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Chronic aerobic exercise enhances components of the classical and novel insulin signalling cascades in Sprague–Dawley rat skeletal muscle

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Abstract

Aim: The aim of this study was to provide a more extensive evaluation of the effects of chronic aerobic exercise on various components of the insulin signalling cascade in normal rodent skeletal muscle because of the limited body of literature that exists in this area of investigation.

Methods: Male Sprague–Dawley rats were assigned to either control ($n = 7$) or chronic aerobic exercise ($n = 7$) groups. Aerobic exercise animals were run 3 day week^{-1} for 45 min on a motor-driven treadmill (32 m min^{-1}, 15% grade) for a 12 week period. Following the training period, all animals were subjected to hind limb perfusion in the presence of 500 μU mL^{-1} insulin to determine what effect chronic aerobic training had on various components of the insulin signalling cascade, c-Cbl protein concentration and c-Cbl phosphorylation.

Results: Twelve weeks of aerobic training did not alter skeletal muscle Akt 1/2 protein concentration, Akt Ser 473 phosphorylation, Akt Thr 308 phosphorylation, Akt 1 activity, aPKC-ζ protein concentration, aPKC-λ protein concentration or c-Cbl protein concentration. In contrast, chronic aerobic exercise increased insulin-stimulated phosphatidylinositol 3-kinase, Akt 2 kinase and aPKC-ζ/λ kinase activities, as well as c-Cbl tyrosine phosphorylation, in a fibre type specific response to aerobic training. In addition, chronic aerobic exercise enhanced insulin-stimulated plasma membrane glucose transporter 4 (GLUT4) protein concentration.

Conclusion: Collectively, these findings suggest that chronic aerobic exercise enhances components of both the classical and novel insulin signalling cascades in normal rodent skeletal muscle, which may contribute to an increased insulin-stimulated plasma membrane GLUT4 protein concentration.

Keywords Akt 2 kinase, aPKC-ζ/λ kinase, c-Cbl, c-Cbl tyrosine phosphorylation, GLUT4, PI 3-kinase.

Our laboratory recently reported that chronic resistance exercise training increases rates of insulin-stimulated glucose transport in normal rodent skeletal muscle, as well as normalizes glucose transport in high fat diet-induced insulin resistant rodent skeletal muscle (Krisan *et al.* 2004). These training adaptations were a result of alterations in both the insulin signalling cascade and glucose transporter effector system. In the process of framing our findings within the existing body of literature we found no other reports that detailed the effects of resistance training on components of the insulin signalling cascade in normal rodent skeletal muscle, and thus attempted to compare/contrast the effects of resistance training to those of aerobic exercise training in normal rodents. Surprisingly, we discovered that there were only a limited number of investigations

that had evaluated the effects of chronic aerobic exercise on the insulin signalling cascade in normal rodent skeletal muscle (Kim *et al.* 1999a, Arias *et al.* 2001, Luciano *et al.* 2002), and none of these investigations provided a comprehensive evaluation of the components of the cascade. In contrast, the majority of studies that have evaluated the effects of exercise on components of the insulin signalling cascade in normal rodent skeletal muscle have utilized acute or short-term exercise. These investigations report that when normal rodents are subjected to acute or short-term aerobic exercise that skeletal muscle insulin receptor substrate (IRS-1) associated phosphatidylinositol (PI) 3-kinase activity (Zhou & Dohm 1997, Chibalin *et al.* 2000), Akt phosphorylation and kinase activity (Chibalin *et al.* 2000, Sakamoto *et al.* 2003) and glucose transporter 4 (GLUT4) protein concentration (Ren *et al.* 1994, Jessen *et al.* 2003) are increased, but Akt protein concentration is unaffected (Sakamoto *et al.* 2003). To the best of our knowledge aPKC-ζ/λ protein content and activity have yet to be evaluated in normal rodent skeletal muscle subjected to aerobic exercise. Therefore, the primary aim of this investigation was to provide a more extensive evaluation of the effects of chronic aerobic exercise on various components of the insulin signalling cascade in normal rodent skeletal muscle.

It has been reported that aerobic training can improve insulin-stimulated glucose uptake in skeletal muscle from obese Zucker rats without affecting insulin receptor signalling through the PI 3-kinase pathway (Christ *et al.* 2002), which suggests that aerobic training may be capable of affecting other insulin-activated pathways. It has been demonstrated that activation of PI 3-kinase alone is not sufficient to fully account for all insulin-stimulated glucose transport (Jiang *et al.* 1998) which has led to the identification of a PI 3-kinase independent or 'novel' insulin signalling cascade. In response to insulin the adaptor protein APS (adaptor containing PH and SH2 domains) recruits c-Cbl to the insulin receptor along with a second adaptor protein CAP (Cbl associated protein) (Ribon *et al.* 1998, Liu *et al.* 2002, 2003). c-Cbl is then phosphorylated on multiple tyrosine residues, followed by the disassociation and translocation of the CAP/c-Cbl complex to the lipid raft subdomains (Ribon *et al.* 1998, Liu *et al.* 2002, 2003) which results in the activation of small guanosine 5′-triphosphate (GTP)-binding proteins that facilitate GLUT4 translocation to the plasma membrane (Baumann *et al.* 2000, Chiang *et al.* 2001, Chang *et al.* 2002). While it has been reported in skeletal muscle that both mRNA (Ribon *et al.* 1998) and protein expression (Neudauer *et al.* 1998, Alcazar *et al.* 2004) for various components of this pathway exist, it has not been determined if aerobic exercise alters the novel insulin signalling cascade. Thus, a secondary aim of this investigation was to determine if chronic aerobic exercise alters c-Cbl protein concentration and/or insulin-stimulated phosphorylation in normal rodent skeletal muscle.

Materials and methods

Experimental design

Fourteen male Sprague–Dawley rats approximately 6 week of age were obtained from B & K Universal (Fremont, CA, USA). Each animal was randomly assigned to either a control (CON, $n = 7$) or chronic aerobic exercise (AT, $n = 7$) groups. Both CON and AT animals received standard laboratory chow and water *ad libitum* throughout the investigation. Rats were housed two per cage in a temperature-controlled environment (21 °C) with an artificial 12 : 12 h light-dark cycle.

