Munc18c Heterozygous Knockout Mice Display Increased Susceptibility for Severe Glucose Intolerance

Eunjin Oh,1 Beth A. Spurlin,1 Jeffrey E. Pessin,2 and Debbie C. Thurmond1

The disruption of Munc18c binding to syntaxin 4 impairs insulin-stimulated GLUT4 vesicle translocation in 3T3-L1 adipocytes. To investigate the physiological function and requirement for Munc18c in the regulation of GLUT4 translocation and glucose homeostasis in vivo, we used homologous recombination to generate Munc18c-knockout (KO) mice. Homozygous disruption of the Munc18c gene resulted in early embryonic lethality, whereas heterozygous KO mice (Munc18c+/−) had normal viability. Munc18c−/− mice displayed significantly decreased insulin sensitivity in an insulin tolerance test and a >50% reduction in skeletal muscle insulin-stimulated GLUT4 translocation when compared with wild-type (WT) mice. Furthermore, glucose-stimulated insulin secretion was significantly reduced in islets isolated from Munc18c−/− mice compared with those from WT mice. Despite the defects in insulin action and secretion, Munc18c−/− mice demonstrated the ability to clear glucose to the same level as WT mice in a glucose tolerance test when fed a normal diet. However, after consuming a high-fat diet for only 5 weeks, the Munc18c−/− mice manifested severely impaired glucose tolerance compared with high-fat–fed WT mice. Taken together, these data suggest that the reduction of Munc18c protein in the Munc18c−/− mice results in impaired insulin sensitivity with a latent increased susceptibility for developing severe glucose intolerance in response to environmental perturbations such as intake of a high-calorie diet rich in fat and carbohydrate. Diabetes 54:638–647, 2005

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VAMP2 or SNAP25. It has been proposed that Munc18a orchestrates the conversion of syntaxin 1 to the “open” conformational state, thereby facilitating the interaction among syntaxin 1, VAMP2, and SNAP25 (24–26). Structural studies have led to the general conclusion that the Munc18 proteins share a similar overall structure in which a small folded NH₂-terminal domain mediates their interaction with plasma membrane syntaxins (27,28), whereas the remainder of the COOH-terminal domain carries out the poorly understood effector function that appears essential for fusion. Dulubova et al. (28) have further speculated that a particular loop between domains 2 and 3 of Munc18 proteins may be critical for this effector function, consistent with findings in our own studies showing that an inhibitory peptide directed at this region or a single-point mutation within it alters the syntaxin 4–Munc18c interaction in 3T3L1 adipocytes (29,30). These findings in combination with the functional studies of SNARE vesicle fusion therefore suggest that Munc18c plays a critical role in the transition between the syntaxin 4 open and closed states.

The physiological function of SM proteins has been investigated using overexpression and has generally resulted in inhibition of vesicle exocytosis. This result is likely attributable to these proteins’ ability to bind and sequester their cognate syntaxin. For example, expression of the Munc18a/n-Sec1 isoform in neurons inhibits the association of VAMP and SNAP25 with syntaxin 1 (11). Analogous to Munc18a, increased expression of Munc18c inhibits the priming/fusion of GLUT4 vesicles by blocking the binding of VAMP2 to syntaxin 4 (31,32). We have also investigated the effects of overexpressing Munc18c in vivo, first by overexpressing Munc18c locally in skeletal muscle via adenoviral particle injection (33) and most recently by overexpressing a tetracycline-repressible (tet-off) cytomegalovirus-driven Munc18c transgene that is expressed only in skeletal muscle, adipose tissue, and pancreas of mice (34). In both cases, skeletal muscle GLUT4 translocation was markedly impaired, thus supporting the data collected using 3T3L1 adipocytes in culture. However, in addition to having whole-body insulin resistance, Munc18c-overexpressing transgenic mice have been shown to have markedly diminished insulin secretion, thus demonstrating for the first time that Munc18c plays a role in islet β-cells. Thus, because islet β-cells were previously thought to use the same t-SNAREs as neurons, namely syntaxin 1 and Munc18a, our data now suggest that syntaxin 4 and its binding partner Munc18c regulate insulin secretion as well. Consistent with this conclusion, overexpression of a dominant-interfering syntaxin 4 mutant also inhibits insulin secretion in a pancreatic β-cell line (35).

Although null mutations in the genes encoding the Sec-1, Rop, and neuronal Munc18a proteins cause dramatic reductions in vesicle exocytosis (13,15,36), there have been no in vivo studies on the functional requirement for Munc18c. Using inhibitory peptides, we have shown that the interaction between Munc18c and syntaxin 4 is required for the integration of GLUT4 vesicles into the plasma membrane (30), but the significance of this interaction in vivo remains unknown. Therefore, to examine the physiological requirement for Munc18c in the maintenance of glucose homeostasis, we used homologous recombination to generate mice with a disruption of the Munc18c gene. In this study, we demonstrate that heterozygotic Munc18c-knockout (KO) mice have impaired insulin sensitivity in an insulin tolerance test as well as defects in skeletal muscle insulin-stimulated GLUT4 translocation. Glucose-stimulated insulin secretion from islets isolated from the Munc18c heterozygotic⁻/− mice was also significantly compromised. Moreover, the Munc18c⁻/− mice showed an increased susceptibility to severe glucose intolerance and elevated fasting hyperglycemia after consuming a high-fat diet relative to that of wild-type (WT) mice. Taken together, these data suggest that Munc18c plays an essential role in multiple vesicle exocytosis events that are known to be required for the maintenance of whole-body glucose homeostasis.

