Acylation-stimulating protein/C5L2-neutralizing antibodies alter triglyceride metabolism in vitro and in vivo

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Obesity is a worldwide problem, even more so considering the associations with increased incidence of coronary heart disease, hypertension, diabetes, osteoarthritis, inflammation, and certain cancers (2, 18, 31, 34). The obese state results from an imbalance between energy storage and expenditure. Acylation-stimulating protein (ASP; also known as C3adesArg) is a lipogenic hormone that plays a key role in regulating energy balance (6). ASP is produced and secreted by white adipose tissue, which is well recognized as a metabolically active endocrine organ as well as an energy storage tissue (10). Several factors have been shown to influence ASP production and secretion. During differentiation, adipocytes gradually increase cellular production of ASP (7). In vitro, chylomicrons stimulate ASP production up to 150-fold in human differentiated adipocytes (24). In vivo, adipose tissue ASP production correlates with postprandial chylomicron triglyceride (TG) clearance (38, 40). Finally, insulin also increases ASP production up to twofold (24).

ASP increases energy storage by stimulating TG synthesis through activation of diacylglycerol acyltransferase and increases glucose transport, as demonstrated in 3T3-L1 preadipocytes, adipocytes, and human skin fibroblasts (1, 19, 24, 45). Previous studies (19, 25, 43) showed that ASP stimulates glucose transport in vitro by increasing glucose transporter (GLUT1, GLUT3, and GLUT4) translocation to the cell surface. Although GLUT1 plays a major role in preadipocyte glucose transport, GLUT4 and GLUT3 are the main glucose transporters in adipocytes and muscle tissue, respectively (19, 25, 43). Indirectly, ASP influences lipoprotein lipase (LPL) activity by upregulating TG storage (16, 17). In white adipose tissue, ASP increases in situ LPL activity by facilitating non-esterified fatty acid (NEFA) uptake into adipocytes, thereby preventing product inhibition of LPL. By contrast, ASP decreases LPL activity in skeletal muscle, similar to the effects of insulin (16, 17). Furthermore, ASP inhibits adipose tissue hormone-sensitive lipase activity, decreasing lipid hydrolysis and increasing reesterification of NEFA (44). Overall, ASP promotes fat uptake and storage in adipose tissue.

ASP is derived from complement C3, generated though a series of catalytic processes. C3 knockout (KO) mice (C3−/−) are ASP-deficient (ASPdef KO) mice, since they lack the precursor protein. ASPdef KO mice have significant delays in both postprandial TG and NEFA clearance (27, 29). They are lean with reduced fasting leptin levels (49). Leptin-deficient (ob/ob)/ASPdef double-KO mice had delayed TG clearance and decreased body weight compared with ob/ob KO mice (48), demonstrating that ASP acts independently of leptin.

C5L2, a G protein-coupled receptor (GPCR), was recently identified as an ASP receptor (20, 21). It is expressed in human adipose tissue, liver, brain, spleen, intestine, human skin fibroblasts, and 3T3-L1 cells (21). Gain-of-function studies in human C5L2 stably transfected HEK-293 (HEK-hC5L2) cells (21) showed that TG synthesis and glucose transport were significantly increased upon ASP stimulation compared with untransfected cells. Loss-of-function studies (21) showed that cells endogenously expressing C5L2, treated with antisense or
small interfering RNA treatment, had decreased ASP response. In addition, activation of C5L2 by ASP induces β-arrestin translocation to the plasma membrane and C5L2 phosphorylation (21). It has been demonstrated (23) that phosphatidylinositol 3-kinase, Akt, and protein kinase C are all involved in ASP signaling.

Studies (32) have been initiated on C5L2 KO mice to examine C5L2 function in vivo. As with ASP-def KO mice, C5L2 KO mice have delayed TG clearance, increased food intake, and increased fatty acid oxidation. Furthermore, ex vivo adipose tissue studies have demonstrated that C5L2 KO mice have reduced basal TG synthesis, lipolysis, and fatty acid reesterification.

Neutralizing antibodies have increasingly been used to examine ligand-receptor interaction and are exciting targets for therapy (13, 36). In the present study, ASP/C5L2-neutralizing antibodies were developed, tested in vitro, and used to investigate blocking ASP-C5L2 interaction in vivo. We hypothesized that postprandial TG and NEFA clearance would be delayed by neutralizing antibodies that blocked ASP-C5L2 interaction in vivo.

MATERIALS AND METHODS

Antibody generation and purification. Based on the structure of C5L2 proposed by the ClustalW alignment (http://www.ebi.ac.uk/clustalw/), antigenic peptides to extracellular loops were designed. An 18-amino acid peptide spanning the first extracellular loop (PIARGGHWPYGAVGCRAL, C5L2-L1), a 17-amino acid peptide spanning the first part of the second extracellular loop (AIYRRLHQEHFPARLQC, C5L2-L2.1), a 17-amino acid peptide spanning the second part of the second extracellular loop (LQCVVDYGGSSSTENV, C5L2-L2.2), and a 20-amino acid amino acid peptide corresponding to the third extracellular loop (LTVAAPSNALLARALRAEPL, C5L2-L3) of human C5L2 were synthesized and complexed to produce multiple antigenic peptides to generate antibodies. Polyclonal antibodies were generated in rabbits following multiple subcutaneous immunizations. Titres of antibodies were tested by ELISA against the antigenic peptides. Antibodies were purified by γ-bind plus Sepharose for IgG purification (GE Healthcare, Chicago, IL). An antibody against the NH2-terminal portion of C5L2 and monoclonal and polyclonal antibodies against ASP was prepared as described previously (21, 38).

