Exercise Training Eliminates Age-Related Differences in Skeletal Muscle Insulin Receptor and IRS-1 Abundance in Rats

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Insulin resistance is common in old age, and exercise training can improve insulin sensitivity. The purpose of this study was to determine the influence of age (6 vs 26 months) and exercise training (10 weeks of treadmill running) on insulin signaling protein abundance in skeletal muscle from male Fisher 344 rats. Muscle levels of insulin receptor (IR), insulin receptor substrate-1 (IRS-1), phosphatidylinositol 3-kinase (PI3K), and Akt1, a serine–threonine kinase, were determined. IRS-1 was reduced with aging, IR and PI3K abundance was greater in old rats, and Akt1 was unchanged. IRS-1 was increased by training in old but not young rats, and IR was increased by training in young but not old rats. PI3K tended to increase and Akt1 did not change with training, regardless of age. Aging does not uniformly affect insulin signaling protein abundance, and exercise differentially alters IR and IRS-1 in young and old rats, thereby eliminating age-related differences in these proteins.

A DVANCING age is often associated with insulin resistance (1,2). This outcome may not, however, be inevitable, and it is clearly influenced by aspects of life style, including physical activity (3). Skeletal muscle, an important target tissue for insulin, is highly responsive to exercise-induced adaptations, for example the increased abundance and activity of enzymes in the Krebs cycle (4,5). In addition, at least in muscles from young animals, exercise training can influence skeletal muscle proteins that are involved in insulin signaling and action (6).

In recent years, much has been revealed about the signaling pathway that links insulin to its multiple actions. The first step in the insulin signaling pathway is insulin's binding to its receptor, followed by receptor autophosphorylation on specific tyrosines, which in turn activates the receptor's tyrosine kinase to phosphorylate downstream insulin receptor substrate (IRS) proteins (7). IRS-1 is the predominant IRS protein expressed by skeletal muscle (8-11). Phosphorylation of multiple tyrosine residues on IRS proteins is likened to a "molecular switch" that turns on insulin action: tyrosinephosphorylated motifs within IRS proteins serve as docking sites for binding to downstream signaling proteins that possess SH2 domains (8). One of these downstream proteins, phosphatidylinositol 3-kinase (PI3K), is a heterodimer consisting of a regulatory subunit and a catalytic subunit (12). The PI3K regulatory subunit engages specific, tyrosinephosphorylated motifs of IRS, resulting in enhanced PI3K enzyme activity. A serine-threonine kinase known as Akt (also known as protein kinase B, or PKB) is an important signaling protein that is downstream of PI3K (13,14).

Several studies have addressed the influence of age on insulin binding in skeletal muscle (15–17), but only a few have ad-

dressed age-related changes in the expression of insulin signaling proteins (18,19). Prior to this study, to our knowledge, postreceptor signaling protein abundance in skeletal muscle had not been reported for rats older than 18–20 months of age.

Many studies have indicated that exercise training can enhance insulin sensitivity for glucose disposal by skeletal muscle (20,21). Exercise-induced enhancement of glucose disposal is preserved in 25-month-old rats (22). Recently, investigators have evaluated the effect of exercise training on the abundance of insulin signaling proteins, including the insulin receptor, IRS-1, PI3K, and Akt, in the muscle of young and adult rats (6,23). Previous research with young rats (that were approximately 4-27 weeks old) has indicated that exercise training can result in increased insulin receptor (6) and PI3K (23) abundance in skeletal muscle. Differing results (no change, increased, or decreased levels) have been reported for skeletal muscle IRS-1 abundance after training (6,23). Chibalin and colleagues (6) found no change in Akt levels in muscle after exercise training. However, apparently no studies have been published addressing the effects of exercise training on the abundance of insulin signaling proteins in rats older than 5 months of age. Accordingly, the goal of this study was to determine the influence of exercise training on the abundance of several key insulin signaling proteins (insulin receptor, IRS-1, PI3K, and Akt1) in skeletal muscle from adult (6 months) and old (26 months) rats.

