Age-related differences in skeletal muscle insulin signaling: the role of stress kinases and heat shock proteins

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Gupte AA, Bomhoff GL, Geiger PC. Age-related differences in skeletal muscle insulin signaling: the role of stress kinases and heat shock proteins. J Appl Physiol 105: 839–848, 2008. First published July 3, 2008; doi:10.1152/japplphysiol.00148.2008.—Aging is associated with an increase in insulin resistance in skeletal muscle, yet the underlying mechanism is not well established. We hypothesize that with aging, a chronic increase in stress kinase activation, coupled with a decrease in oxidative capacity, leads to insulin resistance in skeletal muscle. In aged (24 mo old) and young (3 mo old) Fischer 344 rats, 2-deoxyglucose uptake and insulin signaling (as measured by phosphorylation of insulin receptor substrate-1 (IRS-1), Akt (protein kinase B), and Akt substrate of 160 kDa (AS160)) decreased significantly with age. Activation of c-Jun NH2-terminal kinase (JNK), glycogen serine kinase-3β (GSK-3β), and degradation of IkBα by the upstream inhibitor of kappa B kinase (IKKβ), as measured by Western blot analysis, were increased with age in both soleus and epitrochlearis (Epi) muscles. However, much higher activation of these kinases in Epi muscles from young rats compared with soleus results in a greater effect of these kinases on insulin signaling in fast-twitch muscle with age. Heat shock protein (HSP) 72 expression and phosphorylation of HSP25 were higher in soleus compared with Epi muscles, and both parameters decreased with age. Age and fiber type differences in cytochrome oxidase activity are consistent with observed changes in HSP expression and activation. Our results demonstrate a significant difference in the ability of slow-twitch and fast-twitch muscles to respond to insulin and regulate glucose with age. A greater constitutive HSP expression and lower stress kinase activation may account for the ability of slow-twitch muscles to preserve the capacity to respond to insulin and maintain glucose homeostasis with age.

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tyrosine phosphorylation, IRS-1 and IRS-2 bind and activate the enzyme phosphatidylinositol 3-kinase (PI3K). PI3K activation results in serine phosphorylation of Akt/protein kinase B (Akt), and ultimately, stimulation of glucose transport in skeletal muscle and adipose tissue. Previous studies indicate the development of insulin resistance with age occurs downstream of the insulin receptor (2).

IRS-1 is the primary IRS expressed in skeletal muscle and acts as a metabolic switch for the insulin signaling pathway (43). Serine/threonine phosphorylation of IRS-1 can result in suppression of the insulin signaling pathway and increased serine phosphorylation of IRS-1 has been reported in animal models of insulin resistance (46) and in muscles of patients with insulin resistance (5). Previous studies have shown a decrease in tyrosine IRS-1 phosphorylation (11) and a decrease in total IRS-1 protein (2) in aged rats. To our knowledge, only one other study has assessed chronic serine phosphorylation of IRS-1 with age, and they showed an increase in serine IRS-1 phosphorylation in 30-mo-old Brown Norway/Fischer 344 rats compared with 6-mo-old rats (22). These investigators examined the mixed fiber type medial gastrocnemius muscle and therefore could not make any correlations between muscle fiber type-specific insulin signaling and age. Skeletal muscle displays a fiber type-specific response in the activation of key insulin signaling intermediates (41), and given the preferential atrophy of fast-twitch fibers that occurs with age (30), we might expect insulin signaling to be compromised to a greater extent in fast-twitch muscles compared with slow. The present study examined the effect of age on IRS-1 function in fast-twitch and slow-twitch rat skeletal muscles with age.

Obesity-induced insulin resistance is characterized by increased oxidative stress, inflammation, and impaired defense against stress (13, 45). Obesity is linked to a state of chronic inflammation resulting in TNF-α mediated activation of serine threonine kinases, primarily c-Jun NH2-terminal kinase (JNK), inhibitor of kappa B kinase (IKKβ) and PKCθ (45). These serine/threonine kinases phosphorylate IRS-1 on serine residues and suppress the insulin signaling pathway. The important role of JNK and IKKβ in obesity models of insulin resistance is well established, and genetic models that alter JNK and IKKβ expression offer protection against obesity-induced insulin resistance (3, 25). Aging is also characterized by chronic inflammation and oxidative stress (6), yet the extent to which inflammatory mediators affect insulin signaling in aged skeletal muscle is not known.

