Age-related differences in skeletal muscle insulin signaling:

The role of stress kinases and heat shock proteins

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Running title: Age-related differences in insulin signaling
Abstract

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 month-old) and young (3 month-old) Fischer 344 rats, 2-deoxy glucose uptake and insulin signaling (as measured by phosphorylation of IRS-1, Akt and AS160) decreased significantly with age. Activation of c-Jun NH2-terminal kinase (JNK), glycogen serine kinase-3β (GSK-3β), and degradation of IκBα by the upstream inhibitor of kappa B kinase (IKKβ), as measured by Western blot analysis, were increased with age in both soleus and Epi muscles. However, much higher activation of these kinases in Epi muscles from young rats compared to soleus results in a greater effect of these kinases on insulin signaling in fast-twitch muscle with age. Heat shock protein 72 (HSP72) expression and phosphorylation of HSP25 were higher in soleus compared to 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.

KEY WORDS: glucose uptake; IRS-1 serine phosphorylation; stress kinases, HSP72, HSP25
Introduction

Aging is associated with a decrease in the body’s ability to respond to the hormone insulin, a condition referred to as insulin resistance. Insulin resistance represents an independent risk factor in age-related diseases and insulin resistance of skeletal muscle glucose transport represents a key defect in the development of type 2 diabetes. The underlying mechanism for age-related insulin resistance remains poorly defined, yet increased oxidative stress, inflammation and impaired defense against stress are likely involved.

The initial step in the insulin-signaling pathway is binding of insulin to its receptor at the cell surface of tissue such as muscle, fat or liver. Insulin binding stimulates the intrinsic tyrosine kinase activity of the insulin receptor resulting in the tyrosine phosphorylation of its cytosolic substrate, insulin receptor substrate-1 and -2 (IRS1 and IRS2) (47). After 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 month-old Brown Norway/Fischer 344 rats compared to 6 month-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 to slow. The current 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 PKC0 (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. In order 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 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

$^{14}$C Mannitol was obtained from ICN Radiochemicals (Irvine, CA). 2-Deoxy $^{[1,2-3]}$H glucose was purchased from American Radiolabeled Chemicals (St. Louis, MO). Antibodies against phospho-SAPK/JNK(T183/Y185), total SAPK/JNK, phospho-Akt (S473), total Akt, IκBα, phospho-GSK-3 α/β (Ser21/9), and phospho-PKCθ (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 (St.Louis, MO). Anti-PKCθ and goat-anti-rabbit 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-Ser307-IRS-1, phospho-AS160 (Thr 642) and total AS160 antibodies were purchased from Upstate (Lake Placid, NY), anti-phospho-Tyr612-IRS-1 from Biosource (Camarillo, CA) and anti-IRS-1 was from BD Biosciences (Franklin lakes, NJ). Enhanced chemiluminescence reagents were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). All other reagents were obtained from Sigma (St.Louis, MO).

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 months) (49). In addition, considerable background data exists 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 NIA, 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) months 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 h light/dark cycle. All animals were given free access to Purina Rat Chow and water ad libitum. A total of 5 rats per age group were used in the current study. The rats were fasted for 12 h overnight prior to 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 epitrochlearis (Epi) muscles. Soleus muscles consist predominantly of slow-twitch red fibers (84% type I, 16% type II) (1), while the Epi is comprised of primarily fast-twitch white fibers (15% type I, 20% type IIa, 65% type IIb) (36). Soleus muscles were split prior to incubation to allow adequate diffusion of oxygen and substrates. In spite of the sarcopenia associated with age, due to the greater body weight of the aged rats compared to 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% O$_2$/5% CO$_2$. The flasks were placed in a shaking incubator maintained at 35°C. Following recovery, muscles were either treated with 1 mU/ml insulin or left untreated for 30 min and then clamp frozen in liquid nitrogen. For measurement of basal MAP and stress kinase phosphorylation, soleus and Epi muscles from young and aged F344 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 20 min at 29°C in flasks containing 2 ml KHB with 4 mM 2[1,2-$_3^3$H] deoxyglucose (2-DG) (1.5 µCi/ml) and 36 mM [$_{14}$C] mannitol (0.2 µCi/ml), with or without insulin, with a gas phase of 95% O$_2$/5% CO$_2$, 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 epitroclearis muscles as described previously (20). Briefly, muscles were dissected out and homogenized in a mitochondria isolation medium (0.32 M sucrose, 2 mM K\(^+\) EDTA, 10 mM Tris Base, 0.3 % BSA, 1 mM ATP, pH 7.4). After 3 low speed centrifugations (two at 3200 rpm and one 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 three 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 (seconds\(^{-1}\) per milligram of protein).

