Published in final edited form as:

Eur J Appl Physiol. 2010 November ; 110(4): 779–788. doi:10.1007/s00421-010-1559-7.

Aerobic training reverses high-fat diet-induced pro-inflammatory signalling in rat skeletal muscle

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Abstract

High-fat feeding activates components of the pro-inflammatory pathway and increases co-immunoprecipitation of suppressor of cytokine signalling (SOCS)-3 with both the insulin receptor (IR)- β subunit and IRS-1, which together contribute to keeping PI-3 kinase from being fully activated. However, whether aerobic training reverses these impairments is unknown. Sprague-Dawley rats were fed a chow (CON, $n = 8$) or saturated high-fat ($n = 16$) diets for 4 weeks. High-fat-fed rats were then allocated ($n = 8/\text{group}$) to either sedentary (HF) or aerobic exercise training (HFX) for an additional 4 weeks after which all animals underwent hind limb perfusions. Insulin-stimulated red quadriceps 3-*O*-methylglucose transport rates and PI-3 kinase activity were greater ($p < 0.05$) in CON and HFX compared to HF. IRS-1 tyrosine phosphorylation was increased ($p < 0.05$) and IRS-1 serine 307 phosphorylation was decreased ($p < 0.05$) in HFX compared to HF. IR- β subunit co-immunoprecipitation with IRS-1 was increased in HFX compared to HF. SOCS-3 coimmunoprecipitation with both the IR- β subunit and IRS-1 was decreased ($p < 0.05$) in HFX compared to HF. IKK α/β serine phosphorylation, and I κ B α serine phosphorylation were decreased ($p < 0.05$) while I κ B α protein concentration was increased in HFX compared to HF. By decreasing the association of SOCS-3 with both the IR- β subunit and IRS-1 the interaction between IRS-1 and the IR- β subunit was normalized in the HFX, and may have contributed to skeletal muscle PI-3 kinase being fully activated by insulin. Additionally, the reduction in IKK α/β

serine phosphorylation in HFX may have contributed to decreasing IRS-1 serine phosphorylation, and in turn, promoted the normalization of insulin-stimulated activation of PI-3 kinase.

Keywords

Insulin resistance; Insulin signalling; Pro-inflammatory signalling

Introduction

Aerobic exercise is well recognized for its beneficial effects on skeletal muscle glucose metabolism (Hawley and Lessard 2008). In the high-fat-fed rodent model, chronic aerobic exercise has been reported to reverse skeletal muscle insulin resistance (Kraegen et al. 1989; Kim et al. 2000), but in these early studies it was not clear whether the favourable effects of aerobic exercise on glucose metabolism resulted from improvements in either the expression of and/or the activation of components of the insulin signalling cascade. In agreement with these earlier investigations we have reported that chronic aerobic exercise improves skeletal muscle carbohydrate metabolism, as evidenced by insulin-stimulated rates of 3-MG transport being similar in high-fat-fed, exercise-trained and normal diet animals (Lessard et al. 2007; Yaspelkis et al. 2007; Saito et al. 2008). Of particular note, we have found that the aerobic training-induced improvements in rates of insulin-stimulated skeletal muscle glucose transport are not a result of increased expression of components of the insulin signalling cascade but rather due to insulin being able to more effectively activate the insulin signalling cascades (Yaspelkis et al. 2007; Saito et al. 2008). Specifically, insulin-stimulated activation of cytosolic Akt2 and aPKC (protein kinase C) ζ/λ , and plasma membrane-associated aPKC ζ/λ were normalized by aerobic training in the skeletal muscle of high-fat-fed animals (Yaspelkis et al. 2007). However, the mechanism(s) by which aerobic training reverses the high-fat diet-induced impairments in the activation of the insulin signalling cascade has not been fully elucidated.

In rodent skeletal muscle, high-fat feeding impairs insulin-stimulated rates of glucose transport and uptake, in part, due to decreased IRS (insulin receptor substrate)-1 associated PI (phosphoinositide)-3 kinase activity (Tremblay et al. 2001; Yaspelkis et al. 2004; Yaspelkis et al. 2007). While activation of downstream components of the insulin signalling cascade are reduced by high-fat feeding (Herr et al. 2005; Yaspelkis et al. 2007) (e.g., Akt2 and aPKC ζ/λ activities) and leads to decreased GLUT4 translocation to the plasma membrane (Hansen et al. 1998; Tremblay et al. 2001; Krisan et al. 2004; Yaspelkis et al. 2004; Yaspelkis et al. 2007), it appears that these impairments arise from an insufficient activation of PI-3 kinase (Herr et al. 2005; Yaspelkis et al. 2007). Based on these observations we wished to identify potential mechanism(s) that might account for why the provision of a high-fat diet decreases insulin-stimulated PI-3 kinase activity. We have recently reported that high-fat feeding increases the expression of SOCS (suppressor of cytokine signalling)-3 and co-immunoprecipitation of SOCS-3 with both the insulin receptor (IR)- β subunit and IRS-1 (Yaspelkis et al. 2009). Co-localization of SOCS-3 with both the IR- β subunit and IRS-1 may result in a steric hindrance that prevents IRS-1 from interacting with the IR- β subunit thereby reducing PI-3 kinase activity (Emanuelli et al. 2001; Rui et al.