A decline in the body’s best known endogenous defense system, the heat stress response, could play an important role...
in the development of insulin resistance. Aging, hyperlipidemia, and Type 2 diabetes are all associated with a diminished heat stress response, as measured by decreased expression of heat shock protein 72 (HSP72) (13, 40), and recent studies of individuals with Type 2 diabetes demonstrate that reduced skeletal muscle HSP72 mRNA correlates with the degree of insulin resistance (7, 28). Although expression levels of heat shock protein 25 (HSP25) were not reduced in streptozotocin-induced diabetic rats, the ability to increase HSP25 with heat stress in these animals was attenuated (35). In addition to their chaperonin functions, HSPs have been shown to confer resistance to oxidative stress and inhibit stress kinase activation (18, 37). Consequently, HSP72 expression levels and HSP25 activation may play a significant role in the development of age-related insulin resistance in skeletal muscle. HSPs have also been shown to have a protective effect on mitochondrial function (39) and therefore could have a significant role in preserving muscle oxidative capacity with age. To examine potential mediators of insulin resistance of glucose uptake with age, we measured insulin signaling intermediates, stress kinases, and HSPs in fast-twitch and slow-twitch skeletal muscles from young and aged rats. We hypothesize that with aging, a chronic increase in stress kinase activation, coupled with a decrease in oxidative capacity, leads to insulin resistance in skeletal muscle. Comparing the oxidative soleus muscle and the more glycolytic epitrochlearis (Epi) muscle will allow us to determine the extent to which the muscle’s oxidative capacity impacts the development of age-related insulin resistance.

MATERIALS AND METHODS

Materials. [14C]mannitol was obtained from ICN Radiochemicals (Irvine, CA). 2-Deoxy-[1,2-3H]glucose (2-DG) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Antibodies against phospho-stress-activated protein kinase (SAPK/JNK/T183/Y185), total SAPK/JNK, phospho-Akt (S473), total Akt, IkBα, phospho-GSK-3 α/β (Ser21/9), and phospho-PKC0 (T538) were purchased from Cell Signaling (Beverly, MA). Anti-HSP72, anti-phospho-HSP25 (S82), and anti-HSP25 were obtained from Stressgen (Victoria, BC, Canada), and anti-tubulin was obtained from Sigma. Anti-PKC0 and goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while goat anti-mouse HRP-conjugated secondary antibodies were obtained from Bio-Rad (Hercules, CA). Anti-phospho-Ser107-IRS-1, phospho-Akt substrate of 160 kDa (AS160) (Thr472), and total AS160 antibodies were purchased from Upstate (Lake Placid, NY); anti-phospho-Thr642-IRS-1 was purchased from Biosource (Camarillo, CA); and anti-IRS-1 was purchased from BD Biosciences (Franklin Lakes, NJ). Enhanced chemiluminescence reagents were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). All other reagents were obtained from Sigma.

Experimental animals and muscle dissection. The Fischer 344 rat strain provides a suitable aging model due to its relatively short life span (senescence is reached at 24 mo) (49). In addition, considerable background data exist for this particular strain, including the extensive characterization of age-associated changes under many environmental and genetic conditions. Finally, barrier-reared aging colonies of Fischer 344 rats, maintained under the supervision of the National Institute on Aging, are readily available for purchase from reputable commercial breeders. Male Fischer 344 rats were purchased at 3 (n = 6, body mass 238.50 ± 5.11 g) and 24 (n = 6, body mass 393.0 ± 17.72 g) mo of age via the National Institutes of Health. Rats were housed in pairs in large cages in a temperature-controlled facility (24 ± 1°C) with a 12:12-h light-dark cycle. All animals were given free access to Purina rat chow and water ad libitum. A total of five rats per age group were used in the present study. The rats were fasted for 12 h overnight before the following morning’s experiment to remove effects of endogenous insulin on the experiments. During this time, the normal 12-h dark cycle was maintained. The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight) followed by the removal of the soleus and Epi muscles. Soleus muscles consist predominantly of slow-twitch red fibers (84% type I, 16% type II) (1), while the Epi is composed of primarily fast-twitch white fibers (15% type I, 20% type IIa, 65% type IIb) (36). Soleus muscles were split before incubation to allow adequate diffusion of oxygen and substrates. Despite of the sarcopenia associated with age, due to the greater body weight of the aged rats compared with the young rats, the soleus muscles of the aged rats remained larger than those from the young rats. The slightly larger soleus muscles of aged rats were carefully trimmed to remain comparable in size with those of the young soleus. Muscle wet weight was used to determine equivalent size. The thin Epi muscles are well suited for glucose transport, and the Epi does not significantly increase in thickness with age (10); so the intact Epi is used for all experiments. All protocols were approved by the Animal Care and Use Committee of the University of Kansas Medical Center.

Muscle incubations. Following dissection, muscles recovered for 60 min in flasks containing 2 ml of Krebs-Henseleit bicarbonate buffer (KHB) with 8 mM glucose, 32 mM mannitol (recovery medium), and a gas phase of 95% O2-5% CO2. The flasks were placed in a shaking incubator maintained at 35°C. Following recovery, muscles were either treated with 1 mM insulin or left untreated for 30 min and then clamp frozen in liquid nitrogen. For measurement of basal MAPK and stress kinase phosphorylation, soleus and Epi muscles from young and aged Fischer 344 rats were removed and rapidly frozen for Western blot analysis.

