Acylation-stimulating Protein (ASP) Deficiency Induces Obesity Resistance and Increased Energy Expenditure in *ob/ob* Mice*

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Acylation-stimulating protein (ASP)[1] acts as a paracrine signal to increase triglyceride synthesis in adipocytes. ASP administration results in more rapid postprandial lipid clearance. In mice, C3 (the precursor to ASP) knockout results in ASP deficiency and leads to reduced body fat and leptin levels. The protective potential of ASP deficiency against obesity and involvement of the leptin pathway were examined in *ob/ob* C3(−/−) double knockout mice (2KO). Compared with age-matched *ob/ob* mice, 2KO mice had delayed postprandial triglyceride and fatty acid clearance; associated with decreased body weight (4–17 weeks age: male: −13.7%, female: −20.6%, $p < 0.0001$) and HOMA (homeostasis model assessment) index (−37.7%), suggesting increased insulin sensitivity. By contrast, food intake in 2KO mice was +9.1% higher over *ob/ob* mice ($p < 0.001$, 2KO 5.1 ± 0.2 g/day, *ob/ob* 4.5 ± 0.2 g/day, wild type 2.6 ± 0.1 g/day). The hyperphagia/leanness was balanced by a 28.5% increase in energy expenditure (oxygen consumption: 2KO, 131 ± 8.9 ml/h; *ob/ob,* 102 ± 4.5 ml/h; $p < 0.01$; wild type, 144 ± 8.9 ml/h). These results suggest that the ASP regulation of energy storage may influence energy expenditure and dynamic metabolic balance.

Acylation-stimulating protein (ASP)[1] is an adipocyte-derived protein that has potent anabolic effects on human adipose tissue for both glucose uptake and non-esterified fatty acid (NEFA) storage (1, 2). This occurs via translocation of glucose transporters (GLUT1, GLUT3, and GLUT4) from intracellular sites to the cell surface (3, 4) and an increase in diacylglycerol acyltransferase (DGAT) activity (2). These effects appear to be mediated through specific cell surface binding (6, 7), resulting in activation of a signaling pathway that includes protein kinase C (8). In addition, ASP has been shown to inhibit hormone-sensitive lipase in adipocytes, independently and additively to insulin (9). There is a differentiation-dependent increase in ASP binding and ASP response in 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 (6).

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 (10). 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 (11). ASP production increases consequent to adipocyte differentiation (13), and plasma ASP levels are elevated in obesity (14, 15). Chylomicrons *in vitro* stimulate ASP production by adipocytes (16, 17). *In vivo* arterial-venous gradients across a subcutaneous adipose tissue bed in humans demonstrate direct postprandial production of ASP (18). The postprandial increase in ASP is adipose tissue specific and is not observed in the general circulation (19). 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 (20). As ASP is derived through cleavage of complement C3, C3 knockout mice are necessarily deficient in ASP. We have previously demonstrated that genetic deficiency of ASP leads to reduced body fat and decreased leptin levels (21, 22). In addition, male mice have delayed triglyceride clearance (22, 18) although this has not been demonstrated in all studies (23).

To determine the influence of the leptin pathway on ASP action and obesity resistance, we examined the effect of ASP deficiency in *ob/ob* mice, which are leptin-deficient. The *ob/ob* mice have been used to test for protection from obesity in a number of double knockout models, such as the *ob/ob* VLDL receptor(−/−) or the *ob/ob* PAI-1(−/−). In most cases, the decrease in weight, which was evident in the single knockout, was enhanced when examined on the background of the *ob/ob* obese mouse model (24, 25).

Leptin is produced by adipocytes (26) and is involved in the regulation of body fat stores. Leptin is critically involved in the regulation of body energy balance via its central actions on food intake and energy expenditure (27). *Ob/ob* mice lose an important negative feedback on food intake caused by a leptin gene mutation. It is well known that leptin acts via a receptor in the hypothalamus, while there is little evidence that ASP acts via this route. However, leptin also appears to have peripheral actions on substrate fluxes in the adipose tissue (28) and may act directly on adipocytes, where it has been reported to increase lipolysis and impair insulin-mediated lipogenesis (29, 30). Finally, leptin production is regulated by insulin responses to meals (31), an effect that appears to involve increased adi-

[1] The abbreviations used are: ASP, acylation-stimulating protein; HOMA, homeostasis model assessment; BMR, basal metabolic rate; RQ, respiratory quotient; NEFA, postprandial non-esterified fatty acid; 2KO, double knockout mice; ANOVA, analysis of variance.