Cells and culture conditions. HEK-hC5L2 stably transfected cells containing an NH2-terminal human hemaglutinin tag were prepared as previously described (20, 21). Unsorted mouse C5L2 stably transfected Chinese hamster ovaray (CHO) cells (CHO-mC5L2) were generously provided by Dr. Peter Monk (Sheffield, UK). CHO-mC5L2 cells and HEK-hC5L2 cells were sorted by flow cytometry and selected for cells with high-level binding of fluorescently labeled ASP (top 5% of fluorescently labeled cells). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)-F-12 medium with 10% fetal bovine serum at 37°C, 5% CO2. The culture medium was supplemented with 250 µg/ml of G418 for the stably transfected cells. All cells were preincubated for 2 h in serum-free medium before experiments.

3T3-L1 preadipocytes were differentiated into adipocytes as previously described (46). After incubation in culture medium containing 0.5 mM isobutylmethylxanthine, 1.0 µM dexamethasone, and 10 µg/ml insulin for 2 days, the confluent cells were grown in culture medium with 10 µg/ml insulin for an additional 2 days and then incubated in culture medium alone. The cells were used for glucose transport experiments when ≥80% of the cells were differentiated (as determined by microscopic evaluation of lipid droplet formation), usually 6 days after initiation of differentiation.

Cell preparation for flow cytometry. Antibody recognition of human C5L2 was assessed by flow cytometry as described previously (20, 21). Briefly, HEK-hC5L2 cells were grown to 85% confluency in six-well plates and were incubated in serum-free DMEM-F-12 medium for 2 h. Cells were detached with a nonenzymatic cell dissociation solution (Sigma Chemicals, St. Louis, MO), pelleted, and resuspended in 1 ml of PBS with 0.5% BSA containing rabbit anti-C5L2 antibodies (diluted 1:100) and incubated at 4°C for 30 min with gentle rocking. After centrifugation, cells were washed twice with PBS, resuspended, and incubated in 1 ml of PBS with 0.5% BSA containing goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibody (dilution 1:400; Bethyl, Montgomery, TX). Following washing, the cells were fixed in 1 ml of 4% paraformaldehyde and transferred into 0.4% paraformaldehyde.

ASP radiolabeling and binding assay. A siliconized tube was coated with iodogen (100 µl of 100 µg/ml iodogen in chloroform; Pierce Biotechnology, Rockford, IL), followed by the addition of 20 µg of ASP and 2 µCi of Na125I (PerkinElmer Life Sciences, Boston, MA). After 18 min of incubation, Na125I was added to stop the reaction and 125I-labeled ASP was purified by Sephadex G25 gel filtration (GE Healthcare).

For binding assays, adherent cells at 95% confluence in 24-well cell culture plates were incubated with a reaction mixture solution (200 µl) containing a constant amount of 125I-labeled ASP as a tracer (1 nM) and increasing concentrations of unlabeled ASP or antibodies in PBS for 1 h at room temperature. After being washed, the cells were dissolved in 0.1 M NaOH. Aliquots were taken for counting using a gamma counter (Canberra Packard, Waltham, MA). After 10 min of incubation, Na125I was again added to stop the reaction and 125I-labeled ASP was purified by Sephadex G25 gel filtration (GE Healthcare).

For blocking assays, adherent cells at 95% confluence in 24-well cell culture plates were incubated with a reaction mixture solution (200 µl) containing a constant amount of 125I-labeled ASP as a tracer (1 nM) and increasing concentrations of unlabeled ASP or antibodies in PBS for 1 h at room temperature. After being washed, the cells were dissolved in 0.1 M NaOH. Aliquots were taken for counting bound 125I-labeled ASP and measuring cell protein by Bradford protein assay (Bio-Rad, Hercules CA).

TG synthesis and glucose transport assays. TG synthesis and glucose transport assays were performed as previously described in detail (21). For TG synthesis, cells were incubated in serum-free medium for 2 h and then in serum-free medium containing 1 µM antibodies (anti-C5L2-L1, anti-ASP, or nonimmune IgG) for 30 min. After addition of ASP for 2 h, the cells were treated for another 2 h with serum-free medium containing 100 µM oleate spiked with [3H]oleate (PerkinElmer Life Sciences) complexed to albumin (molar ratio 5:1, specific activity 100 dpm/µmol). After being rinsed with PBS, lipids were extracted with isopropyl alcohol/heptane (2:3 vol/vol) and were separated by thin-layer chromatography (Whatman LSK5 silica gel 150A; Mandel Scientific, Toronto, ON, Canada) in a solvent of hexane-ether-acetic acid (75:25:1 vol/vol). TG was visualized by iodine vapor, and the silica gel was scraped and counted. After lipid extraction, cell protein was dissolved in 0.1 N NaOH and measured by Bradford protein assay (Bio-Rad). TG synthesis was measured as pmol [3H]oleate incorporated into TG per milligram of soluble cell protein.

