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Journal
The Journal of biological chemistry, 279(6)

ISSN
0021-9258

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Publication Date
2004-02-01

DOI
10.1074/jbc.m311319200

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 6, Issue of February 6, pp. 4051–4057, 2004
Printed in U.S.A.
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Acylation-stimulating Protein (ASP)/Complement C3adesArg Deficiency Results in Increased Energy Expenditure in Mice*

Received for publication, October 15, 2003
Published, JBC Papers in Press, November 13, 2003, DOI 10.1074/jbc.M311319200

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Acylation-stimulating protein (ASP) acts as a paracrine signal to increase triglyceride synthesis in adipocytes. In mice, C3 (the precursor to ASP) knock-out (KO) results in ASP deficiency and leads to reduced body fat and leptin levels yet they are hyperphagic. In the present study, we investigated the mechanism for this energy repartitioning. Compared with wild-type (WT) mice, male and female C3(−/−) ASP-deficient mice had elevated oxygen consumption (VO_2) in both the active (dark) and resting (light) phases of the diurnal cycle: +8.9% males ($p < 0.05$) +9.4% females ($p < 0.05$). Increased physical activity (movement) was observed during the dark phase in female but not in male KO animals. Female WT mice moved 16.9 ± 2.4 m whereas KO mice moved 30.1 ± 5.4 m, over 12 h, +78.4%, $p < 0.05$). In contrast, there was no difference in physical activity in male mice, but a repartitioning of dietary fat following intragastric fat administration was noted. This was reflected by increased fatty acid oxidation in liver and muscle in KO mice, with increased UCP2 (inguinal fat) and UCP3 (muscle) mRNA expression ($p = 0.005$ and 0.036, respectively). Fatty acid uptake into brown adipose tissue (BAT) and white adipose tissue (WAT) was reduced as reflected by a decrease in the fatty acid incorporation into lipids (BAT −68%, WAT −29%. The decrease of FA incorporation was normalized by intraperitoneal administration of ASP at the time of oral fat administration. These results suggest that ASP deficiency results in energy repartitioning through different mechanisms in male and female mice.

Acylation-stimulating protein (ASP)[1] is an adipocyte-derived protein that has potent anabolic effects on human adipose tissue where it increases glucose uptake and non-esterified fatty acid (NEFA) storage (1, 2) via translocation of glucose transporters (GLUT1, GLUT3, and GLUT4) from intracellular sites to the cell surface (3, 4) and activation of diacylglycerol acyltransferase (DGAT) (2). These effects appear to be mediated through specific cell surface binding (5, 6) resulting in activation of a signal pathway that includes protein kinase C (7). In addition, ASP has been shown to inhibit hormone-sensitive lipase in adipocytes, independently and additively to insulin (8). There is a differentiation-dependent increase in ASP binding and ASP response in human adipocytes (1). The major site of action of ASP is adipocytes, as determined by competitive binding, stimulation of triglyceride synthesis, enhanced glucose transport, and transporter translocation (5).

ASP is identical to C3adesArg, a cleavage product of complement C3. Cleavage of complement C3 is mediated through the alternate complement pathway via the interaction of C3, factor B, and adipsin that generates C3a. Rapid cleavage of the C-terminal arginine of C3a by carboxypeptidase N generates ASP (9). Adipocytes are one of the few cells capable of producing all three factors (factor B, adipsin, and C3) that are required for the production of ASP (10). ASP production increases consequent to adipocyte differentiation (11) and plasma ASP levels are elevated in obesity (12, 13). *In vitro* chylomicrons stimulate ASP production by adipocytes (14, 15). *In vivo* arterial-venous gradients across a subcutaneous adipose tissue bed in humans demonstrate direct postprandial production of ASP (16). The postprandial increase in ASP is adipose tissue specific and is not observed in the general circulation (17). Altogether, these data suggest that ASP and lipid storage are metabolically intertwined.

ASP acts as an adipocyte autocrine factor and we propose that it plays a central role in the metabolism of adipose tissue by increasing the efficiency of triglyceride synthesis in adipocytes, an action that results in more rapid postprandial lipid clearance (18). As ASP is derived through cleavage of complement C3, C3 knock-out mice (C3−/−) are necessarily deficient in ASP. We have previously demonstrated that genetic deficiency of ASP leads to reduced body fat and decreased leptin levels (19, 20). In addition, male mice have delayed triglyceride clearance (16, 20) although this has not been demonstrated in all studies (21). Studies with double knock-out mice (C3−/−, ob/ob) further demonstrated a resistance to development of obesity, delayed triglyceride synthesis and demonstrated that these effects of ASP were independent of leptin.

Strikingly, however, although ASP-deficient mice (C3−/−) have decreased body weight and delayed TG and NEFA clearance, they have a marked increased in food intake. This raised the question: why are ASP-deficient mice leaner if they ingest more caloric energy? We addressed that question by examining

* This study was supported by grants from the National Science and Engineering Council of Canada (NSERC, to K. C.), University of California, Davis, Clinical Nutrition Research Unit, National Institutes of Health Grants DK-50129, DK-35747, and the American Diabetes Association (to P. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ Supported by a visiting scholarship from FRSQ/National Science Foundation of China.

