Muscle fiber type specificity in insulin signal transduction

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Song, Xiao Mei, Jeffrey W. Ryder, Yuichi Kawano, Alexander V. Chibalin, Anna Krook, and Julieen R. Zierath. Muscle fiber type specificity in insulin signal transduction. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R1690–R1696, 1999.—We determined the muscle fiber type-specific response of intracellular signaling proteins to insulin. Epitrochlearis (Epi; 15% type I, 20% type IIa, and 65% type IIb), soleus (84, 16, and 0%), and extensor digitorum longus (EDL; 3, 57, and 40%) muscles from Wistar rats were incubated without or with 120 nM insulin (3–40 min). Peak insulin receptor (IR) tyrosine phosphorylation was reached after 6 (soleus) and 20 (Epi and EDL) min, with sustained activity throughout insulin exposure (40 min). Insulin increased insulin receptor substrate (IRS)-1 and IRS-2 tyrosine phosphorylation and phosphotyrosine-associated phosphatidylinositol (PI)-3-kinase activity to a maximal level after 3–10 min, with subsequent downregulation. Akt kinase phosphorylation peaked at 20 min, with sustained activity throughout insulin exposure. Importantly, the greatest insulin response for all signaling intermediates was observed in soleus, whereas the insulin response between EDL and Epi was similar. Protein expression of the p85α-subunit of PI 3-kinase and Akt kinase, but not IR, IRS-1, or IRS-2, was greater in oxidative versus glycolytic muscle. In conclusion, increased function and/or expression of key proteins in the insulin-signaling cascade contribute to fiber type-specific differences in insulin action in skeletal muscle.

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Slow-twitch oxidative skeletal muscle has greater insulin binding capacity (3) and increased insulin receptor (IR) kinase activity and autophosphorylation (16) compared with fast-twitch glycolytic skeletal muscle. Additionally, the percentage of oxidative muscle fibers is positively correlated with glucose transport and GLUT-4 content, whereas glycolytic muscle fibers display a negative relationship (10). In humans, whole body glucose uptake and muscle glucose transport are positively correlated with type I muscle fibers and negatively correlated with type IIb muscle fibers (13, 27, 36). Thus insulin action is much greater in muscle composed primarily of oxidative versus glycolytic skeletal muscle. However, the relationship between muscle fiber type population and insulin signal transduction in skeletal muscle has not been characterized.

Important substrates of the IR include the insulin receptor substrates (IRS)-1 and -2 (34, 35). After IRS proteins are phosphorylated, they can bind to a number of Src-homology-2 domain-containing signaling proteins, including the p85α-regulatory subunit of phosphatidylinositol (PI) 3-kinase (28, 34). PI 3-kinase is an important signaling intermediate that plays a pivotal role in a diverse range of cellular responses, including glucose transport and glycogen synthesis (28). We have previously reported that insulin signaling through the IRS-1/PI 3-kinase pathway is impaired in skeletal muscle from people with Type II (non-insulin dependent) diabetes mellitus (2). The serine-threonine kinase Akt, a downstream target kinase of PI 3-kinase (28), has been suggested to be involved in insulin-stimulated glucose transport (8, 18, 31). In skeletal muscle from moderately overweight people with Type II diabetes, insulin-stimulated Akt kinase and glucose transport are impaired (20). Whether these signaling intermediates are activated in a fiber type-specific manner is not known.

The percentage of type I muscle fibers is reduced with extreme obesity (13) and inactivity due to paralysis (14). Alterations in skeletal muscle fiber type composition may partly contribute to impaired insulin action in skeletal muscle. Our aim was to determine the muscle fiber type-specific response of various intracellular signaling proteins to insulin. For this, we used epitrochlearis (Epi; 15% type I, 20% type IIa, and 65% type IIb), soleus (84, 16, and 0%), and extensor digitorum longus (EDL; 3, 57, and 40%) muscles from Wistar rats (1). Here we show that skeletal muscle displays a fiber type-specific response in the activation of key components of the insulin-signaling cascade. Fiber type-specific differences include increased insulin-stimu-
lated tyrosine phosphorylation of IR, IRS-1, and IRS-2; increased phosphotyrosine-associated PI 3-kinase activity; and increased Akt kinase phosphorylation in skeletal muscle composed predominantly of type I oxidative skeletal muscle fibers. These functional differences are associated with increased expression of p85α-subunit of PI 3-kinase and Akt kinase, with no difference in IR, IRS-1, or IRS-2 protein noted between oxidative and glycolytic muscles. The time course of the activation of these signaling cascades was similar between muscle fiber types. Thus increased functional activity and/or expression of key proteins in the insulin-signaling cascade may account for muscle fiber type-specific differences in insulin-stimulated glucose uptake and metabolism. However, increased IRS-1 and IRS-2 tyrosine phosphorylation appear to be related to increased insulin receptor tyrosine kinase activity.

