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ASP enhances in situ lipoprotein lipase activity by increasing fatty acid trapping in adipocytes

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Abstract **Acylation-stimulating protein (ASP) increases triglyceride (TG) storage (fatty acid trapping) in adipose tissue and plays an important role in postprandial TG clearance. We examined the capacity of ASP and insulin to stimulate the activity of lipoprotein lipase (LPL) and the trapping of LPL-derived nonesterified fatty acid (NEFA) in 3T3-L1 adipocytes. Although insulin increased total LPL activity (secreted and cell-associated; $P < 0.001$) in 3T3-L1 adipocytes, ASP moderately stimulated secreted LPL activity ($P = 0.04$; 5% of total LPL activity). Neither hormone increased LPL translocation from adipocytes to endothelial cells in a coculture system. However, ASP and insulin increased the V_{max} of in situ LPL activity ([^{3}H]TG synthetic lipoprotein hydrolysis and [^{3}H]NEFA incorporation into adipocytes) by 60% and 41%, respectively ($P \leq 0.01$) without affecting K_m. Tetrahydrolipstatin (LPL inhibitor) diminished baseline, ASP-, and insulin-stimulated in situ LPL activity, resulting in [^{3}H]TG accumulation ($P < 0.0001$). Unbound oleate inhibited in situ LPL activity ($P < 0.0001$) but did not eliminate the ASP stimulatory effect. Therefore, *1*) the clearance of TG-rich lipoproteins is enhanced by ASP through increasing TG storage and relieving NEFA inhibition of LPL; and *2*) the effectiveness of adipose tissue trapping of LPL-derived NEFAs determines overall LPL activity, which in turn determines the efficiency of postprandial TG clearance.**—Faraj, M., A. D. Sniderman, and K. Cianflone. **ASP enhances in situ lipoprotein lipase activity by increasing fatty acid trapping in adipocytes. *J. Lipid Res.* 2004.** 45: **657–666.**

Supplementary key words C3adesArg • insulin • triglyceride • acylation-stimulating protein

Dietary fat circulates in the blood in the form of triglyceride (TG)-rich lipoproteins (chylomicrons), which contain an inner core of 80–95% TG (1). Postprandially, plasma TG increases 2–2.5 times above fasting levels (2, 3) and is rapidly cleared from the plasma by the efficient coupling of two events. The first involves hydrolysis by lipoprotein lipase (LPL), and the second involves uptake and metabolic disposition by local tissue. LPL is situated principally in skeletal muscle and white adipose tissue (WAT). In skeletal muscle, the great majority of nonesterified fatty acids (NEFAs) are oxidized, whereas in WAT, they are esterified to form TG [for review, see ref. (4)].

LPL exerts its lipolytic action while attached to the endothelial glycocalyx (5). LPL is synthesized and secreted from underlying parenchymal cells, mainly adipose and muscle tissue, and is then translocated to the endothelial apical surface (6). Studies of factors affecting LPL activity in WAT have focused primarily on the synthesis and secretion of LPL from the adipocytes. Insulin is a major promoter of LPL activity in that it causes both synthesis and secretion to increase. In vitro studies of murine 3T3-L1 cells have shown that insulin increases LPL activity through an increase in protein synthesis, dimerization, cellular secretion, and increased cell surface-associated mass (7–10). The effects of insulin are both time- and concentration-dependent (9, 10). Furthermore, the release of LPL from adipocytes is enhanced by an endothelial cell-secreted factor that is in turn insulin-dependent (10).

The conventional view is that LPL activity in WAT is the rate-limiting step in the hydrolysis of TG-rich lipoproteins and the uptake of derived NEFAs (11). In fact, this has not always been supported by in vivo findings. In a study examining the effect of epinephrine on LPL activity in humans, the extraction of plasma TG across a subcutaneous WAT bed was increased with epinephrine infusion, indicating an increase in WAT LPL activity. However, LPL-derived NEFAs were not taken up into WAT but released into the circulation (12). The authors concluded that the coordinated reciprocal regulation of NEFA influx and NEFA efflux (resulting from the activation of hormone sensitive lipase (HSL) by epinephrine) is an essential de-

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Abbreviations: ASP, acylation-stimulating protein; CPAE, calf pulmonary artery endothelial; DG, diglyceride; LPL, lipoprotein lipase; NEFA, nonesterified fatty acid; TG, triglyceride; THL, tetrahydrolipstatin.

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terminant of the efficient uptake and esterification of LPL-derived NEFAs. This suggests that factors other than the activity of LPL can determine overall TG clearance and the uptake of released NEFAs.

LPL activity is known to be inhibited by NEFAs (the main product of lipolysis) (13–17). Therefore, an alternative hypothesis is that the rate-limiting step in the hydrolysis and clearance of TG-rich lipoproteins by endothelial LPL is the capacity of underlying adipocytes to take up, esterify, and trap generated NEFAs. That is, effective or in situ LPL activity in WAT will not only be determined by LPL activity in the adipocytes but also by the effectiveness of fatty acid trapping within them. Factors that enhance NEFA uptake and esterification into the adipocytes would then relieve LPL inhibition by NEFAs. The aim of the present study is to determine whether acylation-stimulating protein (ASP) acts in this way.

ASP (also known as C3adesArg) is a lipogenic hormone secreted by adipocytes (18, 19), and the more differentiated the adipocytes, the greater the secretion of ASP (18). The lipogenic effect of ASP has been demonstrated in many cell models, including human skin fibroblasts, human preadipocytes and adipocytes, and 3T3-L1 cells (20–25). ASP increases TG synthesis in a concentration- and time-dependent manner in two ways. First, ASP stimulates the activity of diacylglycerol acyltransferase, the last and possibly the rate-limiting enzyme involved in TG synthesis. Hence, ASP indirectly increases NEFA uptake and esterification without increasing the delivery of NEFAs or acyl-CoA to microsomal enzymes (26, 27). Second, ASP directly increases glucose uptake in cultured human skin fibroblasts (24) and human adipocytes (28). Moreover, ASP also decreases NEFA release from human adipocytes (29) by increasing the fractional reesterification of NEFAs (to the same extent as insulin) and decreasing lipolysis (to a lesser extent than insulin). Thus, the ASP pathway in the adipocytes interacts in a positive feedback mechanism that increases ASP secretion, increases lipogenesis, and decreases lipolysis, the net effect being increased NEFA trapping (i.e., TG storage) within the adipocytes. This effect may be mediated through a recently identified ASP receptor, C5L2, that is expressed in WAT and 3T3-L1 cells, cell models that are responsive to ASP action (25).