For glucose transport, following pretreatment (serum-free medium for 2 h, antibody treatment for 30 min, and then ASP stimulation for 2 h as described above), the cells were rinsed twice with 37°C PBS and then incubated for 10 min in PBS containing [3H]-deoxyglucose (50 µM, specific activity 60–120 dpm/µmol; PerkinElmer Life Sciences) at 37°C. After being washed twice with cold PBS, the cells were dissolved in 0.1 M NaOH. Aliquots of NaOH solution were taken for counting and cell protein measurement (as described above). Glucose transport was measured as picomoles [3H]-deoxyglucose uptake per microgram of soluble cell protein.

Blocking ASP-C5L2 interaction in vivo. Twenty-one C57BL/6 wild-type male mice (8–12 wk old, 25–29 g) were purchased from Charles River Laboratories (Wilmington, MA), weighed, and randomly divided into three groups (n = 7). One group of mice was injected with nonimmune IgG as control; the other two groups of mice were injected with either anti-ASP antibody or anti-C5L2-L1. The mice were housed individually in a sterile barrier facility with a 12:12-h light-dark cycle at the animal center facility. The mice were maintained on a normal chow diet (10% kcal fat; Charles River Laboratories). Nonimmune IgG, anti-ASP, and anti-C5L2-L1 solution
(1 mg/ml) were prepared in sterile PBS and stored at –20°C. Before use, antibody was warmed to room temperature. Antibodies (200 µl) were administered intraperitoneally once a day for 10 days. Mouse body weight and food intake were recorded on days 0, 1, 5, 8, 9, and 10. All protocols were approved by and were conducted in accordance with the Canadian Council on Animal Care and Laval University animal care guidelines.

Intragastric fat administration. Fat load assays were performed as previously described (27, 29). On day 10, after an overnight fast (16 h), the last injection was administered and fasting blood samples were collected. All mice were then administered 250 µl of olive oil by gastric gavage [12-cm curved ball tipped feeding needle (28)], followed by 100 µl of air. Blood samples (40 µl) were taken by leg vein bleeding at 0, 2, 3, and 4 h and were collected in 2% EDTA. Mice were killed by cardiac puncture at 5 h, and blood was collected. Skeletal muscle (quadriceps), liver, and gonadal and perirenal adipose tissue were excised, weighed, frozen in liquid nitrogen, and stored at –80°C for later analysis. Plasma was separated by centrifugation at 8,000 g for 5 min and stored at –20°C for further analysis.

Plasma assays. Plasma TG and NEFA were measured using colorimetric enzyme kits (Roche Diagnostics, Indianapolis, IN, and Wako Chemicals, Richmond, VA, respectively). Glucose was measured using a Trinder glucose kit (Sigma). Leptin, adiponectin, and insulin were measured by radiolabeled immunoassay kits (Linco Research, St.Charles, MO).

Plasma nonimmune IgG antibody concentrations were determined by using a Bio-Dot Microfiltration Apparatus (Bio-Rad). Nitrocellulose membrane (Bio-Rad) was soaked in Tris-buffered saline (TBS; pH 7.4) and the fat layer discarded. Mice samples were homogenized in 1 ml of homogenization buffer containing 50 mM Tris (pH 7.4), 1 mM EDTA, 10% (wt/vol) glycerol, 0.02% (vol/vol) Brij-35, 1 mM dithiothreitol, protease inhibitor (Sigma), and phosphatase inhibitor (Sigma). The homogenates were centrifuged at 10,000 g for 20 min at 4°C and protein content measured by Bradford assay (Bio-Rad). Using AMARA, a synthetic peptide, as substrate (Upstate), AMPK activity was performed in a 25-µl reaction solution (80 mM HEPES-NaOH, pH 7.0, 160 mM NaCl, 1.6 mM EDTA, 1 mM MgCl₂, 16% glycerol, and 200 µM AMARA) containing 2 mM ATP spiked with γ-32P ATP (specific activity 500 dpm/pmol; PerkinElmer Life Sciences) and 5 µg of homogenate at 30°C for 15 min. The reaction was stopped using the addition of 3% H₂PO₄. An aliquot (10 µl) was spotted onto p81 phosphocellulose paper, washed, and dried. 32P-phosphorylated AMARA was determined using standard liquid scintillation procedures. AMPK activity was expressed as picomoles of 32P incorporated into AMARA per microgram of tissue protein per minute.