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[1] The abbreviations used are: ASP, acylation-stimulating protein; BAT, brown adipose tissue; WAT, white adipose tissue; FA, fatty acid; TG, triglyceride; KO, knock-out; ANOVA, analysis of variance; WT, wild type; UCP, uncoupling protein; NEFA, non-esterified fatty acid; DGAT, diacylglycerol acyltransferase; RQ, respiratory quotient; AUC, area under curve; VO_2, oxygen consumption; NS, not significant.

energy expenditure and postprandial fat partitioning during resting and active phases of the diurnal (light:dark) cycle.

MATERIALS AND METHODS

Generation and Genotyping of Mice—The genetic background of the mice used in this study was 129Sv. Complement C3 knock-out (C3−/−) mice (129Sv × C57B6 genetic background), as well as wild-type mice, were originally obtained from Dr. Harvey Coulton. Mice were back-crossed 8–10 generations to obtain a homogenous 129Sv genetic background. (C3−/−) 129Sv back-crossed mice demonstrate a distinct delay in triglyceride clearance, which the C57Bl/6 (C3−/−) mice do not, although the reduced leptin and leanness were evident in both back-crossed strains (18). Mice were housed in a pathogen-free barrier facility, 21 °C, 12 h dark/12 h light cycle, and fed regular rodent chow food. All procedures on mice followed the guidelines established by the CACC and were approved by the Royal Victoria Hospital 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 weeks of age (19, 20).

Oxygen Consumption, Respiratory Quotient (RQ), Physical Activity, Body Weight, Food Intake, and Body Composition—Mice were fed a low fat diet (10% of daily caloric intake being provided by fat), which represents a normal mouse chow diet. This diet contained 19.3% protein, 67.3% carbohydrate, and 4.3% fat w/w. The mice were weighed weekly, and the food was weighed three times weekly to determine food intake. Body composition analysis was measured with calculation of percent water, fat, and protein.

Metabolic rate and activity was measured using the Integra ME System from AccuScan Instruments, Inc. (Columbus, OH). The system consists of an O_2 analyzer, CO_2 analyzer and a PhysioScan analyzer, which monitors all vertical, horizontal, and ambulatory movement via light beam interruptions. A flow controller/channelyzer allows for flow rate adjustments and for sequential channeling of the airflow from the air reference line and the four animals chambers through the CO_2 and O_2 analyzer. The plexiglass animal chambers were 16 × 16 × 8 inches. Animals were provided with their standard food and water while in the chambers, and were acclimated to the chambers for 18 h immediately prior to the 24 h data collection period. The Integra ME software includes an O_2 and CO_2 analyzer calibration program and data collection and analysis programs. Reported values include O_2 consumption, CO_2 production, RQ, heat production, total ambulatory movement and total rest time.

Intragastric Fat Administration—After an overnight fast (16 h), 400 μl of olive oil labeled with 50 μCi of [^{3}H]oleate (followed by 100 μl of air above the oil) was administered by gastric gavage using a feeding tube (12-cm curved ball tipped feeding needle, Ref. 22) according to standard procedures as previously published (19, 20, 23–26). Where indicated, ASP was injected intraperitoneally at the time of fat administration. ASP was purified and injected as described previously (7, 27). Each mouse received intraperitoneal injections of either sterile ASP (500 μg) dissolved in 300 μl of phosphate-buffered saline, pH 7.4, containing 1 mg/ml fatty acid free bovine serum albumin (Sigma) or sterile vehicle (same solution without ASP). Previous experiments have demonstrated that injection of the vehicle solution (1 mg/ml bovine serum albumin in phosphate-buffered saline, 300 μl) had no effect of postprandial TG clearance in the mice compared with the same mice without vehicle (19, 20). Blood was collected at the time points indicated for analysis. Total muscle tissue weight was calculated as 25% of body weight based on previous published data in 129Sv mice (28).

Plasma Assays—Blood was collected (at the indicated ages) for TG and NEFA from overnight (16 h) fasted mice (with water *ad libitum*) or after intragastric fat administration. Blood was separated by centrifugation and stored at −80 °C until analysis. Plasma non-esterified fatty acids (NEFA), triglyceride, and glucose were measured using colorimetric enzymatic kits (Roche Applied Science). Insulin, adiponectin, and leptin were measured by commercial immunoassay (Linco Research, Inc).

Tissue Analysis—The mice were anesthetized with a mixture of 5 ml of Ketamine (100 mg/ml), 2.5 ml of xylazine (20 mg/ml), 1 ml of aceprozamine (10 mg/ml), and 1.5 ml of sterile saline amounting to 0.01 ml/10 g body weight. They were then sacrificed by cervical dislocation. The following tissues were excised: spleen, kidney, heart, skeletal muscle (quadriceps), liver, intrascapular brown adipose tissue (BAT), peritoneal adipose tissue, gonadal adipose tissue, pectoral adipose tissue, inguinal adipose tissue, intestine, and stomach. The samples were weighed and frozen in liquid nitrogen at −80 °C for later analysis. Tissue fragments (10–50 mg) were extracted to measure total lipid and separated from fatty acid oxidation products. Following extraction with 2 ml of chloroform/methanol (2:1), samples were mixed overnight. On the following day, 1 ml of 50 mM $CaCl_2$ was added, and the suspension was centrifuged at 3000 rpm 4 °C for 20 min to achieve a phase separation. The total lipids in the chloroform/methanol layer were separated, evaporated and resuspended in chloroform/methanol (2:1) for separation by thin layer chromotagraphy as previously published to quantify triglyceride, diglyceride, fatty acid, and polar lipids by scintillation counting (29). The aqueous phase containing oxidation products was counted directly.