FIG. 1. **In both male and female 2KO mice had significantly lower body weight than the *ob/ob* group but higher than the wild type group.** Male, −13.7%; female, −20.6% *versus ob/ob* over 4–17 weeks, $p < 0.0001$. The number of subjects (*n*) is 6. Values are shown as mean ± S.E. for $n = 6$ per group. The data were analyzed using ANOVA.
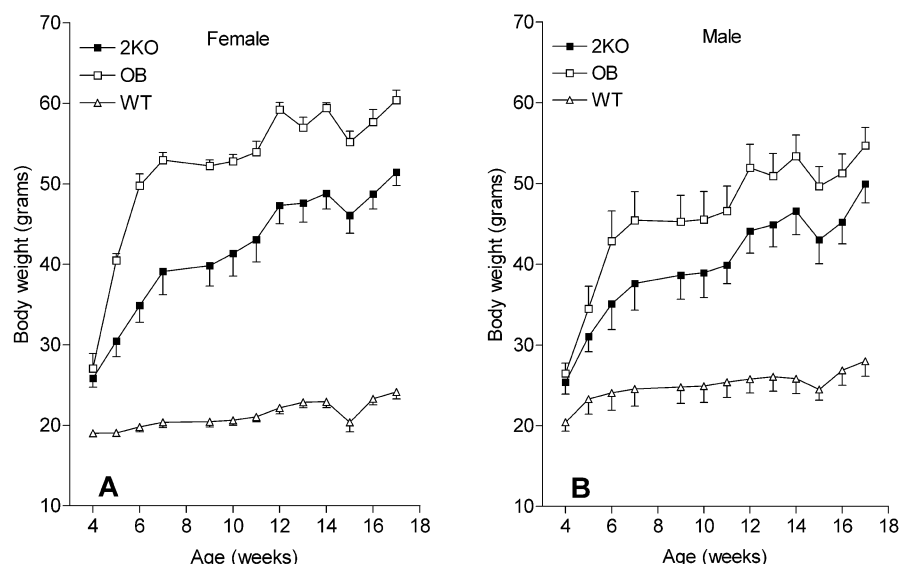
pocyte glucose metabolism (32). Leptin and ASP contrast both in their function and major site of action; however both alter energy disposition through altering either storage or oxidation and the aim of this paper was to determine how the absence of leptin coupled to ASP deficiency alters fat metabolism.

MATERIALS AND METHODS

Generation and Genotyping of Mice—The leptin and complement C3 double knockout (2KO) mouse strain was generated by crossing the Leptin (+/−) mice (C57Bl/6, Jackson Laboratories, Bar Harbor, ME) with complement C3 knockout (C3−/−) (129/Sv genetic background) (previously obtained from Dr. Harvey Coulton) to generate (Leptin+/−, C3+/−) mice. The mixed genetic background mice were bred to generate Leptin(+/−) C3(−/−), Leptin(+/−) C3(+/+). These leptin(+/−) heterozygous mice were used to generate *ob/ob* C3(−/−) 2KO mice as well as *ob/ob* C3 (+/+) and wild type mice. All the mice studied were of a mixed genetic background but littermates were used. Mice were housed in a pathogen-free barrier facility, 21 °C, 12/12 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.

Genotyping—The mice were selected by genotyping with PCR as described previously for C3 (21, 22) and for the leptin gene (33). Mice tail tips were collected when the mice were weaned at 3 weeks of age. Tails were digested with 750 ul of digestion buffer (500 μM Tris, 50 μM EDTA, 0.004% SDS, 40 mM NaCl, 0.028% proteinase K, pH 8.5) at 56 °C overnight with mild rotation. Samples were centrifuged (14,000 × g, 5 min). To the supernatant was added 600 ul of isopropyl alcohol followed by centrifugation for 1 min at 14,000 × g to isolate the DNA pellet. The DNA was washed with 70% ethanol and then re-dissolved in 50 ul of Tris/EDTA buffer. For PCR, 1 ul of DNA was used.

