Differential regulation of fatty acid trapping in mouse adipose tissue and muscle by ASP

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Faraj, May, and Katherine Cianflone. Differential regulation of fatty acid trapping in mouse adipose tissue and muscle by ASP. Am J Physiol Endocrinol Metab 287: E150–E159, 2004; 10.1152/ajpendo.00398.2003.—Acylation-stimulating protein (ASP) is a lipogenic hormone secreted by white adipose tissue (WAT). Male C3 knockout (KO; C3−/−) ASP-deficient mice have delayed postprandial triglyceride (TG) clearance and reduced WAT mass. The objective of this study was to examine the mechanism(s) by which ASP deficiency induces differences in postprandial TG clearance and body composition in male KO mice. Except for increased 3H-labeled nonesterified fatty acid (NEFA) trapping in brown adipose tissue (BAT) of KO mice (P = 0.02), there were no intrinsic tissue differences between wild-type (WT) and KO mice in 3H-NEFA or [14C]glucose oxidation, TG synthesis or lipolysis in BAT, muscle, or liver. There were no differences in WAT or skeletal muscle hydrolysis, uptake, and storage of [3H]triolein substrate [in situ lipoprotein lipase (LPL) activity]. ASP, however, increased in situ LPL activity in WAT (+64.8%; P = 0.02) but decreased it in muscle (−35.0%; P = 0.0002). In addition, after prelabeling WAT with [3H]oleate and [14C]glucose, ASP increased 3H-lipid retention, [3H]TG synthesis, and [3H]TG-to-[14C]TG ratio, whereas it decreased 3H-NEFA release, indicating increased NEFA trapping in WAT. Conversely, in muscle, ASP induced effects opposite to those in WAT and increased lipolysis, indicating reduced NEFA trapping within muscle by ASP (P < 0.05 for all parameters). In conclusion, novel data in this study suggest that ASP differentially regulates in situ LPL activity and NEFA trapping in WAT and skeletal muscle, which may promote optimal insulin sensitivity in vivo.

Acylating-stimulating protein; C3adesArg; fatty acid; triglyceride; lipoprotein lipase; brown adipose tissue

FAT STORAGE OR FATTY ACID TRAPPING in white adipose tissue (WAT) is determined by the equilibrium of two reciprocal metabolic processes, triglyceride (TG) synthesis and lipolysis. Acylation-stimulating protein (ASP; aka C3adesArg) is a WAT-derived hormone (36) that markedly affects the reciprocal rates of both TG synthesis and lipolysis. ASP enhances TG synthesis in a concentration- and time-dependent manner in a number of cell models, including human preadipocytes and adipocytes and murine 3T3-L1 preadipocytes (3, 25, 36, 64). This effect is mediated by two mechanisms. First, ASP stimulates the activity of diacylglycerol acyltransferase, the last enzyme involved in TG synthesis and believed to be the rate-limiting step and, hence, indirectly increases nonesterified fatty acid (NEFA) uptake and esterification (68). Second, ASP directly increases glucose uptake and transport in cultured human skin fibroblasts (25), human adipocytes (36), and rat muscle cells (62). On the other hand, a study in human adipocytes demonstrated that ASP also decreases lipolysis and increases fractional reesterification of NEFA (63). The effects of ASP are likely mediated by a recently identified receptor, C5L2 (aka gpcr 77), that is expressed in human WAT, 3T3-L1 preadipocytes, and human skin fibroblasts, cell models that are responsive to ASP action (30).

C3 knockout (KO) mice are necessarily ASP deficient and manifest many metabolic aberrations indicative of subnormal fat storage. With an acute oral fat challenge, male KO (C3−/−) 129sv-backcrossed mice demonstrate a distinct delay in TG and NEFA clearance, whereas female KO mice (C3−/−) or C57Bl/6 mice (C3−/−) do not (15, 42, 43). Acute administration of ASP by intraperitoneal injection enhances postprandial TG clearance not only in male KO mice (42) but also in WT C57Bl/6 mice (41) and two obese mice models examined (ob/ob and db/db) (54). In contrast, the long-term effect of ASP deficiency on fat storage is evident in altered body composition in all KO mice examined (regardless of sex or genotype). Female KO mice have reduced body weight and WAT mass, lower leptin concentration, and higher insulin sensitivity (15, 40). Male KO mice also have reduced WAT mass, lower glucose and leptin concentrations, and higher insulin sensitivity (15, 43). Both male and female KO mice have decreased feed efficiency compared with WT mice, which results in less body weight gain for greater food intake (40, 43).

In a double-KO model that is ASP and leptin deficient (ob/ob, C3−/−), both male and female mice have delayed postprandial TG and NEFA clearance compared with ob/ob mice, despite higher insulin sensitivity, and they have reduced body weight despite greater food intake (66). Decreased body weight in double-KO mice in the face of hyperphagia is counterbalanced by increased energy expenditure in these mice (66).