Measurement of glucose transport activity. The muscles were rinsed for 30 min at 29°C in 2 ml of oxygenated KHB containing 40 mM mannitol, with or without insulin. After the rinse step, muscles were incubated for 2 min at 29°C in flasks containing 2 ml KHB with 4 mM 2-DG (1.5 μCi/ml) and 36 mM [14C]mannitol (0.2 μCi/ml), with or without insulin, with a gas phase of 95% O2-5% CO2 in a shaking incubator. The muscles were then blotted, clamp-frozen, and processed for determination of intracellular 2-DG accumulation and extracellular space as described previously (19, 48).

Measurement of cytochrome oxidase activity. Mitochondria were isolated from soleus and Epi muscles as described previously (20). Briefly, muscles were dissected out and homogenized in a mitochon- drial isolation medium (0.32 M sucrose, 2 mM KCl, EDTA, 10 mM Tris base, 0.3% BSA, and 1 mM ATP, pH 7.4). After three low-speed centrifugations (2 at 3,200 rpm and 1 at 11,000 rpm), the final supernatant was layered on a Ficoll gradient (10–7.5%) and subjected to ultracentrifugation at 27,500 rpm for 45 min. The mitochondrial pellet was resuspended in BSA-free isolation buffer. Protein concentrations were determined with a Bradford assay. For the cytochrome oxidase assay, a cuvette containing 50 μg of protein, potassium phosphate buffer (20 mM, pH 7.0), and dodecyl maltoside (20 μl of 10 mg/ml stock solution) was warmed to 30°C for 3 min. The reaction was initiated by addition of 25 μM reduced cytochrome c, which brought the total cuvette volume to 1 ml. The oxidation of the reduced cytochrome c was followed for 2 min at 550 nm on a DU series spectrophotometer (Beckman Coulter, Fullerton, CA). To facilitate calculation of enzyme activity, the maximally oxidized cytochrome c absorbance was determined by adding a few grains of potassium ferricyanide. The activity was calculated and normalized to the amount of protein (per second per milligram of protein).

Western blotting. Clamp-frozen soleus and Epi muscles were homogenized in a 12:1 (volume/weight) ratio of ice-cold buffer from Biosource containing 10 mM Tris·HCl (pH 7.4); 100 mM NaCl; 1 mM each of EDTA, EGTA, NaF, PMSF, and 2 mM Na2VO4; 20 mM Na3P04; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.5% deoxy-
Glucose uptake in isolated soleus and Epi muscles was measured using 2-DG uptake analysis. Figure 2 shows the basal (non-insulin-stimulated) and insulin-stimulated 2-DG uptake. #Significant difference between young Epi and soleus basal glucose uptake, $P < 0.05$. *Significant difference between young and aged Epi basal glucose uptake, $P < 0.05$. **$P < 0.001$.

Statistical analysis. Two-way ANOVA was used when both age and muscle fiber type differences were studied. This was followed by a post hoc comparison using the Student-Newman-Keuls test when necessary. Statistical significance was set at $P < 0.05$.

RESULTS

Insulin-stimulated glucose uptake. Glucose uptake in isolated soleus and Epi muscles was measured using 2-DG uptake analysis. Figure 2 shows the basal (non-insulin-stimulated) and insulin-stimulated 2-DG response in soleus and Epi muscles from young and aged rats. Basal 2-DG uptake was higher in soleus muscles compared with Epi from both young and aged rats. Basal 2-DG uptake was maintained with age in the soleus muscle, whereas a significant decrease in basal glucose uptake was observed in Epi muscles with age (32% decrease from young). To determine the muscle glucose uptake in response to insulin, soleus and Epi muscles were incubated in the presence of 1 mU/ml insulin. As shown in Fig. 2, insulin-stimulated glucose uptake was higher in young soleus compared with young Epi muscles. In aged rats, insulin-stimulated glucose uptake was significantly decreased in both the soleus and Epi muscles. Although the decrease in insulin-stimulated 2-DG uptake with age was slightly greater in the soleus muscles (35% decrease from young) compared with Epi muscles (26% decrease from young), the insulin-stimulated 2-DG uptake remained greater in soleus muscles compared with Epi.