**RESEARCH DESIGN AND METHODS**

The rabbit anti-GLUT4 and rabbit anti-Munc18c antibodies were obtained as previously described (32). The syntaxin 4, VAMP2, SNAP-23, and transferrin receptor antibodies were purchased from Chemicon (Temecula, CA), Synaptic Systems (Gottingen, Germany), Affinity Bioreagents (Golden, CO), and Zymed (South San Francisco, CA), respectively. The Akt and phospho-Akt (Ser 473)-specific Akt antibodies were purchased from Cell Signaling (Beverly, MA). Goat anti-rabbit horseradish peroxidase (HRP) and anti-mouse HRP secondary antibodies were purchased from Bio-Rad (Hercules, CA). The rat insulin radioimmunoassay kit was acquired from Linco Research (St. Charles, MO).

**Isolation of murine Munc18c genomic clone.** A panel of PCR oligonucleotide primers was designed spanning the entire murine Munc18c cDNA. PCR amplification of mouse liver DNA using the O/F primer pair (O primer: 5′-G AAAGAAGGCGAATGGGACA; F primer: 5′-TCAAGCTCAGGGTTTGTCTCC) yielded a 506-bp product. DNA sequencing revealed that this matched the cDNA sequence, indicating an absence of intronic DNA. The O and F primers were sent to Genome Systems (St. Louis, MO) for a PCR-based screen of the murine 129/SvJ library and yielded two 120-kb bacterial artificial chromosome (BAC) clones containing the Munc18c gene. Digestion with BamHI yielded a 9-kb fragment of the Munc18c gene. This 9-kb fragment was isolated from each of the two BAC clones, and each was subcloned into pbLueScript (Stratagene, La Jolla, CA) and sequenced in both directions complemented by Southern analysis using the O/F DNA; two exons spanning ~1.4 kb of the genomic sequence were contained in this 9-kb fragment. The O/F DNA was subjected to Fluorescence in situ hybridization (Incyte Genomics, St. Louis, MO), which showed the location of this gene at a position within 15% of the chromosone 19, an area that corresponds to band 19B. A total of 80 metaphase cells were analyzed, with 73 exhibiting specific labeling.

**Generation of the Munc18c targeting vector.** The Munc18c targeting vector, pMunc18c-KO, was constructed using the positive-negative selection vector, pOSUDUFDEL (Gene Targeting, University of Iowa, Iowa City, IA). The 5′ homologous region in the targeting vector was a 4-kb NotI fragment containing all intronic DNA upstream of the exon that includes the start codon, taken from the pbLueScript 94-kb clone using a NotI site from the vector just 20 bp up-stream of the 5′ BamHI site of the 9-kb fragment. This fragment was inserted into the NotI−SalI cloning site upstream of the neomycin gene (neomycin cassette is in reverse orientation) in the pOSUDUFDEL vector. The 3′ homologous region in the targeting vector was generated by PCR amplification of the 568-bp intron separating the two exons in the 9-kb fragment of the sequenced gene, engineered with XhoI−XbaI ends and ligated into the XhoI−XbaI sites of pOSUDUFDEL just downstream of the neomycin cassette. The entire DNA region spanning the 5′ homologous region, the neomycin cassette, and the 3′ homologous region was sequenced for verification. The vector also contained a thymidine kinase cassette distal to the 5′ homologous region. The targeted gene therefore lacked a 1.34-kb region that included the 100-bp exon containing the start codon and the 1 kb of intronic DNA immediately proximal to the exon.

**Generation of the Munc18c knockout mice.** The targeting construct was linearized with NotI and introduced into 2 × 10⁹ pluripotent embryonic stem cells (RI clone) by electroporation (Gene Pulser; Bio-Rad). Embryonic stem clones that were G418- and ganciclovir-resistant were isolated, amplified, and screened for targeting fidelity by Southern blot analysis. Two targeted clones were selected from the 253 that were analyzed, and both were subsequently reconfirmed by Southern blot analysis by hybridizing the Hinfl-digested embryonic stem DNA with the O/F probe. Cells from the two targeted clones (AB46 and AB49) were microinjected into donor C57BL/6J blastocysts and implanted into pseudopregnant recipients. Chimeric animals resulting from
the microinjections were bred to C57BL/6J mice, and agouti pups were screened from germline transmission of the mutant allele. The genotypes from these matings and all subsequent matings were determined by PCR on DNA from tail-biopsy specimens using primers that annealed to the 5′ and 3′ flanking DNA of the neomycin substitution site (AA: 5′-CTTGGTGTCTGGTTGCTTTCC; AH: 5′-GCTCATGTGAGACCAAAAG), whereby the PCR product of the Wt (C57BL/6J) and that of the targeted allele was 1,500 bp.