Statistical analyses. Results are presented as means ± SE. For competition curves, the IC₅₀, standard error values, and nonlinear regression analyses were calculated. Further significance was also evaluated by one-way ANOVA vs. control (no addition of blocking antibodies). The groups were compared by two-way ANOVA or two-way repeated-measures (RM) ANOVA. P value <0.05 was taken as significant, where NS means not significant. Incremental area under the curve (△AUC) was calculated using the trapezoidal method. All graphs and statistical calculations were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

RESULTS

Anti-CSL2-L1 antibodies bind to CSL2 receptor. Antipeptide epitopes to extracellular loops were designed to generate antibodies against human CSL2 receptor based on the structure of CSL2 proposed by the ClustalW alignment (http://www.ebi.ac.uk/clustalw/). Five antibodies were produced: anti-CSL2-N (against NH²-terminal peptide), anti-CSL2-L1 (against the first extra-
showed that polyclonal anti-ASP efficiently blocked ASP binding to C5L2 except for anti-C5L2-L2.1 (a representative scan for anti-C5L2-L1 is shown in Fig. 1A).

Anti-ASP and anti-C5L2-L1 antibody block ASP binding to C5L2. Binding assays were performed in different cell lines: stably transfected HEK-hC5L2 as well as CHO-mC5L2 cells and 3T3-L1 preadipocytes that endogenously express mouse C5L2. The antibodies included nonimmune rabbit IgG and rabbit antibodies against C5L2 (anti-C5L2-N, anti-C5L2-L1, anti-C5L2-L2.2, and anti-C5L2-L3) and against ASP (rabbit polyclonal and monoclonal). As shown in Fig. 1, anti-C5L2-L1 antibody not only significantly blocked ASP binding to C5L2 in HEK-hC5L2 cells (P < 0.0001; Fig. 1B) and CHO-mC5L2 cells (P < 0.001; Fig. 1C) but also significantly blocked ASP binding to endogenous C5L2 expressed in mouse 3T3-L1 cells (P < 0.0001; Fig. 1D) with IC₅₀ values of 212, 185, and 760 nM, respectively. Anti-C5L2-N, anti-C5L2-L2.2, and anti-C5L2-L3 were unable to block ASP binding to C5L2 (data not shown), although they were immunogenic toward C5L2.

Polyclonal antibody against ASP (anti-ASP) was generated using the entire ASP protein as the antigen, and monoclonal antibody against ASP was produced using a COOH-terminal peptide (amino acid 69–76) of ASP as the antigen. The results showed that polyclonal anti-ASP efficiently blocked ASP binding to C5L2 in HEK-hC5L2 cells (P < 0.0001; IC₅₀ 2.3 nM; Fig. 1B), CHO-mC5L2 (data not shown), and 3T3-L1 cells endogenously expressing mC5L2 (P < 0.0001; IC₅₀ 2.2 nM; Fig. 1D), whereas the monoclonal antibody against ASP did not block ASP binding to C5L2 in any of these cell lines (data not shown). Furthermore, one-way ANOVA analysis indicated that nonimmune rabbit IgG had no effect on ASP binding to C5L2 in CHO-mC5L2 cells (P = NS; Fig. 1C), HEK-hC5L2 cells, and 3T3-L1 cells (data not shown).

Anti-ASP and anti-C5L2-L1 neutralized ASP stimulation of TG synthesis and glucose transport. In vitro, the lipogenic function of ASP has been well documented, including in HEK-hC5L2 cells and 3T3-L1 cells (1, 19, 24, 45). In the present study, we used three cell lines (HEK-hC5L2 cells, 3T3-L1 preadipocytes, and 3T3-L1 adipocytes) to test the neutralizing ability of anti-ASP and anti-C5L2-L1 on ASP function. TG synthesis was increased more than twofold by ASP stimulation in 3T3-L1 preadipocytes (Fig. 2A). This ASP stimulation was significantly reduced by anti-ASP and anti-C5L2-L1 to basal levels (Fig. 2A). In contrast, nonimmune IgG had no effect on ASP stimulation of TG synthesis (Fig. 2A). Furthermore, the addition of nonimmune IgG, anti-ASP, or anti-C5L2-L1 (without adding ASP) to 3T3-L1 preadipocytes had no effect on basal TG synthesis (Fig. 2A). The inhibition of anti-ASP and anti-C5L2-L1 on ASP stimulation of TG synthesis was concentration dependent (data not shown). In addition, TG synthesis assay was also performed in HEK-hC5L2 cells, and the results obtained were similar to those in 3T3 cells (data not shown).

Glucose transport assays were also performed in three cell lines: HEK-hC5L2 cells, 3T3-L1 preadipocytes, and adipocytes. Figure 2, B and C, shows the effective neutralizing ability of anti-ASP and anti-C5L2-L1 on ASP stimulation of glucose transport in 3T3-L1 preadipocytes and adipocytes, whereas nonimmune IgG had no blocking effect (Fig. 2, B and C). Furthermore, the addition of nonimmune IgG, anti-ASP, or anti-C5L2-L1 (without addition of ASP) did not affect basal glucose transport in 3T3-L1 preadipocytes and adipocytes.
Adipocytes (Fig. 2, B and C). In addition, the blocking effects of anti-ASP and anti-C5L2-L1 were concentration dependent (data not shown), and similar results were also obtained with HEK-hC5L2 cells (data not shown). These results clearly demonstrate that anti-ASP and anti-C5L2-L1 antibodies not only blocked ASP-C5L2 interaction but also blocked the functions of ASP, stimulation of TG synthesis, and glucose transport in vitro.