**Western Blotting**

Clamp frozen soleus and Epi muscles were homogenized in a 12:1 (volume to weight) ratio of ice cold buffer from Biosource containing: 10 mM Tris-HCL (pH 7.4), 100 mM NaCl, 1mM each of EDTA, EGTA, NaF, PMSF and 2 mM Na\(_3\)VO\(_4\), 20 mM Na\(_4\)P\(_2\)O\(_7\), 1% Triton X-100, 10% glycerol, 0.1 %SDS, 0.5% deoxycholate, 250 µl/5ml protease inhibitor cocktail. Homogenized samples were rotated for 30 minutes and then centrifuged for 20 minutes at 13,000 rpm at 4°C. The protein concentration of the supernatant was determined by Bradford method (Bio-Rad, Hercules, CA). Samples were prepared in 2 X Laemmli buffer containing 100 mM dithiothreitol and heated in a boiling water bath for 5 minutes.

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 hour at room temperature in TBST 5 % non-fat dry milk followed by an overnight incubation with the appropriate primary antibodies at a
concentration of 1:1000. Antibodies were diluted in Tris-buffered saline, 0.1% Tween-20 (TBST), 5% BSA. Following 3 brief rinses in TBST, blots were incubated in TBST 1% non-fat dry milk supplemented with an HRP-conjugated goat anti-rabbit secondary antibody at a concentration of 1:10,000 for 1 hour at room temperature. Bands were visualized by ECL 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 prior to gel loading. To verify transfer of proteins and equal loading of lanes, the membranes were stained with ponceau S (Figure 1). From the ponceau stain, a protein band was selected at random (~60kDa) 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 does not change with age in soleus and Epi muscles, making this an accurate loading control for age comparisons within each muscle. However, tubulin expression did differ between 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 analysis of variance (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 to Epi from both young and aged rats. Basal 2-DG uptake was maintained with age in the soleus muscle, while 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 Figure 2, insulin-stimulated glucose uptake was higher in young soleus compared to 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 to Epi muscles (26% decrease from young), the insulin-stimulated 2-DG uptake remained greater in soleus muscles compared to 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 Ser 473. Basal, non-insulin stimulated, Akt phosphorylation levels were similar between soleus and Epi muscles from both young and aged rats (Figure 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 Figure 3A, Akt phosphorylation on Ser 473 was increased with insulin to a greater extent in the soleus muscle compared to 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 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 Figure 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**

Akt substrate of 160 kDa (AS160) is a Rab GTPase-activating protein previously shown to be activated by insulin and muscle contraction, the latter most likely via AMPK (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 Thr642. Basal, non-insulin stimulated, phosphorylation of AS160 did not differ between soleus and Epi muscles (Figure 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 tyrosine 612 phosphorylation. Basal, non-insulin stimulated, tyrosine phosphorylation of IRS-1 is negligible in skeletal muscle and therefore this data is not shown. Figure 4A demonstrates that insulin-stimulated phosphorylation of IRS-1 at tyrosine 612 was
significantly greater in soleus muscles compared to 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 serine 307 is phosphorylated (Figure 4B). Serine
phosphorylation of IRS-1 was significantly increased with age in both the soleus and Epi muscles. The increase
in serine 307 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 (Figure 4C). Although total IRS-1 expression is greater in soleus
compared to 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
(Figure 5A). JNK phosphorylation was significantly lower in soleus muscles compared to 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 to 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 (Figure 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 to 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 to Epi muscles from both young and aged rats, indicating a lower level of activation of IKKβ
in soleus. With age, a decrease in IkBα 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 serine 9, a measure inversely related to GSK-3 activity (17). As previously shown with JNK and IKKβ activation, GSK-3 β 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 (Figure 5D). Phosphorylation of GSK-3β on serine 9 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 to Epi in both young and aged rats (Figure 5E). GSK-3β protein expression did not change with age in either muscle type. Phospho-PKC0 levels were similar across soleus and EPI muscles from both young and aged rats (data not shown). There was no change in PKC0 expression with age in either muscle type.

