Acylation-stimulating protein deficiency and altered adipose tissue in alternative complement pathway knockout mice

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1Department of Biochemistry, McGill University, Montreal; 2Centre de Recherche de l’Hôpital Laval, Université Laval, Québec, Canada; and 3Institute of Biomedicine, Sahlgrenska Academy at Göteborg University, Gothenburg, Sweden

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Paglialunga S, Fisette A, Yan Y, Deshaies Y, Brouillette J-F, Pekna M, Cianflone K. Acylation-stimulating protein deficiency and altered adipose tissue in alternative complement pathway knockout mice. Am J Physiol Endocrinol Metab 294: E521–E529, 2008. First published December 26, 2007; doi:10.1152/ajpendo.00590.2007—Acylation-stimulating protein (C3adesArg/ASP) is an adipokine that acts on its receptor C5L2 to stimulate triglyceride (TG) synthesis in adipose tissue. The present study investigated ASP levels in mouse models of obesity and leanness and the effect of ASP deficiency in C3 knockout (C3KO) mice on adipose tissue morphology. Plasma ASP levels in wild-type (WT) mice correlated positively with plasma nonesterified fatty acids (NEFA) (R = 0.664, P < 0.001) and total cholesterol (R = 0.515, P < 0.001). Plasma ASP was increased by 85% in obese ob/ob leptin-deficient mice and decreased in lean diacylglycerol acyltransferase 1 (DGAT1) KO mice (−54%) and C/EBPα−/− transgenic mice (−70%) compared with WT. Mice lacking alternative complement factor B or adipsin (FBKO or ADKO), required for ASP production, were also ASP deficient. Both FBKO and C3KO mice had delayed postprandial TG and NEFA clearance on low-fat (LF) and high-fat (HF) diets, suggesting that lack of ASP, not C3, drives the metabolic phenotype. Adipocyte size distribution in C3KO mice was polarized (increased number of both small and large cells), with decreased adipin expression (−33% gonadal HF), DGAT1 expression (−31% to −50%) and DGAT activity (−41%). Overall, a reduction/deficiency in ASP is associated with an antiadipogenic state and ASP may provide a target for controlling fat storage.

C3adesArg; complement C3; diacylglycerol acyltransferase 1; postprandial lipemia

ACYCLATION-STIMULATING PROTEIN (ASP/C3adesArg) is an adipose tissue-derived hormone that stimulates adipocyte triglyceride (TG) synthesis and glucose transport (20, 43). ASP acts through its receptor C5L2, a G protein-coupled receptor, to stimulate diacylglycerol acyltransferase (DGAT) activity, the rate-limiting enzyme in the TG synthesis pathway, and glucose transport (17, 18).

It has been suggested that ASP is generated through the alternative pathway of complement activation, based on differentiation-dependent expression of adipin (factor D), complement C3, and factor B (FB) in adipose tissue (4, 5); however, this has not been shown directly. C3 convertase of the alternative pathway, a proteolytic complex formed by the interaction of C3b, FB, and adipin (factor D), cleaves the third complement component (C3) into C3a and C3b (8). C3a is a potent anaphylatoxin interacting with its receptor C3aR. However, in the circulation, the terminal arginine of C3a is rapidly cleaved by carboxypeptidase B, inactivating the C3a anaphylatoxic function and generating C3adesArg (ASP) (8). Both ASP/C3adesArg and C3a interact with the receptor C5L2 to effectively stimulate TG synthesis in cultured adipocytes (18).

C3 knockout (C3KO) mice are obligatorily ASP deficient, since they lack the precursor protein. We have previously reported that C3KO mice are lean yet hyperphagic (22, 40); the increased energy intake is being balanced by an increased energy expenditure (40). C3KO mice also displayed an altered plasma lipoprotein profile, mainly characterized by delayed postprandial TG clearance (23, 24, 40). Although initially disputed (38), lipid abnormalities were also found in independently derived C3KO on an LDL receptor/apoE double-knockout background (30).

In humans, ASP levels are increased in obesity, type 2 diabetes, and cardiovascular disease (reviewed in Ref. 8), whereas exercise or weight loss decreases ASP levels (33). Postprandially, subcutaneous adipose tissue increases production of ASP; and this has been shown to correlate with local fatty acid trapping (19, 32). Furthermore, similar to insulin resistance, a deleterious ASP-resistant state has been proposed to also contribute to the disturbed adipose tissue metabolism and dyslipidemia common to diabetes and cardiovascular disease (8, 9).