An additional two PCR screens were performed to verify the orientation of the insertion site of the neomycin cassette. Founder animals were bred to C57BL/6J stock and offspring carrying the targeted allele were outbred for an additional five generations to enrich the C57BL/6J background at the University of Iowa College of Medicine animal care unit, and carried through an additional two generations upon arrival at the Indiana University School of Medicine laboratory animal resources center, all according to animal care guidelines.

**Tissue sampling and immunoblotting.** Tissues were homogenized in a 1% IgEal detergent buffer (25 mmol/l Tris [pH 7.4], 1% IgEal, 10% glycerol, 50 mmol/l sodium fluoride, 10 mmol/l sodium pyrophosphate, 137 mmol/l sodium chloride, 1 mmol/l sodium vanadate, 1 mmol/l phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 5 μg/ml leupeptin) for 15 s then centrifuged at 2,000 g for 5 min. The resulting supernatants were microcentrifuged at 13,500 g for 20 min at 4°C. Proteins were separated on 10 or 12% SDS-PAGE and then transferred to polyvinylidine fluoride or nitrocellulose membranes.

**Diet composition and feeding.** Mice (age 3 months) were individually housed for the 10-week high-fat (HF) diet study. The study was conducted in three separate modules with two or more mice per feeding group (WT-HF, Munc18c<sup>+/−</sup>-HF, WT−normal diet [ND], or Munc18c<sup>−/−</sup>-ND). Each module was conducted at a different time over the span of 9 months in an effort to obtain randomized results from independent litters of mice. Body weight and food intake measurements were taken daily along with the replenishment of fresh food. The proximate profile of the HF diet (cat. #FS282; Bio-Serv, Frenchtown, NJ) was 20% protein, 35.5% fat, 32.4% carbohydrate, 3.7% ash, 0.1% fiber, and <1% moisture. The diet’s fatty acid composition was a mix of 16 g oleic, 89 g palmitic, 44 g stearic, 35 g linoleic, 11 g iodin-cis-hexadecanoic, 5 g myristic, and 4.7 g cis-11-eicosanoic, with all fatty acids at 1.5 g/kg or less. The caloric profile of the HF diet was 16% protein, 50% fat, and 25% carbohydrate with 5.286 kcal/g energy. The caloric profile of the ND (Teklad cat. # 7,017; Harlan, Indianapolis, IN) was 22% protein, 11% fat, and 60% carbohydrate with 3.41 kcal/g metabolizable energy.

**Intraperitoneal glucose tolerance test and insulin tolerance test.** Male Munc18c KO and WT mice (age 4–6 months) were fasted for 18 h before the intraperitoneal glucose tolerance test (IPGTT). After a sample of fasted blood was collected, animals were given glucose (2 g/kg body wt) by intraperitoneal injection; blood glucose readings were then taken at 30-min intervals over 120 min with a glucometer (Beckman Instruments, Fullerton, CA). Hindlimb skeletal muscle was microinjected with 1,300 μl 10 mmol/l HEPES [pH 7.4], 250 μmol/l sucrose, 1 mmol/l EDTA, 5 mmol/l benzamidine, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mmol/l PMSF for 5 min at 4°C, and the supernatant was then centrifuged at 9,000 g for 2 h. Pellets were resuspended and combined with sample buffer for separation by 10% SDS-PAGE and subsequent immunoblotting for GLUT4.

**Isolation, culture, and stimulation of insulin secretion from mouse islets.** Pancreatic mouse islets were isolated as previously described (34). Briefly, islets were separated from male mice (age 8–12 weeks) were digested with collagenase and purified using a Ficoll density gradient. After being isolated, islets were cultured overnight in CMRL-1066 medium. Fresh islets were hand-picked into groups of 10, preincubated in Krebs-Ringer bicarbonate buffer (10 mmol/l HEPES [pH 7.4], 134 mmol/l NaCl, 5 mmol/l NaHCO<sub>3</sub>, 4.8 mmol/l KCl, 1 mmol/l CaCl<sub>2</sub>, 1.2 mmol/l MgSO<sub>4</sub>, and 1.2 mmol/l KH<sub>2</sub>PO<sub>4</sub>) containing 2.8 mmol/l glucose and 0.1% BSA for 2 h, then stimulated with 20 mmol/l glucose for 2 h. Medium was collected to measure insulin secretion and islets were harvested in NP-40 lysis buffer to determine cellular insulin content by radioimmunoassay.