2002). Additionally, we observed that components of the pro-inflammatory pathway are activated in the skeletal muscle collected from high-fat-fed animals (Yaspelkis et al. 2009). Of note was the observation that IKK α/β (I κ B kinase) serine phosphorylation was significantly increased, which may have contributed to increasing IRS-1 serine phosphorylation (Gao et al. 2002), and in turn also prevented PI-3 kinase from being fully activated in response to insulin stimulation (Hirosumi et al. 2002; Yu et al. 2002; Kim et al. 2004; Um et al. 2004). However, it is currently unknown whether aerobic training reverses these impairments in the skeletal muscle of the high-fat-fed rat, and thereby contributes to improving insulin-stimulated PI-3 kinase activation. Therefore, the aim of this investigation was to determine if aerobic training reverses the high-fat diet-induced co-immunoprecipitation of SOCS-3 with both the IR- β subunit and IRS-1, and increased IKK α/β serine phosphorylation. We hypothesized that aerobic training would mediate these positive effects which, in turn, would contribute to improving insulin-stimulated PI-3 kinase activation in high-fat-fed rodent skeletal muscle.

Materials and methods

Ethical approval

All experimental procedures were approved by the Institutional Animal Care and Use Committee at California State University Northridge and conformed to the guidelines for the use of laboratory animals published by the United States Department of Agriculture.

Experimental design

Thirty-two male Sprague-Dawley rats (Harlan, San Diego, CA, USA), ~6 weeks old, were randomly placed into the following groups: normal diet (CON; $n = 8$) or high-fat diet ($n = 16$). The normal diet consisted of 73.1% carbohydrates, 10.5% fat (coconut oil), and 16.4% protein (D12328; Research Diets, New Brunswick, NJ, USA). The high-fat diet contained 25.5% carbohydrates, 58% fat (coconut oil) and 16.4% protein (D12330; Research Diets). Animals were on their respective diets for 4 weeks and allowed to feed ad libitum, which we have previously shown to induce skeletal muscle insulin resistance in male Sprague-Dawley rats (Krisan et al. 2004; Yaspelkis et al. 2004, 2009). During the subsequent 4-week experimental period, high-fat rats continued to eat the high-fat diet and were randomly allocated to one of the following groups: high-fat control (HF, $n = 8$) or exercise trained (HFX, $n = 8$). Exercise training consisted of treadmill running for 1 h day⁻¹, 5 days week⁻¹ at 32 m min⁻¹ on a 15% incline. The speed was gradually increased during the first week of training such that the animals were running at 32 m min⁻¹ by the 5th day of training and continued to run at this pace for the duration of the protocol. We have previously shown that when Sprague-Dawley rats are exercised using this speed and grade, skeletal muscle oxidative capacity is significantly increased (Yaspelkis et al. 1999b). The control rats (CON; $n = 8$) remained on the normal chow diet for the duration of the study (8 weeks). The experimental design employed in the present investigation is consistent with the majority of studies that have placed rats on a high-fat diet and then subjected them to exercise training but did not include a chow fed-exercise trained group. Following the experimental period, animals were fasted for 8–12 h prior to undergoing hind limb perfusion. Exercise-trained animals undertook their last training bout 36–48 h prior to hind limb perfusion, which

should be of sufficient duration to allow any potential inflammation from the last exercise bout to subside (Spangenburg et al. 2006).

Hind limb perfusions

Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (6.5 mg 100 g body wt⁻¹) and surgically prepared for hind limb perfusion as previously described by Ruderman et al. (1971) and modified by Ivy et al. (1989). During the surgical preparation the epididymal fat pads were dissected, placed in a tared weigh boat and the mass of the fat pads were recorded. Following surgical preparation, cannulas were inserted into the abdominal aorta and vena cava, and the animals were killed via an intracardiac injection of pentobarbital as the hind limbs were washed out with 30 ml of Krebs-Henseleit buffer (KHB; pH 7.55). Immediately the cannulas were placed in line with a non-recirculating perfusion system, and the hind limbs were allowed to stabilize during a 5-min washout period. The perfusate was continuously gassed with a mixture of 95% O₂–5% CO₂ and warmed to 37°C. Perfusate flow rate was set at 7.5 ml min⁻¹ during the stabilization and subsequent perfusion, during which rates of glucose transport were determined.

