Increased β-Oxidation but No Insulin Resistance or Glucose Intolerance in Mice Lacking Adiponectin

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Previous reports showed that recombinant fragments of adiponectin (adipo) displayed pharmacological effects when injected into rodents, but the relevance of these observations to the physiological function of adiponectin in muscle (6, 7) is uncertain. We generated Adipo−/− mice by gene targeting. Adipo−/− mice are fertile with normal body and fat pad weights. Plasma glucose and insulin levels of Adipo−/− and Adipo+/− mice are similar under fasting conditions and during an intraperitoneal glucose tolerance test (GTT). Insulin tolerance test (ITT) also produces similar plasma glucose and insulin levels in the two groups of mice. Hyperinsulinemic-euglycemic clamp analysis showed that Adipo−/− and Adipo+/− mice have similar glucose infusion rates to maintain a similar serum glucose. High-fat diet feeding for 7 months led to similar weight gain and similar GTT and ITT responses. We next measured β-oxidation and found it to be significantly increased in muscle and liver of Adipo−/− mice. In conclusion, our study indicates that absence of adiponectin causes increased β-oxidation but does not cause glucose intolerance or insulin resistance in mice.

Adiponectin (adipo, also known as Acrp30, adiponectin, GBP28, and apM1) is a major adipocyte secretory protein of unknown function (1–3). Injection of full-length and partial-length fragments of recombinant adiponectin into rodents was shown to suppress glucose intolerance and insulin resistance as an oral basis. Even after being fed a high-fat diet for 7 months. Unexpectedly, we found that Adipo−/− mice had increased β-oxidation in muscle and liver. This study sheds light on the function of native adiponectin in vivo.

To elucidate the in vivo role of adiponectin, we generated Adipo−/− mice by gene targeting. We found that these mice had no glucose intolerance or insulin resistance as an oral basis. Even after being fed a high-fat diet for 7 months. Unexpectedly, we found that Adipo−/− mice had increased β-oxidation in muscle and liver. This study sheds light on the function of native adiponectin in vivo.

EXPERIMENTAL PROCEDURES

Generation of Targeted Mice—We used a replacement-type targeting vector constructed from a mouse 129/Sv strain bacterial artificial chromosome genomic clone and R1 ES cell line for gene targeting (Fig. 1A). Transfection and ES cell clone selection were as described (9). Nine positive ES clones were injected into blastocysts of C57BL/6J, and chimeric mice were obtained with C57BL/6J mice. Germ line transmission was confirmed by Southern blots using tail DNA.

Northern Blot and Immunoblot Analysis—Northern blots were performed on total RNA isolated from white and brown adipose tissue using a 32P-labeled mouse full-length cDNA probe as described (9, 10). Glycerolaldehyde 3-phosphate dehydrogenase cDNA was used as a control. Polyclonal rabbit antibody was raised against a C-terminal fragment of mouse adiponectin (residues 110–247) fused to glutathione S-transferase. Immunoblotting were performed as described previously using 3 μl of plasma (9).

Plasma Sample Assay—Plasma-free fatty acid, glycerol, triglyceride, total cholesterol, and leptin levels were determined by a NEFA-C test (Wako Chemicals, Neuss, Germany). Triglyceride GPO-Trinder, total cholesterol INFINITY (Sigma) and Quantikine M kit (R & D System Inc.), respectively.

Glucose Tolerance Test and Insulin Tolerance Test—Intraperitoneal glucose tolerance test (GTT), using 1.5 g of glucose/Kg and intraperitoneal insulin tolerance test (ITT) using 1 unit of insulin/Kg were performed in mice fasted for 16 h as described (9, 11).

Hyperinsulinemic-Euglycemic Clamp—We measured in vivo glucose utilization by the hyperinsulinemic-euglycemic clamp method as described previously (12) with slight modification. Mice received an insulin infusion (10 milliliters/kg/min) for 90 min. The infusion rate of glucose solution (4%) was adjusted to a target plasma glucose level of 100 mg/dl. Plasma glucose was monitored every 3 min for 90 min. Total body glucose infusion rate was calculated as described (12).

High-fat High-sucre (HFS) Diet Feeding—For high-fat diet feeding experiments, F6 C57BL/6J Adipo−/− and Adipo+/− mice were fed a high-fat/high-sucrose (per Kg feed containing 210 g of milk fat and 341 g of sucrose) diet from Harlan Teklad (TD 88137) for 7 months.

β-Oxidation—For quantification of β-oxidation, we measured [14C]CO2 production from [1-14C]palmitic acid in isolated soleus muscle as described (13) and in liver homogenates as described (14).

