Glucose Uptake in Rat Extraocular Muscles: Effect of Insulin and Contractile Activity

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PURPOSE. Extraocular muscles show specific adaptations to fulfill the metabolic demands imposed by their constant activity. One aspect that has not been explored is the availability of substrate for energy pathways in extraocular muscles. In limb muscles, glucose enters by way of GLUT1 and GLUT4 transporters in a process regulated by insulin and contractile activity to match metabolic supply to demand. This mechanism may not apply to extraocular muscles because their constant activity may require high basal (insulin- and activity-independent) glucose uptake. The authors tested the hypothesis that glucose uptake by extraocular muscles is not regulated by insulin or contractile activity.

METHODS. Extraocular muscles from adult male Sprague-Dawley rats were incubated with 100 nM insulin or were electrically stimulated to contract (activity); glucose uptake was measured with 2-deoxy-d[1,2-³H]glucose. The contents of GLUT1, GLUT4, total and phosphorylated protein kinase B (Akt), phosphorylated AMP-activated protein kinase (AMPK), and glycogen synthase kinase 3 (GSK3) underwent Western blot analysis.

RESULTS. Insulin and activity increased glucose uptake over the basal rate to 108% and 78%, respectively. GLUT1 and GLUT4 were detectable in extraocular muscles. Phosphorylated AKT/total AKT increased by twofold after insulin stimulation, but there was no change with activity. AMPK phosphorylation increased 35% with activity. Phosphorylated-GSK3/total GSK3 did not change with insulin or activity.

CONCLUSIONS. Glucose uptake in extraocular muscles is regulated by insulin and contractile activity. There is evidence of differences in the insulin signaling pathway that may explain the low glycogen content in these muscles. (*Invest Ophthalmol Vis Sci.* 2010;51:6364–6368) DOI:10.1167/iops.10-6081

Extraocular muscles are the effector arm of the ocular motor system and are involved in all voluntary and reflexive eye movements.^{1,2} The functional demands of extraocular muscles are different from those imposed on other skeletal muscles, leading to unique metabolic adaptations as demonstrated by gene expression profiling and by biochemical and functional studies.^{3–8} For example, extraocular muscles seem to have relatively low adenosine triphosphate (ATP)-buffering capacity, evidenced by decreased reliance on glycogenolysis and less creatine kinase activity.^{9–12} Moreover, extraocular muscles can

reduce lactate to pyruvate for entry into the Krebs cycle to sustain their high activity demand.^{9,11,13} Another potential mechanism for enhancing ATP production in the extraocular muscles is enhanced glucose uptake by the muscle fibers, priming glycolysis and then mitochondrial energy pathways.

In limb skeletal muscles, glucose enters muscle fibers by way of specific membrane transporters (GLUT1 and GLUT4) in a process influenced by insulin and contractile activity to match metabolic supply to demand.¹⁴ GLUT1 transporters mediate insulin- and activity-independent basal glucose uptake. On binding to its membrane receptors, insulin triggers a signaling cascade that activates protein kinase B (AKT), which in turn initiates the translocation of GLUT4-containing vesicles to the membrane, increasing glucose uptake over the basal rate.^{15,16} At the same time, glycogen synthase kinase 3 (GSK3) is activated and phosphorylates glycogen synthase, stimulating glycogen production.¹⁷ Contractile activity has a similar effect on glucose uptake: it induces the translocation of a separate pool of GLUT4-containing vesicles to the membrane through activation of the adenosine monophosphate (AMP)-activated kinase (AMPK) pathway.^{18,19} Given their functional characteristics, extraocular muscles may have very high basal glucose uptake rates, negating the need for further regulation by insulin or contractile activity. Therefore, we tested the hypothesis that glucose uptake into extraocular muscles is not influenced by insulin or contractile activity as in other skeletal muscles.

MATERIALS AND METHODS

Materials

All reagents were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise stated. 2D-³H glucose and ¹⁴C-mannitol were obtained from Perkin Elmer (Boston, MA). SDS-polyacrylamide gels and other electrophoresis-related materials were purchased from Bio-Rad (Hercules, CA). Polyvinylidene difluoride (PVDF) membranes were obtained from Immobilon-FL, Millipore (Billerica MA). Protease and phosphatase inhibitor cocktail (100×) was purchased from Thermo Scientific (Halt; Thermo Scientific, Rockford, IL). Rabbit polyclonal GLUT1 and GLUT4 antibodies were purchased from Abcam (Cambridge, MA). Total AKT and phosphorylated-AKT (Ser473) antibodies and phosphorylated AMPK (Thr172) were obtained from Cell Signaling (Danvers, MA). Total GSK-3 β antibody was purchased from BD Bioscience (San Jose, CA), whereas antibody against phosphorylated-GSK-3 α/β (Ser21/9) was obtained from Cell Signaling. Secondary fluorescence-labeled antibodies were obtained from Invitrogen (Carlsbad, CA).

