Skeletal muscle includes many individual fibers with diverse phenotypes. A barrier to understanding muscle glucose uptake at the cellular level has been the absence of a method to measure glucose uptake by single fibers from mammalian skeletal muscle. This study's primary objective was to develop a procedure to measure glucose uptake by single fibers from rat skeletal muscle. Rat epitrochlearis muscles were incubated ex vivo with [3H]-2-deoxy-D-glucose, with or without insulin or AICAR, before isolation of ~10–30 single fibers from each muscle. Fiber type (myosin heavy chain [MHC] isoform) and glucose uptake were determined for each single fiber. Insulin-stimulated glucose uptake (which was cytochalasin B inhibitable) varied according to MHC isoform expression, with −2-fold greater values for IIA versus IIB or IIX fibers and −1.3-fold greater for hybrid (IIB/X) versus IIB fibers. In contrast, AICAR-stimulated glucose uptake was −1.5-fold greater for IIB versus IIA fibers. A secondary objective was to assess insulin resistance of single fibers from obese versus lean Zucker rats. Genotype differences were observed for insulin-stimulated glucose uptake and inhibitor kβ (IκB)-β abundance in single fibers (obese less than lean), with decrements for glucose uptake (44–58%) and IκB-β (25–32%) in each fiber type. This novel method creates a unique opportunity for future research aimed at understanding muscle glucose uptake at the cellular level. Diabetes 61:995–1003, 2012

RESEARCH DESIGN AND METHODS

Materials. Human recombinant insulin was from Eli Lilly (Indianapolis, IN). AICAR and cytochalasin B (CB) were from Calbiochem/EMD Chemicals (Gibbstown, NJ). Reagents for SDS-PAGE and immunoblotting were from Bio-Rad (Hercules, CA). Bicinchoninic acid protein assay reagent and tissue protein extraction reagent (T-PER) were from Pierce Biotechnology (Rockford, IL). [3H]-2-Deoxy-D-glucose ([3H]-2-DG) was from PerkinElmer Life and Analytical Sciences (Waltham, MA). Collagenase (type II) was from Worthington Biochem (Lakewood, NJ). Trypan Blue was from Invitrogen (Carlsbad, CA). Other reagents were from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Reagents and apparatus for SDS-PAGE and immunoblotting were purchased from Bio-Rad. West Dura Extended Duration Substrate was from Pierce Biotechnology. Anti-APPL1 (adaptor protein containing pH domain, PTB domain, and leucine zipper motif) antibody (no. ab69592) was from Abcam (Cambridge, MA), anti-inhibitor kβ (IκB)-β antibody (no. sc-945) was from Santa Cruz (Santa Cruz, CA), and anti-rabbit IgG horseradish peroxidase (no. 7074) was from Cell Signaling Technology (Danvers, MA).

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Animal treatment. Procedures for animal care were approved by the University of Michigan Committee on Use and Care of Animals. Male Wistar rats (aged 7–10 weeks) were from Harlan (Indianapolis, IN), Lean (Fa/fa) and obese (fa/fa) male Zucker rats (aged 7–8 weeks) were from Charles River Laboratories (Wilmington, MA). Animals were provided with rodent chow ad libitum until 1700 h the night before the experiment when food was removed. The next day, between 1000 and 1300 h, rats were anesthetized (intraperitoneal injection of sodium pentobarbital), and both epimysial muscles were extracted.

Muscle incubation. Isolated muscles underwent a series of incubation steps (see below). Unless otherwise noted, vials were shaken (45 rpm), gassed (95% O2:5% CO2) for 10 min, and then incubated in a water bath at 35°C.