The primers used for *ob/ob* mice genotyping were: Sn, 5′-TGACCT-GGAGAATCTCC-3′ and Asn, 5′-CATCCAGGCTCTCTGGC-3′ for the wild type leptin gene and Sn, 5′-TGACCTGGAGAATCTCC-3′ and Asn, 5′-TGACCTGGAGAATCTCT-3′ for the mutated leptin gene. PCR conditions were: 10 μM each leptin primer, 200 μM dNTP, 2 mM MgCl₂, 10 μM TMAC amplified for 35 cycles of 94 °C, 1 min; 55 °C, 1 min; and 72 °C, 1 min.

For detection of the C3 gene: Sn, 5′-CTTAACTGTCCCACTGCCAA-GAAACCGTCCCAGATC-3′ and Asn, 5′-CTCTGGTCCCTCCCTGTTC-CTGCACCAGGGACTGCCCAAAATTTCGCAAC-3′ were used for the wild type complement C3 gene, and Sn, 5′-ATCGCATCGAGCGAG-CACGTACTCGGA-3′ and Asn, 5′-AGCTCTTCAGCAATATCACGGG-TATCC-3′ were used for neomycin detection in the complement C3 knockout mice. The PCR condition for genotyping were: 32 μM each primer, 200 μM dNTP, 1.5 mM MgCl₂ for 35 cycles of 94 °C, 1 min; 67 °C, 1 min; and 72 °C, 1 min.

All of the recruited mice had body weight determined every week and food intake was measured every 2 days from the 4th week to the 17th week of age.

Fat Load—Fat loads were done as a crossover design experiment. 2KO mice were divided into 2 groups. When the mice were 14 weeks old,

a fat load was given to 2KO mice (half with ASP injection and half without ASP injection), as well as wild type mice (male and female) and *ob/ob* mice (male and female). After an overnight fast (16 h), 400 ul of olive oil (followed by 100 ul of air above the oil) was administrated by gastric gavage using a feeding tube (12-cm curved ball tipped feeding needle (34)) according to standard procedures as previously published (21, 22, 35–38). There was at least a 2-week interval between the fasting blood sample and the fat load. Blood (40 ul) was collected at 0, 2, 4, and 6 h by tail bleeding. Where indicated, ASP was injected intraperitoneally at the time of the fat load. ASP was purified and injected as described previously. Each mouse received intraperitoneally 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 as shown previously (21, 22). When the mice were 16 weeks old, a fat load was given to the 2KO mice again, but the groups were switched. The remaining wild type mice and *ob/ob* mice were given fat loads at the same time. Blood was collected as before.

Plasma Assays—Blood was collected at 14 weeks (for fasting lipids and glucose), 16 weeks (for fat load test), and 22 weeks (to measure insulin) into EDTA-containing tubes by tail bleeding from mice fasted overnight (16 h) with water *ad libitum*. Blood was separated by centrifugation and stored at −80 °C until analysis. Fasting insulin was measured using a rat insulin RIA kit (Linco Inc., St. Charles, MO, cat. RI-13K), which has 100% cross-reactivity to mouse insulin (as described by the manufacturer, Linco Research, Inc.). Glucose was measured using a Trinder glucose kit (Sigma). Plasma non-esterified fatty acids (NEFA) and triglyceride were measured using colorimetric enzymatic kits (Roche Molecular Biochemicals, Laval, Canada).

Oxygen Consumption Measurement—Oxygen consumption and CO_2 production were measured using the Columbus Instrument Oxymax System (Columbus Instruments, Columbus, OH). Each mouse was measured individually over 4 h in a resting state at 21 °C in the absence of food and water. Five time points were sampled, and the experiment was repeated the following day. The average value was used in the analysis.

Statistical Analysis—All results are presented as average ± S.E. Statistical comparisons were by Student's *t* test, ANOVA, 2-way ANOVA, or Pearson Correlation as indicated in the text and figure legends. Statistical significance was set at $p < 0.05$, where *p ns* indicates not significant.