Perfusions were performed in the presence of 500 µU ml⁻¹ insulin, which we have previously shown to produce a submaximal effect in the Sprague-Dawley rat (Yaspelkis et al. 1999a). A submaximal insulin concentration was selected due to concern that if the insulin signalling cascade was maximally activated, then those potential impairments in the cascade might not be identifiable. The basic perfusate medium consisted of 30% washed time-expired human erythrocytes (Ogden Medical Center, Ogden, UT, USA), KHB, 4% dialyzed bovine serum albumin (Fisher Scientific, Fair Lawn, NJ, USA) and 0.2 mM pyruvate. The hind limbs were washed out with perfusate containing 1 mM glucose for 5 min in preparation for the measurement of glucose transport. Glucose transport was measured over an 8-min period using an 8-mM concentration of non-metabolized glucose analogue 3-O-methylglucose (3-MG; 32 µCi 3-[³H] MG mM⁻¹, PerkinElmer Life Sciences, Boston, MA, USA) and 2 mM mannitol (60 µCi-[1-¹⁴C] mannitol mM⁻¹, PerkinElmer Life Sciences). Rates of insulin-stimulated skeletal muscle 3-MG transport were calculated as previously described (Yaspelkis et al. 2001; Krisan et al. 2004; Yaspelkis et al. 2004). Immediately after the transport period portions of the red (RQ) quadriceps were excised from both hind limbs, blotted on gauze dampened with cold KHB, freeze-clamped in liquid N₂ and stored at –80°C for later analysis.

Muscle lysate preparation

Muscle samples were weighed and homogenized 1:10 in homogenization buffer (HB) that contained 50 mM Hepes, 150 mM NaCl, 200 mM sodium pyrophosphate, 20 mM α-glycerolphosphate, 20 mM NaF, 2 mM sodium vanadate, 20 mM EDTA, 1% IGEPAL, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 1 mM MgCl₂, 1 mM CaCl₂, 10 µg mL⁻¹ leupeptin and 10 µg mL⁻¹ aprotinin. Muscles were homogenized on ice using a glass Pyrex homogenizer and centrifuged at 18,300×g for 15 min at 4°C (Micromax RF, International Equipment Co., Needham Heights, MA, USA). The supernatant was collected and quantified for protein content by the Bradford Method using a Benchmark microplate reader (BioRad, Richmond, CA, USA).

Insulin signalling cascade

Western blotting: Insulin receptor- β subunit (IR- β) and IRS-1 protein concentration was determined using lysate samples. Samples were combined (1:1) with Laemmli sample buffer and heated at 100°C for 5 min. Seventy microgram of muscle lysate protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) run under reducing conditions on 10% resolving gels (IR- β) or 7.5% (IRS-1) using a Mini-Protean III dual slab cell (BioRad). Resolved gels were equilibrated in a Bjerrum and Schafer-Nielson Transfer Buffer for 30 min. Resolved proteins were then transferred to polyvinylidene difluoride (PVDF) membranes using a semidry transfer unit (10 V for 30 min). The membranes were blocked in 5% non-fat dry milk–Tween 20 Tris buffered saline (NFD–TTBS) blocking solution and then incubated with either affinity purified rabbit polyclonal anti-IR- β (Cat# 07-724, Millipore, Temecula, CA, USA) or anti-IRS-1 (Cat# 06-526, Millipore) followed by an incubation with a species specific secondary antibody conjugated to horse radish peroxidase (HRP). Antibody binding was visualized using enhanced chemiluminescence (ECL) in accordance with the manufacturer's instructions. Images were captured using a ChemiDoc system (BioRad) equipped with a CCD camera and saved to a Macintosh G4 computer. Bands were quantified using Quantity One analysis software (BioRad). Each gel run contained samples from each of the experimental groups. The density of the bands was determined and the data were expressed as a percentage of the same muscle standard that was run on each gel such that comparisons could be made across gels. The representative autoradiographs presented in the figures are duplicates of a sample that best reflected the mean value of each experimental group.

IR- β tyrosine phosphorylation (IR- β pY), IRS-1 tyrosine phosphorylation (IRS-1 pY) and IRS-1 serine phosphorylation (IRS-1 pS): Phosphorylation of the IR- β subunit and IRS-1 was determined using immunoprecipitation followed by western blotting. Lysate protein samples (500 μ g for IR- β pY, 500 μ g for IRS-1 pY and 800 μ g for IRS-1 pS 307) were incubated with 4 μ g of either anti-IR- β subunit (Cat#6-492, Millipore) or anti-IRS-1 (Cat#6-248, Millipore) at 4°C. Following an overnight incubation, 80 μ L of Protein-A Sepharose Type CL-4B (PRO-A; Cat# 17-0789-01; GE HealthCare, Piscataway, NJ, USA) bead slurry were added to the samples and incubated for 1.5 h at 4°C with rotation. Samples were then centrifuged, washed, and 30 μ L of Laemmli sample buffer was added to the PRO-A bead slurry. Samples were heated at 100°C for 5 min. Ten microliters of sample was loaded in duplicate onto a SDS-PAGE gel (10% for IR- β pY, 7.5% for IRS-1 pY and IRS-1 pS 307). Proteins were resolved and transferred as described above. Membranes were incubated overnight at 4°C with anti-pY (Cat#6-247, Millipore) for IR- β pY and IRS-1 pY and anti-IRS-1 pS 307 (Cat# 07-247, Millipore) for IRS-1 pS 307. After incubation with a species-specific secondary antibody conjugated to HRP, images were acquired and quantified as described above.