Taken together, these findings indicate that ASP deficiency leads to significant alterations in carbohydrate and lipid metabolism that result in deficient TG clearance and storage in WAT. The clearance of postprandial lipemia, however, involves the activity of endothelial lipoprotein lipase (LPL). LPL hydrolyzes TG-rich lipoproteins and releases NEFA, which are taken up and used by underlying tissue for their specific metabolic needs (for review see Ref. 38). The major physiological sites for postprandial LPL regulation are WAT and skeletal muscle (13). In WAT, NEFA are mainly esterified and stored as TG. In working skeletal muscle, although NEFA may...
temporarily enter the TG pool, they are mainly routed toward oxidation to accommodate the energy needs of the muscle and other tissues (38). In vivo in the postprandial period, LPL activity is upregulated in WAT and downregulated in muscle, and the reverse is true in the fasting period (11, 23, 49). This reciprocal regulation of LPL activity in WAT and muscle is believed to be mediated by reciprocal effects of insulin (11, 49). Male KO mice have delayed postprandial TG clearance despite higher insulin sensitivity. This, we hypothesize, is secondary to ASP deficiency in these mice that results in defective hydrolysis of TG-rich lipoproteins and reduced esterification and storage of the generated NEFA. Thus the overall objective of the present work was to investigate the mechanism by which ASP deficiency leads to subnormal postprandial TG clearance and fat storage ex vivo in male KO mice.

MATERIALS AND METHODS

Materials. [3H]triolein (glycerol tri-[9,10,12-3H]oleate, 0.5 μCi/μl), [3H]oleate ([9,10-3H]oleate, 5 μCi/μl), [3H]-[1,2,4-3H]glucose (1 μCi/μl), and [3H]O2 standard (1.1 μCi/ml) were from PerkinElmer (PerkinElmer Life Sciences, Boston, MA). Triolein, oleate, egg yolk L-α-phosphatidylcholine, and bovine milk LPL were from Sigma (Sigma-Aldrich Canada, Oakville, ON, Canada). α-Methylbenzylamine, 99% (CO2 trapping agent), was from Aldrich (Milwaukee, WI). Fatty acid-free bovine serum albumin (BSA) was obtained from ICN Biomedicals (Aurora, OH). Calcium-free Krebs-Ringer buffer (KR buffer), pH 7.2, contained (in mM) 5 glucose, 0.51 MgCl2, 4.56 KCl, 119.8 NaCl, 0.7 Na2HPO4, 1.3 NaH2PO4, and 15.0 NaHCO3, and 1% fatty acid-free BSA. ASP was prepared by an in-house purification method from fasting human plasma, as previously described (37, 55). Tissue culture plates were from Falcon (Becton Dickinson, Franklin Lakes, NJ). Thin-layer chromatography plates (TLC plates LK5 silica gel 150 Å) were from Whatman (Clifton, NJ).

Animals. Experiments were conducted on six wild-type (WT) and six ASP-deficient (C3−/−) male mice (14–15 wk old). Complement C3 KO (C3−/−) mice (on 129sv × C57Bl/6 strain), as well as WT mice, were originally obtained from Dr. Harvey Colton [Dept. of Pediatrics, Washington U., St. Louis, Missouri (42, 43)]. Mice were backcrossed 8–10 generations to obtain a homoogenous 129sv genetic background (15). Mice were housed in a pathogen-free barrier facility at 21°C on a 12:12-h dark-light cycle and provided with water and standard chow ad libitum. All procedures on mice followed the guidelines established by the Canadian Animal Care Center and were approved by the McGill University Health Centre Animal Care Committee. The mice were selected by genotyping with PCR as described previously for C3, using tail tips collected when the mice were weaned at 3 wk of age (40, 43).

Tissue extraction. On the day of the experiment, one pair of WT and KO male mice (fasted for 16 h) was killed by cervical dislocation. Epididymal WAT, vastus lateralis muscles, BAT, and liver were isolated and maintained in ice-cold KR buffer until all tissues were extracted. Tissues were cut into small pieces (25–35 mg except BAT, which weighed 10–20 mg), blotted on filter paper and weighed. Preliminary experiments showed a linear increase in the rate of TG synthesis, lipolysis, and NEFA and glucose oxidation over this range of tissue weight. Tissue incubations under all experimental conditions were conducted in a covered, shaking water bath (100 oscillations/min) at 37°C under continuous 95% O2-5% CO2 flow of 5 liters/h.

Hydrolysis, uptake, and storage of synthetic TG-rich lipoproteins by WAT and skeletal muscle. Ex vivo tissue differences between WT and KO mice in the hydrolysis of [3H]TG-rich lipoproteins and the incorporation of generated [3H]fatty acids [referred to as in situ LPL activity, as previously described (21)] were assessed by incubating WAT and muscle pieces with synthetic [3H]TG-rich lipoproteins. The [3H]TG-rich lipoprotein substrate is a modification of that originally described by Nilson-Ehle and Schotz (45) to measure in vitro LPL activity. It contained 1.41 mM triolein, 0.08 mM phosphatidylcholine, and 2.5 μCi [3H]triolein (specific activity 1.77 μCi/mM TG), which were dried and emulsified by sonication in an aqueous buffer (0.54 M Tris-HCl, pH 7.2, 5.1% BSA, 7.5% fasting human serum) (21). This generates large phospholipid micelles with a TG-to-phospholipid molar ratio of 17.3:1 (i.e., 95% TG, which is similar to TG content of a chylomicron particle) (26, 61). Fasting human serum, a source of the LPL activator apolipoprotein CII, was heat inactivated at 56°C for 1 h to inactivate any endogenous LPL. The molar ratio of TG to BSA was maintained at ≤2:0 to avoid limiting hydrolysis of the [3H]triolein substrate due to lack of NEFA binding sites to BSA (maximum NEFA-to-BSA molar ratio of 6) (7). Background 3H-NEFA in the [3H]TG substrate constituted 0.15 ± 0.09% of total counts.