UCP mRNA Expression—Separate sets of wild-type and KO mice were sacrificed for RNA analysis. Tissues were collected and immediately frozen in liquid nitrogen, then stored at −80 °C until analysis. The primers used for testing were: UCP-1: sense 5′-AGCAA GAGGAAGG-GACGCTC-3′ (38–57 bp), antisense 5′-TTCGGAAGTTGTCGGGTT TC-3′ (255–237 bp). UCP-2: sense 5′-GTTTCGTCTCCCAGCCATTT-T-3′ (61–80 bp), antisense 5′-TGATTTCCTGCTACCTCCCA-3′ (290–271 bp) and UCP-3: sense 5′-ACTGTATGCTGAAGATGGTGGC-T-3′ (925–946 bp), antisense 5′-AATGTTAGGCATCCAACCG-3′ (1315–1287 bp). 18 S was used as housekeeping gene (Ambion).

Total RNA was isolated from the four tissue samples from each mouse, using the TRIzol method (Invitrogen, Life Technologies, Inc.). Reverse-transcription was performed using 3 μg RNA and Moloney murine leukemia virus reverse transcriptase in buffer containing dNTPs, RNase inhibitor and oligo(dT) primer (Invitrogen, Life Technologies, Inc.). The resulting cDNA (RT mixture) was subjected to PCR. Briefly, 2 μl of cDNA and UCP-1, 2, or 3 primers (1 μM final concentration) were mixed with 0.5 units of TaqDNA polymerase/reaction tube in standard buffer (0.2 mM dNTPs, 100 μM TMAC, and $MgCl_2$ diluted to 2 mM). The cDNA was amplified for 5 min at 95 °C followed by 30 cycles of (1 min 95 °C, 1 min 60 °C and 1 min 72 °C), followed by 7 min 72 °C. The PCR components (*Taq* polymerase, $MgCl_2$, PCR buffer, primers, 10^{-4} M TMAC (tetramethylammonium chloride)) were all obtained from Invitrogen, Life Technologies, Inc.

The amplified cDNA was separated by electrophoresis on 7.5% polyacrylamide gel in 1.5 mM Tris-HCl, pH 8.8 buffer at 200 V, with silver staining (BioRAD) and dried. Quantification was done by gel scanning, using the Molecular Analyst kit (BioRAD), referenced against DNA concentration standards (BioRAD) that were electrophoresed with every gel. Results were expressed as a ratio of specific UCP/18 S RNA.

Statistical Analysis—All results are presented as average ± standard error (S.E.). Statistical comparisons were by Student's *t* tests or ANOVA, as indicated in the text and figure legends. Statistical significance was set at $p < 0.05$, where NS indicates not significant.

RESULTS

We have previously reported that C3(−/−) ASP-deficient mice (KO) have reduced body weight, adipose tissue mass, and plasma leptin but consume 30% more energy compared with WT mice (19, 20). This was also true of the present study where KO mice had a reduced body weight and increased food intake. Leptin levels were reduced: WT 6.23 ± 1.45 *versus* KO 4.23 ± 0.70 ng/ml, $p < 0.05$. Body composition analysis demonstrated decreases in percent fat in KO as compared with WT: WT male 23.15% ± 1.64 *versus* KO male 16.02% ± 0.77 $p < 0.05$ and WT female 18.28% ± 1.16 *versus* KO female 14.74% ± 0.88 $p < 0.05$, g fat/g carcass weight, ANOVA = 0.003.

In addition, we have previously demonstrated that ASP-deficient C3(−/−) mice have increased insulin sensitivity as reflected by decreased HOMA (homeostatic model assessment) based on fasting insulin and glucose levels (19, 20), even when the mice are backcrossed onto an obese (*ob*/*ob*) background (30). ASP-deficient mice also demonstrate enhanced clearance of glucose following an oral fat load and an oral glucose tolerance test (19, 20). In the present study, the KO mice also demonstrated decreases in insulin relative to glucose and lower HOMA where the values were: WT 3.48 ± 0.19 *versus* KO 3.64 ± 0.14 mM of glucose, WT 9.46 ± 1.22 *versus* KO 7.90 ± 0.56 μu/ml insulin and WT 1.46 ± 0.22 *versus* KO 1.21 ± 0.11 mM·μu/ml for HOMA.

WT and KO mice were randomly selected except for the experiments with intragastric fat administration on male mice. In this case, we deliberately matched the body weights of the