Short-term administration of anti-ASP and anti-C5L2-L1 antibodies in vivo does not affect body weight, food intake, or basal hormone levels. C57BL/6 wild-type mice were used for a short-term (10 days) study to evaluate the effects of anti-ASP and anti-C5L2-L1 antibodies in a physiological setting. One group of mice was injected with nonimmune IgG as control; the other two groups of mice were given injections of anti-ASP and anti-C5L2-L1 antibodies. Body weight, food intake, and hormone levels are given in Table 1. After 10 days of administration of nonimmune IgG, anti-ASP, or anti-C5L2-L1, body weight, food intake (Table 1), and tissue weights for liver, muscle, gonadal adipose tissue, and perirenal adipose tissue (data not shown) were not significantly different among the three groups of mice. In addition, antibody injection did not affect plasma levels of insulin, leptin, or adiponectin (Table 1).

Antibodies have equal tissue distribution. Plasma levels of injected antibody and antibody concentrations in the targeted tissues were examined following the 10-day treatment period. In plasma, the concentrations of the rabbit antibodies in the three study groups were comparable: nonimmune IgG 8.0 ± 0.6 μg/ml, anti-ASP 8.6 ± 0.6 μg/ml, and anti-C5L2-L1 8.0 ± 0.9 μg/ml (NS, 1-way ANOVA). To test the availability of antibodies among the four targeted tissues, 125I-labeled nonimmune IgG and 125I-labeled anti-C5L2-L1 were injected in two separate groups of mice. The concentrations of 125I nonimmune IgG in gonadal adipose tissue, perirenal adipose tissue, liver, and muscle were 8.86 ± 3.31, 9.86 ± 2.36, 5.97 ± 1.15, and 4.73 ± 0.74 μg/g tissue, respectively, with no significant differences across tissue distribution. The concentrations of 125I anti-C5L2 were 5.80 ± 0.85, 5.53 ± 1.28, 4.96 ± 0.90, and 2.16 ± 0.33 μg/g tissue, respectively, with no significant differences across tissues. Thus any potential differences in function among the four tissues could not be explained by differences in antibody access.

Short-term administration of anti-ASP and anti-C5L2-L1 antibodies delays TG clearance and increases NEFA levels after oral fat load administration. Following 10 days of antibody injection the mice were fasted overnight and given an oral fat load, and serial blood samples were taken to measure plasma TG, NEFA, and glucose levels. As shown in Fig. 3A, plasma TG levels were significantly higher in the mice treated with neutralizing antibodies (anti-ASP and anti-C5L2-L1) than the control mice (nonimmune IgG) at 2 h, with significant increases in iAUC (iAUC: 2.0 ± 0.3 mM/5 h nonimmune IgG control; 11.8 ± 1.5 mM/5 h anti-ASP, P < 0.001 vs. control; 10.4 ± 0.9 mM/5 h anti-C5L2-L1, P < 0.001 vs. control; Fig. 3A, right). In addition, two-way RM ANOVA analysis indicated significant differences across tissue distribution. Plasma levels of injected antibody and antibody concentrations in the targeted tissues were examined following the 10-day treatment period. In plasma, the concentrations of the rabbit antibodies in the three study groups were comparable: nonimmune IgG 8.0 ± 0.6 μg/ml, anti-ASP 8.6 ± 0.6 μg/ml, and anti-C5L2-L1 8.0 ± 0.9 μg/ml (NS, 1-way ANOVA). To test the availability of antibodies among the four targeted tissues, 125I-labeled nonimmune IgG and 125I-labeled anti-C5L2-L1 were injected in two separate groups of mice. The concentrations of 125I nonimmune IgG in gonadal adipose tissue, perirenal adipose tissue, liver, and muscle were 8.86 ± 3.31, 9.86 ± 2.36, 5.97 ± 1.15, and 4.73 ± 0.74 μg/g tissue, respectively, with no significant differences across tissue distribution. Thus any potential differences in function among the four tissues could not be explained by differences in antibody access.

Table 1. Body weight, food intake, and hormone levels

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<tr>
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<th>NI-IgG Mice</th>
<th>Anti-ASP Mice</th>
<th>Anti-C5L2-L1 Mice</th>
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<tr>
<td>Body weight (baseline), g</td>
<td>27.1 ± 0.4</td>
<td>27.5 ± 0.4</td>
<td>26.0 ± 0.6</td>
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<tr>
<td>Body weight (posttreatment), g</td>
<td>27.5 ± 0.5</td>
<td>28.2 ± 0.4</td>
<td>26.7 ± 0.6</td>
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<tr>
<td>Food intake, g/mouse • 1-day⁻¹</td>
<td>3.8 ± 0.3</td>
<td>4.0 ± 0.2</td>
<td>4.4 ± 0.3</td>
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<tr>
<td>Insulin (posttreatment), ng/ml</td>
<td>0.5 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>0.6 ± 0.2</td>
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<tr>
<td>Leptin (posttreatment), ng/ml</td>
<td>4.7 ± 0.2</td>
<td>5.0 ± 0.2</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>Adiponectin (posttreatment), μg/ml</td>
<td>4.3 ± 0.3</td>
<td>4.1 ± 0.3</td>
<td>4.6 ± 0.5</td>
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Values are presented as average ± SE. (n = 7/group). NI-IgG, nonimmune IgG; anti-ASP, antibody against acylation-stimulating protein; anti-C5L2-L1, antibody against C5L2 receptor.
cated that anti-ASP- and anti-C5L2-L1-neutralizing antibodies significantly delayed postprandial TG clearance ($P < 0.0001$).