Many in vitro and in vivo lines of evidence link ASP to the regulation of postprandial plasma TG clearance. Human studies demonstrated that postprandial local production of ASP from a subcutaneous WAT bed correlated positively with postprandial TG clearance and NEFA trapping across the same bed (30, 31). C3 knockout mice (obligate ASP-deficient) have delayed postprandial TG and NEFA clearance compared with wild-type mice (32, 33). Furthermore, intraperitoneal injection of ASP accelerated postprandial TG clearance in knockout mice as well as wild-type, *db/db*, and *ob/ob* mice (32–36).

In vitro ASP increases NEFA esterification and storage in adipocytes. In vivo observations of *i*) delayed postprandial TG clearance in ASP-deficient mice, *ii*) correction of postprandial clearance with administration of ASP, and *iii*) correlation of WAT ASP production with TG clearance in humans suggest that ASP has a positive effect on TG clearance and hence an effect on LPL activity. Accordingly, in the present study, we tested the capacity of ASP to *a*) directly increase LPL activity or translocation and *b*) influence TG clearance by increasing NEFA trapping/TG storage of LPL-derived NEFAs in 3T3-L1 adipocytes. Our hypothesis was that ASP would increase in situ LPL activity (i.e., TG hydrolysis and NEFA release, uptake, and incorporation) in 3T3-L1 adipocytes by increasing NEFA trapping within the adipocytes, thereby relieving product inhibition of LPL activity.

MATERIALS AND METHODS

Materials

3T3-L1 preadipocytes and calf pulmonary artery endothelial (CPAE) cells were obtained from the American Tissue Culture Collection (ATCC CL-173 and CCL-209, respectively) and frozen in aliquots in liquid N_2. FBS and DMEM/nutrient mixture F12 (DMEM/F12; 1:1 mixture) were obtained from Gibco BRL (Life Technologies, Burlington, Ontario, Canada), and tissue culture plates and six-well plate inserts (polyethylene terephthalate membrane base containing 8×10^5 pores/cm^2; 3 μm pore size) were from Falcon (Becton Dickinson, Franklin Lakes, NJ). Fatty-acid free BSA was obtained from ICN Biomedical (Aurora, OH). [^{3}H]Triolein (glycerol tri-[9,10(N)-^{3}H]oleate) was from Life Sciences, Inc. (Boston, MA), and insulin, dexamethasone, 3-isobutyl-1-methylxanthine, triolein, sodium oleate, egg yolk L-α-phosphatidylcholine, and bovine milk LPL were from Sigma-Aldrich Canada, Ltd. (Oakville, Ontario, Canada). TLC plates (LK5 silica gel 150 A) were from Whatman, Inc. (Clifton, NJ). The LPL inhibitor tetrahydrolipstatin (THL) was a kind gift from Hoffman-La Roche (Mississauga, Ontario, Canada). ASP was prepared by an in-house purification method from fasting human plasma as described previously (30, 37). Cell viability was assessed by the CytoTox 96 nonradioactive cytotoxicity assay (Promega, Madison, WI).

3T3-L1 and CPAE cell culture

3T3-L1 preadipocytes were maintained in growth medium (10% FBS in DMEM/F12 medium) supplemented with streptomycin (100 mg/l) and penicillin (100,000 U/l). Cells were passaged at less than 70% confluence a maximum of five times and plated at a seeding density of 10^5 cells/well in six-well plates (unless otherwise indicated). 3T3-L1 differentiation was induced at 2 days postconfluence by supplementing the growth medium with 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 1.74 μM (10 μg/ml) insulin for the first 2 days followed by 1.74 μM insulin alone for the remaining 5–6 days of differentiation. Adipocytes were used for experiments on the 7th or 8th day of differentiation when complete differentiation was achieved.

For coculture experiments, CPAE cells were grown separately on 4.2 cm^2 inserts (seeding density = 10^6 cells/insert) and maintained in 20% FBS in DMEM/F12 medium supplemented with streptomycin (100 mg/l) and penicillin G (100,000 U/l) for 3–4 days. CPAE cells were cocultured with adipocytes at 1 day after confluence by transferring the CPAE inserts into the six-well plates containing the adipocytes.

ASP/insulin effects on secreted and cell-associated LPL activity in 3T3-L1 adipocytes

Twenty-four hours before the experiment, 3T3-L1 adipocytes were preincubated to remove the insulin. This was accomplished

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by rinsing the adipocyte monolayer and then incubating it with 1% charcoal-stripped, heat-inactivated FBS in DMEM/F12 twice for 20 min (10) and incubating it overnight in the same medium. The next day, adipocytes were washed and incubated with 1% charcoal-stripped FBS in DMEM/F12 with the indicated concentrations of ASP or insulin for the indicated times. The culture medium was then collected and frozen at −80°C for measurement of LPL activity. To measure cell-associated LPL activity, fresh medium (1% charcoal-stripped FBS) containing 100 μg/ml heparin was immediately added to the cells for 45 min at 37°C to release LPL (10). Medium was collected and stored at −80°C for later determination of LPL activity (cell-associated LPL activity).

For coculture experiments, inserts with CPAE cells were preincubated for 4 h in 1% charcoal-stripped FBS in DMEM/F12. Inserts were then transferred to six-well plates containing 3T3-L1 adipocytes incubated in 1% charcoal-stripped FBS in DMEM/F12 with 3% BSA. For treatment, insulin and ASP were added to both chambers (upper endothelial cells and lower adipocytes). After the incubation period, medium in both chambers was collected separately and stored at −80°C for measurement of LPL activity. For the measurement of LPL activity translocated to the endothelial cells, inserts were transferred to an empty six-well plate, and heparin (100 μg/ml) was added for 45 min at 37°C to displace cell-associated LPL; the medium was then frozen at −80°C. Adipocytes were treated as described above. At the end of each coculture experiment, the endothelial cells were stained with 2% toluidine blue to verify the uniformity of the monolayer (6).