Co-localization of IRS-1 pY with IR- α : Sixty microliters of PRO-A bead slurry was incubated with 5 μ g of anti-IR- β (Cat# 07-724, Millipore) antibody and rotated overnight at 4°C. Following the overnight incubation PRO-A/antibody complex was washed three times with phosphate-buffered saline (PBS). Subsequently, 500 μ g of lysate protein was added to the PRO-A/antibody complex and incubated overnight at 4°C with rotation. Following the

overnight incubation the PRO-A/antibody complex was washed once with PBS, once with 2% Triton X-PBS and a final PBS wash before the addition of 30 μ l of Laemmli sample buffer. Samples were heated at 100°C for 5 min. Ten microliter of eluted sample was loaded in duplicate and subjected to SDS-PAGE run under reducing conditions on a 7.5% resolving gel. Resolved proteins were transferred to PVDF membranes and blocked with 5% NFDM–TTBS. The membranes were then incubated overnight at 4°C with anti-phospho-IRS-1 (Cat# 07-848, Millipore) primary antibody followed by incubation with species-specific secondary antibody conjugated to HRP. Antibody binding was visualized and quantified as described above.

IRS-1 associated PI3-kinase activity: Between 100 and 150 mg protein of insulin-stimulated RQ were weighed frozen and homogenized as previously described (Singh et al. 2003; Krisan et al. 2004). The supernatant was collected, quantified for protein and IRS-1 associated PI 3-kinase activity was determined as we have detailed previously (Krisan et al. 2004).

Proinflammatory signalling and SOCS-3

Skeletal muscle TNF- α : TNF- α concentration was determined in lysate samples. Sixty microliter of PRO-A bead slurry was incubated with 5 μ g of anti-TNF- α antibody (Cat# ARC3012, Biosource, Camarillo, CA, USA) overnight at 4°C with rotation. Following the overnight incubation the PRO-A/anti-TNF- α complex was washed three times with PBS. Eight hundred micrograms of lysate protein was then incubated overnight at 4°C with rotation with the PRO-A/anti-TNF- α complex. Following the overnight incubation the bead slurry was washed once with PBS, once with 2% Triton X-PBS and a final PBS wash before the addition of 30 μ l of Laemmli solution. Ten microliters of supernatant was loaded in duplicate and subjected to SDS-PAGE run under reducing conditions on a 15% resolving gel. Resolved proteins were transferred to PVDF membranes and blocked with 5% NFDM–TTBS. The membranes were then incubated overnight at 4°C with affinity purified rabbit polyclonal anti-TNF- α (Cat# ARC3012, Biosource) primary antibody followed by incubation with species-specific secondary antibody conjugated to HRP. Antibody binding was visualized and quantified as described above.

Analysis of select components of the I κ B/NF- κ B proinflammatory pathway: Skeletal muscle lysate samples were used for western blot analysis of IKK α , IKK β , IKK α/β pS 176/180 and I κ B α . One hundred micrograms of muscle lysate protein was subjected to SDS-PAGE run under reducing conditions on a 7.5% resolving gel and transferred to PVDF membranes. The PVDF membranes were blocked using a 5% NFDM–TTBS solution followed by incubation with either anti-IKK α (Cat# 2682, Cell Signalling, Beverly, MA, USA), anti-IKK β (Cat# 2370, Cell Signalling), anti-IKK α/β pS 176/180 (Cat# 2694, Cell Signalling) or anti-I κ B α (Cat# 4812, Cell Signalling) primary antibody solutions followed by incubation with species specific secondary antibody conjugated to HRP. Images were captured and quantified as described above.

I κ B α serine phosphorylation: Phosphorylation of I κ B α pS 32/36 was determined using immunoprecipitation followed by Western blotting. Lysate protein samples (800 μ g) were

incubated with 5 µg of anti-I κ B α pSer 32/36 (Cat# 9246L, Cell Signalling) at 4°C. Following an overnight incubation, 60 µL of PRO-A bead slurry was added to the samples and incubated for 1.5 h at 4°C with rotation. Samples were then centrifuged, washed and 30 µL of Laemmli sample buffer was added to the PRO-A bead slurry. Samples were heated at 100°C for 5 min. Ten microliters of supernatant was loaded in duplicate onto a 10% SDS-PAGE gel. Proteins were resolved and transferred as described above. Membranes were incubated with anti-I κ B α pSer 32/36 (Cat# 9246L, Cell Signalling) primary antibody overnight at 4°C. After incubation with species-specific secondary antibody images were acquired and quantified as described above.