Insulin-stimulated phosphorylation of Akt. Akt is a serine kinase located downstream of IRS-1/PI3K in the insulin signaling pathway. Akt phosphorylation in the absence and presence of 1 mU/ml insulin was measured by Western blot analysis with a phosphospecific antibody that recognizes Akt when phosphorylated on Ser473. Basal, non-insulin-stimulated, Akt phosphorylation levels were similar between soleus and Epi muscles from both young and aged rats (Fig. 3A). The pattern of Akt phosphorylation in response to insulin displayed a similar pattern to the 2-DG transport in soleus and Epi muscles. As shown in Fig. 3A, Akt phosphorylation on Ser473 was increased with insulin to a greater extent in the soleus muscle compared with the Epi in both young and aged muscles. Insulin-stimulated Akt phosphorylation was significantly decreased with age in both soleus and Epi muscles. The relative decrease in Akt phosphorylation with age is greatest in young and aged rats. Basal 2-DG uptake was determined by Bradford method (Bio-Rad, Hercules, CA). Samples were prepared in 2X Laemmli buffer containing 100 mM dithiothreitol and heated in a boiling water bath for 5 min.

The 30–100 μg of protein was subjected to SDS-PAGE (6.25–10% gel) followed by a wet transfer to a nitrocellulose membrane for 90 min (200 mA/tank). Membranes were blocked for 1 h at room temperature in Tris-buffered saline, 0.1% Tween 20 (TBST)-5% nonfat dry milk followed by an overnight incubation with the appropriate primary antibodies at a concentration of 1:1,000. Antibodies were diluted in TBST-5% BSA. Following three brief rinses in TBST, blots were incubated in TBST 1%-nonfat dry milk supplemented with an HRP-conjugated goat anti-rabbit secondary antibody at a concentration of 1:10,000 for 1 h at room temperature. Bands were visualized by enhanced chemiluminescence and quantified using densitometry. To allow direct comparisons between age groups and muscle types, young and aged, soleus and Epi samples were run on the same gel, and identical conditions (including transfer time, washing conditions, antibody dilutions and chemiluminescence detection) were maintained across gels. Equal amounts of protein were loaded onto each gel lane, and to ensure accuracy, protein concentration was determined in triplicate before gel loading. To verify transfer of proteins and equal loading of lanes, the membranes were stained with Ponceau S (Fig. 1). From the Ponceau stain, a protein band was selected at random (~60 kDa) and quantified in each lane via densitometry. This band was directly proportional to the amount of protein loaded, and there was no significant difference in band intensity across soleus and Epi muscles. Blots were stripped and probed for tubulin to further assess equal protein loading and transfer. Tubulin expression did not change with age in soleus and Epi muscles and therefore could not be used as a loading control for soleus and Epi muscle protein comparisons.

Statistical analysis. Two-way ANOVA was used when both age and muscle fiber type differences were studied. This was followed by a post hoc comparison using the Student-Newman-Keuls test when necessary. Statistical significance was set at $P < 0.05$.

RESULTS

Insulin-stimulated glucose uptake. Glucose uptake in isolated soleus and Epi muscles was measured using 2-DG uptake analysis. Figure 2 shows the basal (non-insulin-stimulated) and insulin-stimulated 2-DG response in soleus and Epi muscles from young and aged rats. Basal 2-DG uptake was higher in
Epi muscles (35% decrease from young) compared with soleus muscles (22% decrease from young). Basal and insulin-stimulated total Akt protein expression was measured, and as shown in Fig. 3B, there were no differences in total Akt expression in response to insulin. However, muscle fiber type differences in total Akt expression were observed with soleus muscle Akt expression greater than Epi in young rats. Somewhat surprisingly, there was no decrease in total Akt expression with age in either the soleus or Epi muscles.

Insulin-stimulated phosphorylation of AS160. AS160 is a Rab GTPase-activating protein previously shown to be activated by insulin and muscle contraction, the latter most likely via AMP-activated protein kinase (8, 27). We measured AS160 phosphorylation in the absence and presence of 1 mU/ml insulin by Western blot analysis with a phosphospecific antibody that recognizes AS160 when phosphorylated on Thr\textsuperscript{642}. Basal, non-insulin-stimulated phosphorylation of AS160 did not differ between soleus and Epi muscles (Fig. 3C). Although
basal phosphorylation of AS160 increased slightly with age in the soleus muscle, no significant age differences in basal AS160 phosphorylation were observed in either muscle fiber type ($P < 0.08$ between young and aged basal AS160 phosphorylation). In response to insulin, phosphorylation of AS160 was increased to the same extent in soleus and Epi muscles. An age-related decrease in insulin-stimulated AS160 phosphorylation was observed in both soleus and Epi muscle (37 and 37.5% in soleus and Epi, respectively). Figure 3D demonstrates basal and insulin-stimulated total AS160 protein expression, and similar to Akt, there were no effects of insulin on total protein expression of AS160 in soleus and Epi muscles. Total AS160 protein expression did not differ between soleus and Epi muscles, however, there was a significant effect of age in both muscles. Total AS160 protein expression decreased by 22 and 33% in soleus and Epi muscles, respectively.