**RESULTS**

**Generation of the Munc18c knockout mice.** To determine the importance of Munc18c protein on glucose homeostasis in vivo, we cloned a 9-kb piece of the murine Munc18c gene that contained the start AUG codon and constructed a targeting vector that replaced 1.1 kb of the Munc18c genomic sequence. The ATG-coding exon was removed and replaced with a neomycin resistance cassette oriented in the opposite orientation of the endogenous Munc18c gene (Fig. 1A). Of the 253 neomycin-resistant embryonic stem cell clones analyzed, two demonstrated the correct recombination as determined by PCR screening and Southern blot analysis (Fig. 1B and C). Both clones (AB46 and AB49) were used to generate chimeric founder mice. Targeted embryonic stem cell clones and DNA from F1 generation heterozygous mice tail snips exhibited the appearance of the 1,500-bp band in addition to the 1,300-bp endogenous Munc18c band, indicating the addition of the neomycin cassette insertion into the targeted allele (Fig. 1B). Southern blotting using an exon probe confirmed the presence of the 3.3-kb band of the targeted allele in addition to the endogenous 2.9-kb band of the Munc18c gene (Fig. 1C). Real-time PCR was used to further confirm the reduction in Munc18c mRNA in the Munc18c heterozygous KO mice (data not shown). Heterozygotic offspring of the F1 generation were crossed in an effort to obtain homozygotic offspring; of >150 offspring, none were homozygotic, suggesting that homozygotic disruption of the Munc18c gene resulted in embryonic lethality. Subsequent analysis of embryonic development revealed that the Munc18c<sup>−/−</sup> embryos died before embryonic day 7.5, and that by embryonic day 9.5, ~30% of the embryos were WT and 70% were heterozygotic. Subsequent studies were performed using the AB49 line of Munc18c heterozygous KO mice, enriched for the C57BL/6J strain background by more than seven generations of outbreeding into the C57BL/6J strain.

The underexpression of Munc18c protein in the heterozygous<sup>−/−</sup> KO mice was directly compared with endogenous levels in WT littermate mice in heart, skeletal muscle (hindlimb), liver, fat (epididymal), and pancreas by immunoblot analysis (Fig. 2A). Other tissues such as kidney, lung, brain, and spleen also showed reduced expression of Munc18c protein (data not shown). Quantitation by optical density scanning analyses of immunoblots showed Munc18c to be reduced in tissues by 50 ± 9% in the Munc18c heterozygous<sup>−/−</sup> KO mice. In contrast, no differences in abundance of the other SNARE proteins—syntaxin 4, SNAP-23, or VAMP2—were detected in any of the tissue homogenates. No changes in the abundance of the GLUT4 protein were detected in insulin-responsive tissues that displayed characteristic tissue-specific expression such as the heart, skeletal muscle, and fat, but not the liver (Fig. 2B). Similarly, there were no alterations in the abundance of the GLUT4 vesicle cargo protein insulin-responsive aminopeptidase (data not shown).

**Impaired insulin sensitivity and skeletal muscle GLUT4 translocation in the Munc18c knockout mice.** To investigate if the reduction in Munc18c protein, particularly in skeletal muscle and adipose tissues, would reduce the insulin sensitivity of the Munc18c<sup>−/−</sup> mice, we subjected Munc18c<sup>−/−</sup> and WT mice to an ITT. Mice were fasted for 6 h and fasting glucose levels were determined before
insulin for the ITT was injected (Fig. 3). Munc18c\(^{-/-}\) mice had elevated levels of blood glucose at all time points after the insulin injection compared with WT mice, and calculation of the area under the curve of the ITT data revealed the reduction in insulin responsiveness of the Munc18c\(^{-/-}\) mice to be statistically different from that of the WT mice \((P < 0.05)\). These data demonstrate that the Munc18c\(^{-/-}\) mice had significantly impaired insulin sensitivity.

FIG. 1. Generation of heterozygous Munc18c knockout mice by homologous recombination in embryonic stem (ES) cells. A: Schematic representation of the 9-kb region encompassing the start codon of the murine Munc18c gene; two exons are shown in numbered, filled boxes, the first encoding the AUG. The 4-kb Not I–Xho I fragment (5’ homology) and the 568-bp Xho I–Xba I fragment (3’ homology) that allowed homologous recombination with the genomic locus are indicated as flanking the neomycin resistance gene, which replaced the AUG-coding exon “1.” The relative position of the diagnostic probe is indicated, and the 5’ and 3’ primers used in the diagnostic PCR are depicted for the targeted allele. B: PCR analysis of ES and mouse tail DNA using the 5’ and 3’ primers flanking the neomycin insertion. C: Southern blot analysis of ES cell DNA using the diagnostic probe. The WT and targeted alleles generated 2.0- and 3.3-kb HindIII fragments, respectively.

FIG. 2. Protein expression in Munc18c\(^{-/-}\) knockout mice. Heart, hindlimb skeletal muscle (Musc), liver, lung, spleen, epididymal adipose (Fat), kidney (Kid), and pancreas (Panc) tissues were isolated from Munc18c heterozygous (\(-/-\)) and WT littermate (\(+/-\)) mice and homogenized as described in RESEARCH DESIGN AND METHODS. Proteins were resolved by SDS-PAGE and immunoblotted with antibodies for Munc18c, syntaxin 4 (Syn 4), SNAP-23, or VAMP2 (A) or GLUT4 (B). Data are representative of at least six independent sets of tissues.