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 U.S. Department of Health and Human Resources.

Aerobic training

Animals from the AT group trained 3 day week^{-1} for 12 week on a motor-driven treadmill (Quinton Instruments, Seattle, WA, USA) set at a 15% grade. Training began at 20 m min^{-1} for 20 min and continued to increase in both speed and duration over the first 4 weeks. Treadmill running was maintained for 45 min at 32 m min^{-1} for the remainder of the training period.

Surgical preparation and hind limb perfusions

After the 12 week aerobic training period and approximately 40 h after the last exercise bout animals were anaesthetized with an intraperitoneal injection of pentobarbital sodium [6.5 mg (100 g body weight)$^{-1}$] and surgically prepared for hind limb perfusion as we have described elsewhere (Yaspelkis *et al.* 2002, Krisan *et al.* 2004). Perfusions were carried out in the presence of 500 μU mL^{-1} insulin. After completion of the perfusion, portions of the red (RG) and white (WG) gastrocnemius were removed from both hind limbs, freeze clamped in liquid N_2 and stored at −80 °C for later analysis. Body mass, and muscle mass of the CON and AT animals have been published elsewhere (Yaspelkis *et al.* 2002), where we reported that while muscle mass was not different between the CON and AT animals, body mass of the AT animals (439 ± 17.1 g) was less than that of the CON animals (487.0 ± 8.6 g) at the end of the training period. A basal group was not included in the original experimental design as a

number of investigations, including two from our group, have shown that chronic exercise does not increase non-insulin stimulated rates of glucose uptake, glucose transport or affect basal activity of components of the insulin signalling cascade in skeletal muscle (Ivy *et al.* 1983, Ploug *et al.* 1990, Rodnick *et al.* 1992, Slentz *et al.* 1992, Brozinick *et al.* 1993, Ren *et al.* 1994, Etgen *et al.* 1997, Zhou & Dohm 1997, Chibalin *et al.* 2000, Kirwan *et al.* 2000, Christ *et al.* 2002, Luciano *et al.* 2002, Yaspelkis *et al.* 2002, Jessen *et al.* 2003, Krisan *et al.* 2004).

Western blotting

Muscles were cut, weighed frozen and homogenized as previously described by Singh *et al.* (2003). The supernatant was collected and protein concentration was determined by the Bradford method (Bradford 1976) using a Benchmark microplate reader (BioRad, Richmond, CA, USA). Methods used to determine IRS-1, Akt 1/2, and aPKC-ζ/λ protein concentration have been previously described (Krisan *et al.* 2004). Antibody binding was visualized by enhanced chemiluminescence in accordance to the manufacturer's instructions (West Femto, Pierce Chemical Company, Rockford, IL, USA). Images were captured using a CCD camera in a ChemiDoc system (BioRad) and saved to a Macintosh G4 computer. Bands were quantified using the freehand contour tool in the Quantity One analysis software (BioRad). The density of the bands were determined and expressed as a percentage of a skeletal muscle standard run on each gel.

IRS-1 associated PI 3-kinase activity

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

Akt phosphorylation

Lysates from insulin-stimulated skeletal muscles were used for the determination of Akt Ser 473 and Thr 308 phosphorylation as we have previously described (Krisan *et al.* 2004). Values were expressed as a percentage of an insulin-stimulated skeletal muscle standard run on each gel.

aPKC-ζ/λ activity

The aPKC-ζ/λ activity was determined as previously described (Krisan *et al.* 2004). Following the 12 min reaction period, samples were briefly centrifuged and 5 μL of supernatant was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) run under reducing conditions on a 20% resolving gel. Following SDS-PAGE the gel was wrapped in plastic wrap and exposed to a phosphor screen for 2 h. Images were captured and quantified as described above.

Akt kinase activity

Three hundred micrograms of insulin-stimulated muscle lysate protein was combined with either 4 μg of anti-Akt 1 [no. 07–416, Upstate Biotechnology (UBT), Charlottesville, VA, USA] or anti-Akt 2 (no. 07–372, UBT) and incubated overnight at 4 °C. One hundred microlitres of a protein A-sepharose slurry was then added to each immunoprecipitate and incubated with rotation at 4 °C for 1.5 h. Following incubation, samples were centrifuged at 18 300 ***g*** at 4 °C for 10 min and the supernatant was discarded. Ten microlitres of assay dilution buffer (UBT) was added to the immunocomplex in addition to protein kinase A (PKA) inhibitor peptide (UBT), and 10 μCi [γ-^{32} P] ATP (Perkin Elmer Life Sciences, Boston, MA, USA). Kinase reactions were initiated by the addition of the crosstide substrate oligopeptide (UBT), which contains a sequence homologous to glycogen synthase kinase-3, and warmed to 37 °C with constant mixing for 10 min. The reaction was terminated by the addition of 40 μL of Tris/Tricine sample buffer and heating at 95 °C for 5 min. Fifteen microlitres were loaded onto a 20% Tris/Tricine polyacrylamide gel in duplicate and electrophoresed for 130 min at 100 V using a MiniProtean 3 electrophoresis system (BioRad). Following electrophoresis, gels were wrapped in plastic wrap and exposed to a phosphor screen overnight. Images were captured and quantified as described above.