METHODS

Materials

Pure nitrocellulose membranes (Osmonics, Inc.) were purchased from Fisher Scientific (Itasca, IL). Anti-IRS-1

(06-248) and anti-PI3K p85 (06-497) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and anti- β -insulin receptor (sc-711) and anti-Akt1 (sc-1618) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Enhanced chemiluminescence (ECL) kits were purchased from Amersham Pharmacia Biotechnology (Piscataway, NJ). Total protein concentrations were performed by using the bicinchoninic acid method (BCA; Pierce, Rockford, IL), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Treatment of Rats

Young (age 3 months) and old (age 23 months) male Fischer 344 rats were obtained from the National Institute on Aging (Bethesda, MD) and housed two per cage. The animals were maintained on a 12:12 (hour) light–dark cycle and provided with Purina rat chow and water ad libitum. Upon arrival at the animal facility at the University of Buffalo, the animals were acclimated to their surroundings for 5 days (24).

Young and old rats were randomly assigned to either untrained controls (young untrained or YUT, n = 9; old untrained or OUT, n = 8) or exercise-trained animals (young trained or YT, n = 8; old trained or OT, n = 7). The training protocol was similar to that previously described (24). Animals were familiarized to treadmill walking (8–10 m/min) over a 1-week period. Following this period, the rats were weight matched and randomly assigned to untrained controls or exercise-trained groups. The exercise-trained groups were subjected to a 10-week progressive training regimen, ultimately running at speeds corresponding to \sim 70% of maximal oxygen consumption for 45 min/day, 5 days/week (25). During the final 4 weeks of training, YT and OT rats were running 27 and 15 m/min, respectively, on a 15% incline. The animals were anesthetized, 24-48 hours following the final exercise bout, with a mixture of xylazine (10 mg/kg) and ketamine (60 mg/kg) given intraperitoneally (0.1 ml/100 g). Upon loss of pedal reflexes, plantaris muscles were quickly excised, frozen in liquid nitrogen, and stored at -80° C until processing.

Muscle Preparation

Frozen plantaris muscle was weighed, transferred to prechilled sterile polypropylene tubes, and homogenized (Tissumizer; Tekmar Co., Cincinnati, OH) in ice-cold lysis buffer (1 ml/100 μ g of muscle tissue). The buffer contained 50 mM N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic] acid (HEPES), pH 7.4, 1% Triton X-100 (vol/vol), 10 mM ethylenediamine tetra-acetic acid, 10 mM Na pyrophosphate, 100 mM NaF, 10 mM Na₃VO₄, 5 µg/ml leupeptin, 0.5 µg/ ml pepstatin, 10 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Homogenates were transferred to microfuge tubes, rotated for 1 hour at 4°C, and then centrifuged at $12,000 \times g$ for 1 hour to remove insoluble material. Rat plantaris muscles to be used for standard curves (pooled standard) were also prepared in the same fashion, and supernatants were pooled and stored in frozen aliquots. For insulin receptor and IRS-1, muscles from eight male Fischer 344-Brown Norway rats (~8 months old; sedentary) were used for the pooled standard. A second pooled standard was similarly prepared from four male Brown Norway rats (retired breeders of indeterminate age; sedentary) and used for PI3K and Akt1 measurements. Supernatants were stored at -80° C until used. Protein concentrations of the supernatants were determined by the BCA method (26).