RESULTS

We first examined body weight in these double knockout *ob/ob* C3(−/−) (2KO) mice. Compared with the *ob/ob* mice of the same age, 2KO have lower body weight. As shown in Fig. 1, this is especially pronounced during the 4–12 week age group. Over the total time (4–17 weeks) there was a 13.7% reduction of body weight in the males, and a 20.6% reduction in the

FIG. 2. **NEFA clearance after a fat load.** Wild type, *ob/ob* and 2KO were given an oral fat load, serial blood samples were obtained, and serum NEFA measured. 2KO were significantly different from wild type ($p < 0.01$). ASP administration (intraperitoneal ASP) restored the NEFA profile to normal. (as *versus* wild type or *ob/ob* mice). Values are shown as mean $\pm$ S.E. for $n = 12$. The data were analyzed using 2-way ANOVA.
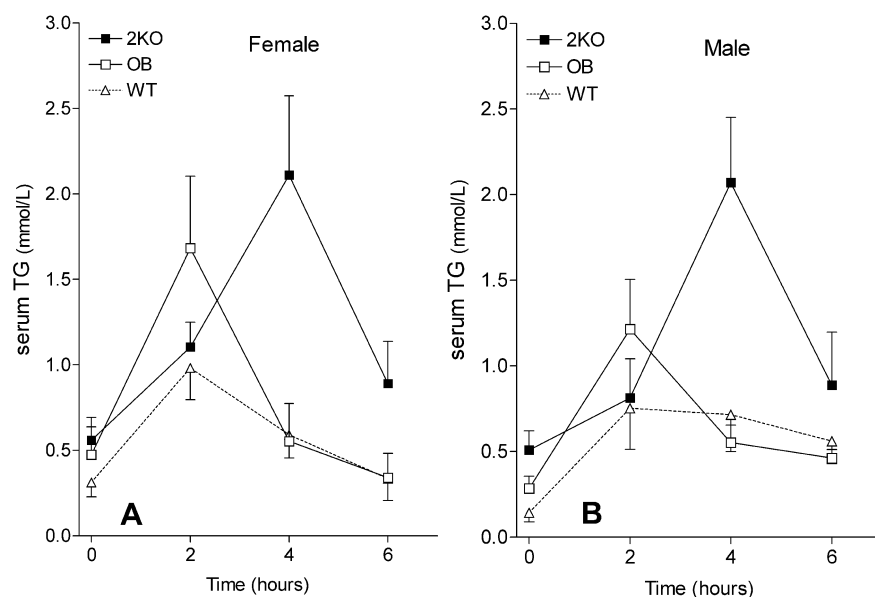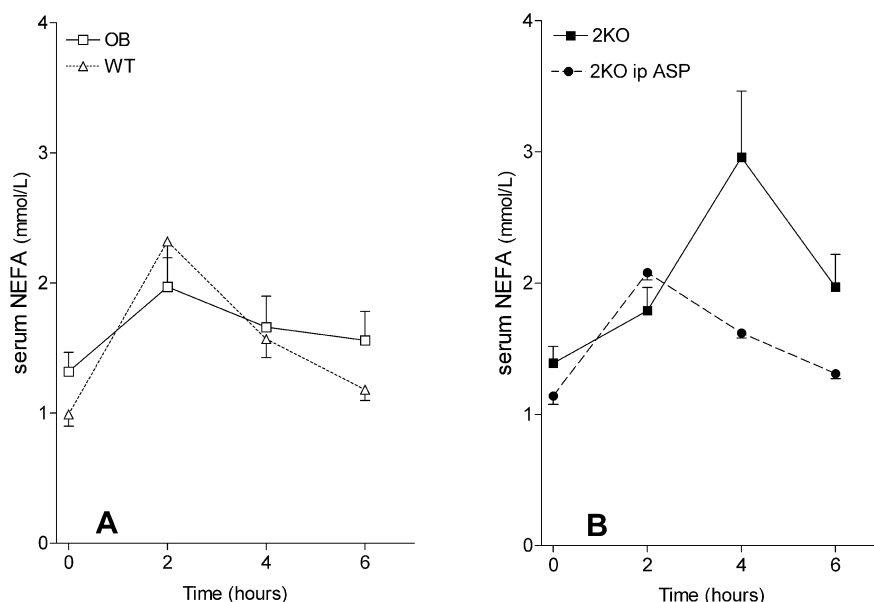

FIG. 3. **Both male and female 2KO mice had delayed postprandial triglyceride clearance compared with *ob/ob* mice or wild type mice.** The *ob/ob* mice had a profile similar to the wild type mice (p value NS) while the 2KO were significantly different ($p < 0.01$). Values are shown as mean $\pm$ S.E. for $n = 6$ per group. The data were analyzed using 2-way ANOVA.

females (both $p < 0.0001$) (Fig. 1). The effect was the same in male and female mice.

