Caveolin-3 knockout mice show increased adiposity and whole body insulin resistance, with ligand-induced insulin receptor instability in skeletal muscle

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Caveolin-3 knockout mice show increased adiposity and whole body insulin resistance, with ligand-induced insulin receptor instability in skeletal muscle. Am J Physiol Cell Physiol 288: C1317–C1331, 2005. First published February 2, 2005; doi:10.1152/ajpcell.00489.2004.—Caveolin-3 (Cav-3) is expressed predominantly in skeletal muscle fibers, where it drives caveola formation at the muscle cell’s plasma membrane. In vitro studies have suggested that Cav-3 may play a positive role in insulin signaling and energy metabolism. We directly address the in vivo metabolic consequences of genetic ablation of Cav-3 in mice as it relates to insulin action, glucose metabolism, and lipid homeostasis. At age 2 mo, Cav-3 null mice are significantly larger than wild-type mice, and display significant postprandial hyperinsulinemia, whole body insulin resistance, and whole body glucose intolerance. Studies using hyperinsulenic-euglycemic clamps revealed that Cav-3 null mice exhibited 20% and 40% decreases in insulin-stimulated whole body glucose uptake and whole body glycogen synthesis, respectively. Whole body insulin resistance was mostly attributed to 20% and 40% decreases in insulin-stimulated glucose uptake and glucose metabolic flux in the skeletal muscle of Cav-3 null mice. In addition, insulin-mediated suppression of hepatic glucose production was significantly reduced in Cav-3 null mice, indicating hepatic insulin resistance. Insulin-stimulated glucose uptake in white adipose tissue, which does not express Cav-3, was decreased by 70% in Cav-3 null mice, suggestive of an insulin-resistant state for this tissue. During fasting, Cav-3 null mice possess normal insulin receptor protein levels in their skeletal muscle. However, after 15 min of acute insulin stimulation, Cav-3 null mice show dramatically reduced levels of the insulin receptor protein, compared with wild-type mice treated identically. These results suggest that Cav-3 normally functions to increase the stability of the insulin receptor at the plasma membrane, preventing its rapid degradation, i.e., by blocking or slowing ligand-induced receptor downregulation. Thus our results demonstrate the importance of Cav-3 in regulating whole body glucose homeostasis in vivo and its possible role in the development of insulin resistance. These findings may have clinical implications for the early diagnosis and treatment of caveolinopathies.

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Cav-1 null mice were generated and maintained by us as previously described (15). For all experiments, 2-mo-old male mice, in the C57Bl/6 genetic background, were used, unless otherwise indicated in the text. At this young age, Cav-3 null mice do not show any myopathic changes in their skeletal muscle fibers (15, 19). In older Cav-3 null mice, only mild myopathic changes are present, and these mice do not show any restrictions in their mobility.

In addition to these functions, numerous studies have shown that the role of caveolin-3 in muscle cells is similar to that of caveolin-1 in other cell types. For instance, with regard to signal transduction, an equivalent cohort of signaling molecules are known to localize to caveolin-3-generated caveolae, including nitric oxide synthase, α- and β-adrenergic receptors, various isoforms of PKC, and Src family tyrosine kinases (13, 39, 40). However, while these molecules have been shown to interact with caveolin-3, very little is known about the role of this protein in cellular processes outside of those related to muscular dystrophy.

Using standard homologous recombination techniques, we (15) and others (19) have recently generated caveolin-3 (Cav-3) null mice. Importantly, older Cav-3 null mice develop a similar mild muscular dystrophy phenotype, as seen in patients with LGMD-1C. However, at age 2 mo, Cav-3 null mice do not show any myopathic changes in their skeletal muscle fibers (15, 19).

With the generation and characterization of Cav-1 null mice it has become quite evident that caveolin-1 serves a critical function in lipid metabolism and insulin signaling. Cav-1 null animals were found to be profoundly resistant to diet-induced obesity, displaying marked abnormalities in plasma lipid profiles (38). Furthermore, these mice showed defective insulin signaling in adipose tissue, as well as alterations in lipid storage and breakdown (8, 9). Because caveolin-3, which is the only caveolin family member expressed in muscle, shares 85% homology with caveolin-1, caveolin-3 may have a metabolic role in skeletal muscle. A basis for this can be found in previous in vitro experiments, which showed that, like caveolin-1, transfection of caveolin-3 into human embryonic kidney 293T cells resulted in augmentation of insulin signaling (50).

Although the role of caveolin-3 has been investigated in multiple cellular and tissue processes, there are no reports describing a direct relationship between caveolin-3 and insulin signaling in muscle tissue.

Therefore, we sought to investigate the consequences of genetic ablation of caveolin-3 on whole body glucose homeostasis and skeletal muscle insulin action. Interestingly, when we followed these mice over a 10-mo period, we observed increased body weight and increased adiposity in Cav-3 null animals. This phenotype was accompanied by marked glucose intolerance and insulin resistance, as evidenced by glucose and insulin tolerance tests, respectively. Using the hyperinsulinemic-euglycemic clamp technique to elucidate the tissue-specific effects of caveolin-3 ablation on glucose metabolism, we now demonstrate alterations in skeletal muscle insulin action, consistent with the role of caveolin-3 as a positive regulator of insulin signaling via stabilization of the insulin receptor at the plasma membrane.