Plasma membrane c-Cbl and c-Cbl phosphorylation

Plasma membrane fractions were prepared from portions of perfused RG muscle samples as described elsewhere (Singh *et al.* 2003). The activity of the plasma membrane marker enzyme 5′-nucleotidase was assessed in plasma membrane fractions and compared with the activity in crude homogenate fractions to insure that purified plasma fractions were being used for analysis. 5′-nucleotidase activity (μmol min^{-1} mg protein^{-1}) indicated that the plasma membrane fractions were purified compared with the crude homogenate (CON: 7.3 ± 1.0 vs. 36.0 ± 5.6; AT: 7.4 ± 1.1 vs. 28.3 ± 3.1). Total c-Cbl protein concentration and c-Cbl phosphorylation were determined in aliquots of plasma membrane fractions. Five hundred micrograms of pro-

tein was immunoprecipitated with 4 μg of anti-c-Cbl [sc-170, Santa Cruz Biotechnology (SCBT), Santa Cruz, CA, USA] overnight at 4 °C. Following the overnight incubation, 100 μL of protein A-sepharose slurry was added to the anti-c-Cbl immunoprecipitates, and rotated for 2 h. The immunocomplex was washed once in buffer A and B and twice in buffer C as previously described (Singh *et al.* 2003). Sample buffer (250 mM Tris HCl (pH 6.8), 10% SDS, 10% β-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue) was added to each sample, and boiled at 100 °C for 5 min. Samples were then centrifuged at 18 300 ***g*** for 5 min at 4 °C. Following centrifugation, 300 μg of supernatant protein for total c-Cbl and 150 μg of supernatant protein for phosphorylated c-Cbl were subjected to SDS-PAGE run under reducing conditions. The resolved proteins were transferred to a polyvinylidene fluoride (PVDF) membrane as previously described in the western blotting section. The membranes were then incubated with either affinity purified polyclonal anti-c-Cbl (sc-170, SCBT) or anti-phosphotyrosine (anti-pY) (no. 06–427, UBT) for 1.5 h followed by incubation with either bovine anti-goat (sc-2350, SCBT) or goat anti-rabbit IgG conjugated HRP (sc-2004, SCBT). Antibody binding was visualized and quantified as described above.

Plasma membrane GLUT4 protein concentration

Insulin-stimulated GLUT4 protein concentration in plasma membrane fractions obtained from the RG was determined as previously described (Singh *et al.* 2003).

Statistical analysis

A one-way analysis of variance (ANOVA) was used on all variables to determine whether significant differences existed between CON and AT 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, Cary, NC, USA) and all values are expressed as mean ± SE.

Results

3-O-Methylglucose transport

Rates of insulin-stimulated 3-O-methylglucose (3-MG) transport in the RG of the AT animals (10.96 ± 0.9 μmol g^{-1} h^{-1}) were significantly greater than the CON animals (6.99 ± 0.3 μmol g^{-1} h^{-1}). No differences in 3-MG transport rates were found in WG between the AT (2.39 ± 0.6 μmol g^{-1} h^{-1}) and CON (1.78 ± 0.4 μmol g^{-1} h^{-1}) animals.

The possibility existed that chronic aerobic exercise might have increased basal rates of glucose metabolism and accounted, in part, for some of the training adaptations observed. To address this concern we evaluated a limited subset of CON and AT animals perfused in the absence of insulin and found that basal rates of 3-MG transport rates in the RG (CON: 3.15 ± 0.3 μmol g^{-1} h^{-1} vs. AT: 3.28 ± 0.3 μmol g^{-1} h^{-1}) and WG (CON: 1.95 ± 0.1 μmol g^{-1} h^{-1} vs. AT: 2.03 ± 0.1 μmol g^{-1} h^{-1}) were identical. As this observation was consistent with an extensive body literature that has already reported chronic aerobic exercise does not increase non-insulin stimulated rates of glucose transport or affect insulin signalling activity we did not further analyse this tissue.

Classical insulin signalling cascade

Chronic aerobic exercise increased insulin-stimulated IRS-1 associated PI 3-kinase activity in the RG but did not affect the activity of this enzyme in the WG (Fig. 1). Training induced improvements in PI 3-kinase activity were not because of alterations in total IRS-1 protein concentration as the total protein concentration remained unchanged in both the RG and WG (Fig. 2a).

We next assessed the effect of chronic aerobic exercise on proteins downstream from PI 3-kinase in normal rodent skeletal muscle. We observed that 12 week of aerobic training did not alter Akt 1/2 protein concentration (Fig. 2b), aPKC-ζ protein concentration (Fig. 2c), aPKC-λ protein concentration

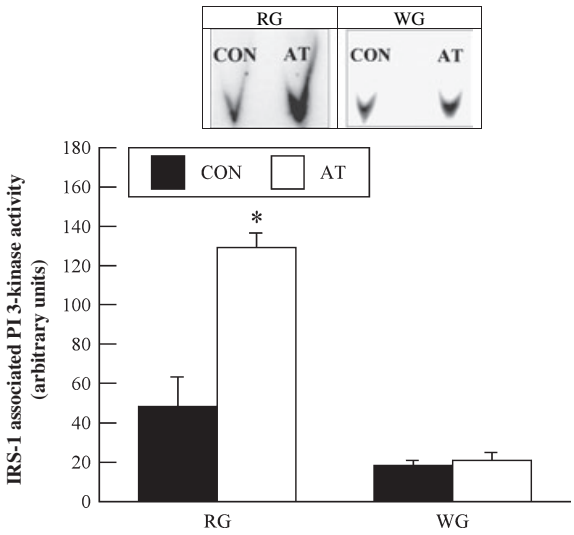

Figure 1 IRS-1 associated PI 3-kinase activity in skeletal muscles from control (CON) or aerobically trained (AT) animals. RG, red gastrocnemius; WG, white gastrocnemius. *Significantly different from CON ($P < 0.05$). Values are mean ± SE.