RESULTS

Targeted Disruption of the Adiponectin Gene in Mice—Targeted mice were produced by the strategy shown in Fig. 1, A and B. Germ line transmission was confirmed by Southern blotting (Fig. 1C). Initially, six Adipo−/− lines were obtained and we bred three of the lines into C57BL/6J. Experiments were performed with identical results in two independent lines. Homologous recombination removed exon 2 of Adipo−/− mice, which contained the translation initiation codon. Adipo mRNA was absent in the knock-out mice (Fig. 1D), and adiponectin protein was undetectable in the plasma of knockout mice (Fig. 1E). As the immunoblot was performed with an antibody against the C-terminal fragment of adiponectin, together with the Northern blot, it excludes the expression of a truncated adiponectin from a downstream translation initiation codon. Adipo−/− mice were fertile, and the null allele was transmitted to the progeny in a Mendelian pattern. Adipo−/− mice were backcrossed into C57BL/6 for six generations. Adipo−/− and wild-type mice had identical body weights (data not shown) and similar fat pad weights.
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Do Not Develop More Insulin Resistance than Wild-type Controls—To bring out potential subtle changes in Adipo−/− mice that were not evident under basal conditions, we fed these mice a HF/HS diet for 7 months. Plasma lipids of Adipo−/− and Adipo+/+ mice were similar (mean ± S.E., triglyceride: –/–, 73.08 ± 10.81 mg/dl; +/-, 79.60 ± 9.23 mg/dl; cholesterol: –/–, 238.88 ± 28.84 mg/dl; +/+, 261.71 ± 24.94 mg/dl; free fatty acids: 1.67 ± 0.53 mm; +/+, 1.69 ± 0.24 mm) while they were maintained on this diet. They also had similar body weights throughout the 7-month feeding period (data not shown). Both wild-type and knock-out mice developed mild fasting hyperglycemia and fasting hyperinsulinemia (compare Figs. 2 and 3), indicating that the diet-induced weight gain produced similar degrees of mild insulin resistance in mice that produced adipo as well as those that lacked the protein. The plasma glucose and insulin response of both types of mice to GTT was also similar (Fig. 3, A and B). Although the plasma insulin in wild-type mice was higher than that of Adipo−/− mice during the GTT, the difference was not significant (Fig. 3B). We next performed an ITT, which revealed that Adipo−/− mice and their Adipo+/+ littermates had a similar plasma glucose response to insulin (Fig. 3C). Therefore, there was no difference in insulin sensitivity (or resistance) in Adipo+/+ or Adipo−/− mice. This was true irrespective of whether the comparison was made while the animals were on regular chow or after they were fed a HF/HS diet for 7 months.

Absence of Adiponectin Stimulates β-Oxidation—As recombinaent fragments of adiponectin were reported to stimulate β-oxidation in muscle of rodents (6, 7), we measured the β-oxidation activity of soleus muscle isolated from Adipo−/− and Adipo+/+ mice. Unexpectedly, we found that β-oxidation rate was significantly increased (by ~47%) in the muscle of Adipo−/− mice compared with wild-type littermate controls (Table IC). The β-oxidation rate in liver homogenates of these animals was also determined and was found to be ~30% higher in Adipo−/− mice than that in wild-type littermate controls. Therefore, the absence of adiponectin stimulates β-oxidation.

**DISCUSSION**

While we were preparing this manuscript for publication, Kubota et al. (15) reported that F1 and F2 Adipo knockout mice displayed insulin resistance; they did not examine β-oxidation in their animals. The reason for the difference between our study and that of Kubota et al. (15) is unclear, but may be related to environmental factors or genetic background. We did not detect any significant difference in glucose homeostasis in F2 and F3 mice produced in our laboratory. The variation among F2 and F3 mice was higher than that among F6 C57BL/6J mice, presumably because of the inhomogeneous genetic background in the former groups. None of these differences were found to be significant in large groups. To rule out the mixed genetic background as a factor that might have masked differences between knock-out and wild-type mice, we bred them into C57BL/6J background and re-examined the mice at the F6 generation. Again, there was no difference in glucose tolerance or insulin sensitivity between Adipo−/− and Adipo+/+ mice (Figs. 2 and 3). This lack of a difference was evident in regular chow-fed mice as well as in mice fed a HF/HS diet for 7 months. The high-fat diet did induce a mild, but similar, degree of glucose intolerance and insulin resistance in Adipo−/− and Adipo+/+ mice (Fig. 3). Interestingly, both Kubota et al. (15) and we observed no difference between knock-out and wild-type mice in their plasma insulin level in the postabsorptive, i.e. fasting state, or after a glucose load. In both studies the plasma insulin during a GTT tended to be (insig-
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TABLE I
Characterization of Adipo⁻/⁻ mice

Data represent mean ± S.E.