MATERIALS AND METHODS

Materials. Antibodies and their sources were as follows: guinea pig anti-insulin (polyclonal antibody; PAb; a gift from Dr. Peter Arvan, Albert Einstein College of Medicine), mouse anti-Actin (monoclonal antibody; MAb; Sigma, St. Louis, MO), rabbit anti-CD-36 (PAb; Cayman Chemical, Ann Arbor, MI), and mouse anti-GLUT4 (MAb; Genzyme, MA). Anti-GSK-3β (MAb) was from BD Biosciences-Pharmingen (San Jose, CA). Anti-insulin receptor-β (C-19; PAb) and anti-phospho-GSK-3-β (Ser9; PAb) were both from Santa Cruz Bio-technology (Palo Alto, CA). Anti-phosphocaveolin-1, anti-phospho-glucokinase (Ser641; PAb), anti-PKB/Akt (PAb) and anti-phospho Akt (Ser473; PAb) were from Cell Signaling. Anti-phospho-tyrosine IgG (4G10; MAb), and anti-IRS-1 (PAb) were obtained from Upstate Biotechnology (Lake Placid, NY). Alternatively, we used anti-phospho-tyrosine IgG (rabbit PAb) from BD Biosciences-Pharmingen. Human recombinant insulin was purchased from Novo Nordisk (Princeton, NJ). Anti-caveolin-3 (MAb) was a gift from Roberto Campos-Gonzalez (BD Pharmingen, San Jose, CA). A variety of other reagents was purchased commercially and were the highest purity grade available.

Generation and husbandry of Cav-3 null mice. Caveolin-3 null mice were created by targeted disruption of the Cav-3 gene in embryonic stem cells and then used to generate knockout mice by standard techniques (28). The protein expression of caveolin-3 was reduced by 85–95% in the young mice. In addition, the expression of caveolin-3 in the young mice was completely reduced in skeletal muscle, while the expression of caveolin-1 remained almost unchanged. The expression of caveolin-3 in the young mice was completely reduced in skeletal muscle, while the expression of caveolin-1 remained almost unchanged. The expression of caveolin-3 in the young mice was completely reduced in skeletal muscle, while the expression of caveolin-1 remained almost unchanged.
tissue sections were deparaffinized in xylene, dehydrated through a graded series of ethanol washes, and placed in PBS. After the use of an antigen retrieval kit (DAKO), tissue sections were blocked with 10% fetal bovine serum in PBS for 30 min at room temperature. Primary antibodies were used at the following dilutions: anti-caveolin-3 (1:1,000) and anti-insulin (1:1,000). Fluorescently conjugated secondary antibodies (5 μg/ml) were added to the sections for 45 min at room temperature. After being washed extensively with PBS, the slides were mounted with Slow-Fade antifade reagent (Molecular Probes). The slides were observed with an inverted microscope (model IX 70, Olympus).

Magnetic resonance imaging and spectroscopy. All images were obtained using a 9.4-T magnet (Varian horizontal bore system). Mice (2 and 5 mo old) were first anesthetized with the use of isoflurane inhalation anesthesia at 1.5% in O2. Imaging and spectroscopy studies were conducted using a 25-mm 1H quadrature birdcage coil and routine pulse-acquire (4 signal averages, 5-s delay between scans) and spin-echo imaging pulse sequences (18-ms echo time, 400-ms repetition time, and 2 signal averages per scan). To quantitatively assess whole-body fat and water, the 1H spectra, including the water and fat peaks, were acquired and were integrated with spectrometer software. To evaluate abdominal adiposity, 13 images of 1-mm thickness, with a 0.5-mm gap between slices, spanning the abdominal region were acquired. Images were analyzed by histogram analysis using MATLAB-based software. Total body and abdominal fat are presented as a percentage of total tissue.

Food intake. Mice (n = 5, for each genotype) were placed in individual cages with ad libitum access to both food and water for a period of 8 days. Food weight was measured daily for the last 4 days of the experiment.

Plasma analysis. Plasma samples were collected by bleeding the tail of each mouse as indicated. Fasting blood samples were collected after a 12-h fast at 12 PM. Post-prandial blood samples were collected 3 h after the beginning of the room’s dark cycle. Glucose, triglycerides and nonesterified free fatty acid (NEFA) levels were measured using standard enzymatic colorimetric assays (Sigma and Wako Biochemical). Insulin levels were determined by radioimmunoassay (RIA; Linco Research). Similarly, leptin and adiponectin levels were determined according to previously described methods (25, 35). And samples were collected 3 h after the beginning of the room’s dark cycle.

Determination of pancreatic islet area. The pancreas was removed from wild-type and Cav-3 null mice (5 mo old), embedded in paraffin, and sectioned into 5-μm-thick sections as previously described (3). Every 50th section was hematoxylin-and-eosin stained and sampled for islet area. A total of 80 islets for each group were analyzed and the mean islet area was quantitated using NIH Image J Software.

Analysis of tissue glycogen levels. Tissue glycogen levels were determined according to previously described methods (25, 35). Briefly, hindlimb muscle (gastrocnemius) and liver from 6-h fasted mice were treated with 1 N NaOH at 80°C. Upon “digestion,” an aliquot was used for protein determination using the bicinchoninic acid reagent (Pierce). Protein precipitation was achieved by adding an equal volume of 2 N TCA. Glycogen was precipitated from the supernatant with ethanol (2:1 vol/vol). The precipitate was washed with 80% ethanol and solubilized in water. An aliquot of the glycogen precipitate was digested to glucose with 1 mg/ml amyloglucosidase (Sigma) in 0.5 M Na acetate buffer, pH 4.5, and the amount of glucose was determined using a glucose assay. Glycogen levels were determined as the difference between amyloglucosidase-treated and untreated samples.