To date, there are very few data available on the regulation of ASP in murine models (37). In the present study, ASP plasma levels were evaluated in FBKO and adipin (AD)KO mice and in several mouse models of obesity and leanness, including obese leptin-deficient ob/ob mice, lean diacylglycerol acyltransferase 1 (DGAT1)KO mice, and transgenic (Tg) CCAAT enhancer binding protein-α (C/EBPα)−/− mice. To address the question of whether it is the lack of ASP or its precursor C3 that drives the altered lipid metabolism in C3KO mice, FBKO mice were evaluated on the basis of lipid clearance and adipose tissue morphology, with simultaneous confirmation of postprandial abnormalities in an independently derived C3KO mouse model. Therefore, the aims of the current study were to 1) evaluate plasma ASP levels in wild-type (WT) mice and in a number of Tg mice deficient in key proteins implicated in adipose tissue metabolism and 2) investigate the effect of ASP deficiency in FB knockout mice and an additional independently derived C3KO model, with detailed examination of adipose tissue morphology.

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Methods

Mice. Mouse plasma samples from leptin deficient ob/ob mice were obtained from a previous study (39). DGAT1KO mouse plasma was provided by Dr. H. Chen (34), and C/EBPβ−/− Tg mouse plasma was obtained from Dr. Y. H. Lee, Taipei, Taiwan (3). Age and sex matched WT mice (C57Bl/6/Sv129 mixed background) were simultaneously provided from these sources. Additional WT mice, C57Bl/6 and Sv129, were obtained from Charles River Laboratories (Wilmington, MA). Mixed C57Bl6/Sv129 mice were bred to 10 generations.

The development of C3KO and FBKO mice has been previously described in detail elsewhere (Refs. 28 and 29, respectively). ADKO mice were a gift from Dr. Ma (41). All three sets of Tg mice were back-crossed onto a C57Bl/6 background, and WT C57Bl/6 mice were used as controls for these studies. Body weight and plasma samples were taken over 2–8 mo. Mice were kept in a sterile-barrier facility under 12:12-h light-dark cycle and housed individually. At 8 wk, mice were placed on either a standard low-fat diet (LF, 10% kcal fat; Charles River Laboratories, Wilmington, MA) or a high-fat diet (HF, 45% kcal fat; Research Diets, New Brunswick, NJ). Body weight and food intake were measured two to three times per week for 8 wk, mice were placed on either a standard low-fat diet (LF, 10% kcal fat; Charles River Laboratories, Wilmington, PA) or a high-fat diet (HF, 45% kcal fat; Research Diets, New Brunswick, NJ).

Body weight and food intake were measured two to three times per week for 12 wk and mice were killed at 20 wk of age. Before the mice were euthanized, adiposity was assessed following an intramuscular general anesthesia injection. Total percent body fat was measured by dual-energy X-ray absorptiometry (DEXA) analysis using the Piximus mouse densitometer (Lunar, Madison, WI). All protocols were approved and conducted in accordance with the CCAC guidelines and the University Animal Care Committee.

Chemicals. [3H]oleate was purchased from PerkinElmer Life Sciences (Boston, MA), and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Hormone measurements. ASP levels were determined by dot-blot as previously described (37) or using a modified in-house direct ELISA assay. Briefly, 50 µl of plasma was acidified to 1.0 M HCl, and the remaining supernatant was neutralized with NaOH and diluted in 1.33% DTT in 0.22 M Tris·HCl to yield a final concentration of 0.8% DTT. This solution was added (100 µl) to a 96-well immunoplate and incubated overnight at 4°C to bind protein. The following day, the plate was washed and blocked with 3% BSA in TBS for 1 h at 37°C, followed by the primary rabbit anti-mouse ASP antibody (1:500; in-house antibody) and incubated for 1 h at 37°C. Then, the secondary goat anti-rabbit IgG peroxidase conjugate antibody (1:3,000) was added for 1 h at 37°C. ASP was detected by a colorimetric reaction as previously described (32). For both assays, commercially available mouse serum (Harlan, Indianapolis, IN) was used as sample control and normalized to a standard of human ASP. C3KO mouse plasma samples (negative control) were used to evaluate experimental background values. Insulin, leptin, and adiponectin were measured using commercially available RIA kits from Linco (St. Charles, MO).