**Insulin-stimulated tyrosine and basal serine phosphorylation of IRS-1.** Our observations of a decrease in glucose uptake and phosphorylation of Akt and AS160 with age suggest a defect in insulin signaling. IRS-1 functions as a metabolic switch in skeletal muscle, with tyrosine phosphorylation resulting in downstream activation of PI3K and serine phosphorylation of Akt. To determine whether differences in insulin-stimulated IRS-1 tyrosine phosphorylation exist between young and aged soleus and Epi muscles, muscles were exposed to 1 mU/ml insulin for 30 min. Western blots were performed on muscle homogenates with an antibody that recognizes IRS-1 Tyr612 phosphorylation. Basal, non-insulin-stimulated, tyrosine phosphorylation of IRS-1 is negligible in skeletal muscle, and therefore these data are not shown. Figure 4A demonstrates that insulin-stimulated phosphorylation of IRS-1 at Tyr612 was significantly greater in soleus muscles compared with Epi for both young and aged muscles. Insulin-stimulated tyrosine phosphorylation of IRS-1 was decreased with age in both soleus and Epi muscles (57 and 60% decrease from young, respectively). Serine phosphorylation of IRS-1 inhibits tyrosine IRS-1 phosphorylation, and chronic increased serine phosphorylation could prevent activation of the normal insulin signaling cascade. For this reason, we measured basal, non-insulin-stimulated, serine phosphorylation of IRS-1 to determine the extent to which serine phosphorylation of IRS-1 could impact insulin action. Basal IRS-1 serine phosphorylation was measured with an antibody that recognizes IRS-1 when Ser307 is phosphorylated (Fig. 4B). Serine phosphorylation of IRS-1 was significantly increased with age in both the soleus and Epi muscles. The increase in Ser307 phosphorylation in soleus muscles with age was significantly greater than that observed in the Epi muscles (160 and 56% increase above young, respectively). Our findings also reveal a significant decrease in total IRS-1 levels in both muscles with age (Fig. 4C). Although total IRS-1 expression is greater in soleus compared with Epi in young rats, the relative decrease in IRS-1 expression with age is similar in both muscles (65 and 64% decrease from young, respectively).

**Modulation of serine/threonine kinases.** A number of serine/threonine kinases have been identified with the potential to serine phosphorylate IRS-1, with JNK and IKKβ being the most commonly associated with insulin resistance. We measured basal phosphorylation levels of JNK with Western blot analysis in soleus and Epi muscles from young and aged rats (Fig. 5A). JNK phosphorylation was significantly lower in
soleus muscles compared with Epi muscles in young and aged rats. In both soleus and Epi muscles, JNK phosphorylation increased with age but to a much greater extent in the soleus compared with the Epi (184 and 70%, respectively). Total JNK protein expression was measured, and no significant differences exist between soleus and Epi muscle total JNK expression (Fig. 5B). Total JNK decreased significantly with age only in the Epi muscles. These data demonstrate that, regardless of age, JNK phosphorylation is greater in the fast Epi muscles compared with the soleus.

IKKβ activation results in degradation of the downstream target IκBα, and IκBα degradation is considered a marker of increased IKKβ activity. Figure 5C demonstrates that IκBα expression levels were higher in soleus muscles compared with Epi muscles from both young and aged rats, indicating a lower level of activation of IKKβ in soleus. With age, a decrease in IκBα protein levels occurred in both soleus and Epi muscles, with a greater degradation in the Epi (27 and 40% decrease, respectively).

We measured phosphorylation of GSK-3β on Ser9, a measure inversely related to GSK-3 activity (17). As previously shown with JNK and IKKβ activation, GSK-3β activation is lower in the slow soleus muscle, indicated by increased GSK-3β phosphorylation in young and aged soleus compared with young and aged Epi muscle (Fig. 5D). Phosphorylation of GSK-3β on Ser9 is decreased with age in both soleus and Epi muscles to a similar extent (34 and 31% decrease, respectively). Total GSK-3β protein expression is dramatically higher in soleus muscle compared with Epi in both young and aged rats (Fig. 5E). GSK-3β protein expression did not change with age in either muscle type. Phospho-PKCβ levels were similar across soleus and EPI muscles from both young and aged rats (data not shown). There was no change in PKCβ expression with age in either muscle type.