Measurement of in vitro LPL activity

LPL activity was assayed in vitro by a modification of the method originally described by Nilsson-Ehle and Schotz (38). Medium LPL activity was measured in duplicate by adding 150 μl of sample to 100 μl of fresh [^{3}H]triolein substrate (synthetic TG-rich lipoprotein) in a 12 × 75 mm glass test tube. The [^{3}H]triolein substrate contained 125 μg of triolein, 0.25 μCi of [^{3}H]triolein, and 6 μg of phosphatidylcholine; it was dried and then emulsified by sonication in 100 μl of aqueous buffer (0.54 M Tris-HCl, pH 8.2, 2.2% BSA, and 7.5% fasting human serum). This generates large phospholipid micelles with a TG/phospholipid molar ratio of 17.3:1 (i.e., 95% TG) (39). Fasting human serum used as a source of LPL activator apolipoprotein C-II (apoC-II) was heat inactivated at 56°C for 1 h before use to inactivate any endogenous LPL. The reaction mixture was gently mixed and incubated in a shaking water bath at 37°C for 60 min to generate [^{3}H]NEFA. The assay was then stopped, and the released [^{3}H]NEFAs were extracted as described by Belfrage and Vaughan (40). Radioactivity in the upper aqueous phase was counted, and LPL activity was calculated based on an LPL standard curve (generated using purified LPL of known activity) (Sigma). LPL activity per well is expressed as micromoles of NEFA released per well per minute. Maximum hydrolysis was less than 15%, which was a requirement for efficient Belfrage extraction (40). To assess [^{3}H]triolein purity, [^{3}H]triolein substrate was extracted with chloroform-methanol (2:1) and separated using thin-layer chromatography. Background [^{3}H]NEFA in the [^{3}H]TG substrate constituted 0.19 ± 0.11% of total counts.

ASP/insulin effects on in situ LPL activity in 3T3-L1 adipocytes

To assess the whole process of hydrolysis of synthetic TG-rich lipoproteins and the uptake and esterification of generated NEFAs in 3T3-L1 (referred to throughout as in situ LPL activity), [^{3}H]triolein substrate was added directly to the 3T3-L1 adipocytes. [^{3}H]Triolein was prepared as described above using 0.54 M Tris-HCl, pH 7.2, in DMEM/F12, 5.1% BSA, and 7.5% fasting human serum. Serial dilutions of the [^{3}H]triolein substrate were then carried out in serum-free medium to achieve the appropriate concentrations [TG concentration = 0.02–1.26 mM and apoC-II concentration = ∼0.26–2.67 μg/ml, estimated from the average human plasma apoC-II concentration of 40 ± 20 mg/l (41)]. BSA concentration was increased to avoid limiting the rate of lipolysis as a result of the lack of available BSA binding sites for NEFAs (six binding sites per albumin molecule) (42). Thus, the number of binding sites on albumin available per molecule of fatty acid (assuming total TG hydrolysis) was maintained at >1 in all concentrations of [^{3}H]triolein substrate used [BSA/NEFA ratio was calculated as mM BSA × 6/mM TG × 3 (42)].

Cells were washed and incubated with [^{3}H]triolein substrate in the presence or absence of ASP and insulin. Medium [^{3}H]NEFA and [^{3}H]NEFA incorporation into the adipocytes were measured. The sum of these two fractions represents measured in situ LPL activity. [^{3}H]NEFA was extracted from medium using 5 volumes of chloroform-methanol (2:1, v/v). [^{3}H]NEFA was separated on TLC plates, and spots were identified (based on standards), scraped, and counted. Adipocytes were washed three times with ice-cold PBS, and cell ^{3}H-labeled lipids were extracted with heptane-isopropanol (2:3). Aliquots were dried down, redissolved in chloroform-methanol (2:1), and directly counted (for total lipids) or separated by TLC for measurement of intracellular [^{3}H]TG, [^{3}H]diglyceride (DG), and [^{3}H]NEFA.

Inhibition of LPL activity in 3T3-L1 adipocytes by THL, absence of serum, and high concentrations of NEFAs

THL is a specific mammalian lipase inhibitor (43, 44). THL was prepared before each experiment by dissolving in 94% ethanol and slowly diluting with warm serum-free medium (37–40°C). THL solution was mixed with the [^{3}H]triolein LPL substrate (0.63 mM substrate concentration at V_{max}) at 37°C and left to stand for 5–10 min before addition to the adipocytes. The final cellular ethanol concentration was 0.2%. The inhibition of LPL activity by the absence of the LPL cofactor/activator apoC-II (45) was examined by preparing the [^{3}H]triolein substrate in the absence of serum.

Finally, the inhibition of in situ LPL activity was examined using oleic acid (0.35–4.15 mM) that was added free or bound to 0.69 mM BSA (i.e., at increasing oleate/BSA molar ratios of 0.5–6). Oleic acid was added to the adipocytes concomitantly with the [^{3}H]triolein substrate (0.28 mM substrate concentration at V_{max}) and incubated for 2 h, and in situ LPL activity was measured as described above. Incubation medium was extracted with chloroform-methanol, and [^{3}H]TG was separated on TLC plates to measure [^{3}H]triolein substrate left after the incubation period as a further verification of the change in the amount of [^{3}H]triolein substrate hydrolyzed. As free NEFAs have a detergent effect that may increase adipocyte permeability and eventually death, cell viability in the presence or absence of free NEFAs was determined using a commercial kit (CytoTox 96 nonradioactive cytotoxicity assay). This assay measures the amount of released lactate dehydrogenase (LDH), which is a cytosolic enzyme released upon cell death. The amount of background LDH release under each incubation condition was compared with that after total cell death induced by 1% Triton X-100.

Statistical analyses

Data presented represent averages of three or more experiments (measured in duplicate) and are expressed as averages ± SEM. Nonlinear regression curves for in situ LPL activity were generated using an equation for hyperbolic data (one-binding site), and V_{max} and K_m were calculated using GraphPad Prism software (version 3.03). Curves were analyzed for significance by two-way ANOVA, and bars were analyzed by unpaired Student's *t*-test. Significance was set at $P \leq 0.05$ and a power of ≥80%.