Glucose tolerance test and intragastric fat meal administration. For glucose tolerance tests, following an overnight fast, an intraperitoneal glucose injection (2 mg/g body wt) was given, and blood samples were taken at 0, 30, 60, and 120 min from the tail vein. For oral fat meal tests, olive oil (300 µl with 100 µl of air above the oil) was administered by intragastric gavage feeding tube after an overnight fast. Tail vein blood samples were taken at 0, 1, 2, 3, 4, and 6 h after fat. Isolated adipose tissues were collected in 2% EDTA, separated by centrifugation at 5,000 g for 5 min, and stored at −20°C. Plasma glucose was measured by a glucose TRINDER assay (Sigma). Plasma TG and nonesterified fatty acids (NEFA) were measured using enzymatic colorimetric kits from Roche Diagnostics (Indianapolis, IN) and Wako Chemical (Richmond, VA), respectively.

Adipose tissue TG extraction and histology. Neutral lipids, including TG, were extracted from gonadal adipose tissue overnight in 500 µl of heptanes-isopropanol (3:2) at 4°C, as previously described (27). For histological analysis, samples of gonadal and inguinal tissues were preserved in 4% paraformaldehyde. Tissue sections were cut 4 µm thick and fixed onto slides using standard histological methods. Slides were stained with hematoxylin and eosin (H&E) dye. Adipocyte circumference (50–100 cells/mouse tissue) was measured using Image-Pro Plus (Media Cybernetics, Bethesda, MD) under ×200 magnification (Olympus).

mRNA analysis. WT and KO mice were killed for tissue analysis. Adipose tissue was collected, immediately frozen in liquid nitrogen, and stored at −80°C. Total RNA was isolated using the TRIzol method (Invitrogen, Carlsbad, CA) and reverse transcription. Transcription of 3 µg was performed as outlined elsewhere (40). The resulting cDNA (4 µl of 100 µl total) was amplified by PCR using the following primer sets for adipin (sense 5'-ATG ATG TGT GTC GAG AGC AAC G-3' and antisense 5'-CCG TTC TTG GCA AAT GAT A A-3'), DGAT1 (sense 5'-TTG AGC GTC TCT TAA AGC TGG-3' and antisense 5'-GCC AGG ACA GGA GTA TTT TTG-3'), and lipoprotein lipase (LPL; sense 5'-ACT CAT CTC GCC ATG CC-3' and antisense 5'-CCA GCT TTC TCC TAG CAA GG-3'). The reaction conditions for LPL were 28 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, as previously described (21). The reaction conditions for LPL were 45 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min (31). Primers for the housekeeping gene 18S were used according to the manufacturer’s instructions (Ambion, Austin, TX). All PCR products were separated by electrophoresis on polyacrylamide gels, stained with silver reagent (Bio-Rad, Hercules, CA), visualized, and analyzed using the Molecular Analyzer program. Results are expressed as specific mRNA/18S RNA in arbitrary units (AU).

DGAT activity assay. DGAT activity was measured in tissue homogenates (250 µg protein) as the incorporation of [3H]oleate (2 mM) into TG. Diolein (125 µM) was used as the substrate in the presence of CoA (0.5 mM), ATP (2.5 mM), MgCl2·6H2O (10 mM), DTT (1 mM), and Tris·HCl (100 mM), as previously described (18), (43). LPL activity was measured as described elsewhere (13).

Statistics. Results are presented as means ± SE. Groups were compared by Student’s t-tests, one- or two-way ANOVA followed by Bonferroni post hoc test as indicated, or two-way repeated-measures ANOVA (two-way RM ANOVA). Statistical significance was set at P < 0.05, where NS indicates not significant. All correlations were performed assuming linear association using Pearson correlation or multiple regression analysis. Analysis of normalized relative frequencies of small (25th %ile) and large (75th %ile) adipocytes were determined based on χ² analysis.

Results

Plasma ASP levels in WT and Tg mice. Fasting ASP levels were measured in WT C57Bl6/Sv129 mixed mice between the ages of 2 and 8 mo in both male and female mice on an LF chow diet. By Pearson analysis, ASP correlated with age, cholesterol, and NEFA (Fig. 1). In multiple linear regression analysis, lipids (TG, NEFA, and cholesterol) all significantly contributed to predicting ASP variance (R² = 0.714, P < 0.001), whereas age and body weight did not contribute additionally. ASP levels in C57Bl6 WT mice were comparable to the C57Bl6/Sv129 mixed mice with similar correlations (data not shown).