SOCS-3 protein concentration: Fifty micrograms of skeletal muscle lysate was subjected to SDS-PAGE run under reducing conditions on a 15% resolving gel. Resolved proteins were transferred to PVDF membranes and blocked overnight at 4°C with 5% bovine serum albumin-TTBS. Membranes were then incubated with affinity-purified rabbit polyclonal anti-SOCS-3 (Cat# sc-9023, Santa Cruz Biotechnology (SCBT), Santa Cruz, CA) primary antibody solutions for 2 h at room temperature. After incubation with species-specific secondary antibody images were acquired and quantified as described above.

Co-immunoprecipitation of SOCS-3 with IR- β or IRS-1: Lysate samples (800 µg of protein) were subjected to immunoprecipitation with 8 µg of either anti-IR- β (Cat# 07-724, Millipore) antibody or anti-IRS-1 (Cat# 06-526, Millipore). Immunocomplexes were allowed to form overnight at 4°C. Following the overnight incubation samples were then loaded with 80 µL of PRO-A bead slurry and rotated for 3 h at 4°C. Samples were then centrifuged, washed, 30 µL of Laemmli sample buffer was added to the PRO-A bead slurry, and were then heated at 100°C for 5 min. Ten microliters of eluted sample was loaded in duplicate onto a 12.5% SDS-PAGE resolving gel. Following electrophoresis, resolved proteins were transferred onto PVDF membranes, blocked with 5% NFDM-TTBS and then probed with anti-SOCS-3 (Cat# sc-9023, SCBT) primary antibody for 2 h followed by incubation with species-specific secondary antibody conjugated to HRP. Antibody binding was visualized and quantified as described above.

Statistical analysis: An analysis of variance (ANOVA) was used on all variables to determine whether significant differences existed between groups. When a significant F-ratio was obtained, a Tukey HSD post hoc test was used to identify statistically significant differences ($p < 0.05$) among the means. Statistical analyses were performed using JMP software (SAS Institute Inc., Cary, NC, USA) and all values are expressed as means $\pm$ SE.

Results

Body and epididymal fat pad mass

Body and epididymal fat pad mass of the HF (464 ± 8 ; 10.7 ± 0.6 g) were heavier ($p < 0.05$) compared to the body and epididymal fat pad mass of the CON (436 ± 6 ; 6.2 ± 0.3 g) and HFX (417 ± 5 ; 6.8 ± 0.4 g) animals. We have reported serum glucose, insulin, adiponectin, FFA and skeletal muscle lipid content for these animals previously (Lessard et al. 2007).

Skeletal muscle 3-MG transport

Insulin-stimulated ($500 \mu\text{U ml}^{-1}$ insulin) rates of 3-MG transport in the RQ of the CON animals ($\text{CON } 9.00 \pm 0.75 \text{ l mol g}^{-1} \text{ h}^{-1}$) and HFX animals ($\text{HFX } 7.78 \pm 0.64 \text{ l mol g}^{-1} \text{ h}^{-1}$) were greater than that of the HF animals ($\text{HF } 5.63 \pm 0.61 \mu\text{mol g}^{-1} \text{ h}^{-1}$; $p < 0.05$).

Insulin signalling cascade

IR- β subunit protein concentration and tyrosine phosphorylation: IR- β protein content was similar among the CON, HF and HFX animals (Fig. 1). Tyrosine phosphorylation of the IR- β subunit was also similar among the CON, HF and HFX animals (Fig. 1).

IRS-1 protein content, tyrosine phosphorylation and serine 307 phosphorylation: IRS-1 protein concentration was similar among the CON, HF and HFX animals (Fig. 2). IRS-1 pY (Fig. 2) was reduced by 59% in the HF animals compared to CON animals ($p < 0.05$). IRS-1 pS (Fig. 2) was elevated 83% in the HF animals compared to CON animals ($p < 0.05$). Exercise training reversed the high-fat diet-induced reduction in tyrosine phosphorylation and increase in serine phosphorylation of IRS-1 in the HFX animals (Fig. 2).

Co-immunoprecipitation of IR- β with IRS-1: IRS-1 co-immunoprecipitation with IR- β (Fig. 3) was reduced 61% in the HF group compared to CON animals ($p < 0.05$). Exercise training reversed this co-immunoprecipitation as noted by no differences existing between the CON and HFX groups.

IRS-1 associated PI3-kinase activity: IRS-1 associated PI3-kinase activity (Fig. 4) was reduced 48% in the HF group compared to CON animals ($p < 0.05$). Exercise training restored PI3-K activity as noted by no differences existing between the CON and HFX groups.