Analysis of tissue triglyceride content. Hindlimb muscle (gastrocnemius) and liver triglycerides were extracted according to the methods of Dole and Meinerz, as modified by Carpéné (6). Briefly, each tissue was removed from 6-h fasted mice, cleaned of all surrounding tissue, and promptly frozen in liquid nitrogen. Tissues were homogenized in 2 ml of homogenization buffer (20 mM Tris, pH 7.3, 1 mM EDTA, and 1 mM β-mercaptoethanol). One milliliter of this homog-enate was placed in a glass tube and 2 ml of Dole and Meinerz extraction buffer (78% vol/vol isopropanol, 20% vol/vol heptane, and 2% vol/vol 1 N sulfuric acid) was added and mixed vigorously. Next, 2 ml of heptane was added, mixed, and the extracts were allowed to stand until the two phases separated. Triglycerides were measured colorimetrically in the upper organic phase, according to the manufacturer’s instructions (Wako Chemicals, Nuss, Germany).

Glucose tolerance test. Food was removed in the morning and the experiments were performed in the afternoon after 6 h of fasting. Blood glucose was measured before and at 30, 45, 60, 120, and 180 min after an oral gavage with a solution of glucose (1 g/kg). Blood samples were collected from the tail vein and glucose concentrations were determined colorimetrically, whereas insulin levels were determined by RIA.

Insulin tolerance test. Insulin tolerance tests (ITT) were performed on fasted mice, as described above for the glucose tolerance test (GTT) assays. Blood glucose was measured before and at 15, 30, 45, 60, 120, and 240 min after an intraperitoneal injection of human recombinant insulin (0.75 U/kg). In vivo assessment of insulin action and glucose metabolism. After an overnight fast, hyperinsulinemic-euglycemic clamps were conducted in awake, 8- to 10-wk-old mice, as previously described (24). Briefly, a 2-h hyperinsulinemic-euglycemic clamp was conducted with a prime-continuous infusion of human insulin (15 pmol·kg−1·min−1; Eli Lilly, Indianapolis, IN), and a variable infusion of 20% glucose to maintain euglycemia. Insulin-stimulated whole body glucose metabolism was estimated using 3-[3H]glucose infusion during clamps, and tissue-specific glucose uptake was assessed with 2-deoxy-D-[1-14C]glucose injection as previously described (24). At the end of the clamps, tissues were taken for biochemical analysis.

Intraperitoneal stimulation with insulin for Western blot analysis. For acute insulin stimulation, 2-mo-old male mice were fasted for 6 h and injected intraperitoneally with 1 U/kg body wt of human recombinant insulin. Fifteen minutes later, the animals were euthanized and skeletal muscle samples (gastrocnemius) were collected and immediately frozen in liquid nitrogen.

Statistical analysis. Results are represented as the means ± SE. Statistical significance was determined using an unpaired two-tailed Student’s t-test, with P < 0.05 being considered significant.

RESULTS

Caveolin-3 null mice show increased body weight with increased whole body adiposity. For all experiments, 2-mo-old male mice, in the C57BI/6 genetic background, were used unless otherwise indicated in the text. At this young age, Cav-3 null mice do not show any myopathic changes in their skeletal muscle fibers (15, 19).

Gross phenotypic evaluation of Cav-3 null mice and their corresponding wild-type counterparts revealed a tendency of Cav-3 null mice to be larger than wild-type mice of the same age. Longitudinal evaluation and quantitation of this finding demonstrated that, at 4 wk of age, there was no statistical difference between the weights of male wild-type and Cav-3 null mice (Fig. 1A). However, starting at 8 wk of age and continuing out to 40 wk of age, we noted that Cav-3 null male mice were significantly larger (~12–20%) than their age- and sex-matched wild-type counterparts (Fig. 1A).

To determine the cause of this increased body mass, we chose to assess the degree of adiposity in wild-type and Cav-3 null mice using magnetic resonance imaging techniques (38). Representative abdominal cross-sections clearly show that Cav-3 null mice have measurably increased visceral adiposity (Fig. 1B). Quantitative analysis of the ratio between total body fat and total body tissue reveals a ~2-fold increase in the total
Fig. 1. Caveolin-3 (Cav-3) null mice show increased body weight with increased whole body adiposity. A: total body mass of age-matched wild-type (WT) and Cav-3 null male mice was followed from 4 to 40 wk of age (1–10 mo of age; n ≥ 8 mice, for each group). Results are reported as means ± SE. *P < 0.05. B: representative magnetic resonance imaging (MRI) cross-sections of the abdominal region from WT and Cav-3 null mice are shown. Red arrows point at visceral/abdominal fat. For orientation, the location of the kidneys (K) is also indicated. Quantitative analysis revealed that the whole body adiposity and abdominal adiposity are increased in Cav-3 null mice (see Table 1). Results with 2- and 5-mo-old mice are shown. C: visualization of paraffin-embedded hematoxylin and eosin (H&E)-stained skin biopsies reveals an increased thickness of the hypodermal fat layer (see arrows) in Cav-3 null mice. Original magnification, ×10. Results with 2- and 5-mo-old mice are shown. D: cohorts of 2-mo-old WT and Cav-3 null mice were housed in single cages for a period of 8 days and daily food intake was monitored for the last 4 days of the experiment (n = 5 mice, for each group). Results are reported as means ± SE (P = 0.3).
body fat of Cav-3 null mice, as well as a ~2-fold increase in abdominal fat (Table 1). Furthermore, histopathological exam-
ination of other fat depots, i.e., the hypodermal fat layer, revealed an increase in total fat pad thickness, as well as an increase in adipocyte size in Cav-3 null mice (Fig. 1C). Similar results were observed in both 2- and 5-mo-old mice (Fig. 1 and Table 1). Importantly, these changes occurred without any differences in food intake between wild-type and Cav-3 null mice (Fig. 1D).

Cav-3 null mice display postprandial hyperinsulinemia and alterations in plasma adipokine levels. To determine whether any measurable physiological changes occur concurrently with the increased adiposity in Cav-3 null mice, we next examined several plasma metabolites involved in energy metabolism (Fig. 2). Analysis of plasma triglyceride, nonesterified fatty acid (NEFA), glucose, and insulin levels in the postprandial and fasted state, revealed no alterations in any of these markers, with the exception of insulin. Interestingly, insulin was found to be significantly elevated in the postprandial state in Cav-3 null mice. The identification of this alteration possibly indicates glucose intolerance and peripheral insulin resistance, as observed in other mouse models of insulin resistance (30).