MATERIALS AND METHODS

Animals and muscle incubations. The study was approved by the local Animal Ethics Committee. Male Wistar rats (90–130 g) were purchased from B & K Universal (Sollentuna, Sweden) and housed at the animal facility of the Karolinska Institute for 1 wk before experimentation. Rats were maintained on a 12:12-h light-dark cycle and were provided free access to water and standard rodent chow. After an overnight fast, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). Epi, soleus, and EDL muscles were removed. Soleus and EDL muscle were split into two equal portions as previously described (11). Muscles were incubated for 60 min at 30°C in 2 ml oxygenated Krebs-Henseleit buffer containing 32 mM mannitol, 8 mM glucose, and 0.1% BSA (RIA Grade, Sigma, St. Louis, MO) as described (11). Thereafter, muscles were incubated in identical medium without or with 120 nM insulin (Novo Nordisk Copenhagen, Denmark) for 3–40 min. The gas phase in the vials was maintained at 95% O2-5% CO2. Incubations were terminated by immediately freezing the muscle specimens with aluminum tongs cooled in liquid nitrogen.

Muscle preparation and insulin signaling assays. Epi, soleus, or EDL muscles were homogenized in 0.6 ml ice-cold buffer containing (in mM) 135 NaCl, 1 MgCl2, 2.7 KCl, 20 Tris pH 8.0, 0.5 Na2VO4, 10 NaF, 0.2 phenylmethylsulfonyl, and 1% Triton X-100, 10% vol/vol glycerol, and 10 μg/ml leupeptin. Insoluble material was removed by centrifugation at 12,000 g for 10 min at 4°C. Protein was determined using the Bradford method (Bio-Rad, Richmond, CA). Aliquots of supernatant were immunoprecipitated overnight at 4°C with antibodies against phosphotyrosine for (IR and PI 3-kinase analysis; Transduction Laboratories, Lexington, KY), IRS-1 (for IRS-1 phosphotyrosine analysis; a kind gift from Professor Ton Maassen, Leiden University, Leiden, The Netherlands), or IRS-2 (for IRS-2 phosphotyrosine analysis; a kind gift from Drs. Morris White and Martin Myers, Joslin Research Center) as described previously (11). Membranes containing IRS-1 or IRS-2 immunoprecipitates were incubated with antiphosphotyrosine antibody (RC20; Transduction Laboratories). After incubation, proteins were visualized by enhanced chemiluminescence (Amersham). Proteins were visualized by enhanced chemiluminescence and quantified by densitometry.

Statistics. Results are presented as means ± SE. Statistical differences for the time course studies were determined by two-way ANOVA. Statistical differences for the protein expression studies were determined by one-way ANOVA. The ANOVA resulted in a significant F ratio (P < 0.05), the location of the significance was determined by the Tukey test.