Tissue pieces were preincubated in 24-well plates with 1 ml of warm KR buffer (previously kept at 37°C under 95% O2 flow) for 20 min and then transferred to wells containing 1 ml of warm [3H]triolein substrate and incubated for 4 h with shaking. Buffer was collected and frozen at −80°C, and tissue pieces were washed twice with 2 ml of ice-cold KR buffer and extracted overnight at room temperature with 1 ml of heptane-isopropanol (2:3) on a shaking rotor. The organic solvent was evaporated, and lipids were redissolved in chloroform-methanol (2.1, vol/vol). Total tissue 3H-lipids were quantified by direct count of the tissue lipid extract. Lipid fractions representing [3H]TG, [3H]-NEFA, and [3H]diglyceride + monoglyceride (DG + MG) were separated by TLC plates after extraction of the incubation medium with 5× chloroform-methanol (2.1, vol/vol). Lipids were separated by TLC, and the [3H]-labeled lipid fractions were scraped and counted. [3H]-fatty acid released into the medium and incorporated into tissue lipids is expressed as [3H]oleate/mg tissue.

Radiolabeling WAT, BAT, muscle, and liver with [3H]-NEFA and [14C]glucose (pulse). Tissue pieces were preincubated for 20 min with 0.6 ml of KR buffer containing 5 mM glucose and 1 mM oleate-BSA (5:1 molar ratio). Incubation medium was then supplemented with an equal volume of KR buffer containing 1 mM [3H]oleate-BSA and 5 mM [14C]glucose (final volume of 1 ml with 1 μCi/mM [3H]-NEFA and 1 μCi/mM [14C]glucose) and incubated for an additional 90 min. The reaction was stopped by washing the tissue pieces twice with warm KR buffer (37°C), after which the incubation medium was collected and stored at −80°C. [3H]-NEFA and [14C]glucose oxidation and incorporation into tissue lipids were measured in one-third of the tissue pieces (see below).

For chase experiments, after incubation of the tissue pieces with [3H]oleate and [14C]glucose and washing them with warm KR buffer, tissue pieces were incubated for an additional 2 h with 1 ml of unlabeled 5 mM glucose in the absence or presence of ASP (1 μM). The fate of [3H]-NEFA and [14C]glucose incorporated in tissue in the pulse period was followed in the following: 1) [3H]- and [14C]-lipids remaining in the tissue (total lipids, TG, DG + MG, and NEFA pools), 2) oxidized [3H]-NEFA and [14C]glucose recovered in the incubation medium, and 3) [3H]-NEFA released into the medium. The percent chase/pulse for [3H]- and [14C]-labeled tissue lipids was calculated as [3H] label in chase/[3H] label in pulse × 100 in each measured lipid fraction (total lipids, TG, DG + MG, and NEFA).

Measurement of substrate oxidation and lipolysis. The complete oxidation of [3H]-NEFA was assayed by measuring [3H]2O release into the incubation medium with modification of the method described by Lee et al. (34). The medium was precipitated twice with 60% trichlo-roacetic acid (final concentration 10%) to remove excess [3H]oleate-BSA. The supernatant was transferred into 1.5-ml microcentrifuge tubes (with caps cut off), which were placed in 16-ml scintillation vials to which 2 ml of unlabeled distilled water had been previously added. The scintillation vials were capped tightly, wrapped individually with foil, and incubated in an oven (55°C) for 18 h to allow equilibrium of [3H]2O vapor in the microcentrifuge tubes with unlabeled [3H]2O.
beled H₂O vapor in the scintillation vials. Counts in H₂O in the scintillation vials were measured. To determine H₂O equilibrium coefficient, 250 μl of H₂O standard (in KR buffer) were analyzed in parallel with the experimental samples, and data were corrected accordingly. On average, 72.1 ± 2.2% of H₂O standard counts in the microcentrifuge tubes were recovered in the water in the scintillation vials.

The complete aerobic oxidation of [¹⁴C]glucose was assayed by measuring ¹⁴CO₂ dissolved in the incubation medium by a modification of the technique used by Muato et al. (39). Medium (250 μl) was transferred to a 12 × 75-mm glass test tube, and a piece of saturation pad (1.1 × 2.5 cm²) with 150 μl of methylenebenzylamine (CO₂ trapping agent) was fixed vertically between the inner walls of the tube. Test tubes were stoppered, 100 μl of 70% perchloric acid were injected into each tube (not touching the saturation pads), and tubes were incubated overnight at 37°C in a shaking water bath to release ¹⁴CO₂. Saturation pad pieces with trapped ¹⁴CO₂ were collected and dropped into 10 ml of scintillation fluid and counted.

In the chase period, lipolysis in WAT and muscle was assessed by the release of glycerol into the medium (12, 63) by use of a modification of a commercial kit by Xyogen (Xyogen Diagnostic, Burgessville, ON, Canada). Sensitivity of the assay was increased whereby samples in the range of 16.6–333 nmol glycerol/ml could be measured. Total NEFA mass released into the medium was measured by a commercial kit (Wako Chemicals, Richmond, VA). ³H-NRFA release was measured by extraction of the medium with chloroform-methanol (2:1) as described above. [³H]oleate standard (in KR buffer) was analyzed in parallel with the experimental samples, and data were corrected accordingly. On average, 75.0 ± 3.8% of [³H]oleate standard counts were recovered in the organic phase.