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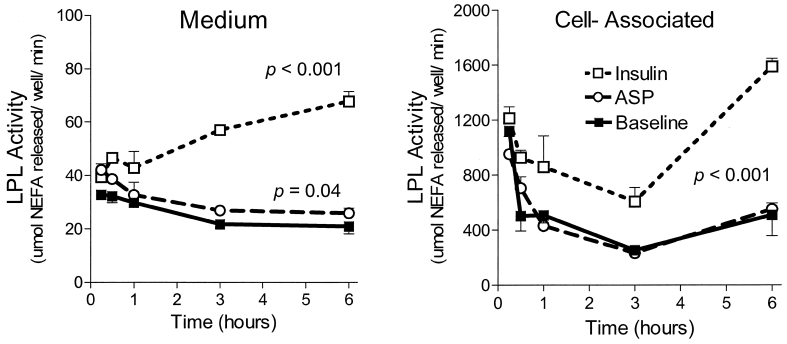

Fig. 1. Time curves of the effects of acylation-stimulating protein (ASP) and insulin on in vitro lipoprotein lipase (LPL) activity in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated over the time course of 15 min to 6 h with 1% charcoal-stripped FBS (baseline) or with ASP (1 μM) or insulin (1 nM). In vitro LPL activity was measured in medium and cell-associated fractions (heparin wash). $P = 0.04$ for ASP (medium) and $P < 0.001$ for insulin (medium and cell-associated) versus baseline. NEFA, nonesterified fatty acid. Error bars represent ± SEM.

RESULTS

LPL activity in 3T3-L1 adipocytes

We first compared the effects of insulin and ASP on secreted (medium) and cell-associated (heparin-releasable) LPL activity in 3T3-L1 adipocytes over a period of 6 h (**Fig. 1**). Medium containing secreted LPL activity was collected after the incubation period, cell-associated LPL was dissociated from the cell surface using a heparin wash, and LPL activity was measured in vitro in both compartments. Insulin (1 nM) markedly increases LPL activity both secreted by and associated with 3T3-L1 adipocytes ($P < 0.001$) in a time-dependent manner. On the other hand, ASP (1 μM) had only a small effect on secreted LPL activity (medium) (increased by 21.0 ± 6.4%; $P = 0.04$) and did not affect cell-associated LPL activity. On average, the amount of LPL activity secreted by the adipocytes (medium) represented a small fraction (5.7 ± 0.5%) of total LPL activity (medium plus cell-associated). In addition, neither insulin nor ASP changed the percentage distribution of cell-associated or secreted LPL activity (average percentage secreted LPL activity/total LPL activity through all time points: baseline = 5.7 ± 0.5% versus ASP = 6.7 ± 0.7% and insulin = 5.6 ± 0.7; P = NS). As the effect of ASP on secreted LPL activity was early (15 min) and constant over time, we examined whether ASP stabilized LPL activity after its secretion from the adipocytes (i.e., whether ASP prevents LPL inactivation at 37°C). Medium bathing the adipocytes was collected and incubated with or without ASP and insulin over a range of time points (0, 5, 15, 30, 60, 120, and 240 min). Neither ASP nor insulin prevented the heat inactivation of adipocyte-secreted LPL at any time point examined (percentage LPL activity remaining after 240 min of incubation: baseline = 44.5 ± 15.6% versus ASP = 30.6 ± 2.7% and insulin = 26.3 ± 2.7%; P = NS).

The effect of increasing concentrations of ASP and insulin on LPL activity in 3T3-L1 adipocytes at 3 h is shown in **Fig. 2**. Whereas insulin (≥1 nM) markedly increases LPL activity in both measured fractions in a concentration-dependent manner by 240% at the highest concentration used, ASP slightly increased medium LPL activity but did not affect cell-associated and thus total LPL activity in 3T3-L1 adipocytes at any concentration used.

LPL activity in a coculture system of 3T3-L1 adipocytes and CPAE cells

In vivo, LPL is secreted by adipocytes and is translocated to the endothelial cell surface. Using a double-chamber coculture system of adipocytes (lower chamber) and endothelial cells (upper chamber), we examined whether ASP or insulin would increase the translocation

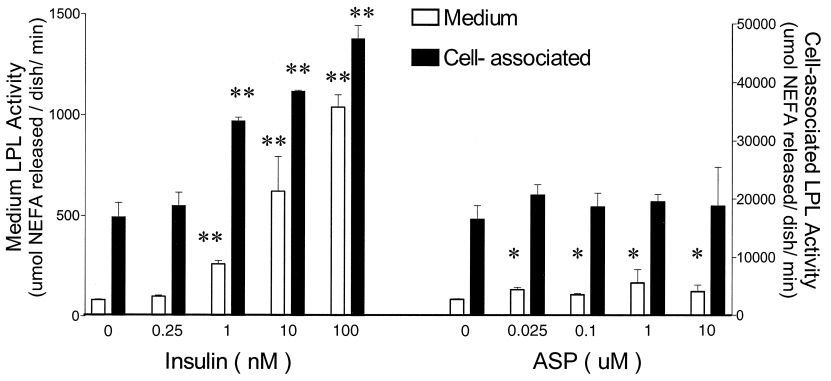

Fig. 2. ASP and insulin effects on in vitro LPL activity in 3T3-L1 adipocytes. 3T3-L1 adipocytes (100 mm dishes) were incubated for 3 h with 1% charcoal-stripped FBS (baseline) or with ASP or insulin at the indicated concentrations. In vitro LPL activity was measured in medium and cell-associated fractions (heparin wash). * $P < 0.05$ for ASP (medium) and ** $P < 0.001$ for insulin (medium and cell-associated) fractions at concentrations of ≥1 nM versus baseline. Error bars represent ± SEM.

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of LPL activity from adipocytes to endothelial cells. At the end of the incubation period, LPL activity in the medium of both chambers and cell-associated LPL activity (adipocytes and endothelial cells) was measured in vitro and compared with LPL activity in adipocytes cultured without endothelial cells.