Next, we examined Tg mice known to have altered adipose tissue and compared their ASP values to WT mice. Table 1 shows the age, body weight, and lipid parameters for genetically obese leptin-deficient (ob/ob) mice, lean DGAT1 KO mice and Tg C/EBPα KO mice overexpressing the β-locus (C/EBPαβ Tg) with their appropriate age- and sex-matched WT mice (on the same genetic background). As expected, ob/ob mice had elevated body weight due to a significant increase in adipose tissue mass and plasma glucose with a
corresponding increase in ASP levels (Table 1 and Fig. 1D). DGAT1 KO mice displayed reduced plasma NEFA, glucose, and ASP levels despite similar body weight (Table 1 and Fig. 1D). In young C/EBPαTg mice, plasma ASP was significantly lower than in WT controls matched for age and body weight, whereas TG, NEFA, and glucose levels were elevated (Table 1 and Fig. 1D). Plasma ASP was also measured in alternative complement system KO mice. By definition, C3KO mice are ASP deficient, since they lack the ASP precursor protein C3. As shown in Fig. 1D, no ASP was detected when plasma from FBKO, ADKO, and C3KO mice was measured, despite the elevated C3 levels previously reported in ADKO mice (41). WT mice on the HF diet demonstrated a 322% increase in plasma ASP levels over LF-fed mice, comparable to the levels detected in ob/ob mice (Fig. 1D and Table 2). However, even on the HF diet there was no detectable plasma ASP in C3KO or FBKO mice (Table 2).

Body weight, adiposity, and lipid values of C3KO and FBKO mice. To demonstrate that the C3KO phenotype is consequent to the absence of ASP and not C3, physiological studies were evaluated in FBKO mice, which lack ASP as shown above. Since FBKO mice have normal or increased expression of C3 but under normal, nonstressed physiological conditions are ASP deficient, we evaluated whether their phenotype was similar to that of C3KO mice. C3KO, FBKO, and WT mice (all back-crossed on C57Bl/6 background) were placed on LF and HF diets for 12 wk starting at 8 wk of age. There were no significant differences in body weight between both KO groups and WT mice on LF (Table 2). Mouse adiposity was examined at the end of the diet regimen by DEXA analysis. Consistent with body weight, there were no differences in percent body fat between LF-fed mice (WT LF: 21.0 ± 1.3%; C3KO LF: 26.2 ± 3.0%; FBKO LF: 24.5 ± 2.5%; NS, n = 6–8 mice per group). As expected, the HF diet increased mouse body weight and adiposity compared with the LF diet; however, there were no differences among the three genotypes fed the HF diet (WT HF: 37.6 ± 1.2%; C3KO HF: 39.7 ± 1.5%; FBKO HF: 35.2 ± 3.1%; NS, n = 6–8 mice per group).

Fasting plasma glucose and insulin levels were similar, as were fasting leptin, adiponectin, and lipid levels, in C3KO, FBKO, and WT mice on the LF diet (Table 2). There were also no genotype-related differences in circulating insulin, leptin, or adiponectin levels.

Table 1. Age, lipid, and glucose values for WT, ob/ob, DGAT1 KO and C/EBPαTg mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Age, mo</th>
<th>Weight, g</th>
<th>ASP, nmol/l</th>
<th>TG, mmol/l</th>
<th>NEFA, mmol/l</th>
<th>Chol, mmol/l</th>
<th>Glucose, mmol/l</th>
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<tr>
<td>WT1, n = 33</td>
<td>3.7 ± 0.1</td>
<td>26.7 ± 0.9</td>
<td>7.63 ± 1.08</td>
<td>0.77 ± 0.07</td>
<td>0.93 ± 0.07</td>
<td>1.78 ± 0.16</td>
<td>8.82 ± 0.50</td>
</tr>
<tr>
<td>ob/ob, n = 6</td>
<td>4.8 ± 0.6</td>
<td>54.1 ± 1.2</td>
<td>14.15 ± 1.58*</td>
<td>0.79 ± 0.07</td>
<td>0.99 ± 0.16</td>
<td>1.81 ± 0.17</td>
<td>17.02 ± 1.56</td>
</tr>
<tr>
<td>DGAT1 KO, n = 8</td>
<td>3.5 ± 0.5</td>
<td>23.6 ± 1.6</td>
<td>3.52 ± 0.57</td>
<td>0.66 ± 0.2</td>
<td>0.38 ± 0.04†</td>
<td>1.50 ± 0.38</td>
<td>2.02 ± 1.29†</td>
</tr>
<tr>
<td>WT2, n = 8</td>
<td>2.2 ± 0.0</td>
<td>21.5 ± 1.2</td>
<td>12.28 ± 2.72</td>
<td>0.53 ± 0.06</td>
<td>0.58 ± 0.11</td>
<td>1.96 ± 0.37</td>
<td>6.64 ± 0.54</td>
</tr>
<tr>
<td>C/EBPαTg, n = 5</td>
<td>1.5 ± 0.0†</td>
<td>20.0 ± 1.1</td>
<td>3.71 ± 0.39*</td>
<td>1.29 ± 0.27†</td>
<td>0.99 ± 0.15*</td>
<td>2.11 ± 0.25</td>
<td>9.63 ± 0.72†</td>
</tr>
</tbody>
</table>