FIG. 3. Impaired insulin sensitivity in Munc18c\(^{-/-}\) knockout mice. Insulin tolerance testing of Munc18c\(^{-/-}\) \((n = 8)\) and WT male mice \((n = 8)\) was performed by intraperitoneal injection of insulin (0.75 units/kg body wt) into male mice (age 4–6 months) fasted for 6 h. Blood glucose was monitored before and 15, 30, 60, and 90 min after injection, as described in RESEARCH DESIGN AND METHODS. Data are the mean percent ± SE of basal blood glucose concentration. Area under the curve analysis showed overall increased blood glucose levels in Munc18c\(^{-/-}\) mice. \(*P < 0.05\) vs. WT mice by unpaired Student’s \(t\) test.
The mechanism for glucose uptake into peripheral tissues involves initiation of the insulin signaling cascade, which leads to the translocation of GLUT4-containing vesicles to the cell surface membranes. We have previously shown that underexpression of syntaxin 4 and Munc18c together in skeletal muscle of syntaxin 4/H11002 KO mice results in inhibition of insulin-stimulated GLUT4 translocation (38), as assessed by skeletal muscle homogenization coupled with sucrose velocity sedimentation to separate sarcolemma/transverse tubule membrane fractions (P1 and P2) and sucrose velocity sedimentation fractions (10 fractions). Proteins were resolved using SDS-PAGE for immunoblotting for GLUT4 (A), or syntaxin 4 (Syn4) and TnFR (B). C: GLUT4-specific P2 fractions on immunoblots from three independent experiments were quantitated using optical density scanning, and GLUT4 translocation into the plasma membrane fraction was normalized to WT = 1. P < 0.05 vs. WT mice. D: Whole hindquarter tissue homogenates were prepared from mice stimulated with or without insulin and proteins separated as described for Fig. 2 for analysis of insulin signaling by immunoblotting for activation of Akt (phosphorylation of Akt at Ser 473). Total Akt was used as a loading control.

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FIG. 4. Insulin-stimulated GLUT4 translocation is impaired in skeletal muscle from Munc18c heterozygous knockout mice. Mice were fasted for 16 h and left untreated or injected with 21 units/kg body wt insulin, as described in RESEARCH DESIGN AND METHODS. Hindquarter muscles were homogenized and centrifuged to partition muscle into sarcolemma/transverse tubule membrane fractions (P1 and P2) and sucrose velocity sedimentation fractions (10 fractions). Proteins were resolved using SDS-PAGE for immunoblotting for GLUT4 (A), or syntaxin 4 (Syn4) and TnFR (B). C: GLUT4-specific P2 fractions on immunoblots from three independent experiments were quantitated using optical density scanning, and GLUT4 translocation into the plasma membrane fraction was normalized to WT = 1. P < 0.05 vs. WT mice. D: Whole hindquarter tissue homogenates were prepared from mice stimulated with or without insulin and proteins separated as described for Fig. 2 for analysis of insulin signaling by immunoblotting for activation of Akt (phosphorylation of Akt at Ser 473). Total Akt was used as a loading control.

FIG. 5. Insulin secretion is impaired in islets from Munc18c heterozygous knockout mice. Islets were isolated as described in RESEARCH DESIGN AND METHODS and placed in culture for 15 h at 37°C in CMRL medium. Islet cells were preincubated for 2 h in low-glucose Krebs-Ringer bicarbonate buffer (2.8 mmol/l) then incubated for 2 h under basal (2.8 mmol/l glucose) or stimulated (20 mmol/l glucose) conditions. A: Insulin secretion was measured by radioimmunomassay, and data were subsequently normalized to glucose-stimulated WT = 100%. Data are the average ± SE of three independent experiments, each performed in triplicate. P < 0.01 for Munc18c^{−/−} vs. WT. B: Average insulin content per 10 islets from WT or Munc18c^{−/−} male mice left untreated or stimulated with glucose.
the enrichment of the plasma membrane protein syntaxin 4 and the endosome-localized transferrin receptor (TnFR) was examined (Fig. 4B). Syntaxin 4 was found to be enriched in the P1 and P2 fractions, with little to no detection in the sucrose fractions, as has been previously described (33,39). TnFR was enriched in early sucrose fractions, distinct from the pattern of the GLUT4-containing vesicles, a finding that is consistent with the original publication of this fractionation method (37). Optical density quantitation of GLUT4 immunoblots confirmed that the skeletal muscle of Munc18c−/− KO mice translocated significantly less GLUT4 to the P2 fraction in response to insulin compared with skeletal muscle of WT mice (Fig. 4C). In contrast, proximal insulin signaling was unaffected, as Munc18c−/− and WT mice injected with insulin showed equivalently increased levels of Ser 473 phosphorylation and expression of protein kinase B/Akt in skeletal muscle tissue homogenates (Fig. 4D). Taken together, these data demonstrate that the Munc18c−/− mice had significantly impaired skeletal muscle insulin-stimulated GLUT4 translocation, independent of effects on proximal insulin-signaling events.