There was no LPL activity detected in endothelial cells cultured alone before their incubation with 3T3-L1 adipocytes. After 3 h of incubation with the adipocytes, LPL activity was detected in the endothelial cell chamber (mostly heparin wash), indicating a translocation of LPL from underlying adipocytes. However, the total LPL activity translocated to endothelial cells (both medium and cell-associated) represents only a small fraction of total adipocyte LPL activity (1–4%). This finding is consistent with previous reports by Saxena, Klein, and Goldberg (6) using ^{125}I-LPL. In the coculture system, as with the adipocytes alone, insulin increased total LPL activity of 3T3-L1 adipocytes but ASP did not. However, neither insulin nor ASP enhanced the translocation of LPL activity to the endothelial cell monolayer (percentage translocation of total LPL activity in 3T3-L1 adipocytes to endothelial cells: baseline = 3.9 ± 0.6% versus ASP = 3.2 ± 0.6% and insulin = 2.7 ± 0.5%; P = NS). It should be noted, however, that our results are not in disagreement with the previously reported observation that insulin increases the fast release (maximum effect at 15 min) of an endothelial factor that in turn increases the mobilization of LPL from adipocytes (10). The effect of insulin on the translocation of LPL activity from adipocytes to endothelial cells was not determined in that study. Whether insulin does increase the early (15 min) translocation of LPL from adipocytes to endothelial cells remains to be determined.

[^{3}H]Triolein substrate hydrolysis and [^{3}H]NEFA release and incorporation in 3T3-L1 adipocytes

We then examined the effect of ASP and insulin on in situ LPL activity in the 3T3-L1 adipocytes. Adipocytes were incubated with increasing concentrations of [^{3}H]triolein substrate for 4 h at 37°C, and the amounts of [^{3}H]NEFAs released into the medium and incorporated into the adipocyte lipid fractions were measured (**Fig. 3**). In situ LPL activity is defined as medium [^{3}H]NEFA plus cellular [^{3}H]lipid (in the adipocytes), which represent measured [^{3}H]NEFAs liberated from the hydrolysis of the [^{3}H]triolein substrate.

In situ LPL activity increased in a substrate concentration-dependent manner (Fig. 3). Maximum in situ LPL activity (V_{max}) over the period of 4 h was calculated to be 118.0 ± 10.3 μmol NEFAs released/mg protein at baseline. Both ASP and insulin significantly increased in situ LPL activity (V_{max}) by 60% and 41%, respectively (ASP, V_{max} = 188.5 ± 17.9 μmol NEFA/mg protein, $P < 0.0001$; insulin, V_{max} = 166.1 ± 15.2 μmol NEFA/mg protein, $P = 0.01$). On the other hand, K_m (TG concentration at half-maximal velocity of LPL activity) was not affected by either ASP or insulin treatment (0.07 ± 0.03 mM TG versus baseline = 0.05 ± 0.02 mM; P = NS). It is important to point out that the hydrolysis of the [^{3}H]triolein substrate

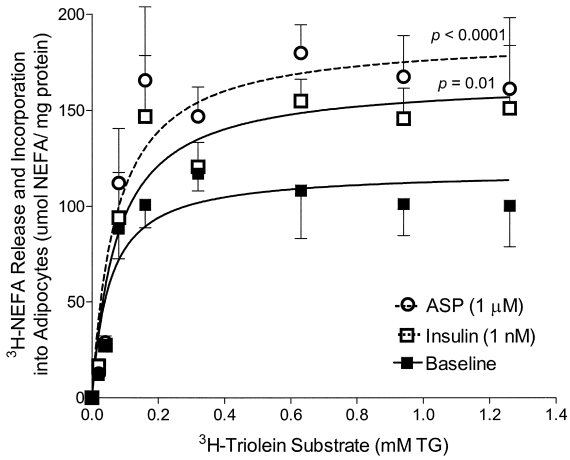

Fig. 3. Effect of ASP and insulin on in situ LPL activity in 3T3-L1 adipocytes. Cells were incubated for 4 h with [^{3}H]triolein substrate at the indicated concentrations in DMEM/nutrient mixture F12 (F12) alone (baseline) or with ASP (1 μM) or insulin (1 nM). In situ LPL activity represents the sum of [^{3}H]NEFA released and [^{3}H]NEFA incorporated into the adipocytes. Curves were generated using a nonlinear regression equation for hyperbolic data. Calculated ASP V_{max} = 188.5 ± 17.9 μmol NEFA/mg protein ($P < 0.0001$) and insulin V_{max} = 166.5 ± 15.2 μmol NEFA/mg protein ($P = 0.01$) both versus baseline V_{max} = 118.0 ± 10.3 μmol NEFA/mg protein. TG, triglyceride. Error bars represent ± SEM.

above 0.6 mM TG was on average only 10.83 ± 0.92% for ASP and insulin treatments; thus, [^{3}H]triolein substrate remaining after 4 h was not limiting for LPL activity.

The time course for the ASP and insulin effects on in situ LPL activity is shown in **Fig. 4**. [^{3}H]Triolein substrate at a concentration of 0.63 mM (TG concentrations > 2× K_m) was added to the adipocytes with or without ASP (1 μM) or insulin (1 nM). [^{3}H]Triolein substrate hydrolysis coupled to [^{3}H]NEFA incorporation into the adipocyte lipids was very efficient, such that, even at 15 min, ~70% of total recovered ^{3}H label was in the cellular lipid extract, mainly as cell [^{3}H]TG (range, 65–70% of total intracellular lipids). Both the ASP- and insulin-induced increases in in situ LPL activity were time-dependent (ASP, 69% increase at 6 h versus 24% at 1 h; $P = 0.003$). Stimulation of in situ LPL activity by ASP was significant in medium and all cellular lipid fractions measured (TG, DG, and NEFA; $P < 0.01$).