Table 1. MRI analysis of adiposity in WT and Cav-3 KO mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age, months</th>
<th>Whole Body Adiposity, %</th>
<th>Abdominal Adiposity, %</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2</td>
<td>11.25±0.85</td>
<td>8.53±0.69</td>
<td>1.89</td>
</tr>
<tr>
<td>Cav-3 KO</td>
<td>2</td>
<td>31.24±1.90*</td>
<td>33.70±2.71*</td>
<td>2.36</td>
</tr>
<tr>
<td>Fold change</td>
<td></td>
<td>2.50*</td>
<td>15.00±1.64*</td>
<td>1.76</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 for each experimental group. WT, wild type; Cav-3, caveolin-3; KO, knockout. Whole body adiposity (%) = (total body fat/total body tissue) × 100. Abdominal adiposity (%) = (total abdominal fat/total abdominal tissue) × 100. *P < 0.05.

As Cav-3 null mice display markedly increased adiposity, next analyzed their plasma adipokine levels (Table 2). Interestingly, we found that leptin levels were increased nearly threefold in Cav-3 null mice compared with wild-type littermates. In addition, plasma adiponectin levels were found to be significantly decreased in Cav-3 null animals. Such changes in plasma adipokine levels are often seen in the setting of increased whole body fat mass and are, thus consistent with the observed phenotype of Cav-3 null animals.

Cav-3 null mice display glucose intolerance and insulin resistance. We next performed oral glucose and intraperitoneal insulin tolerance tests (GTT and ITT). Cav-3 null mice displayed significantly elevated plasma glucose levels at 30-min postglucose load (Fig. 3A), which remained elevated for 120 min. In addition, ITT results showed a blunted glucose response in the Cav-3 null mice compared with the wild-type mice (Fig. 3B).

To further explore this phenotype, insulin levels were measured throughout the time course of the GTT. Interestingly, we found that plasma insulin levels were significantly elevated in Cav-3 null mice as early as 15 min after glucose gavage and remained elevated until the end of the experimental time period (Fig. 3C).

Pancreatic islets are markedly larger in Cav-3 null mice. As insulin levels were found to be dramatically increased in Cav-3 null mice, we next examined the overall pancreatic tissue morphology for any variations from wild-type mice. Remarkably, whereas no abnormalities were noted in the exocrine portion of this organ, we found that in Cav-3 null mice, pancreatic islets were markedly larger than in wild-type mice (Fig. 4A). Quantitative analysis of >80 islets per group re-
revealed a ∼4-fold increase in the mean islet area in Cav-3 null animals.

In addition, immunofluorescence microscopy and Western blot analysis were undertaken to determine whether normal pancreatic islets express caveolin-3, and whether loss of caveolin-3 expression could account for the histological abnormalities described above. Both methodologies revealed that caveolin-3 is not expressed in this tissue type (Fig. 4, B and C), thus suggesting that hyperplasia of islets may be secondary to glucose intolerance. Several groups have analyzed pancreatic islet size in obese and hyperglycemic mice, showing that islet size, but not islet number is increased in these animals (3, 10, 18, 20). Such changes appear to be occurring in the setting of Cav-3 null mice, as well.

**Insulin resistance in Cav-3 null mice as assessed by hyperinsulinemic-euglycemic clamp.** To determine the mechanism of glucose intolerance, a 2-h hyperinsulinemic-euglycemic clamp was conducted in awake wild-type and Cav-3 null mice. The plasma glucose concentrations were maintained at ∼100 mg/dl, whereas plasma insulin concentrations were raised to 720 pM during the clamps. Consistent with the GTT and ITT results, Cav-3 null mice were insulin resistant as reflected by a ∼30% decrease in steady-state rates of glucose infusion during clamps compared with the wild-type mice (Fig. 5A). This was mostly attributed to 20% and 40% decreases in insulin-stimulated whole body glucose uptake and whole body glycogen plus lipid synthesis, respectively. In contrast, insulin-stimulated whole body glycolysis was not affected by loss of caveolin-3 (Fig. 5B).

Tissue-specific insulin action and glucose metabolism were assessed using [3-3H] glucose infusion and 2-deoxy-D-[1-14C]glucose injection during clamps in awake mice. Because skeletal muscle, where caveolin-3 is abundantly expressed, is responsible for ∼75% of glucose disposal during the insulin-stimulated state, insulin-stimulated glucose uptake was examined in the gastrocnemius muscle (Fig. 5C). Insulin-stimulated glucose uptake and glycolysis in skeletal muscle showed a tendency to be reduced in Cav-3 null mice compared with the wild-type mice (166 ± 17 vs. 203 ± 46 in wt for glucose uptake, P = 0.24, 160 ± 17 vs. 193 ± 46 in wt for glycolysis, P = 0.26; Fig. 5C). Insulin-stimulated glycogen synthesis in skeletal muscle was significantly reduced by ∼40% in Cav-3 null mice compared with wild-type mice (6.0 ± 1 vs. 10.0 ± 2 in wt; P < 0.05; Fig. 5C). Surprisingly, insulin-stimulated glucose uptake in white adipose tissue, which does not express caveolin-3, was decreased by ∼70% in Cav-3 null mice compared with wild-type mice (21 ± 4 vs. 79 ± 22 in wt; P < 0.05; Fig. 5D). In contrast, insulin-stimulated glucose uptake in brown adipose tissue was not altered in Cav-3 null mice (data not shown). In addition, insulin-stimulated glucose uptake in the heart, which expresses an abundance of caveolin-3, was not