The increased postprandial plasma NEFA levels of mice treated with anti-ASP or anti-C5L2-L1 were also significantly higher compared with control mice at 2 h (Fig. 3B), with significantly elevated NEFA iAUC (iAUC: 1.3 ± 0.3 mM/h control; 7.2 ± 0.5 mM/h anti-ASP, $P < 0.001$ vs. control; 6.9 ± 0.3 mM/h anti-C5L2-L1, $P < 0.001$ vs. control). Furthermore, two-way RM ANOVA analysis indicated that anti-ASP- and anti-C5L2-L1-neutralizing antibodies significantly delayed postprandial NEFA clearance ($P < 0.0001$).

Plasma glucose levels during the fat load indicated that mice treated with anti-ASP- and anti-C5L2-L1-neutralizing antibodies had higher glucose levels at 2 h compared with control mice, with significantly higher glucose iAUC (iAUC: 30.1 ± 2.4 mM/5 h control; 37.6 ± 1.3 mM/5 h anti-C5L2-L1, $P < 0.05$ vs. control; 36.2 ± 0.6 mM/5 h anti-ASP, $P < 0.05$ vs. control; Fig. 3C). Two-way RM ANOVA analysis showed that anti-ASP- and anti-C5L2-L1-neutralizing antibodies induced a plasma glucose increase ($P < 0.05$).

**Anti-ASP and anti-C5L2-L1 alter TG content in skeletal muscle, liver, and perirenal adipose tissue.** The effect of antibody treatment on tissue TG mass was evaluated following the 10-day injection period. As shown in Fig. 4A, tissue TG mass was altered by treatment with neutralizing antibodies. The mice treated with anti-ASP or anti-C5L2-L1 had a 38.4 ($P < 0.001$) and 18.8% ($P < 0.01$) reduction in TG mass per gram of perirenal adipose tissue, with a trend to decrease in
gonadal tissue as well (−21.6% anti-ASP and −15% anti-C5L2-L1). In liver, there was a small, but not significant, decrease in TG content with anti-ASP (−23%), with a more pronounced decrease with anti-C5L2-L1 (−42.8%, P < 0.05; Fig. 4B). In contrast, in muscle TG content was significantly increased by anti-C5L2-L1 (+128%, P < 0.05), with less of an effect with anti-ASP (+42.8%, NS; Fig. 4B). Total TG content per tissue depot as well as the distribution across the four tissues is presented in Table 2. Compared with control mice, anti-ASP- and anti-C5L2-L1-treated mice had the following changes: in perirenal, anti-ASP and anti-C5L2-L1 treatment resulted in a reduced TG distribution (P < 0.001 anti-ASP and P < 0.05 anti-C5L2-L1). In liver, there was a significant reduction by anti-C5L2-L1 only (P < 0.05). The neutralizing antibodies had their greatest effects in muscle, with both anti-ASP and anti-C5L2-L1 treatment significantly increasing total muscle TG mass (+82%, NS, and +179%, P < 0.001, respectively) and %TG partitioning (+62%, P < 0.05, and +109%, P < 0.001, respectively).

**LPL activity and AMPK activity ex vivo**. As indexes of lipid storage and energy utilization, LPL and AMPK activities, respectively, were measured in tissues collected at the end of the fat load, representing a postprandial state. In gonadal adipose tissue, neutralizing antibodies did not affect LPL activity (data not shown). By contrast, in perirenal adipose tissue, where significant TG mass reduction was observed, anti-ASP and anti-C5L2-L1 decreased LPL activity by 75% (P < 0.05) and 72% (P < 0.05), respectively, compared with IgG control (Fig. 4C). On the other hand, in muscle, anti-ASP and anti-C5L2-L1 significantly increased LPL activity (+164%, P < 0.05, and +226%, P < 0.001, respectively) compared with IgG control (Fig. 4C).

Next, AMPK activity was examined, as shown in Fig. 4D. In both gonadal and perirenal adipose tissues, there were no changes in AMPK activity between anti-ASP- and anti-C5L2-L1-neutralizing antibody-treated mice and control mice. AMPK activity in liver was increased 34% (P < 0.01) by anti-ASP and was increased 17% (NS) by anti-C5L2-L1 compared with nonimmune IgG. In skeletal muscle, there was a pronounced increase in AMPK activity of 53.9% (P < 0.05) with anti-ASP and 71.1% (P < 0.01) with anti-C5L2-L1.