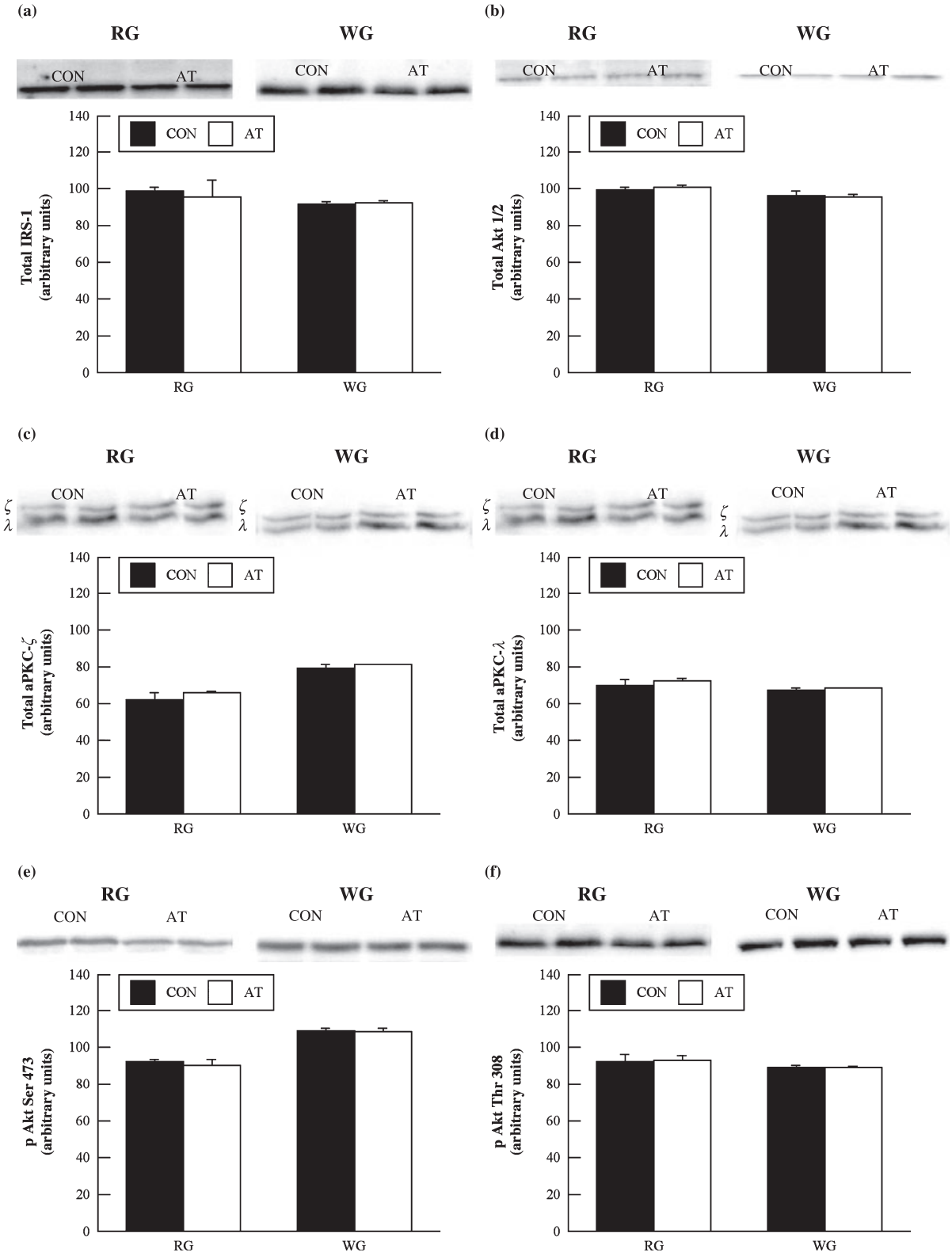

Figure 2 (a) IRS-1 protein concentration, (b) Akt 1/2 protein concentration, (c) aPKC-ζ protein concentration, (d) aPKC-λ protein concentration, (e) pAkt Ser 473, and (f) pAkt Thr 308. CON, control animals; AT, aerobically trained animals; RG, red gastrocnemius; WG, white gastrocnemius. No differences existed within muscles among experimental groups.

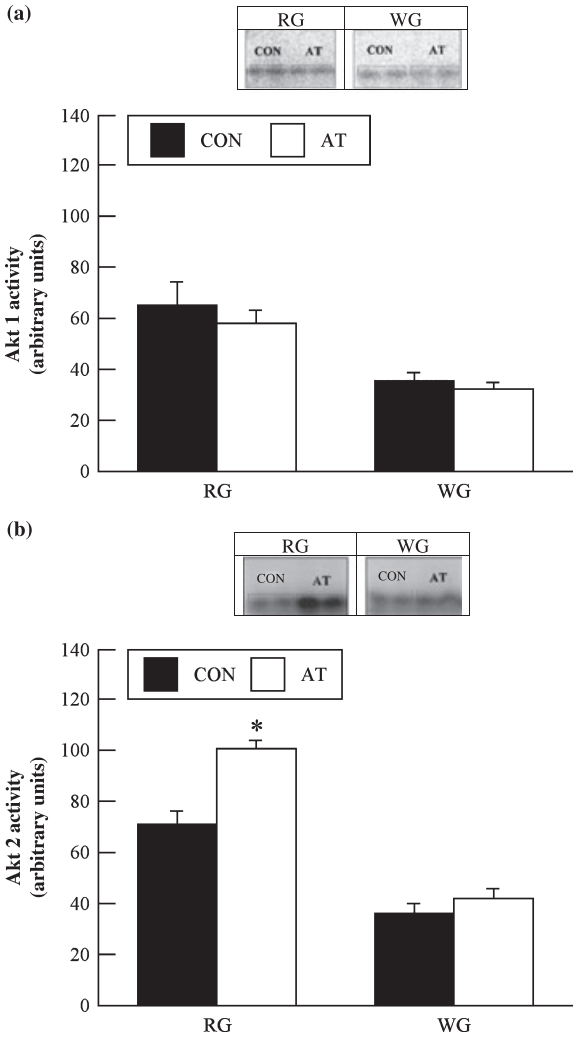

Figure 3 (a) Akt 1 kinase activity and (b) Akt 2 kinase activity in skeletal muscles from control (CON) or aerobically trained (AT) animals. RG, red gastrocnemius; WG, white gastrocnemius. *Significantly different from CON ($P < 0.05$). Values are mean ± SE.