SOCS and proinflammatory signalling cascade

Skeletal muscle TNF- α : Skeletal muscle TNF- α concentration was significantly ($p < 0.05$) elevated 148% in the HF animals (140.70 ± 17.5 arbitrary units) compared to CON animals (56.73 ± 6.6 arbitrary units). Exercise training reversed the high-fat diet-induced increase of TNF- α concentration in skeletal muscle of the HFX animals (50.28 ± 6.4 arbitrary units).

IKK α and IKK β protein concentration and serine phosphorylation: IKK α and IKK β protein concentration was similar among the CON, HF and HFX animals (Fig. 5). Phosphorylation of IKK α/β serine 176/180 was increased 137% in the HF animals ($p < 0.05$; Fig. 5) compared to CON animals. Exercise training normalized skeletal muscle serine phosphorylation of IKK α/β in the HFX animals.

I κ B α protein concentration and I κ B α serine phosphorylation: High-fat feeding significantly decreased skeletal muscle I κ B α protein concentration by 40% ($p < 0.05$; Fig. 6). To further investigate the decrease in I κ B α protein content, I κ B α serine phosphorylation was evaluated as phosphorylation on the serine residues targets I κ B α to the proteosomal degradation pathway. I κ B α serine phosphorylation was increased 174% in HF animals ($p < 0.05$; Fig. 6) compared to CON group. Exercise training reversed the high-fat diet-induced

increase in I κ B α concentration and serine phosphorylation as it was observed that no differences existed between the HFX and CON animals.

SOCS-3 protein concentration and co-immunoprecipitation of SOCS-3 with IR- β and IRS-1: High-fat feeding significantly ($p < 0.05$) increased SOCS-3 protein concentration by 95% (Fig. 7). Our results reveal that exercise training restores SOCS-3 protein concentration to normal levels. SOCS-3 co-immunoprecipitation with IR- β and IRS-1 (Fig. 8) was increased (123 and 74%, respectively, $p < 0.05$) in the HF animals compared to CON animals, and that exercise training reduced this co-immunoprecipitation of SOCS3 with IR- β and IRS-1.

Discussion

Aerobic training reverses the effects of high-fat diet-induced skeletal muscle insulin resistance (Kraegen et al. 1989; Kim et al. 2000; Lessard et al. 2007; Yaspelkis et al. 2007; Saito et al. 2008). Our observation that the high-fat-fed exercise-trained animals exhibited improved insulin-stimulated skeletal muscle carbohydrate metabolism, as evidenced by increased rates of 3-MG transport, is consistent with these previous reports. The novel finding of this study though was that aerobic training reversed the high-fat diet-induced co-localization of SOCS-3 with both the IR- β subunit and IRS-1 and the increased IKK α/β serine phosphorylation in rodent skeletal muscle.

Several recent studies have implicated suppressor of cytokine signalling (SOCS) proteins as being capable of impairing insulin signalling (Howard and Flier 2006; Ronn et al. 2007). Emanuelli et al. (2000, 2001) using 3T3-L1 adipocytes and COS7 cells have observed that insulin stimulation causes translocation of SOCS-3 to the plasma membrane where it interacts with phosphotyrosine 960 in the juxtamembrane region of the insulin receptor (IR). Co-localization of SOCS-3 with the insulin receptor β -subunit prevents association of IRS-1 with IR- β resulting in decreased activation of the downstream components of the insulin signalling cascade (Emanuelli et al. 2001). It has been reported that over-expression of SOCS-3 in liver decreases insulin-stimulated PI-3 kinase activity (Ueki et al. 2004a). Additionally, SOCS-3 can bind to tyrosine phosphorylated IRS-1 (Rui et al. 2002), which may also contribute to impaired insulin signalling. SOCS-3 expression is higher in adipocytes and liver of ob/ob and db/db mice (Emanuelli et al. 2001; Ueki et al. 2004b), while in high-fat-fed rodent skeletal muscle SOCS-3 mRNA (Steinberg et al. 2004) and protein concentration (Ropelle et al. 2006) is also elevated. Taken collectively, these findings suggest that insulin resistance may result from a greater SOCS-3 protein concentration that in turn increasingly binds to the IR- β and IRS-1 resulting in suppressed insulin signalling in skeletal muscle.

Consistent with these observations we have recently reported that in the skeletal muscle of the high-fat-fed rat that SOCS-3 protein is increased, and co-localization of SOCS-3 with both the IR- β subunit and IRS-1 is elevated (Yaspelkis et al. 2009). We postulated that this might provide steric hindrance that prevents IRS-1 from interacting with the IR- β thereby resulting in PI-3 kinase activation being reduced in the skeletal muscle of the high-fat-fed rat. Thus, our first line of inquiry was directed towards determining whether aerobic training

reduced SOCS-3 protein concentration in the skeletal muscle of the high-fat-fed rat, which we noted did occur. To the best of our knowledge the effects of chronic aerobic exercise on SOCS-3 protein concentration in the skeletal muscle of high-fat-fed rats has not previously been evaluated. However, it has been reported that chronic aerobic training does not reduce the high-fat diet-induced SOCS-3 mRNA rodent skeletal muscle (Steinberg et al. 2004) and increases SOCS-3 mRNA in the skeletal muscle of normal rodents (Spangenburg et al. 2006). While it is difficult to reconcile the discrepancies among studies, it is possible that the SOCS-3 mRNA content is not representative of the actual skeletal muscle SOCS-3 protein concentration.