Standard incubation protocol. Isolated muscles were incubated in vials containing 2 mL media 1 (Kreb's Henseleit buffer [KHB] supplemented with 0.1% bovine serum albumin [BSA] [KHB-BSA], 2 mM L-sodium pyruvate, and 6 mM L-mannitol) with or without 12 mM L-insulin for 20 min. Each muscle was then transferred to a second vial for a 60-min incubation in 2 mL media 2 (KHB-BSA, 1 mM L-LYS-DG [specific activity of 2.25 mCi/mmol [3H]-2-DG], and 9 mM L-mannitol [specific activity of 0.62 mCi/mmol [3H]-2-DG]) and the same insulin concentration as the previous step. Muscles underwent 3× 5-min washes (100% washes in ice-cold KHB-BSA to clear extracellular space of 2-DG (22). Muscles were placed in collagenase media (Ca2+-free KHB and 1.5× type II collagenase) for 60 min for enzymatic digestion of collagen (enzymatically digested muscles are hereafter referred to as fiber bundles).

Some muscles were incubated with CB (25 mM in 0.4% DMSO). One muscle per rat was incubated with CB for sequential incubations in media 1 and media 3. The contralateral muscle served as a control (vehicle, 0.4% DMSO). CB or vehicle replacement media (micromoles per microgram of protein for CB incubation) was added to separate vials containing 10 mL scintillation cocktail. [3H]-2-DG distribution counter. 3H dpm accumulation was calculated as

$$\text{correction factor} = \frac{\text{corrected total} \times \mu\text{mol} \text{2-DG per mL incubation media}}{\text{current concentration} \times \mu\text{mol} \text{L}}$$

Corrected total 3H dpm per fiber was calculated using a three-point MHC standard curve from soleus and extensor digitorum longus muscles lysate loaded on each gel. A similar amount of MHC protein for each fiber sample was loaded onto 9% SDS-PAGE. Following electrophoretic transfer to nitrocellulose, gels were stained with Coomassie Brilliant Blue, and posttransfer MHC bands were visualized by enhanced chemiluminescence and quantified by densitometry, as previously described (26). After immunoblotting the membranes, the immunoreactive protein bands (Ieβ and APPL1) were visualized by an enhanced chemiluminescence and quantified by densitometry, as previously described (27). Ieβ and APPL1 protein levels for each fiber were expressed relative to the fiber’s respective posttransfer MHC density.

Muscle and fiber-bundle homogenization. Intact muscles and fiber bundles for 2-DG uptake were processed in 1 mL ice-cold lysis buffer using glass-bead homogenizing tubes. Homogenates were removed (4°C, 1 h) before being centrifuged (12,000g, 10 min, 4°C). Aliquots of supernatant used for 2-DG uptake were pipetted into vials for scintillation counting, and 2-DG uptake was determined. A portion of supernatant was used to determine protein concentration (bicinchoninic acid protein assay). 2-DG uptake was normalized to either muscle weight (micromoles per gram for intact muscles) or protein content (micromoles per microgram of protein for fiber bundles). Remaining supernatant was stored at −80°C until further analysis.

Statistical analysis. Data are expressed as means ± SEM. Differences between two groups were determined by the Student t test. The Pearson correlation coefficient was used for correlation analysis. One-way ANOVA was used to evaluate the effect of fiber type on 2-DG uptake. Two-way ANOVA was used for the CB experiment and the Zucker rat experiment. The Tukey post hoc t test was applied to determine the source of significant variance. A P value of <0.05 was considered statistically significant.

RESULTS

2-DG values were similar for whole muscles using revised versus standard protocols. The standard incubation protocol for 2-DG uptake uses [14C]mannitol to calculate extracellular space, which is used to calculate extracellular 2-DG, which is subtracted from whole-muscle 2-DG to calculate intracellular 2-DG accumulation (24). To confirm that the standard versus revised protocols resulted in similar values for 2-DG uptake, paired muscles from Wistar rats were studied with both protocols. Both muscles from some rats were incubated with insulin, and both muscles from other rats were incubated without insulin. KHB was supplemented with [3H]-2-DG and [14C]mannitol for one muscle, whereas KHB was supplemented with [3H]-2-DG only for the contralateral muscle. The muscle incubated in [3H]-2-DG and [14C]mannitol and [3H]-2-DG was immediately freeze clamped following incubation, whereas the contralateral muscle (incubated in [3H]-2-DG only) underwent three rinses (5 min each) in ice-cold KHB. 2-DG uptake did not differ significantly between muscles incubated using the standard versus revised protocol (Fig. 1A). 2-DG values were significantly correlated (basal: R = 0.88, P < 0.05; insulin stimulated: R = 0.91, P < 0.05) between paired muscles undergoing the standard versus revised protocol (Fig. 1B). 2-DG uptake for muscles undergoing the standard protocol also can be expressed as nanomoles per microfiber: basal 1.86 ± 0.41 nmol/μL and insulin stimulated 5.89 ± 0.82 nmol/μL.