Immunoblotting

Aliquots of supernatants (100 µg protein) were solubilized in sodium dodecyl sulfate (SDS) sample buffer, boiled for 3 minutes, and subjected to 7% (insulin receptor and IRS-1) or 10% (PI3K and Akt1) SDS-polyacrylamide gel electrophoresis. A five-point standard curve using pooled standard (50-150 or 75-125 µg of total protein) was also loaded on gel and used to normalize signal intensities between blots. Resolved proteins were transferred to nitrocellulose paper in electrotransfer buffer overnight at a constant current of 150 mA/transfer apparatus in electrotransfer buffer (20 mM Tris, pH 8.0, 150 mM glycine, 0.025% SDS, 10% methanol). Nitrocellulose blots were incubated in blocking solution, which consisted of 5% nonfat milk protein (Blotto, Santa Cruz Biotech., Inc, Santa Cruz, CA) and 4% bovine serum albumin (BSA) in phosphate-buffered saline plus 0.05% Tween-20 (PBST), pH 7.5, for 2 hours at room temperature or overnight at 4°C. Blots were then washed $(1 \times 15 \text{ min}, 2 \times 5 \text{ min})$ in PBST and subsequently incubated in a 1% Blotto/0.8% BSA PBST solution with anti-B-IR (1:1000), anti-IRS-1 (1:1000), anti-PI3K (1:1000), or anti-Akt1 (1:1000) overnight at 4°C. Blots were then washed again and incubated with an appropriate secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG or anti-goat IgG, 1:5000) for 2 hours at room temperature. Blots were again washed of excess antibody, subjected to ECL, and immunoreactive protein was quantified by densitometry. Protein abundance is reported as relative concentration based on linear regression from the standard curves that were run with each gel.

Statistical Analysis

All data are expressed as mean \pm standard error. Differences among treatment groups were determined by a twoway (Age \times Training) analysis of variance using Sigma-STAT (SPSS, Inc., Chicago, IL). A value of $p \leq .05$ was considered statistically significant. Pairwise multiple comparison procedures (Tukey's post hoc test) were used to identify the source of significant variance among groups.

RESULTS

An important strength of this study was that protein abundance was quantified based on a standard curve included with each Western blot. The amount of pooled standard loaded on each gel was linearly related to the relative densitometric units for each of the proteins evaluated: insulin receptor ($R^2 = .930-.998$), IRS-1 ($R^2 = .972-.999$), PI3K ($R^2 = .876-.995$), and Akt1 ($R^2 = .904-.996$). Therefore, the magnitude of relative changes can be quantitatively interpreted (e.g., a 25% increase in densitometric units can be interpreted as a 25% increase in immunoreactive protein).

There were significant main effects of age (p = .03) and training (p = .04) on insulin receptor abundance (Figure 1). A post hoc analysis indicated that the 23% greater value for



Figure 1. Insulin receptor (β subunit) protein in skeletal muscle: 100 µg of plantaris muscle homogenate from old untrained (OUT; n = 8), old trained (OT; n = 7), young untrained (YUT; n = 9), or young trained (YT; n = 8) rats were immunoblotted with anti-insulin receptor (β -subunit). Densitometric values were quantitated relative to the standard curve (pooled plantaris muscle extracts, 75–125 µg) and expressed as the mean \pm SE. Age main effect, p = .03; training main effect, p = .04. *p = .02, YUT vs YT; †p = .02, YUT vs OUT, post hoc analysis.

OUT rats compared with YUT rats was significant (p = .02), as was the 22% greater value for YT rats compared with YUT rats (p = .02). Unlike the young rats, in the old group, a post hoc analysis did not reveal a statistically significant effect of training, and no significant difference was detected between the YT and OT groups.

There were significant main effects of age (p < .001) and training (p = .035) on IRS-1 abundance (Figure 2). There was a nonsignificant trend for an Age × Training interaction (p = .08). A post hoc analysis indicated that the 30% decreased value for YUT versus OUT rats was significant (p < .001). The 31% greater IRS-1 level for OT compared with OUT rats was also statistically significant based on the post hoc analysis (p = .01). In contrast with results for old animals, the post hoc analysis did not reveal a significant training effect in the young rats, and no age-related significant difference was detected between the YT and OT groups.

There was a significant main effect of age (p = .01), but not of training (p = .07), on PI3K abundance (Figure 3). A post hoc analysis indicated an aging effect when rats were matched for training status: OUT values were 21% greater than YUT values (p = .05), and OT values were 25% greater than YT values (p = .04).

There were no significant effects of age (p = .28) or training (p = .42) on protein abundance of Akt1 in skeletal muscle (Figure 4).