|                        | Adipo⁺/+ (n = 11) | Adipo⁻/⁻ (n = 11) | Adipo⁻/- (n = 8) |
|------------------------|-------------------|-------------------|-----------------|
| Cholesterol (mg/dl)    | 84.47 ± 7.34      | 89.35 ± 5.55      | 101.24 ± 8.19   |
| Triglyceride (mg/dl)   | 45.43 ± 4.34      | 46.22 ± 2.09      | 43.67 ± 2.92    |
| Free fatty acid (nm)   | 0.97 ± 0.10       | 0.83 ± 0.05       | 0.79 ± 0.08     |
| Glycerol (mg/dl)       | 110.79 ± 17.07    | 107.23 ± 11.00    | 93.93 ± 14.60   |

B. Hyperinsulinemic-euglycemic clamp

|                        | Adipo⁺/+ (n = 13) | Adipo⁻/- (n = 7) |
|------------------------|-------------------|-----------------|
| Glucose infusion rate (mg/Kg/min) | 8.02 ± 0.727    | 10.56 ± 1.318   |
| Serum insulin during clamp (ng/ml) | 18.80 ± 2.949  | 23.44 ± 1.398   |
| Serum glucose during clamp (mg/dl) | 105.98 ± 1.180  | 101.72 ± 0.525  |

C. Fatty acid oxidation

|                        | Adipo⁺/+ | Adipo⁻/- |
|------------------------|----------|----------|
| [¹⁴C]Palmitate → CO₂ (nmol/g/h) | 1.58 ± 0.07 | 2.33 ± 0.394* |
| Muscle                  | 10.32 ± 1.94 | 13.39 ± 2.72b |
| Liver                   | 4.34 ± 46.22 | 7.34 ± 89.35 |

*p ≤ 0.05 (n = 7, Student’s t test, paired).

b p ≤ 0.01 (n = 9, Student’s t test, paired).

Fig. 2. Intraperitoneal GTT and ITT in Adipo⁻/- and Adipo⁺/+ mice (left, males; right, females). Plasma glucose levels (A) and plasma insulin levels (B) in 2-h GTT are shown. C, plasma glucose response during ITT. None of the values in A–C are significantly different between the two types of mice.

Fig. 3. Insulin and glucose dynamics of Adipo⁻/- and Adipo⁺/+ mice fed a high-fat diet for 7 months. Plasma glucose levels (A) and plasma insulin levels (B) during 4-h GTT are shown. C, plasma glucose response during ITT test of 7-month high-fat diet fed mice. None of the values in this figure are significantly different between Adipo⁻/- mice and Adipo⁺/+ mice.

significantly) higher in the wild-type mice than in Adipo⁻/- mice (Fig. 2J in Kubota et al. (15) and Figs. 2B and 3B in this study). These are unlikely scenarios if significant insulin resistance existed. The absence of insulin resistance in Adipo⁻/- mice was also confirmed by the hyperinsulinemic-euglycemic clamp method in the current study (Table IB).

How can we reconcile the absence of insulin resistance in Adipo⁻/- mice and the previously reported effects of full-length and partial-length adipo fragments injected into rodents (6, 7)? The overall phenotype of Adipo⁻/- mice could be the result of the activation or overexpression of compensatory biochemical pathways or molecules in Adipo⁻/- mice that reversed the insulin resistance, if indeed adipo is a natural insulin-sensitizing hormone in vivo. The expression of leptin is largely unchanged in these mice, but there are potentially many other molecules involved in insulin resistance that could be activated in the absence of adipo (16).

Another consideration is the fact that the data obtained in many of the experiments involving the injection of recombinant adipo into rodents may not reflect the normal action of the native protein. Adipo normally exists in plasma as trimers that associate noncovalently to form high molecular complexes, and most of the injected fragments were not competent to reassemble into the native multimeric complexes in vivo (17). Furthermore, adipo undergoes posttranslational modification (17, 18), which does not occur in bacterially derived recombinant adipo. Adipo contains tissue-specific 2,8-linked di/oligosialic acid chains that appear to be adipocyte-specific (18); recombinant adipo undergoes posttranslational modification (17, 18), which does not occur in bacterially derived recombinant adipo. Adipo contains tissue-specific 2,8-linked di/oligosialic acid chains that appear to be adipocyte-specific (18); recombinant adipo produced in non-adipocyte mammalian cells may not contain this specific modification. Such 2,8-linked di/oligosialic acid chains have been shown to be involved in signal transduction as well as other biological activities (19, 20). The fact that increased β-oxidation was observed in mice that received recombinant adipo fragments (6, 7), but also occurred in Adipo⁻/- mice that lacked native adipo, suggests that the injected fragments might have acted as dominant negative molecules that blocked the normal action of native adipo in vivo. Additional experiments will be needed to address this important issue.

While this paper was under review, Maeda et al. (21) reported the production of Adipo⁻/- mice in their laboratory. In agreement with our study, they observed no evidence of insulin resistance when Adipo⁻/- mice were fed a regular chow. However, they found that feeding the Adipo⁻/- mice a HF/HS diet for 2 weeks induced insulin resistance in these animals. The reason for the difference between our study, which involved a 7-month HF/HS diet feeding period, and that of Maeda et al. (21) is unclear.

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