RESULTS

Insulin-stimulated tyrosine phosphorylation and protein expression of the insulin receptor. Isolated Epi, soleus, and EDL muscles were incubated in the absence or presence of 120 nM insulin for 3–40 min. Muscle homogenates were subjected to immunoprecipitation with antiphosphotyrosine antibody followed by immunoblotting with antiphosphotyrosine antibody (RC20). Insulin induced the appearance of a tyrosine-phosphorylated band migrating at 95 kDa (Fig. 1A). Peak tyrosine phosphorylation of insulin receptor was observed at 6 min for soleus and 20 min for Epi and EDL after insulin exposure (Fig. 1B). Phosphorylation was sustained throughout a 40-min insulin exposure. The magnitude of insulin-stimulated receptor tyrosine phosphorylation was significantly greater in soleus compared with EDL and Epi skeletal muscle (P < 0.05). Insulin receptor tyrosine phosphorylation at 6 min was 45–60% lower in Epi or EDL compared with soleus muscle. Basal insulin receptor tyrosine phosphorylation was similar between all muscles. We next determined IR protein expression in skeletal muscle lysates (Fig. 1C). Protein expression of IR tended to be greater in soleus muscle compared with Epi muscle, although this difference did not reach statistical significance.
tyrosine phosphorylation peaked in all muscles (Fig. 2B) and downregulated thereafter. The magnitude of insulin-stimulated tyrosine phosphorylation of IRS-1 was significantly greater in soleus compared with Epi and EDL muscle ($P < 0.01$). After 6 min of exposure to insulin, IRS-1 tyrosine phosphorylation was 35% lower in Epi or EDL compared with soleus muscle. Basal IRS-1 tyrosine phosphorylation was similar among the different muscles. IRS-1 expression in muscle lysate tended to be greater in soleus muscle ($P = 0.18$ soleus compared with Epi or EDL; Fig. 2C).

Insulin-stimulated tyrosine phosphorylation and protein expression of IRS-2. IRS-2 was recently shown to be another important insulin receptor substrate in skeletal muscle (35). Parallel to IRS-1, the pattern of insulin-stimulated IRS-2 tyrosine phosphorylation was similar for oxidative and glycolytic muscles (Fig. 3A).
However, compared with IRS-1, the time course for the insulin responsiveness of IRS-2 tyrosine phosphorylation appeared to be shifted to the left (Fig. 3B). Basal IRS-2 tyrosine phosphorylation was similar in all muscles. Within 3–6 min, maximally insulin-stimulated IRS-2 tyrosine phosphorylation was noted in all muscle fiber types. After 6–10 min, insulin-stimulated IRS-2 tyrosine phosphorylation was downregulated. The magnitude of insulin-induced tyrosine phosphorylation of IRS-2 was significantly greater in soleus muscle relative to Epi and EDL skeletal muscle (P, 0.01). After 3 min, IRS-2 tyrosine phosphorylation was 64% (P, 0.05) and 78% (P, 0.01) lower in Epi and EDL, respectively, compared with soleus muscle. IRS-2 protein expression was similar between all muscle fiber types (Fig. 3C).

Insulin-stimulated PI 3-kinase activity and protein expression of p85α. To further explore the possibility that muscle fiber type-specific differences occur in the insulin signal transduction cascade, PI 3-kinase activity was assessed in antiphosphotyrosine immunoprecipitates of Epi, soleus, and EDL muscle (Fig. 4A). Consistent with our results for tyrosine phosphorylation of IRS-1 and IRS-2, the time course for the increase in antiphosphotyrosine-immunoprecipitable PI 3-kinase activity was similar between Epi, soleus, and EDL muscle (Fig. 4B). Within 6 min, PI 3-kinase activity was maximally stimulated in the three different muscles. Thereafter, PI 3-kinase activity was downregulated. However, insulin-stimulated PI 3-kinase activity remained increased over basal levels from 20 to 40 min. Thus insulin-stimulated PI 3-kinase activity in skeletal muscle can be characterized by a rapid initial activation followed a partial deactivation. However, the absolute magnitude of insulin-stimulated PI 3-kinase activ-

Fig. 3. Time course of insulin-stimulated IRS-2 tyrosine phosphorylation and protein expression in skeletal muscle. Muscles were obtained as in Fig. 2. Equal amounts of protein lysate were immunoprecipitated with anti-IRS-2 antibody. A: representative immunoblot of IRS-2 tyrosine phosphorylation. B: quantified data expressed as 100% of insulin-stimulated value for soleus muscle at 6 min insulin stimulation. Results are means ± SE for 3 or 4 muscles for each time point. P < 0.01 for soleus vs. Epi or EDL. C: protein expression of IRS-2 in muscle lysate. Results are presented as means ± SE arbitrary densitometric units (n = 7). Differences between soleus and EDL or Epi were not significant.