**Modulation of HSPs**

In the present study, HSP72 expression was significantly greater in soleus muscles compared to Epi muscles from both young and aged rats (Figure 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 MAPKAPK2, a kinase downstream of p38MAPK (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 (Figure 6B). Phosphorylation 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 to Epi from young rats and aged rats (Figure 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 (Figure 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 approximately 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 to 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 to 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 current 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 in spite of 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 to 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 month-old male Fischer 344/Brown Norway hybrid rats demonstrated a pattern of lower basal 3-methyl glucose uptake values compared to 3.5 month-old rats, although the differences were not statistically significant. We did not measure
the levels of glucose transporters in the current 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 (Figure 4C). While 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 (IR) 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 month-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 current 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 month-old and 36 month-old compared with 6 month-old Fischer 344/Borwn Norway rats (33). However these investigators did not compare JNK activation or expression levels between the two muscle types. In the current study, we show dramatically greater phosphorylation levels of JNK in Epi muscles compared to soleus (Figure 5A), suggesting JNK may play an important inhibitory role in Epi muscle insulin action. In our findings, IKKβ (as measured by increased degradation of IκBα) and GSK-3 follow this same pattern of increased activation in fast-twitch compared to 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 to the fast-twitch EDL, although these investigators did not measure serine 9 phosphorylation as an estimate of GSK-3 activation in these muscles. Dokken et al. (17) measured GSK-3β serine 9 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 to obese Epi muscles from Zucker rats. These findings along with a study of the spontaneous nonobese model of GK 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, ROS) in the mitochondria. Given the greater mitochondrial content of oxidative muscle fibers, this lipid stress could have a greater impact on oxidative fibers compared to 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). While 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 HSP72mRNA 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 approximately 40% higher than the fast-twitch plantaris muscle. In addition, they reported a 30% decrease in cytochrome oxidase activity in 36-month-old male Fischer 344 Brown Norway hybrids as compared to 6-month-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 MAP kinases, ERK and JNK, are known to negatively regulate the primary HSP transcription factor, 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 pharmacologic 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. While a decrease in insulin signaling and glucose uptake occurs 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 IκBα 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.
Acknowledgements

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41. Song XM, Ryder JW, Kawano Y, Chibalin AV, Krook A, and Zierath JR. Muscle fiber type specificity


Figure legends:

**Figure 1. Western blot loading controls.** Samples from both young and aged soleus and Epi muscles were run together on gels to allow comparison across groups. Following the ponceau S stain, a protein band was selected at random (~60kDa) 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 does not change with age in soleus and Epi muscles, making this an accurate loading control for age comparisons and further verifying equal loading.

**Figure 2. Insulin stimulated glucose transport in muscles from young and aged rats:** Young (3 mo) and aged (24 mo) F344 rats were fasted overnight and the soleus and Epi muscles were dissected out. Muscles recovered in KHB for 1 hr and were then incubated without (open bars) or with (filled bars) insulin (1 mU/ml) in flasks containing KHB with 2[1,2-3H]-deoxy glucose (1.5 µCi/ml) and [14C] mannitol. Insulin stimulation resulted in a significant increase from basal glucose uptake for all muscles shown (P<0.005). Intracellular 2-DG and extracellular space were quantified in a scintillation counter. Values are means of ± SE for muscles from 10-14 muscles per group. Symbols for basal 2-DG values, open bars: Significance between young Epi and soleus basal glucose uptake #P<0.05. Significance between young and aged Epi basal glucose uptake +P<0.05. Symbols for insulin-stimulated glucose uptake, filled bars: *P<0.05, **P<0.001.