![Fig. 3. Cav-3 null mice are glucose intolerant and insulin resistant. A: glucose tolerance test (GTT). Plasma glucose levels were measured in WT and Cav-3 null animals (2 mo of age) before and after an oral glucose bolus (1 g/kg) at the time points indicated (n = 6–7 mice, for each group). Note that plasma glucose levels are significantly elevated in Cav-3 null mice at each time point after the glucose bolus. B: insulin tolerance test (ITT). Wild-type and Cav-3 null mice (2 mo of age) were given an intraperitoneal injection of insulin (0.75 U/kg) and plasma glucose levels were measured at the indicated time points (n = 7–9 mice, for each group). Plasma glucose levels are significantly elevated in Cav-3 null mice at all time points measured after insulin injection. C: plasma insulin levels during the GTT. Plasma insulin levels were determined before and after an oral glucose (1 g/kg body wt) load (n = 7–9 mice, for each group). Plasma insulin levels were measured by RIA. Note that the plasma insulin levels are significantly elevated in Cav-3 null mice during the glucose tolerance test (see A). Results are reported as means ± SE (⁎P < 0.05).](http://ajpcell.physiology.org/)

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### Table 2. Plasma leptin and adiponectin levels in WT and Cav-3 KO mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age, months</th>
<th>Leptin, ng/ml</th>
<th>Adiponectin, μg/ml</th>
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<tbody>
<tr>
<td>WT</td>
<td>2</td>
<td>15.3 ± 3.5</td>
<td>8.6 ± 0.6</td>
</tr>
<tr>
<td>Cav-3 KO</td>
<td>2</td>
<td>41.2 ± 6.9*</td>
<td>4.9 ± 0.3*</td>
</tr>
<tr>
<td>Fold change</td>
<td></td>
<td>2.7</td>
<td>∼1.76</td>
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Values are means ± SE; n = 4 for each experimental group, *P < 0.05.
Fig. 4. Pancreatic islets are significantly larger in Cav-3 null mice. A: the pancreas was removed from WT and Cav-3 null mice (5 mo old), fixed, embedded in paraffin, and 5-μm-thick sections were cut. Every 50th section was hematoxylin and eosin stained and sampled for islet area. Mean islet area was quantitated using NIH Image J Software. Whereas no abnormalities were noted in the exocrine portion of this organ, pancreatic islets from Cav-3 null mice were markedly larger than in WT mice (top). Quantitative analysis of >80 islets per group revealed a ~4-fold increase in the islet area in Cav-3 null animals (bottom). Results are reported as means ± SD (*P < 0.05). B: paraffin-embedded pancreatic tissue sections were doubly immunostained with anti-Cav-3 IgG and anti-insulin IgG. Note that pancreatic islets from WT mice do not show any Cav-3 immunostaining, consistent with the notion that Cav-3 is not normally expressed in the pancreas. IF, immunofluorescence. C: Western blot (WB) analysis. Pancreatic tissue lysates were subjected to immunoblot analysis with anti-Cav-3 IgG. Note that Cav-3 is not expressed in the pancreas at detectable levels. As a control for equal protein loading, we determined the expression levels of β-actin. A positive control with skeletal muscle tissue lysates is also shown for comparison. KO, knockout; MAb, monoclonal antibody.
altered in Cav-3 null mice (data not shown). Furthermore, basal hepatic glucose production was not different among the groups (data not shown), but insulin-mediated suppression of basal hepatic glucose function was significantly reduced by ~40% in Cav-3 null mice (Fig. 5E).

Cav-3 null mice show marked alterations in glycogen storage in both muscle and liver, with an increased triglyceride content in liver. Consistent with decreases in insulin-stimulated glycogen synthesis in Cav-3 null mice, we found that after a 6-h fast, muscle glycogen content was significantly reduced by ~40% in these animals compared with wild-type mice. Hepatic glycogen content was also reduced by ~70% in Cav-3 null mice (Fig. 6A). Insulin resistance in skeletal muscle and the liver has been shown to be associated with increased tissue deposition of fat (4, 23, 32, 36). In agreement with the results presented above, we found that the triglyceride content of the liver was significantly elevated in Cav-3 null mice by ~50%, suggesting that the changes often associated with “metabolic syndrome X” may be occurring in Cav-3 null mice (Fig. 6B). Examination of this parameter in skeletal muscle revealed a tendency toward increased triglyceride content; however, these changes were not statistically different (Fig. 6B).

Cav-3 null mice show normal insulin receptor protein levels at steady state with compensatory changes in downstream signaling molecules. To determine the mechanism of insulin resistance in the Cav-3 null mice, we analyzed the expression and activation state of several insulin signaling molecules in skeletal muscle. We have previously demonstrated a direct role...
for caveolin-1 in insulin signaling in vivo (7); however, to date, a direct role for caveolin-3 in the insulin signaling pathway of skeletal muscle has not been described.

Western blot analysis of skeletal muscle (gastrocnemius) lysates revealed a dramatic compensatory increase in the expression of both GLUT4 (~4-fold increase) and PKB/Akt (~3-fold increase) in Cav-3 null mice compared with wild-type animals (Fig. 7A). Interestingly, these results are similar to those described previously in the perigonadal fat pads of Cav-1 null mice. Although the expression levels of IRS-1, IRS-2, and GSK-3β were unchanged in the skeletal muscle of Cav-3 null animals (Fig. 7A and B).