DISCUSSION

Both aging (18,19,27) and exercise (6,16,22,23,28-31) can influence insulin signaling in skeletal muscle. However, little is known about the influence of exercise training on insulin signaling in older animals (16,22). Most published studies regarding exercise and insulin signaling have focused on very young rats, typically 1–3 months old.



Figure 2. Insulin receptor substrate-1 protein in skeletal muscle from old untrained (OUT; n = 8); old trained (OT; n = 7); young untrained (YUT; n = 9), or young trained (YT; n = 8) rats. Densitometric values were quantitated relative to the standard curve (pooled plantaris muscle extracts, 75–125 µg) and expressed as the mean ± SE. Age main effect, p < .001; training main effect, p = .035. *p = .01, OUT vs OT; $^{\dagger}p < .001$, OUT vs YUT, post hoc analysis.

(6,28,29). Insulin signaling is markedly different in these young, rapidly growing rats as compared with adult or older animals (18,19). Therefore, the primary purpose of this study was to determine the influence of age and exercise training on the abundance of several key insulin signaling proteins (IR, IRS-1, PI3K, and Akt1) in adult (6 months) and old (26 months) rats. The most important findings were that, among these proteins, only IRS-1 was lower in 26-month-old versus 6-month-old rats, and this age-related difference was virtually eliminated in the endurance-trained groups. The difference in insulin receptor in untrained rats (old slightly greater than young) was also essentially eliminated by endurance training.



Figure 3. The p85-phosphatidylinositol 3-kinase (PI3-kinase) protein in skeletal muscle from old untrained (OUT; n = 8), old trained (OT; n = 7), young untrained (YUT; n = 9), or young trained (YT; n = 8) rats. Densitometric values were quantitated relative to the standard curve (pooled plantaris muscle extract, 50–150 µg) and expressed as the mean ± SE. Age main effect, p = .01. *p = .05, YUT vs OUT; †p = .04, YT vs OT, post hoc analysis.

Figure 4. The serine-threonine kinase (Akt1) protein in skeletal muscle from old untrained (OUT; n = 8), old trained (OT; n = 7), young untrained (YUT; n = 9), or young trained (YT; n = 8) rats. Densitometric values were quantitated relative to the standard curve (pooled plantaris muscle extract, 50–150 µg) and expressed as the mean \pm SE.

Influence of Age on the Abundance of Insulin Signaling Proteins in Skeletal Muscle

We found a small but significant increase in the abundance of insulin receptor in plantaris muscle from old compared with young rats. Carvalho and colleagues (18) reported no significant effect of age (2, 5, 12, and 18 months) on insulin receptor of rat skeletal muscle, based on the use of a Western blot analysis. Previous studies in adult rats have demonstrated no age-related change (32,33), or modest reductions in insulin receptor number as assessed by insulin binding measures (15–17). Martineau and colleagues (27) observed no significant age-related changes in insulin receptor protein levels of skeletal muscle from mice at 5, 12, and 25 months of age. Although results have varied somewhat, it is clear that there is not a large change in insulin receptor content of rat skeletal muscle between adulthood and old age.

IRS-1 protein abundance was 36% lower in skeletal muscle from old compared with young rats. Previous research has indicated a substantial reduction in IRS-1 in the muscle of 5-month-old versus 1- to 2-month-old animals (18,23), with no further decreases in IRS-1 abundance in animals that were 5, 12, and 18 months of age (18). Our data, taken together with data from Carvalho (18), suggest that IRS-1 content in rat skeletal muscle declines with advancing age in a biphasic manner: the first reduction occurs during development, and after a long period of stability, the content declines again during old age. The functional consequences of this later decline are uncertain. Several IRS isoforms have been identified (11,34,35), including two in skeletal muscle (IRS-1 and IRS-2). IRS-1 is recognized as the predominant isoform involved with insulin-mediated glucose uptake in skeletal muscle (8,19).