**Impaired insulin secretion in islets isolated from Munc18c knockout mice.** To investigate the effects of the reduced expression of Munc18c on insulin secretion, glucose-stimulated insulin secretion was quantitated from islets isolated from Munc18c−/− and WT mice. Glucose stimulation (20 mmol/l) resulted in a 25-fold increase in insulin release compared with unstimulated islets of WT mice, whereas islets isolated from Munc18c−/− mice showed <40% of this induction (Fig. 5A). No significant alterations of total insulin content of WT or Munc18c−/− islets incubated with or without glucose were detected (Fig. 5B). These data indicate that the islets of the Munc18c−/− mice had significantly impaired responsiveness to glucose.

**Munc18c knockout mice develop severe glucose intolerance with high-fat feeding.** To determine if the reduction in Munc18c protein resulted in aberrant whole-body glucose tolerance, WT and Munc18c−/− mice were
subjected to an IPGTT. Glucose tolerance was monitored in animals that were fasted overnight and subjected to an intraperitoneal injection of glucose (2 g/kg); blood glucose levels were monitored over a 2-h period (Fig. 6). We also tested glucose tolerance in male Munc18c<sup>−/+</sup> and WT littermate male mice (age 3 months) that had been fed the HF diet to exacerbate potential aberrations in glucose tolerance, as high fat intake negatively impacts mechanisms involved in insulin action and secretion. Three separate feeding trials were conducted over a 9-month period for randomization, and glucose tolerance testing was performed after 5 or 10 weeks on the diet. After 5 weeks on the diet, fasting glucose levels were similar in all four groups of mice, as were peak blood glucose levels 30 min after glucose injection (Fig. 6A). In mice fed the normal diet (ND), peak blood glucose was reached 30 min after injection in the WT mice and was cleared over the remaining 90 min. Similarly, the Munc18c<sup>−/+</sup> mice fed the ND demonstrated the ability to clear glucose to the same level as WT mice throughout the experiment. Both WT and Munc18c<sup>−/+</sup> mice had increased serum levels of insulin within 30 min after glucose injection, indicating responsiveness to the glucose injection (data not shown). However, 2 h after the glucose injection, the glucose levels of the Munc18c<sup>−/+</sup> mice fed the HF diet remained significantly higher than those of WT mice on the same diet, showing essentially no clearance of glucose (Fig. 6A). This glucose intolerance worsened over time as these Munc18c heterozygous mice displayed an even greater level of glucose intolerance after an additional 5 weeks on the HF diet, with significantly elevated blood glucose throughout the clearance period compared with the WT mice on the same diet. Serum insulin levels were not determined for the HF-fed mice due to insufficient quantities of serum that could be collected from the tails of these mice during the IPGTT. These data indicate that the Munc18c<sup>−/+</sup> mice could clear glucose normally when fed a balanced diet, but were unable to compensate as well as the WT mice when challenged with the HF diet.

Daily food intake monitoring showed that mice fed the HF diet consumed slightly less food than those fed the ND, although there were no differences in food consumption between WT and Munc18c<sup>−/+</sup> mice fed the HF diet (Fig. 6B). Furthermore, no significant differences in overall body weight were detected between WT and Munc18c heterozygous mice, although those HF-fed mice weighed significantly more than mice fed the ND after 8 weeks of feeding (Fig. 6C). Tissue weights did not differ between Munc18c heterozygous KO and WT mice fed the ND (Table 1), with liver accounting for ~5%, fat for 2%, kidney and hindlimb skeletal muscle for 1.5%, and heart, lung, and spleen for <1% of total body weight. Munc18c heterozygous KO mice fed the HF showed parallel changes in tissue weight compared with HF-fed WT mice, with epididymal fat weight significantly increased to 7% of total body weight and other significant increases in the weight of kidneys and skeletal muscle due to infiltration of fat in the tissue of the HF-fed mice. These data indicate that the Munc18c<sup>−/+</sup> mice had an increased susceptibility to HF diet-induced glucose intolerance, whereas food consumption, body weight gain, and tissue weight redistribution did not differ from that of WT mice.

### Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Normal diet (WT)</th>
<th>Normal diet (Munc18c&lt;sup&gt;−/+&lt;/sup&gt;)</th>
<th>High-fat diet (WT)</th>
<th>High-fat diet (Munc18c&lt;sup&gt;−/+&lt;/sup&gt;)</th>
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<tr>
<td>Body weight (g)</td>
<td>26 ± 1.8</td>
<td>26 ± 0.9</td>
<td>38 ± 2.0</td>
<td>38 ± 1.8*</td>
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<tr>
<td>Heart</td>
<td>0.19 ± 0.02</td>
<td>0.18 ± 0.02</td>
<td>0.22 ± 0.02</td>
<td>0.21 ± 0.01</td>
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<tr>
<td>Lung</td>
<td>0.20 ± 0.01</td>
<td>0.22 ± 0.02</td>
<td>0.22 ± 0.01</td>
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<tr>
<td>Liver</td>
<td>1.32 ± 0.15</td>
<td>1.23 ± 0.07</td>
<td>1.25 ± 0.12</td>
<td>1.29 ± 0.03</td>
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<tr>
<td>Kidney</td>
<td>0.39 ± 0.04</td>
<td>0.40 ± 0.02</td>
<td>0.57 ± 0.02</td>
<td>0.54 ± 0.05*</td>
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<tr>
<td>Spleen</td>
<td>0.10 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>0.12 ± 0.01</td>
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<tr>
<td>Fat (epididymal)</td>
<td>0.57 ± 0.11</td>
<td>0.58 ± 0.07</td>
<td>2.56 ± 0.15</td>
<td>2.65 ± 0.31*</td>
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<tr>
<td>Skeletal muscle</td>
<td>0.31 ± 0.01</td>
<td>0.34 ± 0.02</td>
<td>0.41 ± 0.03</td>
<td>0.43 ± 0.03*</td>
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Data are means ± SE. Weights were collected from wild-type or Munc18c heterozygous knockout (−/+), male mice at the end of the 10-week feeding study for determination of the parameters shown. *P < 0.05 vs. Munc18c heterozygous knockout mice fed normal diet, as determined by unpaired Student’s t test.

### Table 2

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<tr>
<th>Glucose (fasted; mg/dl)</th>
<th>Normal diet (WT)</th>
<th>Normal diet (Munc18c&lt;sup&gt;−/+&lt;/sup&gt;)</th>
<th>High-fat diet (WT)</th>
<th>High-fat diet (Munc18c&lt;sup&gt;−/+&lt;/sup&gt;)</th>
</tr>
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<tbody>
<tr>
<td>Fasted</td>
<td>166 ± 6</td>
<td>161 ± 6</td>
<td>172 ± 4</td>
<td>184 ± 10*</td>
</tr>
<tr>
<td>Fed</td>
<td>1.10 ± 0.42</td>
<td>1.10 ± 0.56</td>
<td>2.27 ± 0.43</td>
<td>3.07 ± 0.46</td>
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</table>

Data are means ± SE. Serum was collected from fasted or fed wild-type or Munc18c heterozygous knockout (−/+), male mice at the end of the 10-week feeding study for determination of the metabolic parameters shown. *P < 0.05 vs. Munc18c heterozygous knockout mice fed normal diet, as determined by unpaired Student’s t test.
differences were seen between WT and Munc18c mice showed slight increases in NEFA levels, but no alterations in glucose homeostasis were no longer sufficient with the addition of Munc18c heterozygous knockout (−/+) mice. Our data clearly show that Munc18c−/− mice may have altered hepatic insulin clearance. Future studies are required to fully comprehend the contributions of the liver to the overall metabolic profile of the Munc18c−/− mice.

It was interesting to observe that the administration of the HF diet uncovered the latent susceptibility of the Munc18c−/− mice to severe glucose intolerance. This may have occurred due to inhibition of glucose transport by free fatty acids and/or hyperglycemia (42). It has also been shown that chronic hyperglycemia induces alterations of the insulin-regulated trafficking of Munc18c in 3T3L1 adipocytes (43). In addition, we recently reported that increased glucosamine leads to O-linked glycosylation of Akt and glycogen synthase kinase-3β (45). Thus, a high-fat/high-calorie diet may alter the glycansylation state or cellular locale of existing Munc18c protein and result in inhibition of GLUT4 translocation by disruption of the VAMP2–syntaxin 4 interaction.

The induction of severe glucose intolerance may also be linked to the inherent deficiencies in insulin-stimulated GLUT4 translocation in skeletal muscle, in addition to reduced glucose-stimulated insulin secretion in pancreatic islets. Although capable of seemingly normal glucose disposal when fed the ideal standard diet, the Munc18c−/− mice became severely glucose intolerant and incapable of glucose clearance after just 5 weeks on the HF diet, which may suggest that compensatory mechanisms used to maintain glucose homeostasis were no longer sufficient with the added stress of the HF diet. These data indicate that Munc18c is an important positive regulator in vivo in multiple cell types critical for insulin secretion and insulin action.

The loss of insulin-stimulated GLUT4 translocation from hindlimb skeletal muscle of Munc18c−/− mice was consistent with the detection of impaired insulin sensitivity as determined by the ITT, although it is unclear as to why the Munc18c−/− mice failed to exhibit a detectable defect in glucose tolerance as measured by the IPGTT under standard conditions. Only with the added perturbation of high-fat feeding did the underlying defects in insulin action and insulin secretion become apparent in the IPGTT assay. Possible explanations for the inconsistency might be 1) Munc18c−/− mice retain expression of 50% of Munc18c protein, which may be sufficient for function in skeletal muscle depots with different cell/fiber types therein (33); 2) the mice experience compensation from GLUT4-mediated glucose uptake, which is insensitive to Munc18c (32); 3) there was a contribution by adipose tissue glucose uptake, as adipocytes in vivo appear to be less sensitive to alterations in protein levels of Munc18c and syntaxin 4 complexes than skeletal muscle in vivo or 3T3L1 adipocytes in culture (34,38,39); or 4) there was enhanced glucose uptake by the liver, as has been demonstrated in the muscle-specific GLUT4 KO mice (40). Taken together with the fact that mice lacking GLUT4 altogether still show insulin-stimulated glucose uptake into soleus muscle (41), our findings strongly suggest that there may be variations in GLUT4-dependent and even GLUT4-independent activation of muscle glucose uptake that might explain the glucose tolerance of the Munc18c−/− mice. Furthermore, alterations in glucose homeostasis clearly detected by hyperinsulinemic-euglycemic clamp analysis have been otherwise missed by the relatively insensitive IPGTT assay (39); future studies of these mice should include this more sensitive analysis as well as ex vivo tissue analyses.