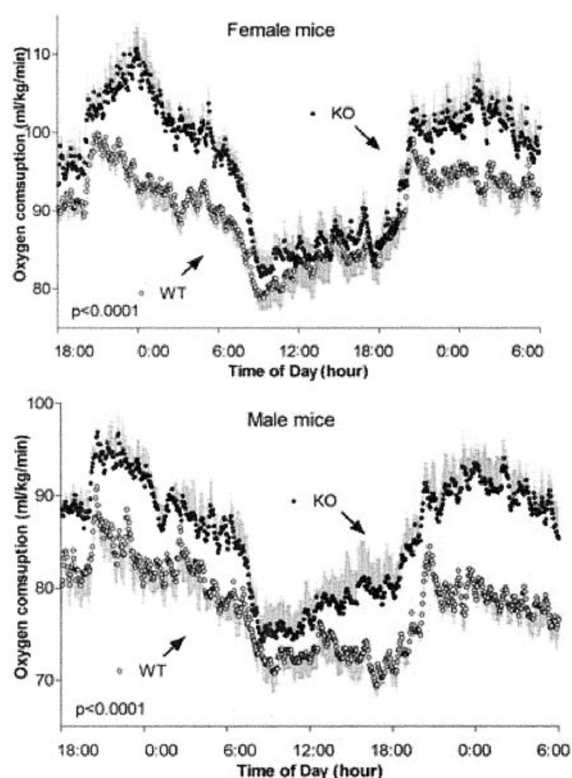

FIG. 1. **KO mice have increased oxygen consumption: oxygen consumption was tested over 36 h.** Samples were recorded every 3 min. Both male and female KO mice had significantly increased oxygen consumption compared with the wild-type group, where the number of mice was: male $n = 5$ (5 KO and 5 WT), female $n = 6$ (6 KO and 6 WT). The data were analyzed using 2-way ANOVA; active phase (20:00–8:00) $p = 0.0039$ males, $p = 0.027$ females; no significant difference in inactive phase (8:00–20:00).

KO and WT mice to avoid a partitioning bias resulting from a difference in adipose tissue mass and body weight (thus the KO mice were, on average, 2 weeks older).

Oxygen Consumption and Physical Activity (Movement) Monitoring—Mice were monitored for 42 h continuously, from 14:00 on day 1 to 8:00 on day 3. To allow for acclimation, only data from the final 24 h are reported (8:00 day 2 to 8:00 day 3). The mice were allowed to eat, drink, and move about freely. Movement was recorded, and the ambient gas was sampled and analyzed every 3 min. As shown in Fig. 1, in wild-type mice (both male and female), oxygen consumption (VO_2 measured as ml/kg/min) was greater in the active phase (20:00–8:00) and less in inactive phase (8:00–20:00) and the KO mice maintained this pattern. Interestingly, female mice had greater oxygen consumption than the male mice (both WT and KO) and this was confirmed in additional experiments where the mice were tested in a pairwise fashion. Both male and female KO mice had higher VO_2 in the active phase compared with wild-type mice (12 h active phase mean VO_2, 2-way ANOVA, $p = 0.0039$ males, $p = 0.027$ females). The differences were also significant when calculated as area under the curve (measured as ml O_2/BW^.67/min; $p = 0.032$ males, $p = 0.031$ females). The KO males had higher inactive phase VO_2 than WT, calculated as area under the curve (AUC) of VO_2 ($p < 0.05$, Table I). VO_2 during the inactive phase was not different between the KO and WT females.

KO female mice showed a substantial (78.4%, $p < 0.05$) increase in active phase movement (physical activity, Fig. 2) compared with WT females, but there were no differences in either active or inactive phase movement between KO and WT male mice. The diurnal pattern of the RQ was similar in the female KO and WT mice, while the KO males had a very slight increase in AUC of RQ during the active phase compared with WT males (Table I). On the other hand, measurement of rectal body temperature did not detect any significant differences between the groups when tested over a range of ages (5–30 weeks) and body weight (20–40 g): WT male 37.8 ± 0.13, KO male 37.7 ± 0.12, WT female 37.7 ± 0.10, and KO female 37.9 ± 0.07 °C, $n = 20$–30 mice per group).

Fat Administration in Male Mice—Three groups of body weight matched male mice were tested: 6 wild-type mice, 6 KO mice and 6 KO mice that received intraperitoneal (IP) injections of ASP at the same time as the intragastric fat bolus. Body weight for the groups was chosen so as to not be different (WT 25.9 ± 0.7 *versus* KO 25.0 ± 0.8 g), thus the KO mice were slightly older. Following an overnight fast, a fat load of olive oil containing [^{3}H]oleate was given to the mice, and serial blood samples was taken over 6 h (0, 1, 2, 3, 4, and 6 h). All the mice were sacrificed at 6 h after the fat load to determine [^{3}H]oleate distribution in various tissues. Even though the KO mice were matched for body weight to WT mice, KO mice had clearly delayed TG clearance (incremental AUC: WT 1.7 ± 0.4 mM·h, KO 6.1 ± 1.2 mM·h, $p < 0.01$; KO with IP ASP 3.8 ± 0.7 mM·h, $p < 0.05$ *versus* KO and *versus* WT) and NEFA clearance (AUC: WT 5.6 ± 0.1 mM·h, KO 7.0 ± 0.5 mM·h, $p < 0.05$; KO with IP ASP 6.0 ± 0.5 mM·h, $p < 0.05$ *versus* KO) after the fat load. Injecting ASP at the time of fat administration (time = 0) partially normalized both TG and NEFA clearance (Fig. 3). There was no significant difference in the amount of [^{3}H]oleate remaining in intestine and stomach in the three groups of animals (data not shown). We have previously reported that ASP deficiency did not alter fat absorption (19, 20).

[^{3}H]Oleate incorporation was measured in spleen, kidney, heart, skeletal muscle, liver, BAT, peritoneal adipose tissue, gonadal adipose tissue, pectoral adipose tissue, and inguinal adipose tissue in all three groups of mice (WT, KO, KO+ IP ASP). Data are reported as total [^{3}H]oleate radiolabel incorporated per organ (Fig. 4). For muscle, total body muscle mass was estimated as 25% of body weight according to previously published data (28). For both WT and KO mice, most of the absorbed radiolabel (average 92%) was present in the combined tissues of liver, muscle, and BAT. For the male KO mice, there was a +63% increase ($p < 0.05$) in muscle radiolabel (WT 5.14 ± 0.39, KO 8.37 ± 0.79, KO with IP ASP 5.66 ± 0.65 million dpm) and a +50% increase in liver radiolabel (WT 2.32 ± 0.27, KO 3.50 ± 0.26, KO with IP ASP 2.80 ± 0.06 million dpm) compared with WT; but a −59% decrease ($p < 0.05$) in ^{3}H radiolabel in BAT (WT 1.75 ± 0.31, KO 0.71 ± 0.25, KO with IP ASP 2.20 ± 0.61 million dpm, $p < 0.05$) (Fig. 4). Administration of ASP in KO mice normalized [^{3}H]oleate tissue uptake in these three tissues (Fig. 4). However, there was no difference in total counts in white adipose tissue (Fig. 4) or in spleen, kidney, or heart for all three groups (data not shown).