Statistical analyses. Experiments were conducted on tissue pieces collected from all 12 mice with two pieces per tissue per mouse for WAT, muscle, and liver and one piece per mouse for BAT. Data are presented as averages ± SE. Statistical significance was determined by paired t-test for within-group differences (within KO or WT) or by unpaired t-test for between-group differences (KO vs. WT), and significance was set at P < 0.05 and power of ≥80%.

RESULTS

Effect of ASP on in situ LPL activity in WAT and muscle. We first examined the difference between WT and KO mice in the hydrolysis of synthetic [³H]TG-rich lipoproteins and the uptake and storage of generated ³H-fatty acids (referred to as in situ LPL activity (21)) in WAT and skeletal muscle over the period of 4 h. In an in vitro study in 3T3-L1 adipocytes, ASP increased maximum velocity (Vₘₐₓ) of in situ LPL (21). Preliminary experiments were conducted to determine the concentration of [³H]triolein substrate needed to reach Vₘₐₓ of in situ LPL activity in WAT and muscle, and a concentration of 1.41 mM TG was thus selected. In situ LPL activity measured as the sum of tissue ³H-lipids and medium ³H-NRFA (Table 1) was equivalent in both WT and KO as well as WAT and skeletal muscle. There was also no difference in ³H label recovered in any tissue lipid fraction examined ([³H]TG, [³H]DG + MG, and [³H]-NRFA) between KO and WT mice (data not shown). Total recovered ³H label in the incubation medium and tissue represented <10% of added [³H]TG substrate and was recovered mainly in tissue lipids (70%). The largest fraction of total tissue ³H-lipids was [³H]TG, which represented a greater fraction in WAT than in muscle (%[³H]TG of total ³H-lipids: WAT = 80.2 ± 2.2% vs. muscle = 73.6 ± 1.6%, P = 0.001). Tissue ³H-NRFA and [³H]DG + MG fractions comprised 2–4% each, and these lipid fractions were higher in WAT than in muscle (P < 0.005).

In situ LPL activity in the presence of ASP was comparable between WT and KO in both WAT and muscle, suggesting that the difference between WT and KO mice is a consequence of the absence of ASP, not an intrinsic difference in the tissue lipid metabolism. Consequently, data were pooled for all mice (KO + WT), as presented in Fig. 1. In WAT, addition of ASP increased the total ³H-lipid tissue extract by 68 ± 19.2% (increase by 3.0 ± 1.3 nmol [³H]-NRFA released/mg tissue, P = 0.02; Fig. 1), as well as [³H]TG and ³H-NRFA fractions (by 58.5 and 44.6% respectively, P ≤ 0.03; Fig. 1). Complementing the stimulatory effect in WAT in situ LPL activity and [³H]TG hydrolysis, ASP decreased the amount of [³H]TG substrate remaining in the medium after the incubation period by 13.4% (decrease of 2.4 ± 0.8 nmol [³H]TG/mg tissue, P = 0.01).

In contrast to its effect in WAT, ASP decreased total muscle ³H-lipid extract by 35.0 ± 4.4% (decrease of 2.3 ± 0.4 nmol [³H]-NRFA/mg tissue, P = 0.0002; Fig. 1) as well as other ³H-lipid fractions (−34.5, −33.0, and −31.1% in muscle [³H]TG, [³H]DG + MG, and ³H-NRFA, respectively, P ≤ 0.006; Fig. 1). Moreover, [³H]TG substrate accumulated in the medium bathing muscle after incubation with ASP ([³H]TG increased by 10.5% or 1.22 ± 0.61 nmol [³H]TG/mg tissue with ASP, P = 0.04), confirming the ASP-mediated decrease in [³H]TG hydrolysis and ³H-fatty acid incorporation in the muscle. There was no effect of ASP on ³H-NRFA recovered in the medium bathing WAT or skeletal muscle [WAT: baseline = 2.6 ± 0.4 nmol ³H-fatty acid/mg tissue, with ASP = 2.4 ± 0.3 nmol ³H-fatty acid/mg tissue; muscle: baseline = 2.6 ± 0.3 nmol ³H-fatty acid/mg tissue, with ASP = 2.5 ± 0.3 nmol ³H-fatty acid/mg tissue, not significant (NS)].

Substrate oxidation and incorporation into lipids in WAT, BAT, skeletal muscle, and liver (pulse period). We then examined the partitioning of exogenous energy sources, given as [³H]oleate and [¹⁴C]glucose, over a labeling period of 90 min in KO and WT mice. The labeling period was chosen on the basis of preliminary experiments where TG storage, lipolysis, and glucose and NEFA oxidation were found to be linear between 30 min and 2 h. The oxidation of ³H-NRFA is measured by the amount of recovered ²H₂O, whereas the oxidation of [¹⁴C]glucose is measured by the amount of recovered ¹⁴CO₂ in the incubation medium. Incorporation of [³H]oleate in tissue lipids labels the NEFA pool and the fatty acid chain of acylglycerols. On the other hand, the incorporation of [¹⁴C]glucose in tissue lipids labels the glycerol moiety of acylglycerol (and not the fatty acid chain). No ¹⁴C label was detected in the NEFA fraction of the tissue lipids, indicating absence of de novo fatty acid synthesis from glucose.