Nevertheless, simply altering the expression of this protein may not directly reverse impaired PI-3 kinase activation. For SOCS-3 to impair the insulin-signalling cascade it must co-localize with phosphotyrosine 960 in the juxtamembrane region of the insulin receptor b-subunit (IR- β), which prevents association of IRS-1 with IR- β and decreases activation of the downstream components of the insulin-signalling cascade (Emanuelli et al. 2000, 2001). We have previously reported (Yaspelkis et al. 2009) and observed again in the present investigation that when we immunoprecipitated the insulin receptor b-subunit from lysates prepared from insulin-stimulated skeletal muscle and probed for SOCS-3 there was a significantly greater co-immunoprecipitation of SOCS-3 with the IR- β subunit in the high-fat-fed skeletal muscle. We also observed that there was a greater co-immunoprecipitation of SOCS-3 with IRS-1 in the high-fat-fed skeletal muscle, which is in agreement with previous reports (Rui et al. 2002; Yaspelkis et al. 2009). Of particular interest was that in the high-fatfed, exercise-trained animals, SOCS-3 co-immunoprecipitation with both the IR- β subunit and IRS-1 was reduced and essentially not different from the control animals despite the fact that the HFX animals remained on the high-fat diet while being trained.

Our data suggest that in skeletal muscle, the chronic consumption of a high-fat diet when combined with aerobic exercise training decreases the expression of SOCS-3 and co-localization of SOCS-3 with both the IR- β subunit and IRS-1 in response to insulin stimulation. This, in turn, possibly removes the physical barrier that impairs IRS-1 from interacting with the IR- β subunit. In support of this hypothesis, we observed that IR- β subunit and IRS-1 co-immunoprecipitation was normalized in insulin-stimulated skeletal muscle collected from the HFX animals. Furthermore, we also showed that PI-3 kinase activity was normalized in the HFX animals. As IRS-1 must associate with the IR- β subunit to activate PI-3 kinase, it is not unreasonable to suggest that normalized co-localization between the IR- β subunit and IRS-1 in the HFX animals was an important factor that contributed to the improved PI-3 kinase activity and normalized rates of 3-MG transport in the aerobically trained animals. It was noted that the body and fat pad mass of the HFX animals was less than that of the CON animals, which may have also partially contributed to enhancing insulin sensitivity in the HFX animals.

It has been suggested that activation of the pro-inflammatory pathway may contribute to skeletal muscle insulin resistance (Shoelson et al. 2003; Bhatt et al. 2006). Itani et al. (2002) showed lipid infusion-induced insulin resistance in humans decreased skeletal muscle I κ B α protein concentration, which is indicative of increased IKK β activation. In contrast, the IKK β knockout mouse does not develop insulin resistance in response to lipid infusion (Kim

et al. 2001; Yuan et al. 2001). Activated IKK β has been shown to directly phosphorylate IRS-1 on serine 307 (Gao et al. 2002). We have previously reported that a high-fat diet increases IRS-1 serine phosphorylation in skeletal muscle (Yaspelkis et al. 2007, 2009) and again show this effect in the present investigation. The observation that IRS-1 serine phosphorylation was increased in the HF animals is of interest as it has been reported that increased IRS-1 serine phosphorylation prevents the insulin-signalling cascade from becoming fully activated (Hirosumi et al. 2002; Yu et al. 2002; Kim et al. 2004; Um et al. 2004) and is related to increased diacylglycerol (DAG) accumulation in the skeletal muscle (Schmitz-Peiffer 2002; Kim et al. 2004). Additionally, we have found that high-fat feeding increases DAG accumulation due to increased rates of palmitate uptake without a concomitant increase in fatty acid oxidation, but that aerobic training reverses this effect (Lessard et al. 2007). Accumulation of DAG in hepatocytes activates components of the pro-inflammatory cascade and contributes to the development of insulin resistance (Boden et al. 2005), although this has not been measured in skeletal muscle. It has been reported that when rodents consume a high-fat diet the skeletal muscle TNF- α content is increased (Borst and Conover 2005; Yaspelkis et al. 2009), and elevated TNF- α levels contribute to increasing IKK activity (DiDonato et al. 1997). Thus, our observation that aerobic training reverses the accumulation of DAG in the high-fatfed animals (Lessard et al. 2007), and that skeletal muscle TNF- α concentration was also decreased provides a plausible explanation to account for why IKK α/β serine phosphorylation was reduced in the skeletal muscle of the exercise-trained animals. This in turn may contribute to the reduced IRS-1 serine phosphorylation that we observed in the skeletal muscle of the high-fat-fed, exercise-trained animals.