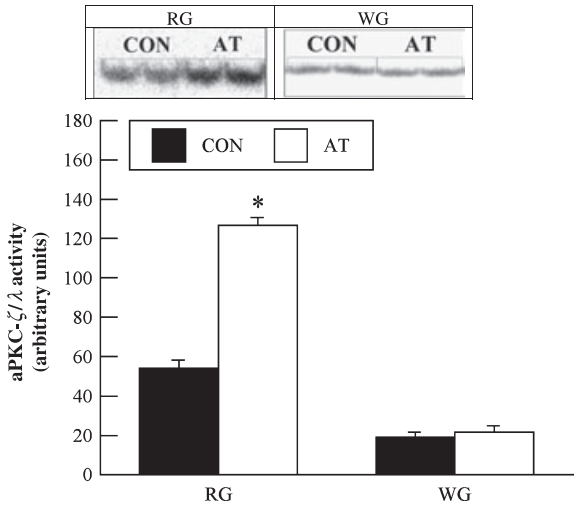

Figure 4 aPKC-ζ/λ kinase activity in skeletal muscles from control (CON) or aerobically trained (AT) animals. RG, red gastrocnemius; WG, white gastrocnemius. *Significantly different from CON ($P < 0.05$). Values are mean ± SE.

(Fig. 2d), insulin-stimulated Akt 1 Ser 473 phosphorylation (Fig. 2e), insulin-stimulated Akt 1/2/3 Thr 308 phosphorylation (Fig. 2f), or insulin-stimulated Akt 1 activity (Fig. 3a) in either the RG or WG. Despite chronic aerobic exercise having no effect on protein expression or phosphorylation on these components of the classical insulin signalling cascade we did find that insulin-stimulated Akt 2 (Fig. 3b) and aPKC-ζ/λ (Fig. 4) activities were significantly increased in the RG from the aerobically trained animals.

Novel insulin signalling cascade

Chronic aerobic exercise did not alter plasma membrane c-Cbl protein concentration in the presence of insulin (Fig. 5a). Although plasma membrane c-Cbl protein concentration was unchanged, insulin-stimulated tyrosine phosphorylation of c-Cbl was significantly increased in plasma membrane fractions that were prepared from the RG of aerobically trained animals (Fig. 5b).

Plasma membrane GLUT4 protein concentration

Insulin-stimulated GLUT4 protein concentration in plasma membrane fractions prepared from aerobically trained skeletal muscle was greater than plasma membrane fractions prepared from control animals (Fig. 6).

Discussion

We chose to initiate our evaluation of the insulin signalling cascade by assessing insulin-stimulated IRS-1 associated PI 3-kinase activity as PI 3-kinase activity was the most commonly measured component of the insulin signalling cascade in the training studies we found (Kim *et al.* 1999a, Arias *et al.* 2001, Luciano *et al.* 2002) and that impairments in PI 3-kinase activity are associated with decreased rates of insulin-stimulated glucose uptake and transport (Singh *et al.* 2003, Krisan *et al.* 2004). It has been observed that PI 3-kinase activity is increased in skeletal muscle of normal rodents subjected to 1 day, 5 day or 6 week of swim training (Zhou & Dohm 1997, Chibalin *et al.* 2000, Luciano *et al.* 2002). In addition, PI 3-kinase activity is increased in normal human skeletal muscle following both short-term (Houmard *et al.* 1999) and chronic (Kirwan *et al.* 2000) exercise. Consistent with these findings we

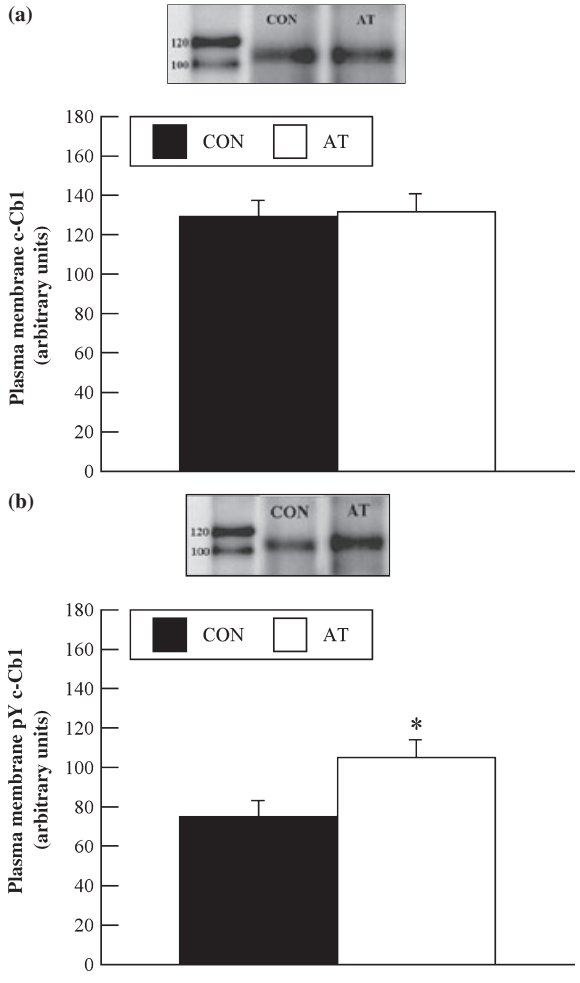

Figure 5 Insulin-stimulated plasma membrane (a) c-Cbl protein concentration and (b) tyrosine phosphorylated c-Cbl from RG of control (CON) or aerobically trained (AT) animals. *Significantly different from CON ($P < 0.05$). Values are mean ± SE.