As a correlate of functional insulin signaling, the phosphorylation state of GSK-3β (Ser9) and Akt/PKB (Ser473) were examined in wild-type and Cav-3 null skeletal muscle tissue. Phosphorylation of GSK-3β on Ser9 by PKB/Akt leads to the inactivation of GSK-3β in response to insulin stimulation (12). Interestingly, here we find that the phosphorylation of GSK-3β and Akt are increased ~3-fold in Cav-3 null animals (Fig. 7A and B), suggesting a compensatory response to defective insulin signaling. However, the total levels of Akt were also proportionally increased, indicating that Akt is not hyperphosphorylated. Thus the increased levels of phosho-Akt at steady state may simply reflect the overall compensatory increase in total Akt levels.

In addition, we examined the expression and phosphorylation state of glycogen synthase (GS) in skeletal muscle. Control of this enzyme is achieved both allosterically by levels of glucose-6-phosphate, as well as by GSK-3β-mediated phosphorylation on Ser641 (11, 42). In response to insulin, GSK-3β is normally inactivated, thus reducing the inhibitory phosphorylation of GS and allowing the storage of glycogen to proceed. Interestingly, in Cuv-3 null mice, we observe reduced expression levels (~5-fold) and a corresponding reduction in the phosphorylation (~2-fold) of GS, compared with wild-type mice. These results provide an explanation for the reduced glycogen content of Cav-3 null skeletal muscle. As the total expression of skeletal muscle GS is decreased, it would be expected that the total activity of this enzyme, and thus the total glycogen storage capability, would also be decreased.

It has been recently reported that CD 36 (a molecule involved in fatty acid transport) colocalizes with caveolin-3 in skeletal muscle fibers (22, 47). However, the expression levels of CD 36 were unchanged between the two genotypes (Fig. 7A).

Cav-3 null mice show decreased insulin receptor protein levels after insulin stimulation, with functional alterations in insulin signaling. Because insulin receptor protein levels appeared normal in caveolin-3 null skeletal muscle (gastrocnemius) at steady state (Fig. 7A), we next assessed the fate of the receptor after acute and chronic insulin stimulation. Figure 8A shows that after 15 min of insulin stimulation (via intraperitoneal injection), the total levels of the insulin receptor in cav-3 null skeletal muscle are significantly reduced (~5-fold), compared with wild-type animals treated identically. Similarly, after 2 h of intravenous administration of insulin (at the end of the clamp studies), insulin receptor protein levels were also dramatically reduced in cav-3 null skeletal muscle (Fig. 8B). However, at both time points, the insulin receptor still underwent tyrosine phosphorylation in Cav-3 null mice. If we normalize for the observed decreases in total insulin receptor protein levels, then it appears that loss of caveolin-3 leads to insulin receptor hyperphosphorylation, possibly explaining its tendency toward increased degradation.

In addition, we observed decreased phosphorylation of Akt, despite an increase in total Akt levels, indicative of an impairment of insulin signaling downstream of the insulin receptor (Fig. 8A). In contrast, the phosphorylation state of IRS-1 is not significantly different between the two genotypes (Fig. 8A); however, we also observed an increase in the total expression levels of IRS-1. If we take into account that total IRS-1 levels are increased ~2-fold, but that the phosphorylation levels appear equivalent, then this indicates that IRS-1 phosphorylation is actually reduced by ~2-fold.

Fig. 6. Analysis of muscle and liver reveals a reduction in glycogen stores and an elevation in triglyceride content in the liver of Cav-3 null mice. A: tissue glycogen content was analyzed in WT and Cav-3 null mice in both the muscle and liver, revealing that loss of caveolin-3 results in a significant decrease in the total glycogen storage of Cav-3 null mice. B: analysis of muscle and liver triglyceride content reveals that Cav-3 null mice display marked accumulation of lipids in the liver compared with WT mice. All mice were 2 mo of age (n = 5 mice, for each group). Results are reported as means ± SE (*P < 0.05).
Taken together, these results suggest that caveolin-3 normally functions to increase the stability of the insulin receptor at the plasma membrane in skeletal muscle, preventing or slowing its ligand-induced downregulation. In support of this notion, we show that the expression levels and the activation state of signaling molecules downstream of the insulin receptor are also clearly affected in caveolin-3 null skeletal muscle.

DISCUSSION

In the present study, we have identified a role for caveolin-3 in the modulation of insulin signaling and glucose metabolism in skeletal muscle. Our initial findings reveal that Cav-3 null mice display markedly increased whole body adiposity evident at a relatively early age, which is not attributed to hyperphagia. In an attempt to explain this phenotype, we analyzed several plasma metabolic parameters and found that Cav-3 null mice exhibit postprandial hyperinsulinemia without alterations in glucose, triglyceride, or NEFA levels. Further experimentation along these lines revealed that Cav-3 null mice display a blunted response to glucose and insulin tolerance tests, suggestive of insulin resistance. Furthermore, histopathological examination of pancreatic islets in wild-type and Cav-3 null mice revealed markedly enlarged islets in Cav-3 null animals, the presence of which are indicative of a compensatory increase in insulin production, typical of a prediabetic state (41).

To address the relative role of liver, fat, and muscle in the observed whole body insulin resistance, we performed a hyperinsulinemic-euglycemic clamp. Interestingly, in Cav-3 null mice, we observe reduced expression levels (~5-fold) and a correlative reduction in phosphorylation (~2-fold) of GS in the skeletal muscle, compared with WT mice. These results provide an explanation for the reduced glycogen content of Cav-3 null muscle. As a control for equal protein loading, we determined the expression levels of β-actin. All mice were 2 mo of age (n = 5 mice, for each group); results with 2 representative mice are shown for each genotype.

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what perplexing. That is, caveolin-3 ablation resulted in whole body insulin resistance that was predominantly due to altered glucose metabolism in organs other than skeletal muscle. In this regard, hepatic insulin action was reduced by 40% in Cav-3 null mice, and insulin-stimulated glucose uptake in white adipose tissue was decreased by 70% in Cav-3 null mice.

