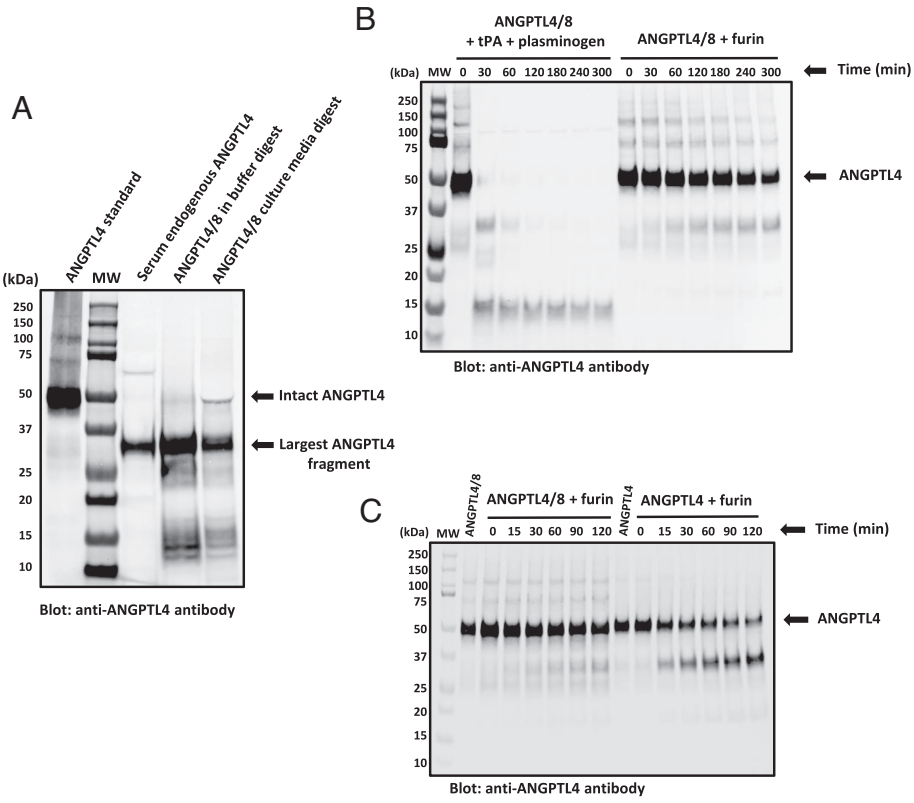


**Fig. 7.** Human serum ANGPTL4 may come from plasmin-mediated ANGPTL4/8 cleavage rather than furin-mediated ANGPTL4/8 or ANGPTL4 cleavage. (*A*) Endogenous ANGPTL4 in human serum was immunoprecipitated using a polyclonal anti-ANGPTL4 antibody. Recombinant ANGPTL4/8 digests with tPA and plasminogen in assay buffer and LPL cell culture media were included for comparison. Proteins were separated electrophoretically and transferred to a PVDF membrane. (*B*) Recombinant ANGPTL4/8 was incubated with tPA + plasminogen or furin. Aliquots were removed at different time points, and the reaction was stopped by adding SDS sample buffer. Samples were separated electrophoretically and transferred to a PVDF membrane. (*C*) Recombinant ANGPTL4/8 complex or ANGPTL4 was incubated with furin for various periods of time. At each time point, the reaction was stopped, and proteins were separated electrophoretically and transferred to a PVDF membrane. All membranes were probed with biotinylated anti-ANGPTL4 antibodies followed by Alexa 680-labeled streptavidin. Results are representative of two independent experiments.

ANGPTL4/8. The result is that LPL activity, which is partially inhibited by ANGPTL4/8 in vitro, is increased after cleavage of the ANGPTL4/8 complex. We observed that substitution of wild-type plasminogen with catalytically inactive plasminogen blocked the ANGPTL4/8-mediated effect, suggesting that conversion of plasminogen to plasmin is crucial for regaining LPL activity, consistent with our data showing the effect of tPA + plasminogen was mimicked by plasmin.