<table>
<thead>
<tr>
<th>Tissue ³H-Lipids, nmol ³H-fatty acid/mg tissue</th>
<th>Medium ³H-NRFA, nmol ³H-fatty acid/mg tissue</th>
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<tbody>
<tr>
<td>WAT</td>
<td>Muscle</td>
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<tr>
<td>WT 6.62 ± 0.71</td>
<td>2.61 ± 0.36</td>
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<tr>
<td>KO 6.25 ± 0.70</td>
<td>2.49 ± 0.45</td>
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Values are means ± SE. LPL, lipoprotein lipase; KO, knockout (C3–/–); WT, wild type; WAT, white adipose tissue; NEFA, nonesterified fatty acid.
As seen in Fig. 2, there was no significant difference between KO and WT mice in the amount of oxidized [14C]glucose or 3H-NEFA or that incorporated in tissue lipids in any tissue examined. Total utilized 3H-NEFA recovered in tissue 13C-lipids and 13H2O in the pulse period was <3%. Of interest, the method of delivery of 3H-fatty acid had an impact on utilization. The uptake and storage of albumin-bound 3H-NEFA was much smaller than TG-derived 3H-NEFA (as [3H]TG substrate) despite the prerequisite LPL-mediated lipolysis step in the latter (~10 times smaller in WAT and 20 times smaller in muscle in both KO and WT mice; Fig. 1 vs. Fig. 2). Moreover, only 23 and 3% of total 3H-NEFA uptake was trapped as [3H]TG in WAT and muscle, respectively, compared with 80 and 74% when 3H-NEFA originated from the [3H]TG substrate.

Effect of ASP on the fate of labeled lipids in WAT, BAT, muscle, and liver (chase period). After the tissue was prelabeled with [3H]oleate and [14C]glucose, both radiolabeled substrates were withdrawn, tissue pieces were washed, and the fate of incorporated [3H]oleate and [14C]glucose was followed over a chase period of 2 h in the presence or absence of ASP. The fate of incorporated [3H]oleate and [14C]glucose in the tissue pieces in the chase period was examined in 1) 14C- and 3H-lipids retained in the tissue, 2) 3H-NEFA released in the medium, and finally 3) 14CO2 and 3H2O in the medium (which represent oxidation products of [14C]glucose and 3H-NEFA).

As shown in Fig. 3A, in the absence of ASP, other than BAT there was no difference between KO and WT mice in the amount of tissue 3H-lipids, released 3H-NEFA, or oxidized 3H-NEFA in all tissues examined. In BAT in KO mice, more 3H-lipids were retained in the chase period (P < 0.05) particularly in the TG pool (WT: 0.24 ± 0.03 vs. KO: 0.49 ± 0.11 nmol [3H]TG/mg BAT, P = 0.02). This is counterbalanced by less 3H-NEFA released into the medium (P = 0.02). Oxidation of endogenous 3H-NEFA represented a minor fraction of 3H-lipids fate in all tissues (<4%). Consistent with the 3H-NEFA data is the similar pattern in KO and WT in the amount
of 14C-lipids retained or oxidized in all tissues examined (Fig. 3B). Again, BAT obtained from KO mice retained more 14C-lipids in the chase period (P = 0.01) and particularly in the TG pool (WT: 0.18 ± 0.03 vs. KO: 0.39 ± 0.06 nmol [14C]TG/mg BAT, P = 0.005). On the other hand, the ratio of [3H]TG to [14C]TG in BAT was not different between KO and WT mice (WT: 1.16 ± 0.04 vs. KO: 1.16 ± 0.08 [3H]TG/ [14C]TG). Because BAT has a considerable amount of glycerol kinase (22, 52) and [14C]glycerol can be reused to synthesize TG, it is hard to distinguish whether the increased radiolabeled lipids in BAT of KO mice are due to increased 3H-NEFA reesterification, reduced lipolysis, or both.

The percent changes in the fate of incorporated labels by ASP in WAT, BAT, muscle, and liver are shown in Fig. 3, C and D. Because there was no difference between KO or WT mice in their response to ASP in any outcome measured or tissue examined, even BAT (data not shown), data were pooled. As with the previous observation in situ LPL activity, ASP had opposing effects on radiolabeled lipid storage in WAT and skeletal muscle in the chase period. In WAT, ASP significantly increased the amount of retained 14C- and 3H-lipids (P < 0.05) and decreased by an equivalent amount the 3H-NEFA released into the medium (P = 0.02). Total NEFA mass released from WAT was also decreased with ASP incubation by 23% (baseline: 3.47 ± 0.29 vs. ASP: 2.64 ± 0.16 nmol NEFA/mg WAT, P = 0.02). Lipolysis could not be assessed in WAT because glycerol release was below the sensitivity range of the assay (<16.6 nmol glycerol/ml).