Values are means ± SE. ASP, acylation-stimulating protein; TG, triglyceride; NEFA, nonesterified fatty acid; Chol, cholesterol; WT, wild type; DGAT, diacylglycerol acyltransferase; KO, knockout; C/EBP, CCAAT enhancer-binding protein; Tg, transgenic. Significance is indicated by *P < 0.05, †P < 0.001 for KO vs. WT as analyzed by 1-way ANOVA (for WT1, ob/ob, and DGAT1 KO mice) or t-test (for WT2 and C/EBPαTg).
adiponectin levels in HF-fed animals. However, both C3KO and FBKO mice fed the HF diet displayed significantly elevated fasting TG levels compared with the HF-fed WT mice (Table 2). Furthermore, the glucose tolerance test revealed that both C3KO LF and FBKO LF mice displayed delayed glucose clearance (Fig. 2, P < 0.0001 by two-way RM ANOVA), consistent with results obtained in ASP receptor-deficient C5L2KO mice (27).

C3KO and FBKO mice have an exaggerated postprandial lipemic response. We have previously reported in a separately established C3KO mouse colony that C3KO mice displayed delayed postprandial TG clearance when challenged with an oral fat load (23, 24, 40). In the present study, using an independently derived C3KO colony (28), we again have demonstrated a similar delay in postprandial lipid clearance (Fig. 3) in both LF- and HF-fed diet mice. FBKO mice had a postprandial response similar to that of C3KO mice on both the LF (Fig. 3A) and HF (Fig. 3B) diet, and the delay in TG clearance compared with WT controls was significant (P < 0.0001 by two-way RM ANOVA). The increase in postprandial NEFA levels closely followed the TG response (Fig. 3, C and D). Postprandial NEFA levels were also similar between C3KO and FBKO mice on either diet and were increased compared with WT controls (P < 0.0001 by two-way RM ANOVA).

Adipose tissue TG content and cell morphology. Although there were no significant differences in adipose tissue mass (data not shown) or gonadal tissue total TG content (WT LF: 1.59 ± 0.33 μmol TG/μg DNA; C3KO LF: 1.06 ± 0.17 μmol TG/μg DNA; FBKO LF: 1.28 ± 0.17 μmol TG/μg DNA, NS, n = 6–8 mice per group), upon closer inspection, there were differences in adipose tissue morphology. Adipsin mRNA expression, a marker of adipocyte differentiation, is shown in Table 3. On the LF diet, gonadal adipin mRNA levels were comparable between WT LF and C3KO LF. Consistent with increased adiposity, adipsin levels were increased by HF feeding in WT mice (Table 3, P < 0.001 by two-way ANOVA).

Histological analysis revealed that in LF-fed mice average gonadal cell size was similar between WT and C3KO mice (WT LF: 191.4 ± 1.7 μm, and C3KO LF: 187.9 ± 2.3 μm, NS, n = 400 cells); however, cell size distribution was significantly different, as shown in Fig. 4. Small (below 25th %ile) and large (above 75th %ile) adipocyte size was based on WT LF cell circumference distribution, as shown in Fig. 4A. C3KO mice displayed an increased number of both small and large cells, resulting in a polarized distribution (Fig. 4B). Gonadal adipose tissue obtained from C3KO LF mice displayed a 40% increase (P < 0.001) in small cells and a 32% increase (P < 0.05) in large cells compared with WT LF by χ² analysis (Fig. 4C). Similar observations were made in the inguinal depot. Although the same average adipocyte circumference was found in both WT and KO mice (WT LF: 168.5 ± 2.6 μm, and C3KO LF: 175.5 ± 3.7 μm, NS, n = 300 cells), the distribution in the C3KO on the LF diet also displayed a polarized adipocyte cell size with an increase in the amount of smaller (+29%) and larger (+37%) cells compared with WT LF (Fig. 4, D and E). In WT and C3KO mice on the HF diet, both gonadal and inguinal mean cell circumference increased compared with LF cohorts; however, although there were no differences in mean cell size, a similar polarized cell distribution was observed in C3KO HF mice (data not shown).