In vivo, LPL is bound to GPIHBP1 on luminal capillary surfaces, and we were able to show that GPIHBP1 did not affect plasmin-mediated cleavage of ANGPTL4/8 or restoration of LPL activity. Interestingly, the plasmin cleavage site of ANGPTL4 in the ANGPTL4/8 complex was identical to the ANGPTL4 furin cleavage site elegantly described by Yin et al. where furin and other proprotein convertases can cleave ANGPTL4 (26, 27). We observed that ANGPTL4/8 was rapidly cleaved by tPA + plasminogen, while only minimal furin cleavage occurred. Furin did cleave ANGPTL4, but more slowly than ANGPTL4/8 was cleaved by tPA + plasminogen, suggesting that some of the predominant ANGPTL4 C-terminal fragment in human serum may come from plasmin-mediated cleavage of ANGPTL4/8.

Fig. 8 shows a model for how this system might control adipose tissue LPL activity based on caloric availability. During fasting (Fig. 8*A*), ANGPTL8 expression is low, and little ANGPTL4/8 is present while ANGPTL4 is highly expressed (24, 25). As shown by the Kersten laboratory, ANGPTL4 binds LPL intracellularly to reduce its secretion (and in the subendothelial space) and inactivates LPL, thus preventing GPIHBP1 from translocating LPL across the capillary endothelium (14–16, 28). Therefore, little LPL reaches capillary luminal surfaces, and TG are routed toward skeletal muscle for use as energy.

After feeding (Fig. 8*B*), newly made ANGPTL8 forms complexes with ANGPTL4, decreasing the amount of free ANGPTL4 produced by adipocytes (1, 2). This reduces ANGPTL4-mediated LPL inactivation. The ANGPTL4/8 complexes are secreted by the adipocytes into the subendothelial space, where they tightly bind LPL and protect it from inactivation by ANGPTL4. However, ANGPTL4/8 moderately inhibits LPL, resulting in LPL being in a partially active state (1, 2). The ANGPTL4/8–LPL complex binds GPIHBP1 on abluminal capillary endothelial surfaces, and GPIHBP1 translocates ANGPTL4/8-bound LPL across the endothelium. While this happens, tPA secreted by endothelial cells binds to the LPL–ANGPTL4/8 complex. As a result, the localized LPL–ANGPTL4/8-tPA complex is displayed on the luminal surface of adipose capillaries. Circulating plasminogen then binds the LPL–ANGPTL4/8-tPA complex, and ANGPTL4/8 acts like fibrin to catalyze tPA-mediated conversion of plasminogen to plasmin, which cleaves ANGPTL4/8 to increase LPL activity.

A limitation of our model is that it is based on biochemical findings, and future investigations will be required to establish its relevance to TG metabolism in vivo. When it is compared to existing reports, there are both consistencies and discrepancies. In a seminal paper, Oldoni et al. showed that serum ANGPTL8 comes from the liver, indicating that circulating ANGPTL4/8 must come from the liver (20). Our observations agree with this concept since they predict that adipose tissue ANGPTL4/8 would be cleaved by plasmin. Our model also suggests that tPA binding to ANGPTL4/8 occurs while ANGPTL4/8 is localized as part of an ANGPTL4/8–LPL complex and before tPA binds its circulating inhibitors. For this reason, ANGPTL4/8 secreted by the liver directly into the circulation would likely not be cleaved by plasmin. The work by Oldoni et al., however, also indicated that ANGPTL4/8 in the adipose tissue protects LPL from circulating