**Figure 3. Effect of insulin on phosphorylation of Akt and AS160 in young and aged muscles:** Soleus and Epi muscles were incubated in the absence (open bars) or presence (filled bars) of insulin (1 mU/ml) for exactly 10 min. Homogenates of clamp frozen muscles were used for measurements of phosphorylation of Akt on serine 437 (A), total Akt (B), phosphorylation of AS160 (C), and total AS160 (D), with Western blot analysis. Insulin stimulation resulted in a significant increase from basal phosphorylation of Akt and AS160 in all muscles shown (P<0.05). The effect of insulin on total Akt and AS160 values was not significant. Values are means of ± SE for
4-9 muscles per group. *P<0.05 and **P<0.001.

Figure 4. Insulin-stimulated tyrosine and basal serine phosphorylation of IRS-1 in young and aged muscles: Soleus and Epi muscles were dissected from overnight fasted rats and incubated with insulin (1 mU/ml) for 30 min and muscle homogenates were then used to determine the insulin-stimulated tyrosine phosphorylation of IRS-1 (A). Alternatively, muscles were incubated in KHB without insulin to assess basal serine phosphorylation of IRS-1 (B), and total IRS-1 (C). Filled bars represent young muscle values and open bars represent aged muscles values. Values are means of ± SE for 4-12 muscles per group. *P<0.05 ** P<0.001.

Figure 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 (A), and total JNK (B), inhibitor I kappa B alpha (IkBα, C), and phosphorylation of GSK-3β (D) and total GSK-3β (E). Filled bars represent young muscle values and open bars represent aged muscles values. Values are means of ± SE for 4-6 muscles per group. *P<0.05 ** P<0.001.

Figure 6. Heat shock protein expression 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 HSP72 (A), phosphorylation of HSP25 (B), and total HSP25 (C). Filled bars represent young muscle values and open bars represent aged muscles values. Values are means of ± SE for 8-15 muscles per group. *P<0.05 ** P<0.001.

Figure 7. Cytochrome oxidase enzyme activity in young and aged muscles. Mitochondria were isolated from soleus and Epi muscles from young and aged rats. Cytochrome oxidase activity was determined spectrophotometrically. Filled bars represent young muscle values and open bars represent aged muscles values. *P<0.05 ** P<0.001.
FIGURE 1

Soleus

Young
Aged

EPI

Young
Aged

Ponceau stain

Tubulin

-- 60 KDa

-- 55 KDa
FIGURE 2

2-Deoxy Glucose Uptake (μmol/ml/20min)

Young
Aged
Young
Aged
Soleus
EPI

**
**
*
#
+

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FIGURE 3

A. Insulin

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Insulin - + - + - + - +

pAkt

[Graph showing pAkt levels with * and ** indicating significance]

B. Insulin

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Insulin - + - + - + - +

Akt

[Graph showing Akt levels with * indicating significance]
C.

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<td>p-AS160</td>
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- 160 KDa

D.

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<td>AS160</td>
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- 160 KDa
FIGURE 4

A. pTyr612-IRS-1

B. pSer307 IRS-1

C. IRS-1
FIGURE 5

A.

B.

C.
D. Young Aged Young Aged

pGSK3 beta--

GSK3 beta--

E. Young Aged Young Aged

Total GSK 3β

Soleus EPI

P-pGSK3β

Soleus EPI

0.0 0.2 0.4 0.6 0.8 1.0 1.2

0.0 0.2 0.4 0.6 0.8 1.0

** *
FIGURE 6

A.

HSP72 - 72KDa

Young
Aged
Young
Aged

Soleus
EPI

HSP70

0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6

Soleus
EPI

B. & C.

p-HSP25 - 25 KDa

Young
Aged
Young
Aged

p-HSP25

0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8

Soleus
EPI

Total HSP25

0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8

Soleus
EPI