Inhibition of in situ LPL activity in adipocytes by THL and absence of LPL activator (apoC-II)

LPL has been shown to anchor lipoproteins and increase their uptake into peripheral tissue, an action that is independent of its lipolytic activity (46, 47). To demonstrate that the measured label is truly the lipolytic product of LPL and that ASP and insulin affect in situ LPL activity and not the uptake of whole particles, we used THL to inhibit LPL activity. THL induces the tetramerization and inactivation of LPL. THL does not affect the binding of LPL to heparin but does increase the binding of LPL to lipid droplets (44). As shown in **Fig. 5** (inset), THL effectively inhibited the activity of purified LPL in vitro. The

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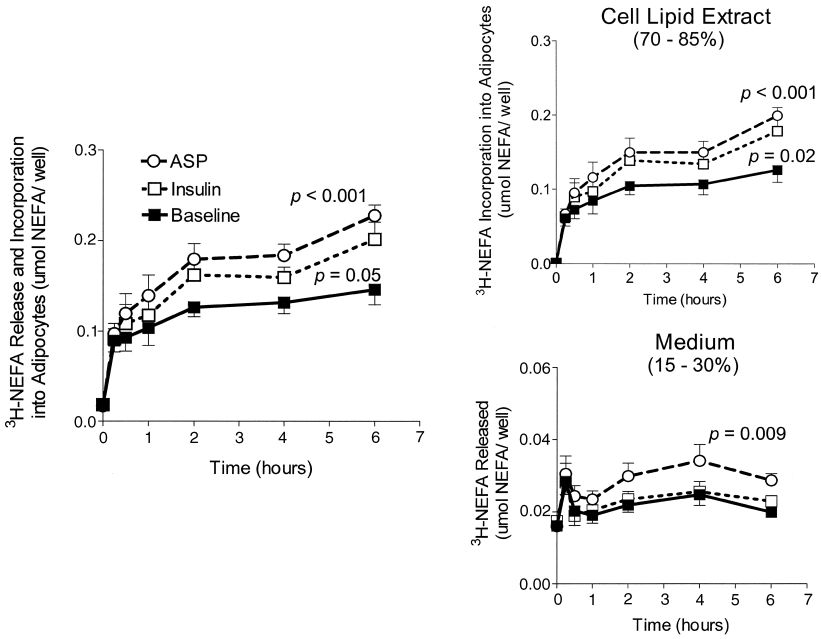

Fig. 4. Time curves of the effects of ASP and insulin on in situ LPL activity in 3T3-L1 adipocytes. Cells were incubated for up to 6 h with [^{3}H]triolein substrate (0.63 mM TG) in DMEM/F12 alone (baseline) or with ASP (1 μM) or insulin (1 nM). The left panel shows the curve for in situ LPL activity. The right panels show the curves for the components of in situ LPL activity: [^{3}H]NEFA incorporated into the adipocytes (top) and [^{3}H]NEFA released into the incubation medium (bottom). $P < 0.01$ for ASP and $P \leq 0.05$ for insulin (except medium) versus baseline. Error bars represent ± SEM.

addition of THL to adipocytes inhibited in situ LPL activity (represented by [^{3}H]NEFA release and incorporation into the adipocytes) by more than 70% at both time points examined (2 and 4 h; $P < 0.0001$ for both) (Fig. 5). Furthermore, THL totally abolished the stimulatory effects of both ASP and insulin on in situ LPL activity. Similarly, the absence of serum (source of the LPL activator apoC-II) equally decreased baseline, ASP-, and insulin-stimulated in situ LPL activity (58%; $P < 0.001$).

Inhibition of in situ LPL activity in adipocytes by NEFAs

We hypothesized that ASP, as an activator of NEFA trapping, increases the hydrolysis of TG-rich lipoproteins in adipocytes by relieving the inhibitory effect of NEFAs on in situ LPL activity. If this is the case, then the addition of high concentrations of NEFAs to the adipocytes should decrease in situ LPL activity measured as the hydrolysis of [^{3}H]triolein and the incorporation of LPL-derived [^{3}H]NEFAs into the adipocytes.

As shown in **Fig. 6** (left panel), we added increasing concentrations of unbound oleate along with 0.28 mM [^{3}H]TG substrate (TG concentration at $V_{max} > 2\times K_m$). The addition of NEFAs (unbound to BSA) inhibited baseline in situ LPL activity (i.e., [^{3}H]NEFA release and incorporation into the adipocytes) in a concentration-dependent manner (up to 38% inhibition of baseline in situ LPL activity at the highest NEFA concentration used; $P < 0.0001$). This suggests that NEFAs decrease the V_{max} of in situ LPL activity. Although free NEFAs decreased ASP-stimulated in situ LPL activity ($P < 0.0001$), in situ LPL activity remained greater with ASP incubation at all concentrations of NEFAs used ($P < 0.0001$). The inhibitory effect of NEFAs on in situ LPL activity was further verified by measuring the amount of [^{3}H]TG substrate remaining in the medium after the incubation period of 2 h (Fig. 6, top right panel). At baseline (without oleate), ~50% of the TG substrate was hydrolyzed. With increasing concentrations of NEFAs added, there was a concentration-dependent increase in the amount of [^{3}H]TG left in the medium ($P < 0.0001$), with NEFAs being less inhibiting in the presence of ASP. It should be noted, however, that although free NEFAs were added to the adipocytes, BSA is available in the incubation medium because [^{3}H]TG substrate is prepared with excess BSA. However, the number of available binding sites on BSA per molecule of NEFA becomes increasingly limited with increasing concentrations of NEFAs. The ratio of BSA to NEFA decreases from 1.1 to 0.19 (from 0 to 4.15 mM NEFA). As free NEFAs may have a detergent effect on the cell, increasing cell permeability and eventually death, we examined the effects of free NEFAs on cell viability. Free NEFAs did not decrease cell viability, as the amount of LDH released with increasing concentrations of free NEFAs did not increase beyond that of background (i.e., in DMEM/F12) (Fig. 6, bottom right panel).

On the other hand, adding increasing concentrations of bound oleic acid (constant BSA of 0.69 mM) before its incubation with the [^{3}H]triolein substrate eliminated the inhibitory effect of NEFAs on in situ LPL activity (baseline = 0.13 ± 0.01 μmol NEFA released/well versus 4.15 mM NEFA = 0.12 ± 0.01 μmol NEFA released/well; P = NS).