LPL and DGAT1 mRNA and activity. Two key adipose tissue enzymes, LPL and DGAT1, were examined in both inguinal and gonadal depots. There were no differences in

### Table 2. Fasting body weight and plasma values

<table>
<thead>
<tr>
<th></th>
<th>WT LF</th>
<th>C3KO LF</th>
<th>FBKO LF</th>
<th>WT HF</th>
<th>C3KO HF</th>
<th>FBKO HF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>30.9±0.6</td>
<td>32.7±1.8</td>
<td>33.9±1.5</td>
<td>38.1±1.1</td>
<td>40.4±1.4</td>
<td>41.0±2.3</td>
</tr>
<tr>
<td>ASP, nmol/l</td>
<td>2.30±0.50</td>
<td>ND</td>
<td>0.07±0.03*</td>
<td>9.72±2.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Leptin, μg/ml</td>
<td>5.89±0.92</td>
<td>8.5±1.13</td>
<td>12.04±3.37</td>
<td>15.54±3.93</td>
<td>22.97±4.92</td>
<td>21.4±7.81</td>
</tr>
<tr>
<td>Adiponectin, μg/ml</td>
<td>12.32±1.86</td>
<td>6.6±1.19</td>
<td>9.11±3.90</td>
<td>13.88±2.11</td>
<td>13.13±2.72</td>
<td>8.68±2.11</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>0.28±0.06</td>
<td>0.35±0.08</td>
<td>0.33±0.09</td>
<td>0.45±0.09</td>
<td>0.68±0.22</td>
<td>0.50±0.18</td>
</tr>
<tr>
<td>TG, mmol/l</td>
<td>0.23±0.05</td>
<td>0.41±0.05*</td>
<td>0.44±0.04*</td>
<td>0.25±0.06</td>
<td>0.58±0.06†</td>
<td>0.52±0.06*</td>
</tr>
<tr>
<td>NEFA, mmol/l</td>
<td>0.63±0.06</td>
<td>0.82±0.16</td>
<td>0.92±0.19</td>
<td>0.57±0.07</td>
<td>1.17±0.23*</td>
<td>0.71±0.12</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>5.82±0.15</td>
<td>6.1±0.40</td>
<td>7.15±0.67</td>
<td>5.4±0.32</td>
<td>7.7±0.77</td>
<td>5.9±0.62</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–10 mice per group. C3KO, C3 knockout; FBKO, factor B knockout; LF, low-fat diet; HF, high-fat diet; ND, none detected. Significance is indicated by *P < 0.05, †P < 0.01 for KO vs. WT counterparts as analyzed by 1-way ANOVA.

Fig. 2. Glucose tolerance test. A: plasma glucose levels following i.p. injection of glucose (2 mg/g) for WT (●), C3KO (○) and FBKO (●) mice on low-fat (LF) diet. **P < 0.05, ††P < 0.01 for KO vs. WT.
inguinal LPL expression and activity between WT and C3KO mice on either LF or HF diet (Table 4). In gonadal adipose tissue, although LPL expression was similar, activity was elevated by 213% in C3KO LF mice compared with WT LF (Table 4). DGAT is the final enzyme in the TG synthesis pathway and plays a key role in TG storage. On both the LF diet (Fig. 5A) and HF diet (Fig. 5B), C3KO DGAT1 mRNA was reduced by 57 and 35% in the inguinal depot and by 31 and 58% in the gonadal depot, respectively. Reduced DGAT1 expression resulted in decreased activity in the inguinal depot (−41%), but not in the gonadal depot (Fig. 5, C and D).