**DISCUSSION**

Obesity is the result of excess TG synthesis and storage in adipose tissue (8), so limiting fatty acid storage is crucial in

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**Table 2. Total tissue TG mass per depot and %distribution among 4 tissues**

<table>
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<tr>
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<th>NI-IgG Mice</th>
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<th>Anti-C5L2-L1 Mice</th>
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<tbody>
<tr>
<td><strong>Gonadal, μmol</strong></td>
<td>216.9±37.3</td>
<td>191.7±13.3</td>
<td>179.4±13.6</td>
</tr>
<tr>
<td><strong>%Distribution</strong></td>
<td>(27.3%±2.5)</td>
<td>(25.7%±1.7)</td>
<td>(26.0%±2.6)</td>
</tr>
<tr>
<td><strong>Perirenal, μmol</strong></td>
<td>52.6±3.0</td>
<td>37.3±3.8</td>
<td>46.9±6.5</td>
</tr>
<tr>
<td><strong>%Distribution</strong></td>
<td>(7.2%±0.6)</td>
<td>(4.9%±0.8)**</td>
<td>(5.4%±1.3)**</td>
</tr>
<tr>
<td><strong>Liver, μmol</strong></td>
<td>205.1±25.7</td>
<td>164.4±14.9</td>
<td>117.4±20.4**</td>
</tr>
<tr>
<td><strong>%Distribution</strong></td>
<td>(28.6%±3.6)</td>
<td>(22.0%±1.8)</td>
<td>(18.1%±2.7)**</td>
</tr>
<tr>
<td><strong>Muscle, μmol</strong></td>
<td>193.8±23.5</td>
<td>361.4±37.6</td>
<td>552.9±131.8*</td>
</tr>
<tr>
<td><strong>%Distribution</strong></td>
<td>(34.2%±3.5)</td>
<td>(48.7%±4.6)*</td>
<td>(56.4%±5.5)**</td>
</tr>
</tbody>
</table>

Values represent total TG mass per tissue depot and are presented as means ± SE (n = 7 per group). *P < 0.05; **P < 0.01. %Distributions relative to total body distribution for the 4 tissues are given in parentheses.
preventing obesity. As a lipogenic hormone, the physiology and pathophysiologic role of ASP associated with obesity is well documented (6). Plasma concentrations of ASP are elevated not only in human obesity but also in type 2 diabetes independent of body weight (10, 22, 26). Acute fasting, chronic hypocaloric diets, or gastric bypass surgery, which result in a decrease in body weight, induce a decrease in plasma levels of ASP (41). However, following a fatload, there is little change in the general circulating level of ASP (11). There is little information available on plasma ASP in mice; however, circulating ASP appears to be lower than in humans (5) (Paglialunga S and Cianflone K, unpublished observations). Interestingly, IL-6-deficient (IL-6−/−) mice have normal body weight when they are young but develop obesity at 6–7 mo old. An elevated plasma ASP level in IL-6−/− mice, which precedes the onset of obesity, was demonstrated as a major contributor to the physical changes (47).

Studies on ASP−/− KO (C3 −/−) mice (49) have shown that lack of ASP resulted in leaner mice with decreased adipose tissue mass. Furthermore, C5L2 KO mice, deficient in the ASP receptor, displayed reduced TG synthesis and fatty acid reesterification (32). Taken together, the KO mice studies have shown that ASP is a critical target for regulating fatty acid storage in vivo. However, one caveat is that these transgenic knockout mice lack ASP stimulation from uterine on, and compensatory mechanisms may have developed. To assess this, in the present study, we used neutralizing antibodies as a tool to prevent ASP stimulation by blocking ASP binding to its receptor C5L2 in wild-type mice.

Antibodies are useful tools to block hormone receptor binding and signaling in cells. Initial studies with antibodies can lead to production of small molecule antagonists for clinical targeting. Antibodies are not only easy to produce, but they are also highly selective with high affinity. Therefore, antibodies are commonly being used to neutralize hormone functions (13, 36). Five antibodies against C5L2 and two antibodies against ASP were generated and tested. In vitro assays identified and validated the specific anti-ASP and anti-C5L2 antibodies potent as ASP/C5L2-neutralizing antibodies by flow cytometry, binding studies, and in vitro functional assays (TG synthesis and glucose transport). However, the C5L2 receptor is also expressed in liver and muscle as well as other tissues (21). Therefore, we cannot rule out that the in vivo effects of anti-C5L2-L1 and anti-ASP antibodies that we obtained are not mediated through direct interaction with liver or muscle.

This in vivo study was designed as a short-term investigation to 1) examine acute effects and specific blocking of ASP/C5L2-neutralizing antibodies, 2) avoid immune reaction against antibodies, and 3) limit body weight change. During treatment with neutralizing antibodies, the experimental mice were healthy and did not present any adverse or immune reaction. Body weight of the mice before and after treatment remained constant, avoiding the potential confounding influence of altered body weight on TG metabolism. Furthermore, the levels of hormones (adiponectin, leptin, and insulin) known to influence lipid metabolism did not change among the three groups of mice; thus these hormones were not the source of altered TG distribution in the treated mice.