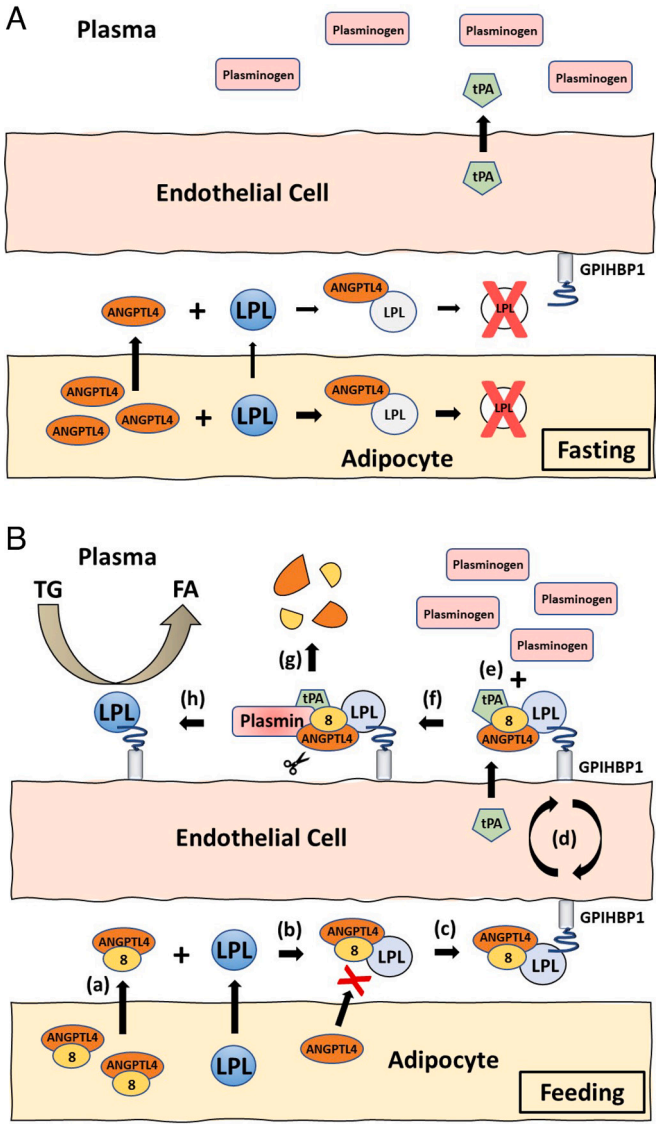


**Fig. 8.** A model for how adipose tissue LPL activity is controlled during fasting and feeding. (*A*) During fasting, ANGPTL8 expression is low, while ANGPTL4 levels are high. ANGPTL4 binds LPL intracellularly to decrease its secretion by adipocytes, binds LPL in the subendothelial space, and irreversibly inactivates LPL. Therefore, LPL cannot be translocated across the capillary endothelium by GPIHBP1. Therefore, LPL levels on the luminal surfaces of capillaries are very low. The endothelium secretes tPA into the circulation, where it can catalyze plasmin-mediated cleavage of any fibrin clots present. (*B*) After feeding, ANGPTL8 increases, causing increased ANGPTL4/8 secretion from adipocytes (a). ANGPTL4/8 binds LPL in the subendothelial space and protects LPL from inactivation by ANGPTL4 but also partially inhibits LPL as shown by the lighter blue color (b). The partially active ANGPTL4/8–LPL complex binds GPIHBP1 on the abluminal surface of the endothelium (c) and is translocated across the endothelium (d). As this occurs, tPA secreted by the endothelium binds ANGPTL4/8, and circulating plasminogen binds the LPL–ANGPTL4/8-tPA complex (e) as it is positioned on the luminal capillary surface (f). ANGPTL4/8 catalyzes tPA-mediated conversion of plasminogen into plasmin, which cleaves ANGPTL4/8 and removes it from LPL (g). This leads to restoration of LPL activity as shown by the darker blue color (h).

inhibitors such as ANGPTL3/8 (20). Yet, our model suggests that while plasmin cleavage of ANGPTL4/8 bound to LPL may initially increase LPL activity, it could also leave LPL exposed to circulating inhibitors that might ultimately decrease LPL activity.