Modulation of HSPs. In the present study, HSP72 expression was significantly greater in soleus muscles compared with Epi muscles from both young and aged rats (Fig. 6A). This is consistent with other results indicating HSP72 expression correlates with muscle oxidative capacity (31). HSP72 expression was significantly reduced in both soleus and Epi muscles with age, although to a greater degree in the Epi (10 and 41% decrease, respectively). The phosphorylation state of HSP25 is thought to play an important role in modifying its function (26). HSP25 is phosphorylated by MAPK-activated protein kinase 2 (MAPKAPK2), a kinase downstream of p38 MAPK (42). Similar to our findings for HSP72 expression, phosphorylation of HSP25 was greater in soleus muscle compared with Epi from both young and aged rats (Fig. 6B). Phosphorylation

Fig. 5. Modulation of serine/threonine kinases in young and aged muscles. Soleus and Epi muscles from young and aged rats were dissected, clamp frozen, and homogenized. Lysates were analyzed by Western blot for JNK phosphorylation (p-JNK; A), and total JNK (B), inhibitor I kappa B alpha (IκBα; C), and phosphorylation of GSK-3β (p-GSK-3β; D) and total GSK-3β (E). Filled bars, young muscle; open bars, aged muscle. Values are means ± SE for 4–6 muscles per group. *P < 0.05. **P < 0.001.
of HSP25 decreased with age in both soleus and Epi muscles to the same extent (32 and 29% in soleus and Epi, respectively). Total HSP25 expression was greater in the soleus muscle compared with Epi from young rats and aged rats (Fig. 6C) but there was no significant change with age in either muscle. This supports previous findings of greater HSP25 expression in oxidative compared with glycolytic muscle (26).

**Cytochrome oxidase activity.** Cytochrome oxidase activity was measured in soleus and Epi muscle homogenates (Fig. 7). Enzyme activities in soleus muscle were 48% higher than Epi muscle from both young and aged rats. Cytochrome oxidase activity decreased with age in both soleus and Epi muscles by ~15%. A decrease in cytochrome oxidase activity is indicative of a reduced mitochondrial content per milligram of muscle.

**DISCUSSION**

The purpose of this study was to determine the role of stress kinases and HSPs in mediating age-related insulin resistance. By comparing the slow-twitch oxidative soleus muscle and the fast-twitch glycolytic Epi muscle, we assessed the impact of muscle insulin and oxidative capacity on the development of insulin resistance with age. Our results demonstrate that decreased insulin-stimulated glucose uptake in soleus and Epi muscles occurs primarily due to decreased total protein expression and increased serine phosphorylation of IRS-1. However, the combination of higher stress kinase activation and lower HSP expression in the Epi muscle compared with the soleus muscle, results in fast-twitch muscle being more susceptible to a decline in insulin action with age. Examination of the mechanisms through which slow-twitch muscles preserve the capacity to respond to insulin and maintain glucose homeostasis with age could have implications for future treatment of insulin resistance and diabetes. Insulin-stimulated glucose uptake was greater in young soleus compared with young Epi muscles, consistent with previous studies indicating insulin-stimulated glucose transport activity is positively correlated with the percentage of oxidative muscle fibers (24, 50). Similarly, insulin signaling intermediates are present and activated
to a greater extent in oxidative muscle fibers (41). Our present findings demonstrating greater phospho-Akt, phospho-tyrosine IRS-1, and total IRS-1 in the oxidative soleus muscle compared with the Epi muscle agree with these previous studies. Oxidative muscles appear to have a greater reserve for insulin signaling and glucose uptake that is not utilized under normal conditions. However, the aging condition may be one in which the reserve capacity for insulin signaling in slow muscles serves a valuable purpose. In the present study, although insulin-stimulated glucose transport decreased significantly with age in both soleus and Epi muscles, glucose transport in aged soleus muscles was still greater than that seen in young Epi muscles. This occurred despite a similar decrease in tyrosine IRS-1 phosphorylation in both soleus and Epi muscles with age, and an even greater increase in serine IRS-1 phosphorylation in soleus muscles with age compared with Epi muscles. The decrease in basal glucose uptake observed in Epi muscles with age further demonstrates the decline in glucose homeostasis that occurs in fast-twitch muscle. In a previous study by Cartee et al. (10), Epi muscles from 25-mo-old male Fischer 344/Brown Norway hybrid rats demonstrated a pattern of lower basal 3-methyl glucose uptake values compared with 3.5-mo-old rats, although the differences were not statistically significant. We did not measure the levels of glucose transporters in the present study; however, a decrease in basal glucose uptake with age could result from decreased GLUT1 protein expression (9). Similarly, a decrease in GLUT4 protein expression with age as previously reported (10) could also contribute to the observed decrease in insulin-stimulated glucose uptake with age in this study. Interestingly, fiber type differences in insulin-stimulated phosphorylation of AS160 were not observed in the present study. Although AS160 phosphorylation decreased with age in both muscles in a manner similar to that seen with Akt, muscle fiber type differences in insulin signaling are not maintained downstream of Akt. The lack of a fiber type difference in total AS160 between soleus and Epi muscles potentially allows for a greater insulin-stimulated phosphorylation of AS160 in Epi muscles. These findings indicate fiber type differences in insulin-stimulated glucose uptake occur upstream of AS160. Results from the present study reveal a significant decrease in total IRS-1 levels in soleus muscle from aged rats compared with young rats (Fig. 4C). Whereas serine phosphorylation of IRS-1 is often thought of as a short-term inhibitory mechanism, degradation of IRS-1 protein could result from long-term insulin resistance. Cellular and oxidative stress and proinflammatory cytokines can induce the degradation of IRS-1 through proteasome-dependent or -independent processes (21). Serine phosphorylation of IRS proteins is considered the primary means of suppressing IRS-1 activity and contributing to insulin resistance (45–47). Previous studies have shown a decrease in tyrosine IRS-1 phosphorylation (11), a decrease in total IRS-1 protein (2), and no change in Akt in aged rats, findings consistent with our results. Although not measured in the present study, Arias et al. (2) previously demonstrated that muscle levels of insulin receptor and PI3K were increased in aged rats, a finding that would suggest these proteins are not involved in age-related insulin resistance. These studies measured a mixture of hindlimb muscles (11) and exclusively plantaris (2) or Epi muscle (10) and thus could not assess muscle fiber type differences in insulin signaling with age. Haddad et al. (22) showed an increase in serine IRS-1 phosphorylation with age in the medial gastrocnemius muscle from 30-mo-old Fischer/Brown Norway rats. Our findings are the first to show an increase in serine IRS-1 phosphorylation in both slow-twitch and fast-twitch skeletal muscle with age.