In ASP-deficient mice, we have previously demonstrated a delayed postprandial triglyceride and non-esterified fatty acids (NEFA) clearance following a fat load in the male mice (21, 22), and we also examined these parameters in the mice. Despite their pronounced obesity, the *ob/ob* mice have a similar NEFA profile to the wild type mice (Fig. 2). However, while the fasting NEFA levels were no different in 2KO mice, they demonstrate clearly a delayed postprandial NEFA clearance compared with both the *ob/ob* group and the wild type group (Fig. 2, $p < 0.01$ by 2-way ANOVA for 2KO *versus* wild type). Intraperitoneal administration of ASP restored a normal NEFA clearance pattern. Similar changes were demonstrated in the triglyceride profiles (as shown in Fig. 3) where again the *ob/ob* mice had a relatively normal postprandial triglyceride clearance, which is markedly delayed in the 2KO mice ($p < 0.01$). Interestingly, the delayed NEFA and triglyceride clearance in the 2KO mice were present in both male and female mice, a difference from previous studies in ASP-deficient mice, where only the male mice demonstrated delayed triglyceride clearance (21, 22).

In addition to lipids, we also measured fasting insulin and glucose in the three groups of mice. The obesity in *ob/ob* mice is usually associated with increased plasma insulin and glucose, as quantitated by the HOMA index (the homeostasis model assessment for insulin resistance, Ref. 12) and this is also shown here. There was no difference in fasting or postprandial glucose between 2KO and *ob/ob.* The fasting glucose was: 2KO 4.1 ± 0.7 mM; *ob/ob* 4.0 ± 0.5 mM; wild type 4.0 ± 0.4 mM. However the 2KO mice had lower insulin levels and a significantly lower HOMA index (-37.7%) as compared with *ob/ob* mice (Fig. 4, *inset*, wild type: 2.7 ± 0.5 mM·milliunits/liter, *ob/ob* 15.8 ± 2.4 mM·milliunits/liter, and 2KO: 9.8 ± 1.5 mM·milliunits/liter, $p < 0.05$), suggesting greater insulin sensitivity in the 2KO mice compared with the *ob/ob.* These changes in HOMA and in insulin are very closely related to the decrease in body weight in the 2KO as shown in Fig. 4A. Again, both male and female 2KO mice were equally improved in this respect.

The analysis of food intake is shown in Fig. 5A (*inset*). Strikingly, although body weight is decreased in the 2KO compared with the *ob/ob* mice, the 2KO mice actually ingest a greater