In attempt to elucidate the molecular mechanisms behind the observed insulin resistance, we next analyzed the glycogen and triglyceride content of several tissues, as the depletion of glycogen stores has been correlated with the early stages of insulin resistance (26, 44, 48). Cav-3 null mice show a significant reduction in the glycogen stores of both liver and muscle, consistent with the insulin resistance of these organs. Interestingly, Cav-3 null mice also show a significant increase in the triglyceride content of the liver, yet normal triglyceride levels in muscle. Taken together, these results are consistent with several epidemiological studies that indicate a strong correlation between tissue lipid content and hepatic and peripheral insulin resistance (29, 36).

Fig. 8. Cav-3 null mice show decreased insulin receptor protein levels after insulin stimulation. After insulin stimulation, mouse skeletal muscle samples were collected. Samples were then subjected to SDS-PAGE and Western blot analysis. Each lane contains an equal amount of total protein. A: intraperitoneal stimulation. Cav-3 null mice were fasted for 6 h and injected intraperitoneally with insulin (1 U/kg). After 15 min, the mice were euthanized and skeletal muscle samples (gastrocnemius) were harvested. Wild-type mice were also processed in parallel and treated identically. Note that insulin receptor protein levels were dramatically reduced in Cav-3 null skeletal muscle. Similarly, the phosphorylation of Akt is also decreased in the Cav-3 null animals (despite an increase in total Akt levels), indicative of impaired insulin signaling in Cav-3 null mice. In contrast, the phosphorylation state of IRS-1 is not significantly different between the two genotypes; however, we also observed an increase in the total expression levels of IRS-1. If we take into account that total IRS-1 levels are increased ~2-fold, but that the phosphorylation levels appear equivalent, then this indicates that IRS-1 phosphorylation is actually reduced by ~2-fold. B: intravenous stimulation. After an overnight fast, a 120-min hyperinsulinenic-euglycemic clamp was conducted with a prime continuous infusion of insulin at a rate of 15 pmol·kg⁻¹·min⁻¹ to raise plasma insulin, within a physiological range (~780 pM). At the end of the clamps, animals were anesthetized and muscle samples (gastrocnemius) were collected. Tissue samples were frozen immediately in liquid nitrogen and stored at ~70°C for analysis. Interestingly, insulin receptor protein levels were significantly reduced in insulin-stimulated Cav-3 null skeletal muscle, compared with WT mice treated identically. In A and B, all mice were ~2 mo of age (n = 5 mice, for each group); results with 2 representative mice are shown for each genotype. Importantly, very similar results were obtained with insulin stimulation using either route of administration. Despite these reductions in total insulin receptor protein levels, insulin receptor tyrosine phosphorylation appeared virtually unchanged in Cav-3 null mice, indicating that the receptor is hyperphosphorylated. Note that for IRS-1, 200 μg of tissue lysate were resolved by SDS-PAGE and then processed for immunoblotting. The phosphorylation state of IR-β or IRS-1 was determined using antiphosphotyrosine antibodies.
The development of this secondary insulin resistant phenotype in the liver and fat is most likely based upon several independent contributing factors. To begin with, the increased triglyceride content of the liver is likely to be secondary to hepatic insulin resistance, which may be due to increased rates of gluconeogenesis and lipogenesis typical of insulin resistant states (2, 14, 43). In addition, our findings demonstrating severely perturbed plasma adipokines levels may also have profound effects on peripheral and liver insulin action. Adipokines levels, which are factors made and secreted by adipocytes, may be altered at least in part due to overwhelming alterations in adipocyte size in Cav-3 null animals. That is, the observed hypertrophy of the adipocytes may lead to decreased adiponectin and increased leptin production, respectively, similar to what has been seen in obese insulin-resistant humans and rodents (31, 37). In particular, the reduction in adiponectin levels in our Cav-3 null mice and the observed whole body and hepatic insulin resistance correlates well with many studies showing a relationship between insulin resistance states and reduced levels of adiponectin (1, 21, 27, 46). However, we can rule out the possibility of leptin resistance because the increased adiposity in Cav-3 null mice is not due to a defect in food intake, as would be expected for an impaired leptin response. The food intake data, instead, suggests that the adiposity of Cav-3 null mice may be due to reduced energy expenditure and increased energy storage.

We determined the expression of several key insulin responsive proteins in caveolin-3 null muscle samples. Initial analysis revealed that the expression of IR-β and GSK-3-β are unchanged in caveolin-3 null muscle at steady state. However, we observed dramatically increased expression of GLUT4 and Akt. Interestingly, the expression of these proteins has also been shown to be increased in the perigonadal white fat of Cav-1 null mice, which display mild postprandial hyperinsulinemia (9). In the setting of caveolin-3 deficiency, the increased levels of GLUT4 most likely represent a compensatory mechanism which may explain the relatively subtle defects found in muscle glucose uptake. That is, the loss of caveolin-3 in skeletal muscle leads to acute defects in glucose uptake and glycogen metabolism which, in response to these factors, are compensated for by an increase in GLUT4 protein expression. In addition, in Cav-3 null mice, the phosphorylation of GSK-3-β and Akt are increased at baseline, probably secondary to the hyperinsulinemia in these mice. The expression and phosphorylation of glycogen synthase are also reduced in the caveolin-3 null muscle, further indicative of an insulin-resistant state in skeletal muscle tissue. In addition, we find reduced phosphorylation of Akt after insulin stimulation, indicative of impaired insulin signaling downstream of the receptor. We also observe increased expression of Akt, at baseline and after insulin stimulation (Figs. 7A and 8A). Thus the increased levels of phospho-Akt at steady state may simply reflect the overall compensatory increase in total Akt levels.