Similarly, our model must be viewed in the context of literature describing the roles of plasminogen and PAI-1 in TG metabolism (29–36). We observed that the addition of PAI-1 to the combination of ANGPTL4/8 + tPA + plasminogen-blocked restoration

of LPL activity. Previous reports have demonstrated that PAI-1 concentrations are directly correlated with TG in humans and that administration of a PAI-1 inhibitor to mice decreased serum TG (29–36). According to our model, though, inhibition of PAI-1 could lead to higher plasmin activity and increased degradation of LPL-bound ANGPTL4/8, thus initially increasing LPL activity, but also leaving LPL in an unprotected state susceptible to circulating inhibitors such as ANGPTL3/8 and ApoC3 that could ultimately decrease LPL activity (1–5, 20, 37).

Our findings, therefore, raise the key question of why the ANGPTL4/8 complex would promote its own degradation. Perhaps ANGPTL4/8 levels should not increase too much after feeding, and if they do, a self-destruction mechanism is activated. Another possibility might be that a two-step mechanism is needed to inactivate and degrade ANGPTL4 in the fed state to disinhibit LPL, with the first step being binding of ANGPTL4 to ANGPTL8, followed by a second step consisting of activation of tPA and plasmin leading to subsequent ANGPTL4 cleavage. The idea that LPL is partially inhibited by ANGPTL4/8 must be viewed with caution because it is based on biochemical data and is not necessarily supported by the available in vivo ANGPTL8 data in adipose tissue (20). Thus, the important question remains of why the adipocyte would make ANGPTL4/8 in the first place if the role of the ANGPTL4/8 complex was to partially inhibit LPL, only to decide that this partial inhibition of LPL is undesirable, resulting in the activation of a mechanism that causes the degradation of ANGPTL4/8. Clearly, further investigations will be required to understand more fully the relevance of our current biochemical observations to TG metabolism in vivo.

From a structural biology perspective, ANGPTL4 is predicted by the artificial intelligence programs AlphaFold2 and Logical Data for Condition Monitoring to exhibit a fibrinogen-like structure (38–40). Our data suggest that once the ANGPTL4/8 complex forms, ANGPTL4 in the complex may functionally resemble fibrin to generate plasmin. Increased plasmin activity has been linked to inflammation, foam cell formation, vascular wall disease, and increased atherogenesis (41–44). In addition, plasmin has the counterintuitive capability to activate the coagulation system, as administration of recombinant tPA for thrombolysis can activate the clotting cascade (45, 46). These reports suggest that chronic increases in plasmin activity may be detrimental. In this context, our observations have uncovered a potentially important connection between the ANGPTL4/8 complex and plasmin generation. Much additional work will need to be performed to understand more fully the implications of our findings.

## Materials and Methods

**Recombinant Proteins, Antibodies, and Reagents.** Commercial reagents were obtained as described in *SI Appendix, Materials and Methods*. Recombinant human C-terminal FLAG-tagged ANGPTL4/8 complex, FLAG-tagged ANGPTL3/8 complex, His-tagged ANGPTL3, His-tagged ANGPTL4, anti-ANGPTL8 monoclonal antibodies, anti-ANGPTL4 (N-terminal specific) monoclonal antibody, anti-ANGPTL4/8 antibody, and GPIHBP1–LPL complex were generated using techniques previously described (47, 48). Recombinant ANGPTL4/8, ANGPTL3/8, and GPIHBP1–LPL were each transiently expressed in chinese hamster ovary (CHO) cells. Anti-FLAG and anti-HIS antibodies were generated in-house. Catalytically inactive plasminogen (S760A) was generated as described in *SI Appendix, Materials and Methods*.