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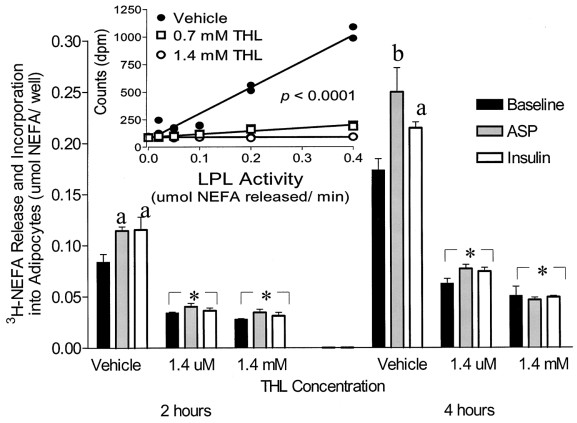

Fig. 5. Tetrahydrolipstatin (THL) inhibition of in vitro LPL activity and in situ LPL activity in 3T3-L1 adipocytes. In situ LPL activity was determined in 3T3-L1 adipocytes incubated with [^{3}H]triolein substrate (0.63 mM TG) for 4 h with or without ASP (1 μM) or insulin (1 nM) in vehicle alone (DMEM/F12 plus 0.2% ethanol) or with the indicated THL concentrations. [a] $P < 0.05$ and [b] $P = 0.004$ for ASP and insulin effects versus baseline, and $*P < 0.0001$ for THL effect versus vehicle under all conditions used. The inset shows in vitro inhibition of purified LPL activity by THL. Purified LPL activity was measured for 60 min in vehicle alone (DMEM/F12 plus 0.2% ethanol) or with the indicated THL concentrations. $P <$ 0.0001 for THL inhibition of LPL activity with both concentrations used. Error bars represent ± SEM.

Excess BSA binding sites are available to NEFAs in this system, as the ratio of BSA to NEFA is maintained at greater than 1.1 at all concentrations of NEFAs used (except at 4.1 mM NEFA, which has a ratio of 0.83).

DISCUSSION

The North American diet includes a considerable amount of fat (50–90 g/day on average) (48). Dietary fat circulating in plasma as TG-rich lipoproteins is either used to meet daily requirements or is stored in WAT as TG. The conventional view is that LPL is the gatekeeper of NEFA distribution, as LPL activity is essential for hydrolysis; it is also the rate-limiting step in the clearance of TG-rich lipoproteins and for the storage of released NEFAs in adipocytes (11).

In the present study, we have shown that insulin, but not ASP, increases cell-associated LPL activity. On the other hand, ASP, through a stimulatory effect on NEFA trapping in situ in 3T3-L1 adipocytes, enhances the hydrolysis of synthetic TG-rich lipoproteins to the same level as insulin. THL markedly reduces [^{3}H]NEFA release and incorporation into the adipocytes and abolishes ASP and insulin stimulatory effects. This result suggests that the stimulatory effects of ASP and insulin are not based on enhanced substrate bridging and uptake of whole nonhydrolyzed particles but on [^{3}H]triolein hydrolysis, which represents a prerequisite for the detection of the measured label. Unquestionably, LPL activity is an indispensable first step in the clearance of TG-rich lipoproteins. However, our data demonstrate that the clearance of TG from the medium can be enhanced by increasing the trapping of LPL-derived NEFAs within the adipocytes without necessarily increasing LPL in the adipocytes. Efficient postprandial TG clearance requires the coordination of both steps, and both insulin and ASP enhance the process through different mechanisms.

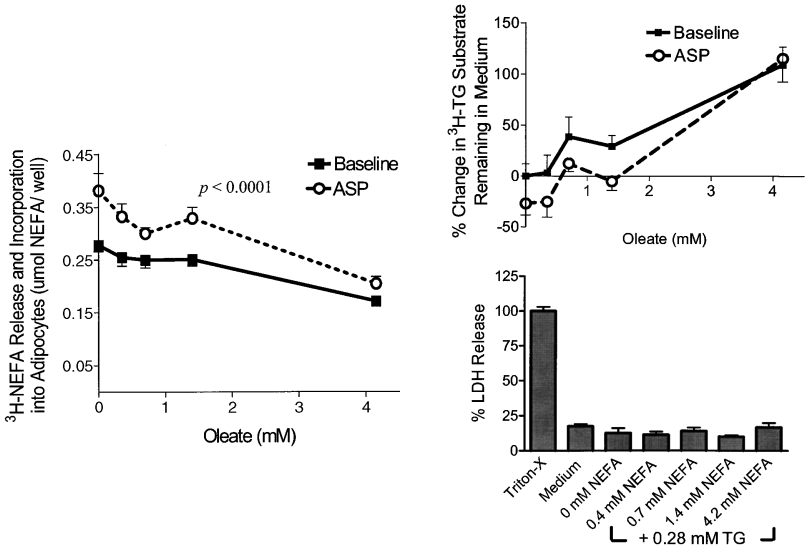

Fig. 6. NEFA inhibition of in situ LPL activity in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated for 2 h with [^{3}H]triolein substrate (0.28 mM TG) and increasing concentrations of free oleate in DMEM/F12 alone (baseline) or with ASP (1 μM). The left panel shows in situ LPL activity at baseline and with ASP ($P <$ 0.0001 for NEFA effect and $P <$ 0.0001 for ASP effect versus baseline). The right top panel shows percentage increase in [^{3}H]TG concentration remaining in medium after the incubation period at baseline and with ASP ($P <$ 0.001 for NEFA effect versus baseline). The right bottom panel shows percentage lactate dehydrogenase (LDH) release at each incubation condition compared with LDH release after total cell death induced by 1% Triton X-100. Error bars represent ± SEM.

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Insulin increases cell-associated LPL activity by ~3-fold, a finding that is consistent with previous data demonstrating that insulin is a primary regulator of LPL activity through different mechanisms involving posttranscriptional effects (increasing mRNA stability) (9) as well as posttranslational effects (increased protein synthesis, shift from inactive to active forms through dimerization and translocation to the cell surface) (49). Indeed, a number of other hormones, such as tumor necrosis factor-α (50), growth hormone (51), and peroxisome proliferator-activated receptor δ agonists (52), as well as nutritional status (49, 50, 53, 54) have been proposed to stimulate or inhibit via similar mechanisms.