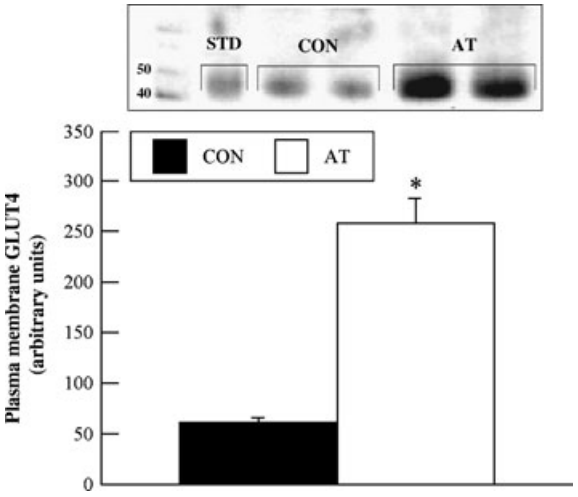

Figure 6 Insulin-stimulated plasma membrane GLUT4 protein concentration from RG of control (CON) or aerobically trained (AT) animals. *Significantly different from CON ($P < 0.05$). Values are mean ± SE.

observed that 12 week of treadmill training increased PI 3-kinase activity in normal rodent skeletal muscle. The improvements in PI 3-kinase activity (as well as for the other kinases assessed) occurred in a fibre type specific manner in response to aerobic training. Specifically, only the RG exhibited training-induced effects, which may be a consequence of recruitment, as evidenced by alterations in citrate synthase activity having occurred in only the RG (Yaspelkis *et al.* 2002). Alternatively, it is possible that the RG adapted to the point where recruitment of the WG was no longer required and that the training adaptation was lost. Nevertheless, it should be noted that Christ *et al.* (2002) has reported that PI 3-kinase activity in skeletal muscle from aerobically trained obese Zucker rats is unaltered suggesting that chronic aerobic exercise may not correct genetic defects in glucose transport.

Our next point of inquiry was to determine if the activation of proteins downstream of PI 3-kinase were affected by chronic aerobic exercise in normal rodent skeletal muscle. The serine/threonine kinase, Akt, has been the subject of study because of its role in transducing signals downstream from PI 3-kinase which may be involved in insulin-stimulated glucose transporter translocation (Shepherd *et al.* 1998, Sano *et al.* 2003). Three isoforms of Akt have been identified and investigations by Birnbaum's group suggest a role for Akt 2 in insulin-stimulated glucose transport (Cho *et al.* 2001a), but not Akt 1 (Cho *et al.* 2001b). It is known that activation of Akt by PI 3-kinase is reliant on phosphorylation of Thr 308 and Ser 473 residues by PDK-1 (Vanhaesebroeck & Alessi 2000) and PDK-2 (Balendran *et al.* 1999), respectively. However, there is some debate concerning the role of Akt in the activation of the glucose transporter effector system in that several investigations have shown phosphorylation of Akt to be impaired (Oku *et al.* 2001, Vollenweider *et al.* 2002) or unaffected in insulin resistant tissue (Kim *et al.* 1999b, Kruszynska *et al.* 2002). With respect to exercise, an acute exercise bout does not alter Akt protein concentration (Chibalin *et al.* 2000) or insulin-stimulated Akt Ser 473 phosphorylation (Sakamoto *et al.* 2003) in normal rodent skeletal muscle, although it has been reported that 5 day of exercise increased insulin-stimulated Akt Ser 473 phosphorylation (Chibalin *et al.* 2000). However, it does not appear that previous investigations have evaluated Akt concentration, insulin-stimulated Akt phosphorylation or insulin-stimulated Akt isoform specific activity in normal rodent skeletal muscle that had been subjected to chronic

treadmill exercise. Therefore, our observation that 12 week of aerobic exercise training did not alter total Akt 1/2 protein concentration, insulin-stimulated Akt Ser 473 phosphorylation, insulin-stimulated Akt Thr 308 phosphorylation or insulin-stimulated Akt 1 activity in normal rodent skeletal muscle appears to be a novel finding. Of greater interest although was our observation that chronic aerobic exercise did increase insulin-stimulated Akt 2 activity, but only in the RG. Brozinick *et al.* (2003) reported that in insulin resistant human skeletal muscle Akt 2 and 3, but not Akt 1, activation is impaired and related to reduced rates of glucose uptake. Additionally, Jiang *et al.* (2003) have observed in 3T3-L1 adipocytes that Akt 2 alone accounts for the majority of insulin-regulated glucose uptake. These data clearly implicate Akt 2 as the key isoform of this serine/threonine kinase and suggests future efforts be directed towards ascertaining how activation of Akt 2 is regulated.

Despite chronic exercise enhancing insulin-stimulated Akt 2 activity, we are unable to reconcile the dissociation from either insulin-stimulated Akt Ser 473 phosphorylation, or Thr 308 phosphorylation. However, this is not the first time that Akt phosphorylation has been dissociated from Akt activity. Tremblay *et al.* (2001) and Krisan *et al.* (2004) have reported a high-fat diet does not alter Akt 1/2 protein concentration, Akt Ser 473 phosphorylation and Akt Thr 308 phosphorylation, but does reduce insulin-stimulated Akt activity in rodent skeletal muscle. Additionally, Kanoh *et al.* (2003) have reported that defective insulin-stimulated aPKC activation in high-fat fed rodent skeletal muscle cannot be explained by diminished PDK-1-dependent Thr 410 phosphorylation and appears to be because of impaired PI-3,4,5-$(PO_4)_3$ responsiveness at activation steps distal to PDK-1-dependent loop phosphorylation. These observations taken collectively clearly indicate that although Akt activation would appear to be unaltered in skeletal muscle, if based solely on evaluation of Akt Ser 473 and Akt Thr 308 phosphorylation, the ability of Akt kinase to phosphorylate a substrate can be significantly altered and suggest that potential errors in data interpretation may occur if only skeletal muscle Akt phosphorylation, but not Akt isoform specific activity, are evaluated.

Recent studies suggest that activation of aPKC-ζ/λ, also a downstream target of PI 3-kinase, is suppressed in insulin resistant skeletal muscle (Vollenweider *et al.* 2002) and appears to be negatively affected by high-fat feeding (Tremblay *et al.* 2001, Krisan *et al.* 2004), but it has not been determined if chronic aerobic exercise training alters either aPKC-ζ/λ protein concentration or insulin-stimulated activation in normal rodent skeletal muscle. After 12 week of training we found insulin-stimulated aPKC-ζ/λ activation to be enhanced in the RG in the absence of an increase total skeletal muscle aPKC-ζ/λ protein concentration. Then again this appears to be a normal training adaptation as Nielsen *et al.* (2003) recently reported that in aerobically trained human skeletal muscle insulin-stimulated aPKC-ζ/λ activity was enhanced with no changes occurring in total aPKC-ζ/λ protein concentration.