Fig. 4. Time course of insulin-stimulated phosphotyrosine-associated phosphatidylinositol (PI) 3-kinase activity and protein expression of the p85α-subunit of PI 3-kinase. Muscles were incubated with or without 120 nM insulin for 3–40 min, and PI 3-kinase activity was determined in antiphosphotyrosine immunoprecipitates as described in MATERIALS AND METHODS. A: representative phosphoimage of lipid products from PI 3-kinase reaction after separation by thin layer chromatography. B: values expressed as 100% of insulin-stimulated value for soleus muscle at 6 min. PI 3-kinase activity was quantified using a PhosphoImager. Results are presented as means ± SE for n = 3–9 muscles for each time point. P < 0.001 for soleus vs. Epi or EDL. C: relative protein expression of p85α-subunit of PI 3-kinase as determined by immunoblot analysis of muscle lysates. Results are means ± SE arbitrary densitometric units (n = 7). P < 0.01 for soleus vs. Epi and P < 0.001 for soleus vs. EDL. PIP, phosphatidylinositol 3-phosphate.
ity was greater in soleus compared with Epi or EDL muscles (P < 0.001). We measured the protein expression of the p85α-regulatory subunit of PI 3-kinase in the different muscles (Fig. 4C). Immunoblot analysis revealed that the p85α-regulatory subunit of PI 3-kinase was most abundant in soleus, with an increase of 2.7-fold over Epi (P < 0.01) and 10.3-fold over EDL muscle (P < 0.001).

Insulin-stimulated serine phosphorylation and protein expression of Akt. The serine kinase Akt is a signaling target of PI 3-kinase (28). We measured Akt phosphorylation by immunoblotting muscle lysates with a phosphospecific Akt antibody that recognizes Akt when phosphorylated on SER 473 (Fig. 5A). Unlike IRS-1, IRS-2, and PI 3-kinase, the downstream component Akt displayed a different pattern for insulin stimulation (Fig. 5B). In all muscle groups, Akt phosphorylation was significantly increased after 3–6 min of insulin exposure. However, maximal insulin-stimulated Akt phosphorylation was not achieved until 20 min, and this level of Akt phosphorylation was maintained over the remaining incubation time. In absolute terms, insulin-stimulated Akt phosphorylation was greater in soleus compared with either Epi or EDL muscle (P < 0.001). Peak Akt phosphorylation measured at 20 min in soleus muscle was 1.8-fold greater than Epi (P < 0.001) and 1.6-fold greater than EDL (P < 0.005). Parallel to the insulin response, protein expression of Akt (Fig. 5C) in soleus muscle was 6.6-fold greater compared with either Epi or EDL muscle (P < 0.001 soleus compared with Epi or EDL).

DISCUSSION

The major finding in the present report is that there are skeletal muscle fiber type-specific differences in the regulation of insulin signal transduction. Skeletal muscle composed primarily of oxidative fibers (soleus) displayed a greater insulin responsiveness for IR, IRS-1 and IRS-2 tyrosine phosphorylation, PI 3-kinase activity, and Akt serine phosphorylation compared with skeletal muscle composed primarily of glycolytic fibers (Epi and EDL). The functional superiority of oxidative versus glycolytic skeletal muscle for insulin signal transduction is likely to be related to increased protein expression of several genes in the signaling cascade, as well as increased functional properties of the signaling intermediates.

Early studies established fiber type-specific differences in insulin action on skeletal muscle glucose uptake and metabolism in humans (22, 23) and rodents (3, 15). Some of these differences may be related to greater insulin binding (3) and increased insulin receptor tyrosine kinase activity (16) in oxidative versus glycolytic muscle. Nevertheless, differences in insulin action between oxidative and glycolytic skeletal muscle are not limited to heterogeneous insulin action at the receptor level, because GLUT-4 expression and insulin-stimulated glucose transport activity are positively correlated with percentage of oxidative muscle fibers (10). Here we show that fiber type-specific differences are also present at the level of IRS-1, IRS-2, and PI 3-kinase, important components of the insulin signal transduction pathway to glucose transport (28, 34, 35). Furthermore, the maximal effect of insulin to increase serine phosphorylation of Akt kinase is fiber type specific. This finding is consistent with a recent report whereby serine phosphorylation and protein expression of Akt kinase were twofold greater in oxidative compared with glycolytic skeletal muscle (32). Nevertheless, the physiological relevance of the dramatic increase in the protein expression of several insulin signaling intermediates is not clear, because full activation of the insulin signaling cascade may not be required for maximal activation of downstream metabolic responses such as glucose transport (12, 30).