We then subfractionated the radiolabel into lipid and non-lipid (oxidation products) components. As shown in Fig. 5, in both BAT and WAT there were significant decreases in [^{3}H]oleate incorporation into the lipid component ($p < 0.05$), which were normalized to the WT level by ASP administration (BAT: WT 1.67 ± 0.20, KO 0.53 ± 0.20, KO with IP ASP 1.33 ± 0.15 million dpm; WAT: WT 0.65 ± 0.05, KO 0.46 ± 0.04, KO with IP ASP: 0.68 ± 0.04 million dpm). This decrease in [^{3}H]oleate incorporation was reflected primarily in a decrease in the triglyceride/diglyceride ratio (BAT: WT 186.1 ± 37.1, KO 84.8 ± 25.0, $p < 0.05$, KO with IP ASP 185.8 ± 31.7 *versus* KO $p < 0.05$; WAT: WT 27.0 ± 5.0, KO 9.8 ± 0.8, $p < 0.05$, KO with IP ASP: 8.3 ± 0.5), which indicated that there was relatively less diglyceride converted into triglyceride in KO in these tis-

TABLE I
Oxygen Consumption (VO_2) and Respiratory Quotient (RQ) in ASP-deficient C3 (−/−) and wild-type male and female mice

		Male		Female	
		Active phase[a]	Inactive phase[b]	Active phase	Inactive phase
VO_2-AUC (L/Kg)	WT	106.5 ± 2.0	73.9 ± 1.3	133.7 ± 1.5	75.4 ± 1.8
	KO	116.0 ± 2.9	81.7 ± 2.9	144.0 ± 2.6	78.3 ± 1.1
	Increase	8.9%	10.6%	9.4%	2.8%
	p	<0.05	<0.05	<0.05	NS[c]
RQ-AUC (min)	WT	1180.0 ± 6.4	645.7 ± 13.0	1237.9 ± 15.9	647.9 ± 16.5
	KO	1210.0 ± 7.2	644.5 ± 13.0	1247.9 ± 15.7	655.8 ± 8.3
	Increase	2.5%	−0.1%	0.8%	1.2%
	p	<0.05	NS	NS	NS

[a] The active phase was from 20:00-8:00.
[b] The inactive phase was from 8:00-20:00.
[c] NS, not significant.

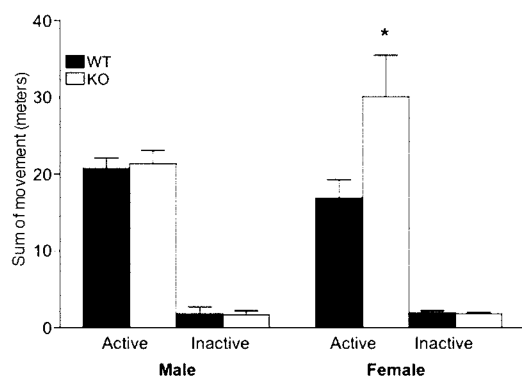

FIG. 2. **Female KO mice have increased movement compared with WT mice during the active phase (20:00–8:00, 78.4%).** There are no differences between male KO and WT mice. All mice had increased movement in active phase. * indicates KO *versus* WT, $p < 0.05$, where the number of mice was: male $n = 5$ (5 KO and 5 WT), female $n = 6$ (6 KO and 6 WT).

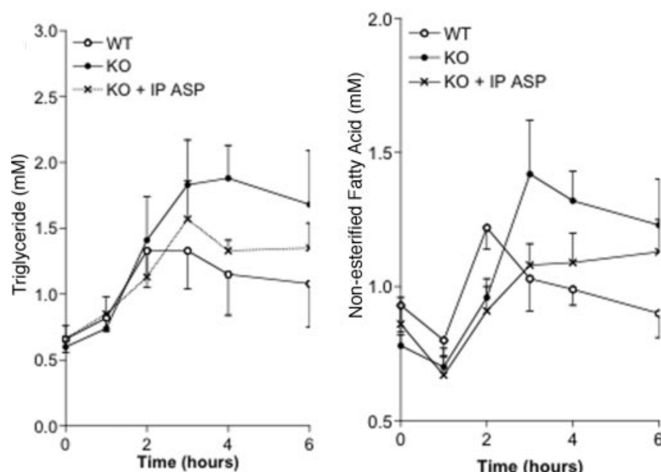

FIG. 3. **Male KO mice have delayed postprandial TG and NEFA clearance compared with WT mice.** A fat load was administered to KO and WT mice. Injection of ASP to one group of KO mice immediately before the fat load partially restores the efficiency of TG and NEFA clearance, where values are shown as for $n = 6$ per group (6 WT, 6 KO, 6 KO with IP ASP). The data were analyzed using ANOVA where $p < 0.05$ for KO *versus* WT.

sues. On the other hand, in these tissues, there was no change in the aqueous phase radiolabel, which represents fatty acid oxidation products (data not shown).