While the majority of exercise studies have evaluated the effects of training on insulin-stimulated IRS-1 associated PI 3-kinase activity and various downstream proteins of this cascade, it has recently been demonstrated that activation of PI 3-kinase alone may not be sufficient to fully account for insulin-stimulated glucose transport (Jiang *et al.* 1998). A second 'novel insulin signalling cascade', or 'PI 3-kinase independent', pathway has been identified and suggested to work in concert with the classical insulin signalling cascade. For this investigation we limited our preliminary evaluation of the novel insulin signalling cascade to c-Cbl, which is an insulin receptor substrate (Ribon & Saltiel 1997). The phosphorylation of c-Cbl by the insulin receptor kinase is facilitated by APS which once tyrosine phosphorylated recruits c-Cbl to the insulin receptor for subsequent phosphorylation of tyrosines 700 and 774 (Liu *et al.* 2002). Although chronic aerobic exercise did not alter the plasma membrane c-Cbl protein concentration, insulin-stimulated c-Cbl tyrosine phosphorylation was significantly enhanced in plasma membranes obtained from the RG of the trained animals. While the activation of the novel insulin signalling cascade has garnered some preliminary evaluation in skeletal muscle obtained from genetic (Wadley *et al.* 2004) and pharmacologically induced models (Thirone *et al.* 2004) of insulin resistance, to the best of our knowledge, the present investigation is the first to assess the activation of a component of this pathway in skeletal muscle obtained from normal rodents that were subjected to aerobic exercise. At the present time the specific role the novel insulin signalling cascade plays in the regulation of insulin-stimulated glucose transport is unclear, although numerous studies suggest that this pathway is involved in the translocation of GLUT4 (Baumann *et al.* 2000, Chiang *et al.* 2001, Liu *et al.* 2002, 2003). While our finding suggests that chronic aerobic training may enhance insulin-stimulated activation of the novel insulin signalling cascade in normal rodent skeletal muscle, which may contribute to enhanced recruitment of GLUT4 to the plasma membrane, additional investigation of this cascade is warranted to better evaluate the functional significance of this observation.

In summary, when normal rodents are subjected to 12 week of chronic aerobic exercise, insulin-stimulated skeletal muscle PI 3-kinase, Akt 2 kinase and aPKC-ζ/λ kinase activities are increased in a fibre type specific

response to aerobic training. In addition, chronic aerobic exercise increased insulin-stimulated c-Cbl tyrosine phosphorylation. Collectively, these findings suggest that chronic aerobic exercise enhances components of both the classical and novel signalling pathway in normal rodent skeletal muscle and appears to contribute to an increased insulin-stimulated plasma membrane GLUT4 protein concentration.

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References

Alcazar, O., Ho, R.C., Fujii, N. & Goodyear, L.J. 2004. cDNA cloning and functional characterization of novel splice variant of c-Cbl-associated protein from mouse skeletal muscle. *Biochem Biophys Res Commun* **317**, 285–293.

Arias, E.B., Gosselin, L.E. & Cartee, G.D. 2001. Exercise training eliminates age-related differences in skeletal muscle insulin receptor and IRS-1 abundance in rats. *J Gerontol A Biol Sci Med Sci* **56A**, B449–B455.

Balendran, A., Cassamayor, A., Deak, M. *et al.* 1999. PDK-1 acquires PDK-2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. *Curr Biol* **9**, 393–404.

Baumann, C.A., Ribon, V., Kanzaki, M. *et al.* 2000. CAP defines a second signalling pathway required for insulin-stimulated glucose transport. *Nature* **407**, 202–207.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

Brozinick, J.T. Jr., Etgen, G.J. Jr., Yaspelkis, B.B. III., Kang, H.Y. & Ivy, J.L. 1993. Effects of exercise training on muscle GLUT4 protein content and translocation in obese Zucker rats. *Am J Physiol Endocrinol Metab* **265**, 419–427.

Brozinick, J.T. Jr., Roberts, B.R. & Dohm, G.L. 2003. Defective signaling through Akt-2 and -3 but not Akt-1 in insulin-resistant human skeletal muscle. *Diabetes* **52**, 935–941.

Chang, L., Adams, R.D. & Saltiel, A.R. 2002. The TC10-interacting protein CIP4/2 is required for insulin-stimulated Glut4 translocation in 3T3L1 adipocytes. *Proc Natl Acad Sci USA* **99**, 12835–12840.

Chiang, S.H., Baumann, C.A., Kanzaki, M. *et al.* 2001. Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation ofTC10. *Nature* **410**, 944–948.

Chibalin, A.V., Yu, M., Ryder, J.W. *et al.* 2000. Exercise-induced changes in expression and activity of proteins involved in insulin signal transduction in skeletal muscle: differential effects on insulin-receptor substrates 1 and 2. *Proc Natl Acad Sci USA* **97**, 38–43.

Cho, H., Mu, J., Kim, J. *et al.* 2001a. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science* **292**, 1728–1731.

Cho, H., Thorvaldsen, J.L., Chu, Q., Feng, F. & Birnbaum, M.J. 2001b. Akt1/PKBα is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J Biol Chem* **276**, 38349–38352.

Christ, C.Y., Hunt, D., Hancock, J. *et al.* 2002. Exercise training improves muscle insulin resistance but not insulin receptor signaling in obese Zucker rats. *J Appl Physiol* **92**, 736–744.

Etgen, G.J. Jr, Jensen, J., Wilson, C. *et al.* 1997. Exercise training reverses insulin resistance in muscle by enhanced recruitment of GLUT-4 to the cell surface. *Am J Physiol Endocrinol Metab* **272**, E864–E869.