Animals

The use of experimental animals was approved by the Institutional Animal Care and Use Committee at the University of Kentucky and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-four adult male Sprague-Dawley rats (300–500 g; Harlan, Indianapolis, IN) were euthanatized by CO₂ asphyxia followed by pneumothorax.

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Supported by National Eye Institute Grant R01 EY12998 (FHA).

Submitted for publication June 22, 2010; revised July 29, 2010; accepted July 29, 2010.

Disclosure: M.L. Garcia-Cazarin, None; T.M. Fisher, None; F.H. Andrade, None

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FIGURE 1. Glucose uptake in rat extraocular muscles. (A) Basal glucose uptake was 1.2 ± 0.3 $\mu\text{mol/g/h}$, comparable to values in other muscles.³⁶ Insulin increased glucose uptake to 2.5 ± 0.3 $\mu\text{mol/g/h}$ (* $P = 0.001$, Insulin greater than Basal). (B) Contractile activity increased glucose uptake in a similar manner: Basal, 1.3 ± 0.2 versus Activity, 2.4 ± 0.4 $\mu\text{mol/g/h}$; * $P = 0.009$. Values are mean $\pm$ SEM; $n = 6$ rats per group.

In Vitro Glucose Uptake

Whole orbits, including the bony posterior wall, were quickly excised from 12 rats immediately after euthanatization and then immersed in Krebs-Ringer's bicarbonate buffer (117 mM NaCl, 4.7 mM KCl, 24.6 mM NaHCO_3 , 1.2 mM KH_2PO_4 , 1.2 mM CaCl_2 , 2.5 mM MgSO_4) bubbled with 95% O_2 and 5% CO_2 . The orbits were carefully dissected to leave the intact extraocular muscles (four rectus and superior oblique muscles) attached to a small bone fragment and a ring of sclera and then tied to a glass rod. Extraocular muscles from one side were used to measure basal glucose uptake ($n = 12$ orbits), and the contralateral extraocular muscles were used to measure the effects of insulin ($n = 6$ orbits) or contractile activity ($n = 6$ orbits). All muscles were first incubated with Krebs-Ringer's bicarbonate buffer with 2 mM pyruvate for 30 minutes at 37°C. Extraocular muscles were then rinsed and incubated with Krebs-Ringer's buffer containing 1 mM 2-deoxy-D-[1,2- ^3H]glucose (2D- ^3H -glucose, 1.5 mCi/mL) and 7 mM D-[1- ^{14}C]mannitol (0.45 mCi/mL) for 10 minutes. Insulin (100 nM) was added to the buffer of the insulin group. Muscles in the activity group were stimulated to contract using 0.5-second stimulus trains at 70 Hz every 10 seconds for 20 minutes before the switch was made to the buffer containing the radiolabeled glucose and mannitol and then for the final 10-minute incubation. Finally, the extraocular muscles were rinsed with plain Krebs-Ringer's buffer. Then the sclera and bone attachments were removed, and the extraocular muscles were blotted dry with filter paper and digested with 250 μL of 1 N NaOH at 80°C for 10 minutes. After neutralizing with 250 μL of 1 N HCl, 350 μL sample was added to the scintillation liquid for dual-label radioactivity counting. Glucose uptake was then determined after calculating the intracellular and extracellular space, as previously described.²⁰

Immunoblotting

To examine signaling events linking insulin and contractile activity to glucose uptake, paired extraocular muscle preparations from eight rats were subjected to the same protocols as those described (insulin, $n = 4$ rats; activity, $n = 4$ rats) in Krebs-Ringer's buffer with nonradioactive glucose and mannitol. In addition, nonstimulated extraocular muscles from four rats were isolated to determine the content of GLUT1 and GLUT4. At the end of the corresponding protocol, extraocular muscles were homogenized (1% NP-40, 0.50% sodium deoxycholate, 0.10% SDS, 50 mM NaCl, 5 mM benzimidazole, 20 mM Tris-HCl [pH 7.6], 5 mM ethylenediamine, 1 mM phenylmethylsulfonyl fluoride, and 1 $\times$ protease and phosphatase inhibitor cocktail) for immunoblotting. Protein concentration was measured with the Bradford protein assay using bovine serum albumin for protein standards.²¹ Extraocular muscle homogenates (15 μg protein) were resolved electrophoretically in 10% to 20% linear gradient SDS-polyacrylamide gels and transferred to PVDF membranes. Ponceau S staining after transfer confirmed equal protein loading. Membranes were blocked for 1 hour with a 1:1 dilution of blocking buffer (Odyssey; LI-COR Biosciences, Lincoln, NE)/phosphate-buffered saline at room temperature. Membranes were then incubated overnight in blocking buffer/PBS/0.2% Tween with primary antibodies against GLUT1, GLUT4, total AKT, phosphorylated AKT, phosphorylated AMPK, total GSK3 β , and phosphorylated GSK3 β . All primary antibodies were used at a dilution of 1:1000. After the membranes were washed with PBS/0.1% Tween, they were incubated with Alexa Fluor 680-conjugated goat anti-rabbit/mouse antibody (1:7500) and then washed again with PBS/0.1% Tween. Membranes were finally rinsed in PBS and scanned (Odyssey Infrared Imaging System; LI-COR Biosciences). Companion software (Odyssey; LI-COR Biosciences) was used to quantify the density of the resultant bands. GLUT1 and GLUT4 contents were evaluated in the basal, nonstimulated conditions. Total AKT, phosphorylated AKT, phosphorylated AMPK, total GSK3, and phosphorylated GSK3 were evaluated in basal conditions and after insulin and electrical stimulation.