**Mass Spectrometry Identification of ANGPTL4/8-Binding Proteins.** Two micrograms of biotinylated anti-FLAG antibody, anti-ANGPTL4/8 antibody, or isotype-matched control antibody were immobilized on Pierce streptavidin high-capacity plates by incubating at room temperature for 1 h. Blank wells served as negative controls. The plate was washed three times using ice-cold PBS, and serially diluted FLAG-tagged ANGPTL4/8 was added to individual wells (dilutions D1 to D4 corresponding to 0.125, 0.25, 0.5, and 1.0 μg per well, respectively). Blank wells with no ANGPTL4/8 added served as an additional control. The plate was incubated at room temperature for 2 h. Afterward, wells were washed with ice-cold PBS, and 200 μL of an equal mixture of pooled normal human serum and PBS were added to each well. The plate was incubated at room temperature for 2 h under gentle shaking. Plates were washed using ice-cold PBS. Bound proteins were eluted using 200 μL of 1% acetic acid, and eluates were dried under nitrogen to remove acetic acid. Proteins were digested overnight at 37 °C using trypsin, after reduction and alkylation with triethylphosphine and iodoethanol, respectively. Digested peptides were characterized as described in *SI Appendix, Materials and Methods*.

**Western Blotting of ANGPTL4/8-Binding Proteins.** Biotinylated anti-FLAG antibody, anti-ANGPTL4/8 antibody, anti-His antibody, or isotype-matched control antibody were immobilized on streptavidin plates. One microgram of FLAG-tagged ANGPTL3/8, FLAG-tagged ANGPTL4/8, His-tagged ANGPTL3, or His-tagged ANGPTL4 were added to selected wells. These experiments were also repeated using anti-ANGPTL3 and anti-ANGPTL4 polyclonal antibodies immobilized on the streptavidin plates. To study plasminogen binding, 200 μL consisting of an equal mixture of pooled human serum and PBS was added to each well. To study tPA binding, 100 μL of 1 μg/mL of tPA in PBS containing 0.2% BSA was added to each well. Samples were incubated at room temperature for 2 h. Afterward, wells were washed with ice-cold PBS, and bound proteins were eluted and dried as described above. Protein eluates were resuspended in SDS-PAGE sample buffer, separated electrophoretically, and transferred to PVDF. A rabbit anti-plasminogen polyclonal antibody and mouse anti-tPA monoclonal antibody were used to detect plasminogen and tPA, respectively. Alexa 680-conjugated anti-rabbit antibody or Alexa 680-conjugated anti-mouse antibody was used for the detection of plasminogen and tPA, respectively. Images were recorded using an Odyssey imaging system (LI-COR Biosciences).

**Plasmin Activity Assays.** The ability of tPA to convert plasminogen to plasmin in the presence of ANGPTL4, ANGPTL4/8, ANGPTL3, ANGPTL3/8, or fibrin was determined as described in *SI Appendix, Materials and Methods*.

**ANGPTL4/8 and LPL Western Blotting on Adding tPA + Plasminogen to LPL Stable Expression Cells.** LPL stable expression cells were incubated for 1 h with 100 nM recombinant ANGPTL4/8 and further incubated in the absence or presence of 10 nM tPA and 30 nM plasminogen. All incubations were carried out at 37 °C using Dulbecco's modified eagle medium (DMEM/F12) supplemented with 0.1% fatty acid-free bovine serum albumin (BSA). For ANGPTL4/8 analysis, aliquots of cell culture media were removed at different times, with reactions stopped by addition of an equal volume of SDS-PAGE sample buffer. For LPL analysis, reactions were stopped by the addition of 50 μM of aprotinin, and LPL was enriched by incubation with heparin–sepharose beads at 4 °C for 30 min. Beads were washed using ice-cold PBS, and LPL was released by heating the beads at 70 °C for 10 min in SDS-PAGE sample buffer without reduction. Samples were separated on separate gels and transferred to separate PVDF membranes. One membrane was probed with two biotinylated anti-ANGPTL4 antibodies. The second was probed with three biotinylated anti-ANGPTL8 monoclonal antibodies. The third was probed with goat anti-LPL antibody. Alex-680 conjugated streptavidin was used for the detection of ANGPTL4 and ANGPTL8, while Alex-680 conjugated anti-goat antibody was used for LPL detection. Images were recorded using a LI-COR Odyssey imaging system. Similar experiments were performed with ANGPTL3, ANGPTL4, and ANGPTL3/8 as described in *SI Appendix, Materials and Methods*.