Finally, we assessed the fate of the insulin receptor after insulin stimulation in caveolin-3 null skeletal muscle. We observed that after 15 min of insulin stimulation, the total levels of the insulin receptor in caveolin-3 null skeletal muscle are significantly reduced, compared with wild-type animals treated identically. Similar results were obtained after 2 h of insulin stimulation.

Because there are no differences in the expression levels of the insulin receptor at baseline in the Cav-3 null mice, the observed poststimulatory reduction in insulin receptor levels in the Cav-3 null animals is clearly ligand dependent (summarized schematically in Fig. 9). There are two possible mechanisms that could explain this phenotype in Cav-3 null mice. One possibility is that an absence of Cav-3 favors ubiquitination of the insulin receptor, after ligand binding. It has been recently described that plasma membrane proteins, in particular receptor tyrosine kinases, can be degraded through the proteasomal pathway (5). As such, Cav-3 may be involved in regulating this pathway. Alternatively, Cav-3-binding and/or skeletal muscle caveolae may stabilize the insulin receptor at the plasma membrane, thereby preventing its degradation via a lysosomal pathway. Finally, if we normalize for the decreases in total insulin receptor protein levels, then loss of caveolin-3 leads to insulin receptor hyperphosphorylation. Such hyperphosphorylation may explain the relatively subtle defects in glucose uptake, as would be expected for an impaired leptin response. The food intake data, instead, suggests that the adiposity of Cav-3 null mice may be due to reduced energy expenditure and increased energy storage.

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phosphorylation may also explain its tendency toward increased degradation. Thus, Cav-3 and skeletal muscle caveolae may normally function to increase the stability of the insulin receptor at the sarcolemmal membrane, thereby preventing its rapid internalization and degradation. These results are consistent with the idea that caveolin-3 is a positive regulator of insulin signaling that acts via insulin receptor stabilization at the plasma membrane by preventing or slowing ligand-induced receptor downregulation.

We speculate that muscle cell caveolae may act to retain the insulin receptor at the level of the plasma membrane, thereby preventing its internalization via clathrin-coated pits. Once this caveolar retention mechanism is disrupted (by loss of caveolin-3), insulin receptor internalization and degradation proceed at a faster rate. In support of this notion, we and others (9, 50) have previously shown that both caveolin-1 and -3 interact directly with the insulin receptor, via a highly conserved caveolin-binding motif located in the insulin-receptor kinase domain. In addition, mutations in this insulin-receptor caveolin-binding motif lead to insulin resistance syndromes in humans (see Refs. 7 and 9 for reviews). Interestingly, unlike caveolin-3, caveolin-1 functions as a “chaperone” to prevent insulin receptor degradation during its biosynthesis and transport to the cell surface in adipocytes and fibroblasts (9). A summary of the insulin/glucose-related phenotypes of Cav-1 null vs. Cav-3 null mice is shown in Table 3.

Another alternative possibility is that caveolin-3 and muscle cell caveolae are actively involved in promoting the recycling of the insulin receptor from the endosomal compartment back to the plasma membrane. Thus an absence of caveolin-3 could also result in defective insulin receptor recycling, e.g., by shunting the insulin receptor from the endosome to the lysosome, instead of back to the plasma membrane (Fig. 9). Additional experiments will be necessary to explore this hypothesis.

In summary, the current study shows that the absence of caveolin-3 leads to whole body insulin resistance and increased adiposity in Cav-3 null mice. The primary defect can be localized to the skeletal muscle and attributed to defective insulin signaling in this tissue (Fig. 9). These changes have profound secondary effects, causing hyperinsulinemia, glucose intolerance, and the development of insulin resistance in the liver and white adipose tissue. These findings could have important implications for the early diagnosis and treatment of Caveolinopathies, such as LGMD-1C, or other related muscular dystrophies.

APPENDIX

While this study was being completed, another study by Oshikawa et al. (33) appeared that described insulin resistance in Cav-3 null mice. Our current results are largely in agreement with their findings; however, these authors did not assess whole body adiposity or perform hyperinsulinemic-euglycemic clamp studies, nor did they demonstrate hyperinsulinemia or evaluate the status of pancreatic islets or examine adipokine levels and glycogen synthesis rates. As such, our current studies provide a more complete assessment of the glucose intolerance phenotype of Cav-3 null mice. Also, these authors did not assess total insulin receptor protein levels after insulin stimulation (by direct Western blot analysis or by immunoprecipitation of muscle cell lysates), an important control. As a consequence, they did not observe the insulin-induced insulin receptor instability that we report here. Thus our current studies provide a fundamentally different mechanism to explain the observed glucose intolerance and insulin resistance in Cav-3 null mice, that is via enhanced ligand-dependent insulin receptor downregulation in the absence of caveolin-3.

Also, in contrast to Oshikawa et al., we showed that the expression levels of other molecules involved in insulin signaling (GLUT4, Akt, and IRS-1) are altered in Cav-3 null mice. The results by Oshikawa et al. regarding decreases in skeletal muscle in vitro glucose uptake are not surprising and are not in contrast with our in vivo findings; we observed a similar trend in vivo, but it was not statistically significant in skeletal muscle. Such differences between in vivo and in vitro results are possibly due to compensatory mechanisms in vivo that allow for normal skeletal muscle glucose uptake, compared with isolated muscle tissue in vitro. However, our in vivo studies did allow us to localize the whole body insulin resistance mainly to fat and liver, with impaired glycogen synthesis in muscle. These issues were not addressed by Oshikawa and colleagues.

Finally, the observation by Oshikawa et al. (33) of a physical interaction between Cav-3 and the insulin receptor in muscle are not in contrast with our findings, but provide further evidence that Cav-3 directly or indirectly has a role in regulating insulin receptor turnover in skeletal muscle.

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REFERENCES


7. Coleman DL. Circulating kinase.