Statistical Analysis

The means of the groups were compared using paired Student's *t*-tests. Group differences were considered significant when $P \leq 0.05$. Statistical software (Prism 5; GraphPad, San Diego, CA) was used for statistical analysis and graph generation.

FIGURE 2. GLUT1 and GLUT4, the glucose transporters typically found in skeletal muscle, were also present in the extraocular muscles. Insets: representative Western blots for both transporters. Each inset shows transporter content from two independent samples.

RESULTS

Insulin Stimulates Glucose Uptake in Extraocular Muscles

The rate of glucose uptake into extraocular muscles was estimated by the incorporation of 2D-³H-glucose under basal conditions (control) and after incubation with 100 nM insulin. As shown in Figure 1A, insulin-stimulated glucose uptake was 108% greater than the basal rate (1.2 ± 0.3 vs. 2.5 ± 0.3 $\mu\text{mol/g/h}$, basal and insulin, respectively; $P = 0.01$; $n = 6$ rats). These results demonstrated that insulin stimulates glucose uptake into extraocular muscles to the same degree as in other skeletal muscles.^{19,22–24}

Activity Increases Glucose Uptake in Extraocular Muscles

To examine the effects of contractile activity on glucose uptake, we tested the response of extraocular muscles to 30 minutes of electrical stimulation (20 minutes before and 10 minutes after adding the radioactive markers). As with insulin, we found glucose uptake was 78% higher (Fig. 1B) in stimulated muscles when compared to the basal rate (1.3 ± 0.2 vs. 2.4 ± 1.4 $\mu\text{mol/g/h}$, basal and activity, respectively; $P = 0.009$; $n = 6$ rats). Taken together, our results indicate both insulin and contractile activity increase glucose uptake in extraocular muscles.

GLUT1 and GLUT4 Are Present in Extraocular Muscles

GLUT1 is the transporter associated with basal glucose uptake, whereas GLUT4 exists in intracellular vesicles that are translocated to the sarcolemma on insulin stimulation or contractile activity to increase glucose uptake.^{25–27} We analyzed the glucose transporter content of four rats by immunoblotting and found that both GLUT1 and GLUT4 are present in extraocular muscles (Fig. 2). These results indicate GLUT4 transporters are responsible for the increase in glucose uptake in extraocular muscles after insulin and contractile activity.^{23,24}

AKT Phosphorylation Increases with Insulin but Not with Activity

The ratio of phosphorylated AKT to total AKT increased twofold in insulin-stimulated muscles ($n = 4$ rats; $P = 0.008$; Fig. 3A). This response to insulin is similar to what has been reported in limb muscles.^{28–31} These results suggest insulin signaling in extraocular muscles is not identical with that of other muscles. The ratio of phosphorylated AKT to total AKT was higher but did not reach significance in the activity-stimulated muscles, indicating an AKT-independent pathway may mediate contractile activity ($n = 4$ rats; $P = 0.28$; Fig. 3B). However, no change was observed in GSK3 β phosphor-

FIGURE 3. AKT signaling in extraocular muscles stimulated with insulin and contractile activity. **(A)** Phosphorylated and total AKT in extraocular muscles under basal conditions and after insulin stimulation were measured by Western blot. The ratio of phosphorylated AKT to total AKT increased twofold in insulin-stimulated muscles (* $P = 0.008$, Insulin greater than Basal; $n = 4$ rats). *Inset:* representative Western blots (Basal, left lane; Insulin, right lane) for phosphorylated AKT (P-AKT, top) and total AKT (middle). The Ponceau S-stained membrane (bottom) demonstrates equal protein loading. **(B)** Phosphorylated and total AKT in extraocular muscles under basal conditions and after activity. Contractile activity did not change the ratio of phosphorylated AKT to total AKT ($n = 4$ rats). *Inset:* representative Western blots (Basal, left lane; Activity, right lane) for phosphorylated AKT (P-AKT, top) and total AKT (middle). The Ponceau S-stained membrane (bottom) demonstrates equal protein loading.