The effect of ASP on the redistribution of prelabeled 3H-lipid fractions (total 3H-lipids, [3H]TG, [3H]DG + MG, and 3H-NEFA) during the 2-h chase period is presented as %change/pulse (Fig. 4), where 100% means that the amount of 3H-lipid present in chase equals that at the end of the pulse period for each fraction. Data were pooled for KO and WT mice, because, again, no genotype difference existed. In WAT at baseline, only the %change/pulse in [3H]TG increased above 100%. As 3H-NEFA was absent from the incubation medium in the chase phase, this increase in %change/pulse in the [3H]TG pool indicates channeling of 3H-NEFA from other intracellular pools into the TG pool. ASP increased the channeling and esterification of 3H-fatty acids as [3H]TG (P = 0.03) as well as the [3H]DG + MG pool (P = 0.03). Together, these lipid pools constitute 75% of total WAT lipids in the chase period ([3H]MG + DG = 19, and [3H]TG = 56% in chase). On the other hand, ASP had no significant effect on the %change/pulse of [14C]TG (baseline: 132.8 ± 13.3 vs. ASP: 141.5 ± 11.7%, NS). Moreover, although there was no difference between the pulse and chase periods in the ratio [3H]TG/[14C]TG, ASP increased this ratio in WAT by 20.9 ± 7.3% (P = 0.005; Fig. 5). In WAT, the amount of glycerol kinase activity in basal, unstimulated conditions is negligible (52); thus [14C]glycerol released from lipolysis cannot be reused. Lipogenesis in this tissue would thus esterify endogenous 3H-NEFA onto prelabeled DG and MG precursors and onto unlabeled glycerol backbone supplied as glucose in the medium. Thus, taken together, these data suggest that, in WAT, 1) there is no effect of ASP on lipolysis or on reesterification of 3H-NEFA onto [14C]DG + MG, as the %change/pulse in [14C]TG was not affected, and 2) the net effect of ASP is to increase the TG synthesis-to-lipolysis ratio favoring reesterification of 3H-NEFA onto an unlabeled glycerol backbone.

As shown in Fig. 3, C and D, in contrast to the effect in WAT, ASP decreased the amount of retained 14C- and 3H-lipids in muscle (P = 0.04) without, however, increasing 3H-NEFA release into the medium. Moreover, in the muscle, as seen in Fig. 4, ASP decreased the %change/pulse of [3H]TG, maintaining it at 100% (P = 0.01), and decreased the intracellular 3H-NEFA pool (P = 0.03). Together, both pools represent 76% of total muscle 3H-lipids in the chase period ([3H]TG = 18% and 3H-NEFA = 58%). This indicates that ASP either decreased 3H-fatty acid reesterification and/or increased lipolysis in muscle. ASP did increase lipolysis, as indicated by the increased glycerol release by 41% (baseline 2.98 ± 0.31 vs. ASP 3.69 ± 0.34 nmol glycerol release/mg muscle, P = 0.05) and decreased the %change/pulse of [14C]TG (baseline 124.9 ± 22.7 vs. ASP 107.0 ± 25.0%, P = 0.03). However, [3H]TG/[14C]TG (P = 0.004; Fig. 5) is also decreased by ASP in muscle, which indicates that the rate of the
decrease in \(^3\)H-fatty acid reesterification as TG exceeds the rate of increases in TG lipolysis. In muscle, two possible sources of glucose for the glycerol backbone of TG are available: unla-
beled glucose in the medium and \(^{14}\)C\glycogen stores that may have been labeled in the pulse period. Thus, all together, these findings suggest that, in skeletal muscle, the net effect of ASP is to 1) increase lipolysis (without compensatory increase in \(^3\)H-fatty acid reesterification on endogenous \(^{14}\)C\glycose or \(^{14}\)C\DG + MG, or else the \%chase/pulse of \(^{14}\)C\TG would remain unchanged) and 2) decrease \(^3\)H-fatty acid reesterification on the unlabeled glycerol backbone. The fate of released NEFA from the endogenous TG lipolysis in the muscle is, however, unclear from the present study, as ASP did not increase the complete oxidation of \(^3\)H-NEFA as measured by assessment of \(^3\)H\_2\_O. Whether, however, ASP increases accumulation of incomplete \(^3\)H-NEFA oxidation products or thermogenesis in muscle remains to be examined.

Similar to muscle, ASP decreased \(^{3}\)H\TG/\(^{14}\)C\TG in BAT (\(P = 0.05\); Fig. 5), whereas no ASP effect was observed on %chase/pulse of either \(^3\)H\_TG or \(^{14}\)C\_TG in BAT (%chase/pulse in \(^3\)H\_TG: baseline 162.1 ± 28.8 vs. ASP 182.1 ± 49.6%; %chase/pulse \(^{14}\)C\_TG: baseline 142.9 ± 22.7 vs. ASP 174.4 ± 41.6%; NS for both labels). As both \(^3\)H-NEFA and \(^{14}\)C\glycerol can be reused for TG synthesis in BAT, it cannot be determined from the present data if the decrease in \(^3\)H\_TG/\(^{14}\)C\_TG is the net result of less \(^3\)H-fatty acid reesterification or more \(^{14}\)C\glycerol reutilization.

ASP had no effect on the metabolism of \(^3\)H-NEFA or \(^{14}\)C\glycerol in the liver in the pulse or the chase periods. Moreover, ASP had no effect on the oxidation of endogenous \(^3\)H-NEFA or \(^{14}\)C\glycerol as measured by \(^3\)H\_2\_O and \(^{14}\)CO\_2 recovered in the medium in the chase period in any tissue measured (data not shown).