Abundance of the p85 regulatory subunit of PI3K was 39% higher in old versus young rats. Carvalho (18) stated that p85-PI3K was not changed with aging (2, 5, 12, and 20 months); however, numerical data (i.e., sample sizes, mean

values, and standard errors) were not provided. In mouse muscle, p85-PI3K protein levels tended to be higher in old (25 months) compared with young (5 and 12 months) groups (27). Collectively, these data demonstrate that PI3K abundance does not decline with aging. Rather, there is evidence for higher values in older animals. Other isoforms of the regulatory subunit of PI3K have been identified (36), but neither these isoforms nor the catalytic subunit of PI3K (37) have been studied in relation to aging.

Of the three isoforms of Akt known to be expressed by skeletal muscle (Akt1, Akt2, and Akt3, also known as PKB α , PKB β , and PKB γ , respectively), insulin activation is greatest for Akt1 (38). Accordingly, we assessed Akt1 and found that its concentration did not differ with age. Apparently, this is the first study to assess Akt1 concentration and aging. The influence of age on muscle levels of Akt2 and Akt3 has not been described.

Influence of Exercise Training on the Abundance of Insulin Signaling Proteins in Skeletal Muscle

We found a small training-induced increase in insulin receptor abundance. Chibalin and colleagues (6) found an increase in receptor abundance after 1 or 5 days of a swimming protocol $(2 \times 3$ hour exercise sessions per day) by female Wistar rats of approximately 6 weeks of age (estimated based on reported body weight). However, Nagasaki and colleagues (23) did not detect an exercise training effect on insulin receptor abundance in muscle from 4- and 27week-old female, Wistar rats that had access to a voluntary running wheel beginning at 2 weeks of age. Several studies have demonstrated an increase in insulin receptor binding with exercise training (treadmill or voluntary wheel running) in young (\sim 3 months) rats (28,29,39). Other studies reported unchanged insulin binding with exercise training (treadmill running) in rats of various ages: \sim 4–5 months (40), 12 months, and 24 months (16). Interestingly, Willis and colleagues (41) found a decline in abundance of insulin receptor β subunit in muscle from 24-month-old mice after they performed voluntary wheel running. However, there is no evidence for reduced insulin receptor quantity in muscle from rats with exercise training, and many studies have found evidence that training elicits increased receptor levels.

Exercise training essentially eliminated the age-related decrease in IRS-1 abundance, with increased levels found in the OT rats. There was no apparent training effect on IRS-1 in young (6-month-old) rats. Nagasaki and colleagues (23) also found the effect of exercise (voluntary wheel running activity) on muscle IRS-1 depended on age: they found increased IRS-1 concentration in muscle from 27-week-old, but not 4-week-old rats. Chibalin reported that 5 days of swim training led to a significant decrease in muscle IRS-1 content in approximately 6-week-old rats (6). Age and perhaps the exercise mode and/or duration are likely determinants of the training response for muscle IRS-1.

Muscle p85-PI3K content tended to be increased with exercise training, regardless of age. Nagasakai and colleagues (23) reported a significant training-induced increase in p85-PI3K abundance in skeletal muscle from 4- and 27-weekold rats. Taken together, these results suggest that exercise





can enhance p85-PI3K content in muscle across a wide range of ages. The influence of exercise on the isoforms of the regulatory and catalytic subunits of PI3K has apparently not been reported.

Akt1 abundance was unaffected by exercise training, regardless of age. Studying much younger rats and using an antibody that reacts with all three isoforms of Akt, Chibalin and colleagues (6) also found no exercise-induced change in protein concentration in muscle.

Implications of Age- and Exercise-Induced Differences in Insulin Signaling Proteins

A reduction in insulin-stimulated glucose disposal (13– 40%) in rats at 18–25 months versus 3–4 months of age has been repeatedly described (2,32,42–44). Would the decrement in IRS-1 we observed be expected to diminish insulin signaling and/or action? There is evidence that a substantial reduction in IRS-1 alone can diminish insulin signaling steps yet not elicit insulin resistance. Transgenic mice that were heterozygous for the null allele in the IRS-1 gene had an ~60% decline in IRS-1 protein expression compared with wild-type littermates, accompanied by a comparable decline in tyrosine phosphorylation of IRS-1 and p85-PI3Kassociated IRS-1 with insulin stimulation (45). However, animals from the two genotypes had similar glucose responses to an insulin tolerance test.