Quite different results were obtained in muscle and liver. As also shown in Fig. 5, in KO mice, compared with WT mice, there was significant increase in [^{3}H]oleate radiolabel in the aqueous phase representing fatty acid oxidation products (muscle: WT 5.07 ± 0.33, KO 7.99 ± 0.47, KO with IP ASP 5.48 ± 0.52 million dpm; liver: WT 0.65 ± 0.04, KO 0.99 ± 0.06, KO with IP ASP 0.62 ± 0.02 million dpm; $p < 0.05$ both in muscle and liver resulting in a +52% increase in the liver and a +60% increase in muscle, both $p < 0.05$. On the other hand, there was no difference in the total lipid fraction, nor was there a difference in the proportion in TG in either liver or muscle: in muscle, WT 63.1% ± 7.4, KO 73.3% ± 5.5, and KO + ASP IP 64.9% ± 4.0, p = NS and in liver, WT 88.1% ± 1.5, KO 87.4% ± 1.9, and KO + ASP IP 87.6% ± 1.0, p = NS). ASP injection at the same time as the intragastric fat administration in male KO mice resulted in a −37% decrease in liver ($p < 0.05$) and a −31% decrease in muscle ($p < 0.05$) oxidation products with no difference found in total lipid phase. These data suggest that in male KO mice the absorbed fat was partitioned differently compared with WT mice, with greater uptakes into muscle and liver. Specifically, oxidation in liver and muscle appear to be increased significantly.

The changes in [^{3}H]oleate radiolabel uptake, especially in fatty oxidation products, point to changes in oxidation/thermogenesis, which may be associated with changes in plasma adiponectin or UCPs. No difference in plasma adiponectin was detected in male KO *versus* WT mice (WT: 46.6 ± 5.8, KO: 48.1 ± 4.3 ng/ml, p = NS). UCP mRNA expression normalized to 18s was tested in muscle, BAT and WAT. As shown in Table II and Fig. 6, UCP1 expression was decreased in BAT, muscle, inguinal, and gonadal fat. These decreases may relate to the lower serum leptin in KO mice (19, 20) also confirmed here, since leptin stimulates UCP1 (31). On the other hand, there were significant increases in inguinal fat UCP2 ($p = 0.02$) and muscle UCP3 ($p = 0.036$, Table II).

DISCUSSION

The results presented here indicate that C3(−) ASP-deficient KO mice, both male and female, have increased oxygen consumption in the active phase and that male mice may also have increased oxygen consumption in the inactive phase. Increased activity, an important source of the energy expenditure change, was observed in the female KO mice, but not in the male KO mice. Both WT and KO male mice had decreased energy expenditure compared with the female mice. Intragastric fat administration resulted in increased fat oxidation in the liver and muscle of male KO mice, consistent with the observed UCP3 overexpression in muscle.

Interestingly, the increased physical activity was only detected in female KO mice, suggesting that the sex hormones (or X-linked genes) may have a function in regulating energy expenditure. Estradiol has been shown to enhance energy expenditure by stimulating voluntary exercise as pointed out by Wade (see review, Ref. 32). Similarly, ovariectomy caused a sharp decrease in running wheel activity in rats and treatment

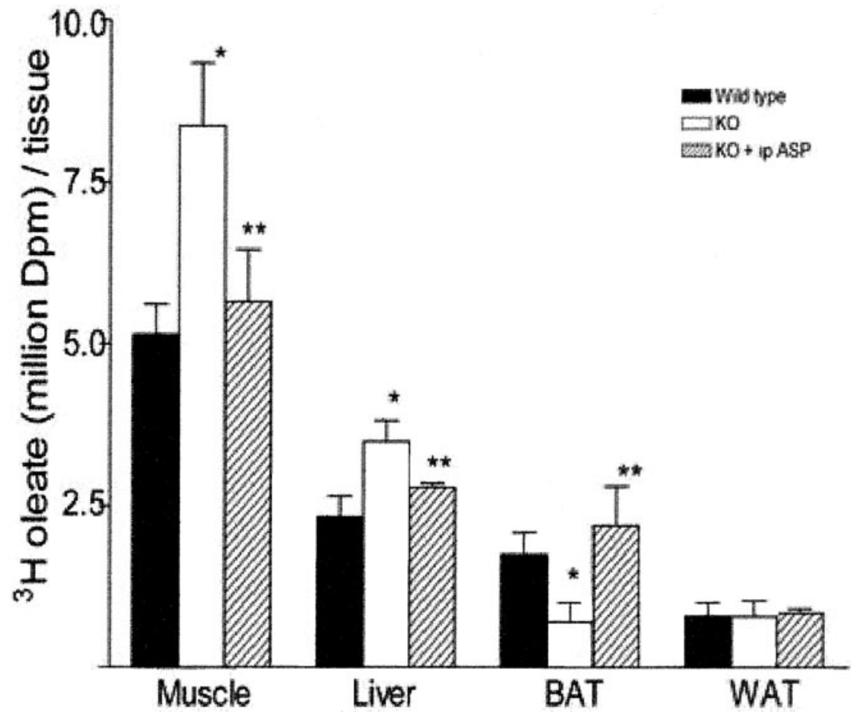

FIG. 4. **Radiolabeled [^{3}H]oleate incorporation into individual tissues in KO, WT, and ASP injected mice at 6 hours.** For the male KO mice, 6 h after fat load, ^{3}H radiolabel was tested in different tissues. There were significant increases in muscle and liver of KO mice compared with WT mice but a significant decrease in ^{3}H radiolabel in BAT. Administration of ASP in KO mice restored the changes. Values are shown as $n = 6$ per group (6 WT, 6 KO, 6 KO with IP ASP) where *, KO *versus* WT, $p < 0.05$; **, KO with IP ASP *versus* KO, $p < 0.05$).