**Western Blotting of ANGPTL4/8 Degradation by tPA + Plasminogen in Assay Buffer and When Incubated with GPIHBP1–LPL and tPA + Plasminogen.** Analyses of recombinant ANGPTL4/8 degradation by plasmin in assay buffer and experiments to determine the effect of ANGPTL4/8 on recombinant GPIHBP1–LPL complex in the absence and presence of tPA + plasminogen were performed as described in *SI Appendix, Materials and Methods*.

**Human LPL Activity Assays.** Human LPL activity assays were performed as previously described (47, 48) with minor modifications as described in the *SI Appendix, Materials and Methods*.

**Characterization of Endogenous ANGPTL4 and Recombinant ANGPTL4/8 Plasmin Cleavage Product.** Endogenous ANGPTL4 protein was immunoprecipitated as described above from 2 mL of sample consisting of equal volumes of pooled normal human serum and PBS using polyclonal anti-ANGPTL4 antibody covalently coupled to tosyl-activated magnetic beads (ThermoFisher Scientific). To prepare ANGPTL4/8 plasmin-generated products in assay buffer, recombinant ANGPTL4/8 (200 nM) was incubated with tPA (0.2 nM) and plasminogen (200 nM) together in Abnova assay buffer at 37 °C for 10 min. The reaction was quenched by adding aprotinin (50 μM). To prepare ANGPTL4/8 plasmin-generated products in culture media from LPL-stable expressing cells, the cells were first incubated with 100 nM of recombinant ANGPTL4/8 for 1 h at 37 °C prior to the addition of 10 nM tPA and 30 nM plasminogen. The cells were incubated at 37 °C for another 30 min. Cell culture media was removed, and the reaction was quenched by adding aprotinin (50 μM). One sample aliquot was used for Western blotting with anti-ANGPTL4 antibodies. Another aliquot was enriched using polyclonal anti-ANGPTL4 antibody covalently coupled to tosyl-activated magnetic beads. Magnetic beads were extensively washed with PBS and captured ANGPTL4 cleavage products were eluted using 1% acetic acid followed by drying the samples under nitrogen to remove acetic acid. Digests of endogenous ANGPTL4 and plasmin-generated ANGPTL4 fragments were characterized as described in *SI Appendix*, *Materials and Methods*.

**Cleavage of ANGPTL4 in the ANGPTL4/8 Complex by Furin versus tPA + Plasminogen.** Recombinant ANGPTL4/8 (200 nM) was incubated at 37 °C for up to 300 min with either tPA (0.2 nM) + plasminogen (200 nM) or furin (200 nM) in assay buffer (50 mM Tris, 100 mM NaCl, 5 mM $CaCl_2$, pH 8.0). In subsequent experiments, 200 nM ANGPTL4/8 or ANGPTL4 was incubated with 67 nM furin for up to 120 min. In each case, at different time points, the reaction was stopped by the addition of an equal volume of SDS-PAGE sample buffer, and proteins were separated electrophoretically and transferred to PVDF membranes. The membranes were probed with biotinylated anti-ANGPTL4 antibodies followed by Alexa 680-labeled streptavidin. Images were recorded using a LI-COR Odyssey imaging system.

**Statistical Analyses.** Statistical analyses were performed as described in *SI Appendix*, *Materials and Methods*.

**Data, Materials, and Software Availability.** All study data are included in the article and/or *SI Appendix*, and raw data have been deposited at MassIVE with the following information: data identifier, MSV000091103; https://doi.org/doi:10.25345/C5X63BF8X; keywords: ANGPTL4/8 and plasminogen.

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