There are a number of serine/threonine kinases capable of inducing serine IRS-1 phosphorylation, and we demonstrated several that are increased with age in the present study. Recent investigations of oxidative stress and inflammatory pathways on insulin signaling have focused on obesity and genetic models of insulin resistance. This has resulted in a gap in the literature concerning the primary inflammatory mediators of age-related insulin resistance. Our findings suggest that JNK, IKKβ, and GSK-3β could play a role in the development of insulin resistance in aged skeletal muscle. Although increased activation of PKCθ and extracellular regulated kinase (ERK) have been implicated in other animal models of insulin resistance, findings from our laboratory indicate these serine/threonine kinases are not increased in skeletal muscles from aged rats (data not shown). As PKCθ has been primarily associated with insulin resistance in the presence of increased lipid availability (45), the lack of an increase in PKCθ in aged, insulin-resistant skeletal muscle potentially differentiates the development of insulin resistance in aging and obesity models of insulin resistance. Future studies designed to inhibit inflammatory-mediated pathways in aging skeletal muscle could profoundly impact the prevalence of insulin resistance and diabetes in this population.

Results from this study are the first to characterize these potential mediators of insulin resistance in fast-twitch and slow-twitch skeletal muscles with age. A previous study demonstrated an increase in basal JNK phosphorylation in soleus and the fast-twitch extensor digitorum longus (EDL) muscles from 30-mo-old and 36-mo-old compared with 6-mo-old Fischer 344/Brown Norway rats (33). However, these investigators did not compare JNK activation or expression levels between the two muscle types. In the present study, we show dramatically greater phosphorylation levels of JNK in Epi muscles compared with soleus (Fig. 5A), suggesting JNK may play an important inhibitory role in Epi muscle insulin action. In our findings, IKKβ (as measured by increased degradation of IkBα) and GSK-3 follow this same pattern of increased activation in fast-twitch compared with slow-twitch muscle, further emphasizing the role these inhibitory stress kinases may play in regulating insulin signaling in fast-twitch muscle. Atherton et al. (4) support our findings of greater GSK-3β expression in soleus muscles compared with the fast-twitch EDL, although these investigators did not measure Ser9 phosphorylation as an estimate of GSK-3 activation in these muscles. Dokken et al. (17) measured GSK-3β Ser9 phosphorylation in soleus and Epi muscles from lean and obese Zucker rats. The results of this study showed a greater GSK-3 activity in obese soleus compared with obese Epi muscles from Zucker rats. These findings along with a study of the spontaneous nonobese model of Goto-Kakizaki rats suggest insulin resistance is more pronounced in skeletal muscle composed predominantly of oxidative fibers (17, 41). Our findings in an aging model suggest a different fiber type effect with a greater impact of insulin resistance on fast-twitch glycolytic muscle. One possible explanation for divergent fiber type–specific effects of insulin resistance with aging and obesity models could be due to age- and obesity-specific metabolic changes that occur in skeletal muscle. A greater impact on insulin signaling in fast-twitch muscle in the aging model could be
due to the greater sarcopenia, muscle atrophy (30), and decrease in expression of insulin signaling intermediates that occurs with age in fast-twitch muscle (2, 11). In contrast, a theory of obesity-induced insulin resistance suggests the metabolic overload and influx of fatty acids that occurs in skeletal muscle with overnutrition results in an increase in β-oxidation without a subsequent increase in tricarboxylic acid cycle flux (32). This dysregulation results in accumulation of metabolic by-products of incomplete fat oxidation (acylcarnitines, reactive oxygen species) in the mitochondria. Given the greater mitochondrial content of oxidative muscle fibers, this lipid stress could have a greater impact on oxidative fibers compared with glycolytic fibers.