FIG. 4. **Insulin, body weight, HOMA and food efficiency in 2KO, *ob/ob* and wild type mice.** *A*, fasting plasma insulin is significantly correlated with body weight ($r = 0.775$, $p < 0.001$). *B*, HOMA is negatively correlated with food efficiency ($r = -0.5223$, $p < 0.01$); HOMA in 2KO mice is significantly lower than *ob/ob* mice ($p = 0.046$), but both are higher than wild type mice ($p < 0.001$). Values are shown mean ± S.E. or as individual values for $n = 12$ mice per group. Correlation was calculated by Pearson analysis.
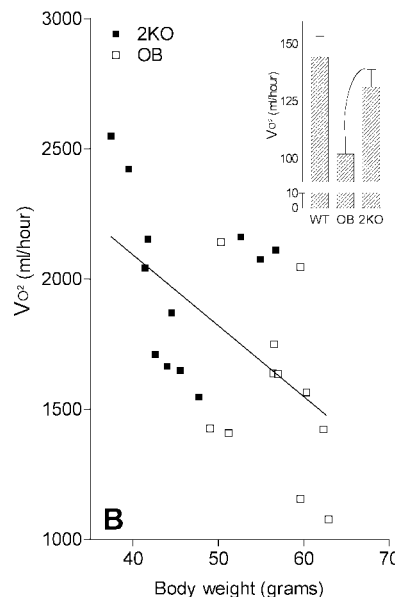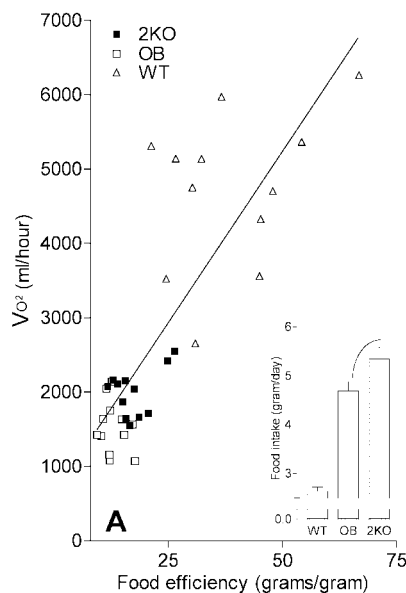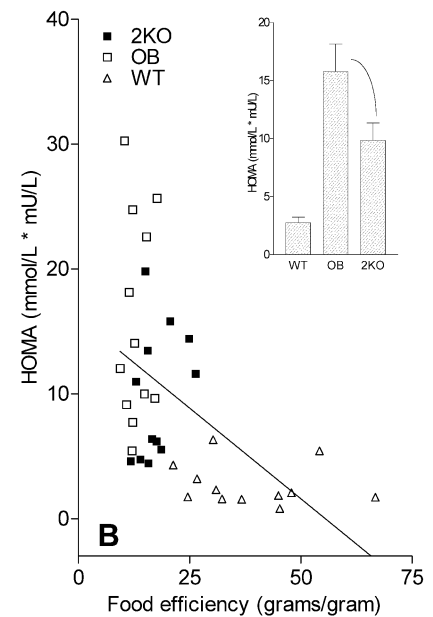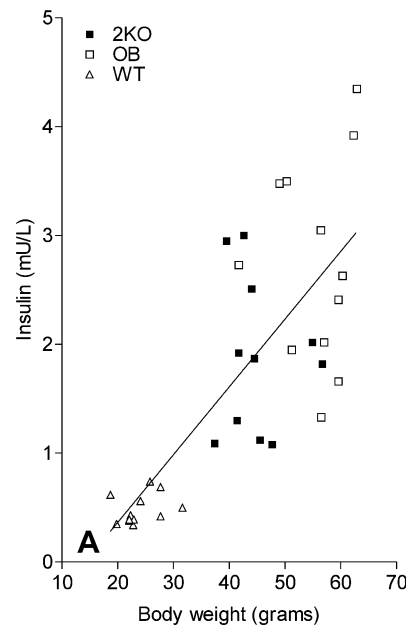

FIG. 5. **Oxygen consumption and relationship to food efficiency and body weight.** *A*, oxygen consumption (VO2) is positively correlated with food efficiency ($r = 0.8162$, $p < 0.0001$). Food intake of 2KO mice was significantly higher than OB mice ($p = 0.001$), and both were higher than wild type mice ($p < 0.001$). *B*, in *ob/ob* and 2KO mice, oxygen consumption (VO2) is negatively correlated with body weight ($r = -0.5580$, $p < 0.01$). Oxygen consumption of 2KO mice was significantly higher than *ob/ob* mice ($p = 0.009$), but there was no significant difference between 2KO mice and wild type mice. Values are shown in mean ± S.E. for $n = 12$ per group. Correlation data were analyzed using Pearson and ANOVA.

caloric amount. In the 2KO group, food intake was 5.3 ± 0.2 g/day, whereas it was 4.7 ± 0.2 g/day in the *ob/ob* and only 2.6 ± 0.1 g/day in the wild type mice (2KO +9.1% increase *versus ob/ob*, $p < 0.001$; +95% increase *versus* wild type, $p < 0.001$). This increase in food intake was seen in both males and females. Thus the decreased body weight is not a result of decreased daily food intake. Consequently, 2KO mice have a significantly reduced food efficiency index: for a 1-g increase in body weight 2KO consume 17.5 ± 1.3 g of food, while the *ob/ob* consume 13.0 ± 0.8 g of food, $p < 0.01$. This parameter (food efficiency) is significantly correlated with HOMA (Fig. 4*B*).