**DISCUSSION**

WAT provides a “buffering” system for the acute increase in NEFA influx derived from the hydrolysis of dietary TG-rich lipoprotein. “NEFA trapping” in WAT as stored TG prevents NEFA circulation at high concentration and efflux to other peripheral tissue beyond the metabolic need of these tissues to assimilate NEFA. Excess NEFA influx to muscle and liver is associated with lipotoxicity and insulin resistance in mice and human models (9, 10, 31, 32, 47, 60). ASP-deficient C3\_/- mice have reduced capacity for TG synthesis in WAT and elevated postprandial NEFA concentration, yet they have enhanced whole body insulin sensitivity, as indicated by lower plasma glucose and insulin concentrations at fasting and after glucose tolerance test (42). These mice, however, have elevated energy expenditure, as shown in both double-KO (C3\_/-\_ob/ob) (66) and single-KO (C3\_/-\_129sv) ASP-deficient mice (67). Thus, despite delayed postprandial TG and NEFA clearance, these mice are protected against the detrimental effects of increased postprandial NEFA influx by upregulating oxidation and energy expenditure (67). It was, however, unknown...
whether this alteration in energy metabolism in C3−/− mice in vivo was a direct or indirect effect of ASP deficiency. To identify the specific mechanism by which ASP deficiency leads to subnormal postprandial TG clearance and fat storage in male KO mice, we examined substrate (glucose and oleate) metabolism in the presence and absence of ASP in tissues that are most active in this process.

Under these experimental conditions, our data suggest that there are no intrinsic tissue differences between KO and WT mice in situ LPL activity, substrate oxidation, TG synthesis, lipolysis, and reesterification in all tissues examined except BAT. In BAT of KO mice, TG storage was enhanced. This finding may explain previous data indicating increased BAT mass in male KO mice (42) and suggests that, in vivo, increased BAT mass in KO mice is not secondary to rechanneling of NEFA from WAT to BAT but to an intrinsic tissue resistance to baseline lipolysis. ASP-deficient KO mice have increased energy expenditure even in the sleeping period (67). Thus, in vivo, increased TG storage in BAT of KO mice may be a mechanism to ensure a larger in situ reservoir for NEFA oxidation, particularly when external energy supply is diminished during fasting. Future studies examining thermogenesis and the activity of uncoupling protein ex vivo in BAT in the absence and presence of ASP could verify this hypothesis.

An interesting observation in this study is that the delivery route of 3H-fatty acid had an impact on utilization. The uptake and storage of albumin-bound 3H-NEFA was much smaller than TG-derived 3H-NEFA in both KO and WT mice. This may be due to differences in the mechanism of their uptake into the tissue. Transport of LPL-derived NEFA into TG-synthesizing tissue (adipose tissue, liver, heart, and skeletal muscle) is suggested to be via lateral movement through a continuum interface from the TG-rich lipoproteins’ surface phospholipid film into the cellular membranes of the parenchymal cell (7, 57–59). On the other hand, it is proposed that albumin-bound NEFA are transported either actively via membrane transporters (1, 2, 6, 56) or passively through a “flip-flop” mechanism that is driven by a concentration gradient across the plasma membrane (28). In fact, it has been previously suggested that, depending on the mode of uptake, TG-derived NEFA and albumin-bound NEFA do not enter the same metabolic pool in the tissue (35). In a recent study on perfused mouse heart, the proportion of oxidized to stored NEFA was increased when albumin-bound NEFA do not enter the same metabolic pool in the tissue (35).

Although there were no intrinsic tissue differences in substrate metabolism in WAT and muscle between WT and KO mice, novel data from the present study suggest that ASP has differential effects on NEFA trapping in these tissues, as represented schematically in Fig. 6. In WAT, ASP increases in situ LPL activity, hydrolysis of [3H]triolein substrate, and trapping of generated 3H-fatty acid in the form of TG, whereas decreased NEFA release from the tissue (Fig. 6A). Conversely, in muscle, ASP decreased in situ LPL activity, hydrolysis of [3H]triolein substrate and trapping of generated 3H-fatty acid, whereas it increased lipolysis (Fig. 6B).

This differential regulation of LPL activity in WAT and muscle is observed in vivo in the fasting and fed states. Several lines of evidence suggest that LPL activity is under nutritional regulation, increasing in WAT and decreasing in muscle with the postprandial rise in insulin, and reciprocal changes are observed during fasting (11, 23, 48). In rats, postprandial heparin-releasable LPL activity increases by 65% above fasting values in epididymal and retroperitoneal WAT while decreasing by 25% in soleus muscle 1 h after the ingestion of a high-carbohydrate meal (49). This effect is partially diminished by inhibition of insulin secretion from the pancreas (49) or reduction of postprandial insulinemia by Intralipid injection and high-fat meal feeding (19, 20). Endothelial LPL in WAT is the key enzyme responsible for TG clearance (38). In addition, postprandial TG concentration has been suggested to be more closely regulated by the rate of TG clearance than the rate of hepatic TG secretion (16–18, 50). Thus reduced in situ LPL activity in WAT with ASP deficiency may contribute to delayed postprandial TG and NEFA clearance in male KO mice and may explain the normalization of TG clearance when ASP is administered. It is important to point out, however, that, compared with WT mice, male KO mice have enhanced insulin sensitivity, as indicated by a decreased insulin response after a glucose tolerance test (43). Yet, despite enhanced insulin sensitivity in KO mice, which might be expected to enhance WAT LPL activity, delayed postprandial TG and NEFA clearance is characteristic of these mice (43). This suggests that insulin action is not the sole regulator of postprandial LPL activity and TG clearance. Insulin-mediated upregulation of postprandial LPL activity in WAT may be overwhelmed by the effects of ASP deficiency on LPL activity and WAT lipogenesis, resulting in delayed TG clearance in KO mice.