By contrast, ASP does not enhance cell-associated LPL activity. Nevertheless, both ASP and insulin increase in situ LPL activity through increased NEFA trapping as stored TG. It should be pointed out, however, that the increase in in situ LPL activity produced by insulin was smaller than anticipated by the increase in in vitro LPL activity alone (41% versus 100% increase, respectively, at an insulin concentration of 1 nM). Thus, LPL activity alone is unlikely to be the rate-limiting step in the clearance of TG-rich lipoproteins; otherwise, the hydrolysis of TG-rich lipoproteins in situ should parallel the amount of available LPL activity in the adipocytes. Furthermore, overloading the adipocytes with unbound NEFAs diminished in situ LPL activity, but to a lesser extent in the presence of ASP. Thus, the efficiency of NEFA trapping by adipocytes appears to contribute to the rate of LPL activity and, consequently, the clearance of TG-rich lipoproteins. Further supporting this finding is the result that the amount of LPL-released NEFAs remaining in the medium is small (15–30%) compared with the amount of NEFAs incorporated into adipocyte lipids (70–85%) even after short incubation times. Thus, these findings emphasize the importance of cellular uptake and storage in the overall regulation of TG clearance. Of interest, binding NEFAs to BSA before their addition to the adipocytes did not have an inhibitory effect on in situ LPL activity. This suggests that in vivo, NEFAs immediately released from TG hydrolysis that is unbound to circulating albumin have a greater inhibitory effect on LPL activity than circulating NEFAs bound to albumin.

Another point that should be emphasized is that the decrease in NEFA release from adipose tissue is suggested to be an important determinant of increased LPL-derived NEFA uptake and esterification (12). Both ASP and insulin decrease NEFA release from human adipocytes by increasing the fractional reesterification of NEFAs (to a similar extent for both ASP and insulin) and decreasing lipolysis/HSL activity (more so for insulin than for ASP) (29). Thus, in addition to increasing TG synthesis by ASP and insulin, decreasing the lipolysis of endogenous TG by both hormones may be another mechanism that would favor the increased trapping of LPL-derived NEFAs.

As with many enzymes, the concentration of substrate, cofactors, and products are important in determining overall lipolytic rate. The concentration and type of substrate (chylomicrons versus VLDLs) affect hydrolysis. Chylomicrons bind LPL more tightly and are hydrolyzed more rapidly and completely than VLDLs (55). ApoC-II and apoC-III are important as activator and inhibitor, respectively (1), and the activating role of apoC-II is well demonstrated in apoC-II-deficient humans (56). Of equal importance, however, is the posttranscriptional regulation of LPL activity by its main product, fatty acids.

In vitro, NEFAs inhibit LPL activity (13, 14, 17, 54), and in murine adipocytes, NEFAs additionally inhibit LPL secretion (17). Furthermore, NEFAs interfere with the binding of LPL both to its activator apoC-II and to heparin sulfate proteoglycan anchors (16), decreasing LPL transport to endothelial cells (6) and displacing it from the endothelial cell surface (15, 54, 57). On the other hand, inhibition of chylomicron and VLDL hydrolysis can block the effect of NEFAs on LPL displacement from endothelial cells (15). In vivo, LPL (primarily inactive) is released from both WAT and muscle according to nutritional status (58). Rapid infusions of lipid emulsions, causing massive increases in plasma NEFAs, displace LPL from the endothelial surface and increase its activity in plasma (59). The displacement of LPL from the cell surface is postulated to prevent the oversupply of NEFAs to peripheral tissues under conditions of excessive lipolysis (59, 60). Thus, there are regulatory mechanisms that operate simultaneously to prevent the flooding of intracellular NEFAs beyond the capacity of cells to assimilate them. The efficient functioning of LPL requires effective channeling of NEFAs into the adjacent cells, such as adipocytes, where they are rapidly esterified to TG. In fact, the channeling of LPL-released NEFAs into adipocytes (and away from the general circulation) is most effective when it is most needed: it is more efficient postprandially than in the fasting state (61). In vivo, postprandial adipose trapping of LPL-generated NEFAs was shown to be close to zero at fasting and close to ~100% at 1 h, decreasing to 10–30% by 6 h (62).

Finally, the deleterious effects of high levels of NEFAs on LPL activity go beyond postprandial TG clearance. In addition to the role of LPL in dietary fat hydrolysis, LPL has functions related to vitamin uptake (especially retinoids) and particle uptake via bridging functions in tissues such as adipose, muscle, liver, and vascular wall (63, 64). It has been suggested that increased LPL-generated NEFAs in the vascular wall may be associated with atherosclerotic complications (65, 66). Increased circulating NEFAs also contribute to insulin resistance. One consequence is compromised WAT LPL, which becomes refractory to insulin activation in the postprandial period in insulin-resistant subjects (67, 68). This leads to fat deposition in other tissues, especially skeletal muscle and liver (67). Whether any of these potential alterations in NEFA flux are associated with changes in the ASP pathway, or whether ASP could potentially counteract any of these effects, is unknown. On the other hand, there is some evidence that ASP "resistance" may contribute to the dysfunction. A number of recent studies demonstrate associations between plasma ASP, C3 (precursor to ASP), NEFAs, and insulin-glucose homeostasis (69). Finally hyper-apoB subjects are characterized by increased apoB, NEFAs, and

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ASP, and a subset demonstrate cellular resistance to the binding and action of ASP (22). The recent description of a cell surface receptor for ASP will shed light in this area (25).

In summary, this study demonstrates that ASP does not directly increase LPL activity in 3T3-L1 adipocytes, as does insulin. Nevertheless, both ASP and insulin increase maximum in situ LPL activity in 3T3-L1 adipocytes (i.e., TG hydrolysis and NEFA incorporation) by increasing the trapping of LPL-derived NEFAs within the adipocytes. Furthermore, it suggests that LPL cannot hydrolyze TG-rich lipoproteins beyond the capacity of the adipocytes to assimilate generated NEFAs and that a major part of the regulatory effect of ASP is to relieve the inhibitory effect of NEFAs on in situ LPL activity in the adipocytes. These findings underscore the coordinated regulation of TG-rich lipoprotein hydrolysis and storage by the adipocytes, the role of WAT in "buffering" the daily influx of dietary fat entering the circulation, and the autocrine role of ASP in this process.

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