The interesting phenomenon that the 2KO mice ate more food than *ob/ob* mice daily, but gain less weight suggests that there are important alterations in energy expenditure and disposition. This was examined by measuring oxygen consumption (VO2) and CO_2 production in the 3 groups of mice (2KO, *ob/ob,* and wild type). The results demonstrate that the *ob/ob* mice have lower oxygen consumption than wild type mice, while oxygen consumption in 2KO mice is almost restored to the same level as the wild type group (wild type, 144.3 ± 9.1

ml/h; *ob/ob,* 102.1 ± 7.6 ml/h; 2KO, 131.2 ± 7.7 ml/h; +28.5% *versus ob/ob*, $p < 0.01$; Fig. 5*B inset*). A similar result was obtained for heat production (wild type, 0.64 ± 0.04 Kcal/h; *ob/ob,* 0.48 ± 0.03 Kcal/h; 2KO, 0.62 ± 0.03 Kcal/h; +28.8% *versus ob/ob*, $p < 0.01$;). These parameters both correlated negatively with body weight (as shown for oxygen consumption, Fig. 5*B*). Oxygen consumption was also significantly correlated with food efficiency (Fig. 5*A*) and HOMA index (not shown). On the other hand, while the RQ (respiratory quotient) was significantly increased in the *ob/ob* mice compared with the wild type mice indicating an increase in the proportion of carbohydrate *versus* fat oxidized, it was not further changed in the 2KO mice.

DISCUSSION

These results extend our knowledge of the role of ASP in energy storage and energy expenditure. Firstly, ASP-deficient *ob/ob* mice demonstrate delayed postprandial triglyceride and NEFA clearance, as demonstrated previously in ASP deficient male mice (21, 22). Note that this is not only seen in the males

ASP Deficiency in ob/ob Mice

(as described before), but in the 2KO mice this is also evident in females. In both cases, for ASP-deficient or 2KO mice, administration of ASP normalizes the postprandial profile.

In addition, HOMA was significantly reduced suggesting that the metabolic changes induced by ASP deficiency lead to increased insulin sensitivity. The very close association between the body weight and HOMA index suggests that it is the decreased weight that results in increased insulin sensitivity.

In the present study we demonstrate increases in food intake and basal metabolic rate in double knockout mice, without any changes in RQ (respiratory quotient). The 9% increase in food intake is more than offset by a 28% increase in energy consumption (oxygen consumption) resulting in an overall decrease in body weight of 17% as compared with the *ob/ob* mice.

The first result of interest to address is the increase in food intake. Previously we demonstrated that in C3(−/−) mice, food intake increased but the mice were leaner. This was coupled to a decrease in adipose tissue mass and decreased circulating leptin levels. This led us to speculate that the increased food intake was a consequence of decreased levels of the satiety factor leptin. However, in the present 2KO mice, the 2KO have a greater food intake than the *ob/ob* mice, a finding that cannot be explained by a decreased leptin, since leptin is absent in both mice. This suggests that the increase in food intake caused by ASP deficiency is not mediated through the leptin pathway. Could ASP or C3a have a satiety effect that is lost in the ASP-deficient and 2KO mice strains? The effects of both ASP (C3adesArg) and C3a have been tested both centrally and peripherally. While there were acute effects on food intake, two studies demonstrated an increase (39, 40) and the other a decrease (41). Neither effect was pronounced and disappeared quite rapidly. Likely the effect of ASP deficiency on food intake is not a direct one.

The second intriguing finding was the increase in basal metabolic rate (BMR) that was quite pronounced in the 2KO mice. The increase in BMR cannot simply be a consequence of hyperphagia, since the *ob/ob* also overeat, but their BMR is substantially lower than normal. Similarly, the change in BMR cannot simply be due to a lack of leptin, since both mutant mice are lacking leptin. Thus the change in BMR must relate (directly or indirectly) to a lack of ASP (as discussed below).

ob/ob have an increased RQ relative to wild type, with an increased shift toward carbohydrate oxidation, probably derived from the high carbohydrate content in the chow diet (46). It is known that leptin acts not only as a satiety factor but also up-regulates energy utilization (42–46), and preferentially increases oxidation of fat in a dose dependent manner (47). In the absence of leptin, energy metabolism shifts toward carbohydrate oxidation. On the other hand, while ASP deficiency induced increases in food intake and BMR, there was no change in RQ compared with *ob/ob*. Thus the increased oxidation in 2KO mice is an increase in both carbohydrate and fat oxidation. This increase in carbohydrate oxidation and glycolysis may help to explain the increase in insulin sensitivity.