Incubation with collagenase did not alter 2-DG uptake. Paired muscles were dissected from Wistar rats. Both muscles from some rats were incubated with insulin, and both muscles from other rats were incubated without insulin. KHB was supplemented with [3H]-2-DG for the second incubation step. After three rinses to remove extracellular 2-DG, one muscle per rat was incubated with collagenase, and the other was incubated without collagenase. Collagenase did not alter 2-DG uptake (Fig. 1C).

Insulin-stimulated 2-DG uptake by single fibers was correlated with 2-DG for fiber bundles. 2-DG that enters muscle fibers is rapidly phosphorylated by hexokinase, whereas resultant 2-DG-6P is trapped intracellularly.
without further metabolization. After collagenase treatment, muscles from Wistar rats were incubated in Ca²⁺-free KHB with Trypan Blue. A bundle of several hundred fibers from each muscle used to harvest single fibers also was processed for 2-DG uptake without insulin or with 12 nmol/L insulin. 2-DG uptake also was determined from ~10–30 single fibers per bundle. Figure 1 illustrates the significant correlations (basal: \( R = 0.68, P < 0.05 \); insulin stimulated: \( R = 0.63, P < 0.05 \)) between mean 2-DG uptake by single fibers from each bundle versus 2-DG uptake by the donor bundle from which fibers were isolated.

**Differences in insulin-stimulated 2-DG uptake by single fibers.** Relative numbers of fibers (%) for each MHC isoform in single fibers isolated from Wistar rats were 9% IIA, 61% IIB, 8% IIX, and 22% IIB/X (see Fig. 2 for representative gel). These values compare with the relative abundance of MHC composition of whole epitrochlearis muscles from 7- to 10-week-old Wistar rats (8% I, 13% IIA, 51% IIB, and 28% IIX) (10). Figure 3 illustrates data from single fibers from Wistar rats used to measure both insulin-stimulated 2-DG uptake and MHC isoform expression. We found no significant fiber-type differences for basal 2-DG uptake. ANOVA revealed a significant difference \( (P < 0.05) \) for insulin-stimulated 2-DG uptake of IIA fibers versus fibers expressing each of the other MHC isoforms, and a significant difference between IIB/X versus IIB fibers (IIB/X greater than IIB).

**Insulin-stimulated 2-DG uptake by single fibers was inhibited by CB.** For 2-DG uptake by single fibers from muscles incubated without insulin, there were significant main effects \( (P < 0.05) \) of CB and fiber type. The only
statistically significant difference (P < 0.05) identified by post hoc analysis was in IIB/X fibers (no CB greater than with CB) (Fig. 3B). For 2-DG uptake by single fibers from insulin-stimulated muscles, there was a significant main effect of CB (P < 0.001). Post hoc analysis (P < 0.001) indicated that values without CB exceeded values with CB for each fiber type. For 2-DG uptake by single fibers from insulin-stimulated muscles, there was a significant main effect of fiber type (P < 0.001) and a significant interaction between CB status and fiber type (P < 0.001). Post hoc analysis indicated that for insulin-stimulated muscles incubated without CB, 2-DG values for IIA fibers were significantly (P < 0.05) greater than values for all other fiber types, and 2-DG values for IIB/X fibers were significantly (P < 0.05) greater than values for IIB fibers. For 2-DG uptake by single fibers from muscles incubated with insulin and CB, post hoc analysis detected no significant differences among fiber types.