**DISCUSSION**

The present study shows that, similar to humans (33, 36), plasma ASP in mice is increased in obesity and reduced with leanness and increased energy expenditure. Interestingly, ASP levels are also reduced with age, a phenomenon observed previously in humans, with children tending to have higher ASP values than adults (7). However, in contrast to leptin, another adipokine that is known to reflect body weight and adiposity, ASP levels in WT mice correlated more strongly with lipid values, with no additional significant contribution provided by age and body weight. Increased levels of both ASP and its precursor C3 have been shown to be associated with metabolic disturbances. ASP and C3 levels are increased in type 2 diabetics independently of obesity (42). Plasma ASP levels are elevated in patients with cardiovascular disease characterized by increased apolipoprotein B and hypertriglyceridemia compared with age- and sex-matched controls (10), and C3 has also been shown to be a marker for myocardial infarction risk (26). More recently, C3 was identified as a robust maker of insulin resistance in an elderly population (25). Increases in inflammatory markers are becoming well recognized in metabolic diseases (diabetes, cardiovascular disease); ASP and C3 are only two examples of these adipose tissue-associated immune molecules.

This is also the first study to show that FBKO and adipsin-KO mice are ASP deficient. Since factor B and adipsin are key players in the alternative complement pathway, these results present direct proof that, under normal physiological conditions, ASP is produced primarily through the alternative pathway of complement activation. The three pathways of complement, the alternative, classical, and lectin systems, all require C3. C3a is generated not only by the alternative pathway but can be produced via interaction of C3 with C2/C4 during activation of the classical and lectin systems. Interestingly, C2 and C4 have recently been shown to be expressed in human adipose tissue (15). Although there is no direct evidence, C2 and C4 are not likely involved in ASP production, as their activation is antibody (classical) or carbohydrate (lectin) dependent (reviewed in Ref. 35). Diet-derived TG-rich chylomicron lipoproteins accelerate the C3 tickover process, increasing ASP formation (14). In addition, the present study has shown that C3KO and FBKO mice

**Table 3. Gonadal adipsin expression**

<table>
<thead>
<tr>
<th>Diet</th>
<th>WT</th>
<th>C3KO</th>
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<tbody>
<tr>
<td>LF diet</td>
<td>1.40±0.13</td>
<td>1.54±0.28</td>
</tr>
<tr>
<td>HF diet</td>
<td>2.66±0.33</td>
<td>1.77±0.26*</td>
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</tbody>
</table>

Values are means ± SE in arbitrary units (AU) of adipsin/18S mRNA expression in gonadal adipose tissue; n = 10–13 measurements. Significance is indicated by *P < 0.05 for KO vs. WT as analyzed by t-test.
manifest similar adipose tissue- and lipid metabolism-related phenotypes. Both C3KO and FBKO mice display altered lipid metabolism and morphological changes in adipocyte distribution. Therefore, it appears that it is the lack of ASP rather than the lack of the precursor C3 that drives the lipid metabolism phenotype observed in these mice.

A detailed investigation of adipose tissue obtained from C3KO mice revealed reduced adipin expression, increased number of smaller fat cells, and decreased TG storage capacity, as reflected in protein expression and morphological and enzyme activity changes. Remarkably, the increase in small adipocytes was also accompanied by an increase in large cells. A polarization in fat cell size has been previously reported in fat-specific insulin receptor KO (FIRKO) mice (2). The underlining mechanism for the two subsets of adipocytes is still unknown; however, the smaller fat cells were shown to have reduced adipogenic transcription factor expression (C/EBPα and SREBP-1) and fatty acid synthase (FAS) expression, suggesting that the smaller adipocytes were protected from excess lipid load, whereas the larger population maintained