What is the mechanism for the increased BMR without a change in RQ in the 2KO? Contributions to BMR can be quantified in terms of oxygen consumption, ATP turnover or uncoupling. About 90% of mammalian oxygen consumption is used by the mitochondria; of this about 20% is uncoupled by the mitochondrial proton leak (through UCP), whereas the other 80% is coupled to ATP synthesis (48). Through proton leaks in the mitochondria, energy is expended without the generation of ATP resulting in heat production. Over the last decade, there has been considerable interest in the role of UCP1, UCP2, and UCP3 in mediating this thermogenesis (49). Increased expression of UCP is associated with increased energy expenditure.

Certainly one mechanism by which leptin increases energy expenditure is through stimulation of UCP1 in brown adipose tissue (50).

However an equally important mechanism of energy expenditure which generates heat through the utilization of ATP is termed substrate cycling. A substrate cycle exists when opposing, non-equilibrium reactions catalyzed by different enzymes are operating simultaneously (for review see (51, 52)). At least one of the reactions must involve the hydrolysis of ATP. Thus, a substrate cycle both liberates heat and increases energy expenditure; yet there is no net conversion of substrate to product. One example of a substrate cycle is the simultaneous breakdown (lipolysis) and re-synthesis (re-esterification) of triglyceride (triglyceride-fatty acid cycling). Other substrate cycles in the pathways of glycolysis and gluconeogenesis also exist (such as the glucose/glucose-6-phosphate cycle, the fructose-1-phosphate/fructose-1,6-diphosphate cycle, and the phosphoenolpyruvate/pyruvate/oxaloacetate). Substrate cycles have been proposed to be important not only in thermogenesis but also in maintaining the sensitivity and flexibility of metabolic regulation (48). Substrate cycling occurs primarily in the liver, muscle and adipose tissue (48). While 20% of total mitochondrial energy is expended via proton leaks (as mentioned above), it has been estimated that a further 20% is expended in substrate cycling (48). Newsholme (53) estimated that if the fructose-1-phosphate/fructose-1,6-diphosphate cycle was fully active, it could account for ~50% of an adult human's daily energy expenditure.

Of particular interest to the present discussion is the triglyceride-fatty acid cycle, which is highly active in adipose tissue. In humans the activity of this cycle ranges enormously from 0 to 100% re-esterification of released fatty acids (54) and is regulated through environmental (diet, exercise) (55), anatomic (gender and adipose tissue site) (56), and hormonal influences (57). A number of hormones, such as norepinephrine and insulin have been shown to modulate the triglyceride/fatty acid cycle in adipose tissue, as well as other substrate cycles in additional tissues (56, 57).

In a recent paper, Reidy and Weber (5) proposed that accelerated triglyceride/fatty acid substrate cycling in adipose tissue provides a new mechanism by which leptin triggers increased metabolic rate. Leptin is thought to increase energy expenditure primarily by driving the hypothalamic-pituitary-thyroid axis to produce more triiodothyronine (T3), a key regulator of standard metabolic rate. T3 in turn increases the expression of uncoupling proteins to reduce the efficiency of ATP production and increase heat. However leptin was also shown to directly activate the triglyceride/fatty acid cycle, lipolysis and fatty acid oxidation, shifting fuel preference from carbohydrate to fat oxidation. Thus, in *ob/ob* mice, the loss of leptin stimulation of UCP1 and triglyceride/fatty acid cycling will simultaneously contribute to the decrease in energy expenditure and the increase in fat storage.

Does ASP influence uncoupling proteins and substrate cycling? Since ASP is known to increase triglyceride synthesis and re-esterification (9, 11) and decrease intracellular triglyceride lipolysis (9), then the effect of ASP, at least in adipose tissue, is to effectively decrease substrate cycling and increase triglyceride trapping. It is possible that ASP deficiency could release the brake on this cycling process, allowing for increased substrate cycling and augmenting energy expenditure. These hypotheses remain to be addressed experimentally.

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