**AICAR-stimulated 2-DG uptake by single fibers.** Figure 4 illustrates data from single fibers used to measure AICAR-stimulated 2-DG uptake and MHC isoform expression. There were no significant fiber-type differences for basal 2-DG uptake. ANOVA revealed a significant difference (P < 0.05) for AICAR-stimulated 2-DG uptake between fiber types. Post hoc analysis indicated a significant difference for IIB fibers versus IIA fibers (IIB greater than IIA).

**2-DG uptake by single fibers from epitrochlearis of OZ versus LZ rats.** Basal 2-DG uptake values by single fibers were not significantly different between OZ and LZ rats, regardless of MHC isoform expression (Fig. 5). For 2-DG uptake by single fibers from insulin-stimulated muscles, there was a significant main effect of genotype (P < 0.001), and post hoc analysis indicated that insulin-stimulated 2-DG values for each fiber type were significantly greater for LZ versus OZ rats (P < 0.05). For 2-DG uptake by single fibers from insulin-stimulated muscles, there also was a significant main effect of fiber type (P < 0.001) and a significant interaction (genotype × fiber type; P < 0.001). Post hoc analysis indicated that within the LZ fibers from insulin-stimulated muscles, 2-DG uptake for IIA fibers exceeded values for all other fiber types (P < 0.05). However, for single fibers from insulin-stimulated OZ muscles, post hoc analysis did not reveal significant differences among fiber types. Relative decrements for 2-DG uptake of insulin-stimulated OZ versus LZ muscles were estimated by calculating differences between the mean LZ value for each fiber type and individual OZ values for the same fiber type. Calculated reductions for insulin-stimulated 2-DG uptake for the fiber types (IIB: 44.4 ± 3.0%, IIA: 58.0 ± 10.0%, IIX: 52.0 ± 8.01%, and IIB/X: 52.3 ± 5.0%) did not differ significantly.

**IkB-β and APPL1 abundance in single fibers.** For IkB-β, we observed a significant main effect of genotype (P < 0.005; LZ greater than OZ), regardless of fiber type. No fiber-type difference for IkB-β was detected, and the calculated reductions in IkB-β content for LZ versus OZ were similar across fiber types (IIB: 24.8 ± 10.7%, IIA: 27.5 ± 7.1%, IIX: 30.0 ± 9.1%, and IIB/X: 31.9 ± 4.0%) (Fig. 6A). For APPL1, there was no significant genotype or fiber-type difference (Fig. 6B).

**DISCUSSION**

Skeletal muscle is a heterogeneous tissue primarily composed of muscle fibers that can have diverse phenotypes. However, there was previously no method to evaluate glucose uptake by single mammalian fibers. The current study filled this gap. It is noteworthy that the novel method includes identification of MHC isoform expression in each fiber used for the glucose uptake assay. The results indicated that insulin-stimulated glucose uptake varied among fibers according to fiber type, with twofold greater insulin-mediated glucose uptake for IIA versus IIB or IIX fibers. These data extend earlier research using tissue analyses in which rat muscles primarily composed of IIA fibers had approximately two- to threefold greater insulin-mediated glucose uptake than muscles mostly composed of IIB fibers (3). The current results also revealed that hybrid fibers, which expressed more than one MHC isoform (IIB/X), had higher insulin-induced glucose uptake than IIB fibers. These results are important because hybrid fibers can be abundant in rat muscle (28,29) and novel because conventional tissue analyses cannot assess the glucose uptake by hybrid fibers. In contrast to the results of insulin-stimulated glucose uptake, AICAR-stimulated glucose uptake was greater in IIB versus IIA fibers. These data coincide with results for whole epitrochlearis (predominantly IIB) that had greater glucose uptake than muscles mostly composed of IIB fibers (3). The relative magnitude of insulin resistance in OZ versus LZ rats was substantial (from 44 to 58% for fibers expressing each of the fiber types evaluated: IIA, IIB, IIX, and IIB/X). Furthermore, for OZ versus LZ rats IkB-β content also was reduced (25–32%) across each fiber type.