Table 4. Inguinal and gonadal LPL expression and activity

<table>
<thead>
<tr>
<th>Mice</th>
<th>Inguinal Adipose Tissue</th>
<th>Gonadal Adipose Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LPL/18S mRNA, AU</td>
<td>LPL Activity, nmol•g⁻¹•h⁻¹</td>
</tr>
<tr>
<td>WT LF</td>
<td>2.21±0.24</td>
<td>341.7±53.4</td>
</tr>
<tr>
<td>C3KO LF</td>
<td>3.33±0.74</td>
<td>317.8±61.7</td>
</tr>
<tr>
<td>WT HF</td>
<td>2.71±0.21</td>
<td>317.7±53.3</td>
</tr>
<tr>
<td>C3KO HF</td>
<td>2.84±0.45</td>
<td>364.5±37.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10–16 measurements. Lipoprotein lipase (LPL) expression and activity measured in inguinal and gonadal adipose tissue. Significance is indicated by †P < 0.001 for KO vs. WT counterpart as evaluated by t-test.
normal TG stores (2). Delayed postprandial TG clearance and altered adipocyte distribution may both impact TG clearance and storage in ASP-deficient mice. LPL, a regulator of lipoprotein TG lipolysis, and DGAT1, a TG synthetic enzyme, were examined to evaluate lipid metabolism in C3KO mice. LPL activity remained largely unchanged in the inguinal adipose tissue depot, with an increase only in gonadal tissue in C3KO LF mice. This increase in LPL activity may partially compensate for decreased adipsin and smaller cell size in C3KO LF gonadal tissue. However, in the postprandial state (during the fat load), the significant increase in NEFA could inhibit LPL function (13), explaining the observed delay in both TG and NEFA clearance. Both DGAT1 expression and total DGAT activity were downregulated in C3KO mice inguinal tissue, whereas only DGAT1 expression was reduced in gonadal tissue, without change in activity. This may be attributed to depot specificity, as adipose tissue depot-specific changes in enzyme activity have been previously reported (1). Overall, decreased (or deficient) ASP levels are associated with reduced TG storage and altered utilization.

Our previous in vivo studies in independently derived C3KO mice obtained from Colten et al. (11) describe the C3KO mice as being lean with reduced leptin levels and increased energy expenditure (22). The changes in adiposity were a reflection of decreased adipose tissue depots, and this was observed in mice with either lower (22, 40) or normal body weight (24). In the present study, we used a separately derived breeding colony of C3KO mice (28) to ascertain and validate the ASP-deficient phenotype. Consistent with previous reports (24, 40), the C3KO mice studied here demonstrated delayed postprandial clearance. The mice were also hyperphagic; however, the hyperphagia was greater than in the previous study [+50% (personal observations) vs. +17% (24)]. Such a large compensation likely explains the maintenance of normal body weight and adiposity, which was not maintained when the mice were crossed to an atherogenic apoE LDL receptor KO background (30). Interestingly, ASP receptor KO mice (C5L2KO) also display delayed postprandial lipemia, hyperphagia, and increased fatty acid oxidation (27). Remarkably, the phenotypes of mice lacking either ASP or its receptor (C3KO and C5L2KO) are remarkably similar with respect to lipemic response and energy expenditure (8, 27, 40).

In the present study, plasma ASP was measured in several mouse models of altered adipose tissue, such as genetically obese and lean mice. As anticipated, leptin-deficient ob/ob mice displayed increased adiposity, hyperphagia, and reduced energy expenditure (39), and ASP levels were elevated compared with lean mice. ASP was also increased in HF-fed WT mice. Increased ASP was also found in another obese rodent model, the interleukin-6 KO (IL6KO) mouse. IL6KO mice develop mature onset obesity, and ASP has been shown to contribute to the development of weight gain, and, in fact, an increase in ASP precedes the weight gain (37). In contrast, DGAT1 KO mice displayed reduced ASP levels. DGAT1 KO mice are lean, have reduced WAT mass and increased energy expenditure, and are resistant to diet-induced obesity (34), and ASP-deficient mice bear a striking resemblance to the DGAT1 KO mice. In addition, C/EBPεTg mice also displayed significantly reduced ASP levels (present study) and adipin levels (3). These Tg mice are characterized by reduced fat mass with decreased adipocyte size and plasma lipid accumulation (3). Furthermore, ASP-deficient mice show reduced TG storage capacity, as evaluated by delayed postprandial TG clearance and DGAT1 activity.

The mouse models investigated here illustrate that as the amount of WAT decreases there is a significant reduction in plasma ASP levels, and vice versa. Altogether, this suggests that increased ASP promotes fat accumulation, and decreased

Fig. 5. DGAT expression and activity in inguinal and gonadal adipose tissue. DGAT1 expression for WT (open bars) and C3KO (hatched bars) on LF (A) and HF (B) diet as well as total DGAT activity for LF (C) and HF (D) diet are shown. Results are expressed as means ± SE; n = 6–8 per group. Statistical differences were determined by t-test, where *P < 0.05, **P < 0.001, ***P < 0.0001, and #P = 0.055.

A

B

C

D

E527

ADIPOSE TISSUE MORPHOLOGY IN ASP-DEFICIENT MICE

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