Our findings regarding fiber type differences in HSP72 and HSP25 expression support what has been previously shown in the literature, with more oxidative fiber types expressing higher levels of HSPs (26, 31). Previous studies demonstrate that skeletal muscle HSP72 expression correlates with the degree of insulin resistance (7, 28) and this is also supported by our data demonstrating a decrease in HSP expression in aged, insulin resistant muscle. The HSP72 content in skeletal muscle has previously been shown to decrease with age in soleus muscle from male Fischer 344 rats (40) and to remain unchanged in the soleus and plantaris muscles of aged female Fischer 344 rats (34). The lack of a decrease in HSP72 with age in the study by Naito et al. (34) could be due to the ability of estrogen to upregulate HSP72 as previously shown in female rat skeletal muscle (44). Although few studies have examined fiber type-specific changes in HSP25 with age, HSP25 expression increased with age in rat mixed gastrocnemius muscle (14) and decreased with age in rat cardiac muscle (15). The downregulation of HSP25 that occurs with unloading suggests activity plays a role in regulation of HSP25 expression in skeletal muscle (29), a theory that is consistent with an age-related decrease in HSP25 phosphorylation shown here.

Previous studies have demonstrated a role for HSPs in protecting mitochondria from oxidative stress (39) and a correlation between HSP72 mRNA expression and mitochondrial enzyme activity (7). Our findings of age and fiber type differences in cytochrome oxidase activity are consistent with observed changes in HSP expression and activation. Muscle fiber type differences in cytochrome oxidase activity demonstrated in the present study are consistent with previous findings. Chabi et al. (12) recently reported that soleus muscle cytochrome oxidase activity was ~40% higher than the fast-twitch plantaris muscle. In addition, they reported a 30% decrease in cytochrome oxidase activity in 36-mo-old male Fischer 344/Brown Norway hybrids compared with 6-mo-old rats. Different muscle and animal strain account for the slight difference in age effect; however, the pattern of cytochrome oxidase changes is evident. Future studies are needed to determine the exact mechanism by which HSPs could protect mitochondria from oxidative damage.

Although the reason for a decrease in HSP expression with age and insulin resistance is not known, it appears that stress kinase activation may play a role. For example, GSK-3, and the MAPKs, ERK and JNK, are known to negatively regulate the primary HSP transcription factor, heat shock factor-1 (HSF-1). Phosphorylation of HSF-1 by GSK-3, ERK, and JNK on serine residues 303, 307, and 363, respectively (16, 23), holds HSF-1 in an inactive state under normal physiological growth conditions. Consequently, overactivity of stress kinases capable of phosphorylating HSF-1 on serine residues may repress HSF-1 activation, and subsequent HSP72 transcription and HSP25 activation, in insulin-resistant tissue.

Induction of HSPs may serve as a powerful tool for preventing insulin resistance with age. A recent publication demonstrated, for the first time, that increased HSP72 expression protects against obesity-induced insulin resistance (13). In the study by Chung et al. (13), heat shock therapy, as well as transgenic and pharmacological means of overexpressing HSP72, all resulted in improved glucose tolerance and insulin action in skeletal muscle. In all cases, this corresponded with a decrease in JNK phosphorylation. It has been proposed that HSP72 binds to JNK, preventing activation of JNK by upstream kinases SEK1 and MKK7 (37). Similar studies revealed that HSP72 inhibits IKKβ, either by direct binding or through an, as yet, uncharacterized signaling intermediate (38). This natural inhibitory effect of HSP72 on JNK, and of HSP25 on IKKβ, could serve as a powerful tool for targeting stress kinase inhibition in the treatment of skeletal muscle insulin resistance. The ability of HSPs to protect against age-related insulin resistance has not yet been tested. In conclusion, our results demonstrate a significant difference in the ability of slow-twitch and fast-twitch muscles to respond to insulin and regulate glucose with age. Although a decrease in insulin signaling and glucose uptake occur in both slow-twitch soleus muscles and fast-twitch Epi muscles with age, the greater reserve capacity of the slow-twitch soleus muscle results in a far smaller decrement in insulin capacity in this muscle with age. Our findings suggest the proportionately lower activation of JNK, GSK-3β, degradation of IkB by IKKβ, and greater expression and phosphorylation of HSP72 and HSP25 play a role in protecting slow-twitch muscle from further insulin resistance with age. Future studies are needed to examine the relationship between HSP expression and stress kinase activation and the impact of these pathways on insulin signaling in skeletal muscle.

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