We performed a series of experiments to validate the procedure for assessing glucose uptake by single fibers. We demonstrated that glucose uptake was unaltered by collagenase treatment and documented that glucose uptake by single fibers was highly correlated with values for donor muscles from which single fibers were isolated. Using CB, a compound that inhibits glucose transporter protein-mediated glucose transport, we established that 2-DG accumulation by single fibers was attributable to transporter-specific glucose uptake.

The MHC isoform profile of single fibers isolated from Wistar rats (9% IIA, 61% IIB, 8% IIX, and 22% IIB/X) compared to the relative abundance of the MHC composition of whole epitrochlearis from Wistar rats (8% I, 13% IIA, 51% IIB, and 28% IIX) (10). It is uncertain why type II fibers were not included among the hundreds of fibers isolated using collagenase. It may relate to the greater collagen levels surrounding type I compared with type II fibers in rat muscle pathology.
largely composed of a particular MHC isoform. Therefore, tissue analysis cannot reveal the glucose uptake of hybrid fibers. 4) Tissue analysis includes the contribution of other cell types in skeletal muscle (vascular, neural, adipose, fibroblasts, etc.). 5) Various conditions alter fiber-type composition of muscles (32–36), confounding interpretation of between-group comparisons. The single-fiber procedure makes comparisons possible between fibers matched for MHC expression.

Full understanding of muscle insulin resistance will require elucidation of insulin resistance at the cellular level. Cultured myocytes provide valuable insights into the mechanisms for insulin resistance (37–39); however, they are imperfect models of fibers in adult muscle. To probe muscle insulin resistance, we compared glucose uptake in single fibers from LZ and OZ rats. Insulin-stimulated glucose uptake is reduced by 67% in isolated intact epitrochlearis (40). Using the perfused rat hind-limb procedure, Sherman
et al. (41) found similar relative deficits for OZ versus LZ rats in glucose uptake for a muscle composed of predominantly type I fibers (soleus, 47% decline), a region of muscle with a high portion of IIA fibers (red quadriceps, 66% decline), and a region of muscle that is mostly IIB fibers (white quadriceps, 54% decline). In single fibers that expressed known MHC isoforms, the relative decrements were similar to these published results for tissue glucose uptake. Furthermore, the relative decrements were similar for each fiber type that was assessed. The results from perfused and isolated muscles indicate that all fiber types contribute to the insulin resistance in OZ rats.

The correspondence for the magnitude of insulin resistance in single fibers compared with published data for isolated epitrochlearis and perfused hind limb (40,41) provides further support for the validity of the single-fiber method. However, various interventions, including exercise, dietary manipulations, or pharmaceutical treatments, may not uniformly modulate single-fiber glucose uptake. The single-fiber method could be especially valuable for experiments using vivo gene delivery (by electroporation or gene gun) to muscle. These approaches have provided important information about the regulation of muscle glucose uptake. However, because gene delivery using

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**FIG. 4.** Mean 2-DG uptake by fibers expressing the same MHC isoform, with (■) or without (□) AICAR stimulation. For basal (no AICAR) fibers, the number of fibers for each MHC type is within parentheses: IIB (27), IIA (6), IIX (6), and IIB/X (1). For AICAR-stimulated fibers, the number of fibers for each MHC type is within parentheses: IIB (51), IIA (21), IIX (13), and IIB/X (15). Data are means ± SEM. ANOVA revealed a significant difference related to MHC expression for AICAR-stimulated fibers ($P < 0.05$). Post hoc analysis of AICAR-stimulated fibers revealed a significant difference. *$P < 0.05$ for IIB vs. IIA fibers.