The incongruity of the data showing the defect in glucose-stimulated insulin secretion of the Munc18c−/− islets with the otherwise normal levels of circulating insulin detected during the IPGTT may also be an indication of altered hepatic function in the Munc18c−/− mice. Because circulating insulin reflects not only insulin secretion but also insulin clearance, these data suggest that the Munc18c−/− mice may have altered hepatic insulin clearance. Future studies are required to fully comprehend the contributions of the liver to the overall metabolic profile of the Munc18c−/− mice.


discussion
In this study, we documented the generation and characterization of Munc18c−/− mice. Our data clearly show that Munc18c−/− mice had an ~50% loss of Munc18c protein and increased susceptibility toward developing severe glucose intolerance. Consistent with this finding, the Munc18c−/− mice showed significantly impaired insulin sensitivity and inherent deficiencies in insulin-stimulated GLUT4 translocation in skeletal muscle, in addition to reduced glucose-stimulated insulin secretion in pancreatic islets. Although capable of seemingly normal glucose disposal when fed the ideal standard diet, the Munc18c−/− mice became severely glucose intolerant and incapable of glucose clearance after just 5 weeks on the HF diet, which may suggest that compensatory mechanisms used to maintain glucose homeostasis were no longer sufficient with the added stress of the HF diet. These data indicate that Munc18c is an important positive regulator in vivo in multiple cell types critical for insulin secretion and insulin action.

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Two of the parameters used to clinically identify the metabolic syndrome are elevated triglyceride levels and impaired fasting glucose. After 10 weeks on the HF diet, the Munc18c−/− mice had significantly increased fasting blood glucose compared with mice on the ND (Table 2) as well as increased fed serum insulin levels (P = 0.06). However, fasting levels of serum triglycerides did not differ between WT and Munc18c−/− mice under standard conditions, nor did levels of serum cholesterol and nonesterified fatty acids (NEFAs) (Table 3). After 10 weeks on the HF diet, both WT and Munc18c−/− mice showed elevated fasting serum cholesterol (174 ± 12) and triglyceride (128 ± 7) levels, with no change in NEFAs. Female mice showed slight increases in NEFA levels, but no differences were seen between WT and Munc18c−/− mice (1.5 ± 0.2 for both). Thus the Munc18c−/− mice had increased susceptibility for developing hyperglycemia without significant aberrations in serum triglycerides as compared with WT mice.

**Table 3**

<table>
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<tr>
<th>Metabolic characteristics of wild-type and Munc18c heterozygous (−/+ ) knockout mice</th>
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<td></td>
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<tr>
<td>Wild-type</td>
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<tr>
<td>Munc18c−/−</td>
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</table>

Data are means ± SE. Serum was collected from wild-type (n = 7) or Munc18c heterozygous knockout (−/+) (n = 4) fasted male mice (age 4–6 months) for determination of the metabolic parameters shown.
the HF diet exerted effects in the current study may be explained by the inhibition of glucose-stimulated insulin granule exocytosis by lipotoxicity and β-cell apoptosis (49,50). However, our WT and Munc18c<sup>−/−</sup> mice fed the HF diet had insulin contents similar to those of the ND-fed mice. Alternatively, consumption of the HF diet may have induced loss of first-phase insulin secretion, as has been previously described (51). Further experiments will be required to establish the role of Munc18c protein in biphasic insulin secretion.

In sum, these data support a model whereby hyperglycemic and hypertriglyceridemic conditions exacerbate the defect in the fusion machinery of the Munc18c<sup>−/−</sup> mice required to facilitate GLUT4 translocation and insulin granule exocytosis, further weakening the glucose homeostatic mechanisms. This is the first report of the importance of Munc18c protein in insulin secretion, a finding that will now warrant further investigation.

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E.O. contributed data for all tables and figures and managed the project. B.A.S. contributed to the collection of data for Figs. 3, 4, 5, and 6A and Tables 2 and 3. D.C.T. isolated the murine Munc18c gene, constructed the targeting vector and contributed to the generation of all the Munc18c heterozygous knockout mice at the University of Iowa in the laboratory of J.E.P. Data in all tables and figures except Fig. 1 were collected at Indiana University School of Medicine in the laboratory of D.C.T.

REFERENCES