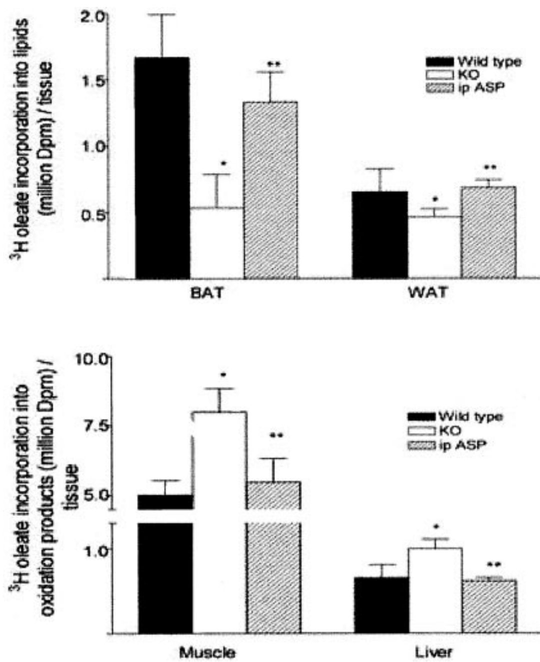

FIG. 5. **Radiolabeled [^{3}H]oleate incorporation into total lipids and oxidation products in specific tissues in KO, WT, and ASP-injected mice at 6 hours.** At 6 h after the fat load, different tissues were extracted and the extractions were separated into total ^{3}H-radiolabeled lipid (*top panel*) and ^{3}H-radiolabeled fatty acid oxidation products (*bottom panel*). Total radiolabeled lipids were significantly decreased in BAT and WAT, while oxidation products were significantly increased in muscle and livers of KO mice. Administration of ASP in KO mice could restore the changes. Values are shown as $n = 6$ per group (6 WT, 6 KO, 6 KO with IP ASP) where *, KO *versus* WT, $p < 0.05$; **, KO with IP ASP *versus* KO, $p < 0.05$).

with estradiol reinstated high levels of voluntary exercise (32, 33). Intracerebral implants of estradiol in or near these estrogen receptor-containing sites also increased activity in ovarectomized rats (32, 34). Lesions that include the medial preoptic area prevented the increases in activity induced by systemic estradiol treatment, but not those induced by other treatments, such as food deprivation or amphetamines (32, 35). Thus, the estradiol stimulation of physical activity may be one way in which the female KO mice compensate and dispose of the excess energy ingested but not stored in adipose tissue. Whether these effects of estradiol are in any way directly related to the absence of ASP is unknown, but there is evidence that estradiol enhances C3 expression (32, 36). As a hypothesis, the effect of estrogen on energy expenditure may be counterbalanced by stimulation of C3 and thus ASP, increasing storage of fatty acid into adipose tissue. The absence of C3 in the KO mice may unbalance the system leading to estrogen increases in voluntary exercise in female KO mice.

With regard to the mechanism underlying the increased energy expenditure in male KO animals, this could not be explained on the basis of increased physical activity, as this was not increased in males, but only in female mice. We observed a clear increase in fatty acid oxidation products in both muscle and liver and specific increases in UCP mRNA expression in certain tissues. It should be noted that there was an increase in oxidation products, but no increase in lipid (especially triglyceride) products, therefore the increased uptake in these tissues would not be expected to lead to lipotoxicity. Absence of ASP results in reduced TG synthesis in adipocytes and leads to delayed NEFA and TG postprandial clearance. This delay in TG and NEFA clearance may be a function of reduced uptake and esterification into TG (as reflected by the decreased [^{3}H]TG in adipose tissues) as well as reduced inhibition of hormone sensitive lipase (both mediated by ASP) (8). Both of these effects will contribute to the increased circulating fatty acid flux, which in turn can lead to local inhibition of lipoprotein lipase (product inhibition). These substrates may then be redirected to muscle and liver for energy production. The accumulation of radiolabeled lipid in ASP-deficient mice into liver and muscle, caused by impaired lipid transportation into adipocytes, appears to simulate what is observed in the environment of high fat diet consumption. The increased energy expenditure would appear to be a systemic response to this challenge. In mice, thermogenesis in brown adipose tissue appears to be an important regulator of high fat diet induced obesity, mediated by UCP1 and UCP2 (37). In the case of KO

TABLE II
Uncoupling proteins (UCP) 1, 2, and 3 expression in muscle and brown adipose tissue (BAT) in male ASP-deficient C3(−/−) knock-out and wild-type mice

	UCP 1			UCP 2			UCP 3		
	WT		KO	WT		KO	WT		KO
Muscle	0.41 ± 0.11		0.18 ± 0.05	1.08 ± 0.15		0.99 ± 0.13	0.82 ± 0.11		1.13 ± 0.13
p		0.05			NS[a]			0.036	
BAT	1.00 ± 0.18		0.61 ± 0.07	1.12 ± 0.17		0.93 ± 0.11	0.80 ± 0.14		0.87 ± 0.12
p		0.03			NS			NS	
Inguinal	0.51 ± 0.15		0.13 ± 0.04	0.30 ± 0.08		0.73 ± 0.20	0.34 ± 0.11		0.30 ± 0.09
p		0.013			0.005			NS	
Gondadal	0.32 ± 0.08		0.13 ± 0.04	0.29 ± 0.07		0.51 ± 0.19	0.17 ± 0.07		0.21 ± 0.08
p		0.025			NS			NS	

[a] NS, not significant.