Although the increased insulin action between oxidative and glycolytic muscle may be related to the coordinated upregulation of key components of the insulin signal transduction pathway, the possibility exists that the degree of insulin receptor phosphorylation may...
partly account for the profound differences in the level of insulin signal transduction between the muscle fiber types. Considering the downstream components PI 3-kinase and Akt, we noted only a twofold difference in the degree of activation between the muscle fiber types, despite an even greater fold increase in protein expression. Furthermore, insulin-stimulated IR, IRS-1, and IRS-2 tyrosine phosphorylation were increased in oxidative soleus versus glycolytic muscle, and these differences could not be accounted for by increased protein expression of each respective IRS protein, although a tendency for increased IRS-1 was noted in soleus muscle. Thus the degree of activation of downstream components of the insulin-signaling cascade appear to correlate more closely with tyrosine phosphorylation of the insulin receptor, rather than the absolute level of expression of intermediate components of the insulin signal transduction cascade.

A comparison of the time course for the activation of several components of the insulin-signaling cascade in Epi, soleus, and EDL muscle revealed a similar response between the different muscle types. Tyrosine phosphorylation of the insulin receptor peaked at 6–20 min, with sustained levels throughout the insulin exposure. In contrast, IRS-1 phosphotyrosine content and phosphotyrosine-associated PI 3-kinase activity peaked within 3–10 min, with subsequent downregulation thereafter. Our findings are in complete agreement with the time course for insulin-stimulated receptor autophosphorylation and PI 3-kinase activity in mouse soleus muscle (12). Interestingly, insulin-stimulated IRS-2 tyrosine phosphorylation peaked earlier than IRS-1 in all muscle fiber types. Insulin-stimulated serine phosphorylation of Akt, a downstream target of PI 3-kinase (8, 18, 31), peaked at 20 min in all muscles. Despite the profound downregulation in insulin-stimulated IRS-1 and IRS-2 tyrosine phosphorylation and PI 3-kinase activity, maximal Akt phosphorylation was sustained throughout the 40-min insulin exposure. The time course for serine phosphorylation of Akt is consistent with the time course for the activation of glucose transport in Epi muscle (33). Although differences in the activation of the key components of the insulin-signaling cascade were observed, muscle fiber type does not appear to have a direct influence over the time course of the activation of these signaling proteins.

Perspectives

Previous studies provided evidence that level of GLUT-4 protein is correlated with insulin responsiveness for glucose transport in skeletal muscle (10, 17), suggesting that the level of GLUT-4 determines the maximal insulin response on glucose transport. Direct evidence for a link between level of GLUT-4 expression and rate of insulin-stimulated glucose transport comes from transgenic animal models in which GLUT-4 protein is overexpressed in skeletal muscle. Transgenic overexpression of GLUT-4 in skeletal muscle can increase insulin action on whole body glucose uptake (4, 25) by increasing cellular glucose transport (4, 9). Nevertheless, our findings provide additional evidence that the increase in glucose transport in oxidative versus glycolytic skeletal muscle is not simply a consequence of increased GLUT-4 expression, because several important proteins in the insulin signal transduction pathway are upregulated in a fiber type-specific manner. The increase in insulin action on glucose uptake and metabolism in oxidative versus glycolytic muscle is likely to be related to increased protein expression-and/or functional activity of several key components of the insulin signal transduction cascade. Functional regulation of these genes will also be important in the modulation of signal transduction. Strategies designed to increase the expression signaling intermediates in type II glycolytic skeletal muscle fibers or to increase the proportion of type 1 oxidative skeletal muscle fibers may be provide a therapeutic means to improve insulin signal transduction and overcome insulin resistance in skeletal muscle.

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