**FIG. 5.** Mean 2-DG uptake by fibers isolated from epitrochlearis muscles from lean Zucker (LZ) (□) or obese Zucker (OZ) (■) rats. For basal (no insulin) fibers from LZ rat muscles, the number of fibers for each MHC type is in parentheses: IIB (51), IIA (10), IIX (8), and IIB/X (11). For basal fibers from OZ rat muscles, the number of fibers for each MHC type is in parentheses: IIB (71), IIA (10), IIX (5), and IIB/X (14). For insulin-stimulated fibers, from LZ rat muscles, the number of fibers for each MHC type is in parentheses: IIB (73), IIA (13), IIX (15), and IIB/X (19). For insulin-stimulated fibers from OZ rat muscles, the number of fibers for each MHC type is in parentheses: IIB (99), IIA (9), IIX (7), and IIB/X (25). Data are means ± SEM. For fibers with insulin, there were significant main effects of genotype (LZ greater than OZ; $P < 0.001$) and fiber type ($P < 0.001$) and a significant genotype × fiber-type interaction ($P < 0.01$). Post hoc analysis of insulin-stimulated fibers revealed 1) #$P < 0.05$ for insulin-stimulated OZ vs. LZ for all fiber types; and 2) for insulin-stimulated fibers within only the LZ group, *$P < 0.05$ for insulin-stimulated IIA vs. IIB, IIX, and IIB/X fibers.
these approaches is not 100% efficient, the interpretation can be imprecise. For example, if gene delivery induces a 50% reduction in glucose uptake, it is possible that only 50% of the fibers were transfected, and glucose uptake was completely inhibited in each transfected fiber. Alternatively, it is possible that most of the fibers were transfected, but glucose uptake was only partly inhibited in each transfected fiber. The single-fiber method can turn this complication into an advantage by allowing isolation of those fibers expressing the gene of interest (facilitated by a fluorescent reporter). Moreover, the nontransfected fibers could provide an invaluable internal control from the same muscle. It also is possible that transfection efficiency will vary by fiber type or that the consequences of transfection will be fiber-type specific. The single-fiber model offers the opportunity to address these possible outcomes.

Obesity-associated inflammation is considered a likely contributor to muscle insulin resistance (42,43). Overactivation of the IkB/nuclear factor-κB (NFκB) pathway is hypothesized to trigger insulin resistance, and reduced IkB-β protein abundance commonly is used as a marker of this activation. For example, IkB-β content was decreased in muscle of insulin-resistant humans with type 2 diabetes (44). Previous studies using whole skeletal muscles or regions of muscles have suggested that activation of the IkB/NFκB pathway may be modulated by obesity in a fiber-type–dependent manner (45,46). The current results demonstrate...
significantly lower IκB-β levels in single fibers from obese rats, regardless of fiber type. When these novel observations are coupled with our demonstrated decline in glucose uptake by single fibers from OZ rats, the results provide new evidence that supports the idea that the insulin resistance found in fibers from the obese rats, regardless of fiber type, is linked to greater activation of the IκB/NFκB pathway. Another novel result was the lack of a significant fiber-type difference for IκB-β levels, indicating that the fiber-type differences in glucose transport are independent of differences in the expression of this protein. APPL1 mediates adiponectin signaling and has been linked to enhanced insulin-stimulated Akt activation, GLUT4 translocation, and glucose uptake (47,48). The lack of significant effects of either obesity or fiber type on APPL1 abundance, taken together with the single-fiber glucose uptake data, demonstrate that neither the obesity-related insulin resistance nor the fiber-type differences in glucose uptake are attributable to differential expression of this regulatory protein.

In conclusion, the current study provides new insights into the relationship between MHC isoform expression and glucose uptake in rat muscle. In addition, the results demonstrated that in OZ rats, the extent of insulin resistance was similar for single fibers expressing different MHC isoforms. More importantly, the study validated an innovative approach that has the potential to provide unique information about muscle glucose uptake to be used for understanding, preventing and treating insulin resistance.

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J.G.M. researched data, contributed to the discussion, and wrote, reviewed, and edited the manuscript. G.D.C. contributed to the discussion and wrote, reviewed, and edited the manuscript. G.D.C. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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