In summary, we found that aerobic exercise training normalizes two independent mechanisms that are altered by high-fat feeding and potentially impair insulin-stimulated skeletal muscle PI-3 kinase activation. Our first observation was the increased SOCS-3 expression, and coimmunoprecipitation of SOCS-3 with both the IR- β subunit and IRS-1, was reversed by aerobic training. By decreasing the association of SOCS-3 with both the IR- β subunit and IRS-1 it appeared to have normalized the interaction between IRS-1 and the IR- β subunit which resulted in PI-3 kinase being fully activated by insulin in the skeletal muscle of the aerobically trained animals. Our second finding was that aerobic training reversed the high-fat diet-induced activation of components of the pro-inflammatory pathway. Specifically, IKK α/β serine phosphorylation was significantly reduced, which likely contributed to decreasing IRS-1 serine phosphorylation, and in turn enabled insulin to fully activate PI-3 kinase. Of particular note, these favourable alterations occurred while the animals continued to consume the high-fat diet while being subjected to aerobic training.

Acknowledgments

This study was supported by grants from the National Institutes of Health (GM-48680, GM-08395 and DK-57625).

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Fig. 1. Insulin receptor beta (IR- β) subunit protein concentration and IR- β tyrosine phosphorylation (pY) in skeletal muscle obtained from control (*CON*), high-fat fed (*HF*) and high fat exercise trained (*HFX*) animals perfused in the presence of 500 $\mu\text{U ml}^{-1}$ insulin

Fig. 2.

IRS-1 protein concentration, IRS-1 tyrosine phosphorylation (*pY*) and IRS-1 serine 307 phosphorylation (*pS*) in skeletal muscle obtained from control (*CON*), high-fat fed (*HF*) and high fat exercise trained (*HFX*) animals perfused in the presence of 500 $\mu\text{U ml}^{-1}$ insulin.

*Significantly different from CON group ($p < 0.05$). #Significantly different from HFX group ($p < 0.05$)

Fig. 3.

Co-immunoprecipitation of IR- β subunit with IRS-1 in skeletal muscle obtained from control (CON), high-fat fed (HF) and high fat exercise trained (HFX) animals perfused in the presence of 500 $\mu\text{U ml}^{-1}$ insulin. *Significantly different from CON group ($p < 0.05$). #Significantly different from HFX group ($p < 0.05$)

Fig. 4.

Insulin-stimulated IRS-1 associated Phosphoinositol 3-kinase (*PI3-K*) activity in skeletal muscle obtained from control (*CON*), high-fat fed (*HF*) and high fat exercise trained (*HFX*) animals perfused in the presence of 500 $\mu\text{U ml}^{-1}$ insulin. *Significantly different from *CON* group ($p < 0.05$). #Significantly different from *HFX* group ($p < 0.05$)

Fig. 5. IKK α , IKK β protein concentration and IKK α/β serine phosphorylation (pS) in skeletal muscle obtained from control (CON), high-fat fed (HF) and high fat exercise trained (HFX) animals perfused in the presence of 500 $\mu\text{U ml}^{-1}$ insulin. Values are mean $\pm$ SE. *Significantly different from CON group ($p < 0.05$). #Significantly different from HFX group ($p < 0.05$)

Fig. 6.

IκBα protein concentration and IκBα serine phosphorylation (*pS*) in skeletal muscle obtained from control (*CON*), high-fat fed (*HF*) and high fat exercise trained (*HFX*) animals perfused in the presence of 500 μU ml⁻¹ insulin. Values are mean ± SE. *Significantly different from CON group ($p < 0.05$). #Significantly different from HFX group ($p < 0.05$)

Fig. 7. SOCS3 protein concentration in skeletal muscle obtained from control (*CON*), high-fat fed (*HF*) and high fat exercised trained (*HFX*) animals perfused in the presence of 500 $\mu\text{U ml}^{-1}$ insulin. Values are mean $\pm$ SE. ^{*}Significantly different from CON group ($p < 0.05$). [#]Significantly different from HFX group ($p < 0.05$)

Fig. 8. SOCS3 co-immunoprecipitation with IR- β and SOCS3 coimmunoprecipitation with IRS-1 in skeletal muscle obtained from control (*CON*), high-fat fed (*HF*) and high fat exercised trained (*HFX*) animals perfused in the presence of 500 $\mu\text{U ml}^{-1}$ insulin. Values are mean $\pm$ SE. *Significantly different from *CON* group ($p < 0.05$). #Significantly different from *HFX* group ($p < 0.05$)