FIGURE 4. GSK3 signaling in extraocular muscles after insulin and contractile activity. **(A)** Phosphorylated and total GSK3 under basal conditions and after insulin stimulation. Insulin did not change the phosphorylated to total GSK3 ratio ($n = 4$ rats). *Inset:* representative Western blots (Basal, *left lane*; Insulin, *right lane*) for phosphorylated GSK3 (P-GSK3, *top*) and total GSK3 (*middle*). The Ponceau S-stained membrane (*bottom*) demonstrates equal protein loading. **(B)** Phosphorylated and total GSK3 under basal conditions and after contractile activity. The phosphorylated to total GSK3 ratio was not changed by activity ($n = 4$ rats). *Inset:* representative Western blots (Basal, *left lane*; Activity, *right lane*) for phosphorylated GSK3 (P-GSK3, *top*) and total GSK3 (*middle*). The Ponceau S-stained membrane demonstrates equal protein loading (*bottom*).

ylation in insulin- or activity-stimulated extraocular muscles ($n = 4$ rats; $P = 0.18$ and $P = 0.7$, respectively; Figs. 4A, 4B).

Activity Increases AMPK Phosphorylation

The phosphorylation of AMPK (Thr172) was increased 35% in the activity-stimulated muscles ($n = 5$; $P = 0.01$; Fig. 5). These results indicate that, as in other skeletal muscles, extraocular muscles uptake glucose through activation of the AMPK pathway during contractile activity.^{19,24}

DISCUSSION

To the best of our knowledge, this is the first study of glucose uptake in extraocular muscles. A number of observations sug-

gested substrate supply, not storage, was likely to be more important in the extraocular muscles.^{14,16,17} In other words, basal glucose uptake would be sufficiently high to satisfy the needs for glycolysis, Krebs cycle, and oxidative phosphorylation. Therefore, we hypothesized that glucose uptake into extraocular muscles would not be influenced by insulin or contractile activity. Our results led us to reject this hypothesis. However, we did find evidence of differences in insulin signaling in the extraocular muscles that may explain their low glycogen content.^{9,11}

As is the case with other skeletal muscles, glucose uptake by extraocular muscles increased significantly in response to insulin stimulation and contractile activity. In brief, basal glucose uptake occurs by way of GLUT1 transporters. Insulin and contractile activity increase glucose uptake by stimulating the translocation

FIGURE 5. AMPK signaling in extraocular muscles after contractile activity. Phosphorylated AMPK under basal and after contractile activity. Phosphorylated AMPK increased 35% in muscles stimulated with contractile activity. (* $P = 0.019$, Activity greater than Basal; $n = 5$ rats). *Inset:* representative Western blots (Basal, *left lane*; activity, *right lane*) for phosphorylated AMPK. The Ponceau S-stained membrane (*bottom*) demonstrates equal protein loading.

of cytosolic vesicles containing GLUT4 after activation of the PI3K/AKT pathway.^{23,32–35} The extraocular muscles have GLUT1 and GLUT4, the same transporters found in other skeletal muscles. We found both insulin and contractile activity significantly increased glucose uptake in the extraocular muscles. Insulin, but not contractile activity, increases AKT phosphorylation in the extraocular muscles compared with the basal unstimulated state. In addition, contractile activity significantly increased AMPK phosphorylation in the extraocular muscles. Therefore, glucose uptake in extraocular muscles may be regulated by mechanisms similar to those in other skeletal muscles.^{28–31}

In most skeletal muscles, glycogen is a readily mobilized storage form of glucose.²⁹ Coupled with their vascularity, the low glycogen content of extraocular muscles suggests these muscles rely more on instantaneous glucose uptake than on glucose derived from glycogenolysis.¹¹ In skeletal muscles, GSK3 is the primary regulator of glycogen metabolism, activating glycogen synthase to increase glycogen formation. Here, we found insulin and contractile activity did not change the ratio of phosphorylated to total GSK3 β . Therefore, glycogen synthesis in the extraocular muscles does not seem to be regulated by insulin or activity; this may explain the low glycogen content in these muscles.

To summarize, insulin and contractile activity increase glucose uptake into extraocular muscles by activating AKT and AMPK, respectively, converging on the eventual translocation of GLUT4 to the plasma membrane. We conclude that extraocular muscles, despite their extreme contractile demands, take up glucose in a manner similar to that of other skeletal muscles. However, GSK3 β is not activated by insulin or activity in these muscles, providing a likely explanation for their low glycogen content.

Acknowledgments

The authors thank Chris White and Andrea Moodhart for superb technical assistance.

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