In this study, mice treated with ASP/C5L2-neutralizing antibodies displayed delayed postprandial TG as well as NEFA clearance. Interestingly, the same phenomena were observed in ASP−/− KO mice and C5L2 KO mice (27, 29, 32). TG clearance is a two-step process, involving 1) lipolysis of TG-rich lipoproteins via LPL to yield NEFA and 2) cellular uptake and intracellular esterification of NEFA to TG. Lack of, or blocking, the ASP stimulation of intracellular TG synthesis inhibits NEFA uptake, which then accumulates in plasma. This, in turn, generates a negative feedback on LPL activity, reducing lipolysis of TG lipoproteins; consequently, TG clearance is delayed.

LPL is a glycoprotein produced by extrahaemepatic tissues, including both adipose tissue and muscle, anchored to the luminal surface of capillary endothelial cells, where it hydrolyzes circulating lipoproteins and releases NEFA (50). Consequently, as shown in the present study, when ASP/C5L2-neutralizing antibodies blocked ASP-C5L2 interaction, ASP stimulation of TG synthesis was inhibited (in vitro) and adipose LPL activity and TG mass were reduced (in vivo). Interestingly, these effects were more pronounced in the perirenal (visceral) adipose tissue than in the gonadal (subcutaneous-like) adipose tissue. Because increased visceral adipose tissue is associated with a more disadvantaged metabolic profile (12), this effect is encouraging.

By contrast, in muscle, ASP/C5L2-neutralizing antibodies increased TG mass and LPL activity. Previous studies have demonstrated that ASP plays a different role in regulating LPL activity in muscle compared with adipose tissue. In muscle, ASP decreases overall LPL activity and NEFA esterification, resulting in increased lipolysis of lipoproteins (16, 17). Therefore, ASP favors dietary fat trapping in adipose tissue for storage rather than in muscle for utilization; thus both LPL and ASP play critical roles in energy substrate partitioning. Consistent with that, ASP is increased in obesity (9, 26, 45), and some studies (14, 15, 30, 35) have shown that LPL activity is chronically elevated in adipose tissue but reduced in muscle obtained from obese people. Blocking ASP-C5L2 interaction, such as in the present study, reversed the ASP function and increased LPL activity and TG mass in muscle. Recently, it has been demonstrated (37) that increased muscle TG mass in obese diabetic subjects is associated with a potential lipotoxicity. On the other hand, muscle TG mass is also increased in athletes, who have a greater capacity for energy expenditure (3). Thus the consequences of an increased muscle LPL activity and TG mass, which may be associated with increased capacity for energy expenditure (discussed below), are not necessarily negative.

AMPK is a central mediator in energy metabolism sensing ATP requirements (42). In the present study, ASP/C5L2-neutralizing antibody treatment significantly increased skeletal muscle AMPK activity. Interestingly, this increase was accompanied by elevated TG accumulation in the muscle. Previous studies in ASP−/− KO mice have shown that, in the skeletal muscle, not only was fatty acid uptake increased but fatty acid oxidation was also upregulated, whereas adipose tissue TG storage was reduced (49). We believe this compensatory mechanism was developed in response to altered fuel partitioning (less adipose tissue storage and increased muscle oxidation). This shift in fuel partitioning has been further evaluated in C5L2 KO mice, where reduced TG synthesis in adipose tissue was associated with increased muscle fatty acid oxidation (32). This was demonstrated by increased ex vivo NEFA oxidation, upregulation of key proteins involved in fatty acid handling (CD36, cytochrome c, and phosphorylated acetyl-CoA carbox-
ylase), and reduced in vivo respiratory quotient, suggesting preferential fat over carbohydrate oxidation (32). Furthermore, AMPK activity was elevated in C5L2 KO mice in response to a high-fat diet. Thus neutralizing antibody treatment resulted in a metabolic profile comparable with ASP-deficient or C5L2 KO mice with reduced adipose TG storage and increased muscle TG mass and AMPK activity. Therefore, the lack of ASP/C5L2 interaction may contribute to a compensatory shift in substrate partitioning and utilization.

Administration of ASP/C5L2-neutralizing antibodies resulted in decreased TG mass and increased AMPK activity in liver. It has been shown (21) that C5L2 is expressed in the liver, yet its function remains unknown. Thus the effects demonstrated here may be a direct or indirect effect of the neutralizing antibodies. The direct effects of ASP and C5L2 on lipid metabolism in liver remain to be investigated.

In conclusion, ASP is an important factor for regulating dietary fat partitioning among different tissues. In the present study, ASP/C5L2-neutralizing antibodies blocked ASP function in vivo, resulting in enhanced TG depots in muscle rather than adipose tissue with reduced hepatic lipid accumulation. These effects were accompanied by changes in dietary fat clearance and altered LPL and AMPK activities. The positive results of these acute studies are encouraging for longer-term studies using agents to block ASP stimulation that would not cause an immune reaction, such as Fab fragments of ASP/C5L2-neutralizing antibodies or blocking peptides. Furthermore, interference of ASP/C5L2 interaction may provide important tools to enhance fat oxidation and decrease storage.

GRANTS

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