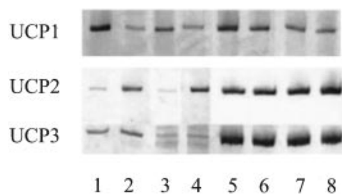

FIG. 6. **Expression of uncoupling proteins UCP1, UCP2, and UCP3 in WT and KO mice.** Representative RT-PCR blots are shown for each protein: UCP1, UCP2, and UCP3 where *1*, WT inguinal adipose tissue; *2*, KO inguinal adipose tissue; *3*, WT gonadal adipose tissue; *4*, KO gonadal adipose tissue; *5*, WT brown adipose tissue; *6*, KO brown adipose tissue; *7*, WT muscle; *8*, KO muscle.

mice lacking ASP, brown adipose tissue does not appear to function as efficiently as in WT mice since UCP1 is decreased. Unlike UCP1, UCP2 is widely expressed (38), whereas UCP3 is predominantly expressed in skeletal muscle (22, 23). In the ASP-deficient mice, other tissues (muscle and fat) may partially compensate for reduced BAT function through increases in UCP2 and UCP3 expression. It has been clearly shown, both in rodents and humans, that administration of fatty acids increases muscle UCP3 expression (39, 40). However, UCP3 is also regulated by several factors, including thyroid hormone, β_3-adrenergic agonists and leptin as well as fat feeding in rodents (24–26). One or more of these factors may be responsible for the up-regulation of UCP3 in the ASP-deficient mice.

There is a striking difference in the metabolic adaptations in mice with decreased adipose tissue triglyceride resulting from partial reduction in stored energy (*i.e.* lean mice), as in the present study, *versus* those mice that completely lack adipocytes to store lipid (lipodystrophic mice). Mice that lack adipose tissue completely are uniformly insulin resistant and hyperinsulinemic with markedly increased plasma triglycerides, decreased or absent leptin and accumulation of triglyceride in non adipose tissue sites such as liver and muscle (41, 42). In contrast, lean mice (as in the present study) are typically insulin sensitive with normal or increased insulin sensitivity, and normal plasma and liver triglycerides (see review, Ref. 43). There is a multiplicity of mechanisms that can result in leanness, but they all generally result in an imbalance in the energy equation such that energy storage and energy expenditure change (review in Refs. 43 and 44). Four mechanisms by which this is achieved include: (i) reduced energy availability through decreased intestinal absorption, (ii) increased peripheral energy metabolism in muscle, adipose tissue or elsewhere, (iii) altered CNS regulation, or (iv) primary defect in peripheral adipose tissue storage. In the first instance, energy input is reduced, while in the remaining three the decreased energy storage is a consequence of substrate re-partitioning and sequestering in other tissues, oxidizing energy that would otherwise be stored.

The intricate balance between energy intake, energy storage and energy oxidation is intriguing. Various CNS-mediated gene targets result in a lean phenotype, often with increased metabolic rate but rarely a compensatory increase in food intake. Such examples are overexpression of leptin (45), melanin concentrating hormone knock-out (46) and muscarinic M3 receptor knock-out (47, 48). Many transgenic or knock-outs, such as UCP1 and UCP3 overexpression (49, 50), muscle LPL overexpression (51, 52) and RIIβ subunit of protein kinase A knock-out (53, 54) that result in increased muscle or adipose tissue oxidation and substrate repartitioning are partially compensated by CNS-mediated increases in food intake, although adipose tissue mass still remains below normal.

In addition, disturbances directly in adipose tissue storage capacity, induced by disruption of a number of genes, including acetyl-CoA carboxylase 2 (55), acyl-CoA diacylglycerol acyltransferase (56) and complement C3(−/−) ASP deficiency as reported here, all result in a reduced ability to store TG, or enhanced lipolysis. What is particularly noteworthy, is that this reduced energy storage consistently results in increased energy expenditure due to repartitioning of energy substrates, to the point where increased food intake cannot compensate.

The second striking point is that despite decreased substrate storage in adipose tissue, the increased flux to other tissues does not appear to result in deleterious consequences, such as accumulation of muscle or liver TG or increased lipoprotein production, but in fact the leanness appears to be associated, if anything, with increased insulin sensitivity and improved lipid profiles (43). In many of these lean models of primary adipose tissue dysfunction, the site of increased energy expenditure remains unknown but may be mediated through increased uncoupling proteins or increased substrate cycling. Future studies will need to focus on the mechanisms responsible for the increased metabolism through *in vitro* experiments in tissues such as muscle and liver.

In conclusion, ASP is an important factor in regulating metabolic balance. The absence of ASP production results in increased energy expenditure in both male and female mice. Male and female KO mice employ different mechanisms to deal with the substrate not stored in adipose tissue resulting from impaired TG synthesis. However, animals of both genders exhibit a lean, insulin sensitive phenotype despite significant hyperphagia.

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