Although the reduction in IRS-1 we observed was likely insufficient to greatly diminish insulin action, this change together with other deficits in the insulin signaling pathway might be important. When mice that were double heterozygous for null alleles in both the IRS-1 and insulin receptor genes were studied, the transgenic mice became hyperinsulinemic compared with wild-type controls, and the magnitude of hyperinsulinemia increased dramatically with increasing age (2 vs 4–6 months of age) (10). Approximately 40% of the older double heterozygotes became diabetic, demonstrating an interaction between age and downregulation of multiple insulin signaling proteins. Kido and colleagues (46) further assessed the interactions among insulin signaling genes by producing transgenic mice with combined heterozygous null mutations in the insulin receptor, IRS-1, and/or IRS-2 genes. Metabolic differences emerged as mice became older (between 2 and 6 months of age; data not reported for older animals). Carvalho and colleagues (18) found, in 18- to 20-month-old compared with 2-monthold rats, that a decline in IRS-1 was accompanied by a relatively much greater decline in tyrosine phosphorylation of IRS-1 and IRS-1-bound PI3K in insulin-stimulated muscle. The disproportionate change in signaling, relative to the decline in IRS-1, suggests that IRS-1 was not the only relevant change. Our results indicate that the other putative agerelated changes are not reductions in abundance of insulin receptor, p85-PI3K, or Akt1.

In old rats, the most striking exercise effect was an increase in IRS-1 content. In addition, we found that p85-PI3K content tended to be increased by exercise training. Han and colleagues (22) found that exercise-trained old (25 months of age) rats had increased IRS-1-bound p85-PI3K in insulinstimulated muscle. Most (22,47,48), but not all, studies (49) have found that exercise training does not increase the muscle GLUT4 glucose transporter content in old (24-28 months of age) rats. In contrast, many studies have indicated that exercise training led to increased GLUT4 content in muscle from young to middle-aged ($\sim 1.5-16$ months of age) rats (6,47,48,50-52). Apparently several key proteins for insulin action, including GLUT4, insulin receptor, and IRS-1, respond to training differently in young and old rats. Despite the lack of increased GLUT4 content in old rats, Han and colleagues (22) found that exercise training led to enhanced insulin sensitivity, as assessed with a hyperinsulinemiceuglycemic clamp. Our results suggest that increased abundance of key insulin signaling proteins, including IRS-1, may contribute to the enhanced insulin action in exercisetrained old rats. It should be noted that, unlike most studies with rats, a training-induced increase in GLUT4 abundance of muscle has been reported for older (\sim 60–65 years) humans (53-55) and old (24 months) mice (41).

It is essential to recognize that the functional activities of signaling proteins (e.g., IRS-1-associated PI3K activity or Akt1 activity) may be altered in the absence of changes in their abundance. The influence of age and exercise on these aspects of insulin signaling should be assessed in future experiments.

Conclusions

Aging did not elicit a uniform decline in insulin signaling protein abundance. Among the proteins studied, a reduction in old rats occurred only for IRS-1, and this decrease was accompanied by an increase in p85-PI3K (the binding partner of IRS-1) and insulin receptor concentration. The decline in IRS-1 may contribute to the insulin resistance that occurs in rats between approximately 6 and 26 months of age, but changes in proteins other than the insulin receptor, p85-PI3K, and Akt1 are probably also necessary for impaired insulin action. The exercise effect on insulin signaling protein abundance was different in young and old rats. IRS-1 levels were increased with training in the old rats, but unaffected in young rats. Conversely, insulin receptor abundance was increased in YT but not OT rats. Regardless of age, abundance of p85-PI3K tended to be higher after training, and Akt1 was unresponsive to exercise. Previous studies have demonstrated that both young and old rats can achieve enhanced insulin sensitivity with exercise training (22,56-58), and the current findings suggest that the underlying mechanisms may not be identical.

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