Despite the well-documented regulation of LPL activity by insulin and nutritional interventions, the mechanism by which insulin elucidates its tissue-specific effects on LPL activity, particularly in muscle, is poorly understood. In WAT, it is suggested that the short-term effect of feeding and insulin on LPL activity is mediated by posttranslational modulation that includes increased LPL activation, increased active LPL release, and decreased LPL degradation (4, 14, 65). More recently, it was proposed that, during fasting, a gene is activated that downregulates LPL activation without affecting LPL synthesis (5). Recent in vitro data from our laboratory demon-
strated that both ASP and insulin increase in situ LPL activity in 3T3-L1 adipocytes, as measured by increasing synthetic [3H]TG-rich lipoprotein hydrolysis and 3H-NEFA incorporation into the adipocytes (21). ASP stimulates this effect on synthetic [3H]TG-rich lipoprotein hydrolysis by stimulating TG synthesis in adipocytes and, hence, by removal of NEFA inhibition of LPL, despite the lack of any direct stimulatory effect of ASP on heparin-releasable LPL activity (as opposed to the stimulatory effect of insulin on heparin-releasable LPL activity) (21). In the present work, a similar mechanism may be employed in WAT and a reciprocal one in skeletal muscle. In the muscle, increased endogenous TG lipolysis and decreased lipogenesis by ASP may exert a feedback inhibition on in situ LPL activity. The tissue-specific mechanism and signal transduction pathways by which ASP regulates the balance between lipolysis and lipogenesis in skeletal muscle are yet to be examined.

The decrease in muscle TG synthesis by ASP may have specific long-term metabolic consequences. Increased intramyocytic TG content is associated with concurrent onset of insulin resistance in many human and mouse studies (9, 31, 47). Mice overexpressing muscle LPL have a threefold increase in muscle TG and are insulin resistant (51). The metabolic abnormalities associated with increased muscle TG are proposed to be secondary to increased metabolites long-chain acyl-CoA and diacylglycerol, which inhibit glycolytic enzymes and glucose utilization and interfere with insulin signaling and insulin-mediated glucose uptake (51). Pioglitazone (an enhancer of muscle action) administration to insulin-resistant hypertriglyceridemic mice increases LPL production in WAT but not in skeletal muscle (29). It was concluded that this differential tissue effect of the drug may help prevent the development of muscle lipotoxicity and insulin resistance in these mice (29). Thus, in vivo, the reciprocal regulation of NEFA trapping in WAT and muscle by ASP, in addition to insulin, may be a mechanism to target NEFA accumulation in the physiological depot. This would help ensure the maintenance of optimal insulin sensitivity.

It should be pointed out, however, that this is not in disagreement with our previous in vivo findings that ASP-deficient mice have increased whole body insulin sensitivity (40, 43, 66). In vivo, ASP deficiency leads to reduced WAT mass, and the tight correlation of insulin sensitivity and body weight is likely to explain the increased insulin sensitivity in KO mice. In addition, ASP-deficient mice have increased energy expenditure, which would channel NEFA to oxidation, prevent the exposure of nonadipose tissues to long-term NEFA efflux and the accumulation of muscle and liver TG. This may thus mask the effect of ASP deficiency and defective NEFA trapping in WAT on insulin resistance. Confirming this, fasting plasma NEFA and TG levels are normal in C3/KO mice, even on a high-fat diet (43). Plasma TG and NEFA are elevated in these mice only in the postprandial period following a fat load (43), i.e., when the effect of defective WAT NEFA trapping is more readily perceived with a sudden high influx of dietary fat.

However, a defective ASP pathway may exacerbate the development or maintenance of insulin resistance when increased total body NEFA input is not counterbalanced by increased NEFA disposal, i.e., in positive energy balance and obesity models. Although the defects underlying the etiology of abnormal lipid metabolism and insulin resistance in obese subjects are unknown, it is generally accepted that elevated plasma NEFA concentrations play a major role (8). A number of studies show that the balance between insulin-stimulated intracellular lipolysis and lipogenesis is defective in obese individuals, with a shift toward increased net NEFA efflux from WAT (18, 24, 27). In addition, it was demonstrated that the habitual insulin-mediated rise in WAT LPL activity in the postprandial period is defective in obese and type 2 diabetic humans, which is suggested to exacerbate postprandial hypertriglyceridemia (18, 44, 46, 53). Increased circulating NEFA, fasting and postprandial TG, and decreased NEFA oxidation in muscle are factors proposed to increase muscle lipotoxicity and insulin resistance (33). However, although obesity is clearly a risk factor for diabetes, many obese individuals never develop any abnormal metabolic profile; in fact only ~15–20% of obese subjects ultimately become diabetic (9). Thus a defective ASP pathway may contribute to the development of the metabolic abnormalities and insulin resistance associated with some types of obesity through delayed postprandial TG clearance, inefficient NEFA trapping in WAT, and elevated NEFA influx and storage in skeletal muscle.

GRANTS

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