Houmard, J.A., Shaw, C.D., Hickey, M.S. & Tanner, C.J. 1999. Effect of short-term exercise training on insulin-stimulated PI 3-kinase activity in human skeletal muscle. *Am J Physiol* **277**, E1055–E1060.

Ivy, J.L., Young, J.C., McLane, A., Fell, R.D. & Holloszy, J.O. 1983. Exercise training and glucose uptake by skeletal muscle in rats. *J Appl Physiol* **55**, 1393–1396.

Jessen, N., Pold, R., Buhl, E.S. *et al.* 2003. Effects of AICAR and exercise on insulin-stimulated glucose uptake, signaling, and GLUT-4 content in rat muscle. *J Appl Physiol* **94**, 1373–1379.

Jiang, T., Sweeney, G., Rudolf, M.T. *et al.* 1998. Membrane-permeant esters of phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* **273**, 11017–11024.

Jiang, Z.Y., Zhou, Q.L., Coleman, K.A. *et al.* 2003. Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. *Proc Natl Acad Sci USA* **100**, 7569–7574.

Kanoh, Y., Sajan, M.P., Bandyopadhyay, G. *et al.* 2003. Defective activation of atypical protein kinase C zeta and lambda by insulin and phosphatidylinositol-3,4,5-$(PO_4)_3$ in skeletal muscle of rats following high-fat feeding and streptozotocin-induced diabetes. *Endocrinology* **144**, 947–954.

Kim, Y.-B., Inoue, T., Nakajima, R. *et al.* 1999a. Effect of long-term exercise on gene expression of insulin signaling pathway intermediates in skeletal muscle. *Biochem Biophys Res Commun* **254**, 720–727.

Kim, Y.-B., Nikoulina, S.E., Ciaraldi, T.P., Henry, R.R. & Kahn, B.K. 1999b. Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. *J Clin Invest* **104**, 733–741.

Kirwan, J.P., Del Aguila, L.F., Hernandez, J.M. *et al.* 2000. Regular exercise enhances insulin activation of IRS-1-associated PI3-kinase in human skeletal muscle. *J Appl Physiol* **88**, 797–803.

Krisan, A.D., Collins, D.E., Crain, A.M. *et al.* 2004. Resistance training enhances components of the insulin signaling cascade in normal and high-fat-fed skeletal muscle. *J Appl Physiol* **96**, 1691–1700.

Kruszynska, Y.T., Worall, D.S., Ofrecio, J. *et al.* 2002. Fatty acid-induced insulin resistance: decreased muscle PI3K activation but unchanged Akt phosphorylation. *J Clin Endocrinol Metab* **87**, 226–234.

Liu, J., Kimura, A., Baumann, C.A. & Saltiel, A.R. 2002. APS facilitates c-Cbl tyrosine phosphorylation and GLUT4 translocation in response to insulin in 3T3-L1 adipocytes. *Mol Cell Biol* **22**, 3599–3609.

Liu, J., DeYoung, S.M., Hwang, J.B., O'Leary, E.E. & Saltiel, A.R. 2003. The roles of Cbl-b and c-Cbl in insulin-stimulated glucose transport. *J Biol Chem* **278**, 36754–36762.

Luciano, E., Carneiro, E.M., Carvalho, C.R. *et al.* 2002. Endurance training improves responsiveness to insulin and modulates insulin signal transduction through the phosphatidylinositol 3-kinase/Akt-1 pathway. *Eur J Endocrinol* **147**, 149–157.

Neudauer, C.L., Joberty, G., Tatsis, N. & Macara, I.G. 1998. Distinct cellular effects and interactions of the Rho-family GTPase TC10. *Curr Biol* **8**, 1151–1160.

Nielsen, J.N., Frosig, C.C., Sajan, M.P. *et al.* 2003. Increased atypical PKC activity in endurance-trained human skeletal muscle. *Biochem Biophys Res Commun* **312**, 1147–1153.

Oku, A., Nawano, M., Ueta, K. *et al.* 2001. Inhibitory effect of hyperglycemia on insulin-induced Akt/Protein kinase B activation in skeletal muscle. *Am J Physiol Endocrinol Metab* **280**, E816–E824.

Ploug, T., Stallknect, B.M., Pederson, O. *et al.* 1990. Effect of endurance training on glucose transport capacity and glucose transporter expression in rat skeletal muscle. *Am J Physiol Endocrinol Metab* **259**, E779–E786.

Ren, J., Semenkovich, C., Gulve, E., Gao, J. & Holloszy, J. 1994. Exercise induces rapid increases in GLUT4 expression, glucose transport capacity, and insulin-stimulated glycogen storage in muscle. *J Biol Chem* **269**, 14396–14401.

Ribon, V. & Saltiel, A.R. 1997. Insulin stimulates tyrosine phosphorylation of the proto-oncogene product of c-Cbl in 3T3-L1 adipocytes. *Biochem J* **324**, 839–845.

Ribon, V., Printen, J.A., Hoffman, N.G., Kay, B.K. & Saltiel, A.R. 1998. A novel, multifuntional c-Cbl binding protein in insulin receptor signaling in 3T3-L1 adipocytes. *Mol Cell Biol* **18**, 872–879.

Rodnick, K.J., Henriksen, E.J., James, D.E. & Holloszy, J.O. 1992. Exercise training, glucose transporters, and glucose transport in rat skeletal muscle. *Am J Physiol Cell Physiol* **262**, C9–C14.

Sakamoto, K., Aschenbach, W.G., Hirshman, M.F. & Goodyear, L.J. 2003. Akt signaling in skeletal muscle: regulation by exercise and passive stretch. *Am J Physiol Endocrinol Metab* **285**, E1081–1088.

Sano, H., Kane, S., Sano, E. *et al.* 2003. Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J Biol Chem* **278**, 14599–14602.

Shepherd, P.R., Withers, D.J. & Siddle, K. 1998. Phosphoinositide 3-kinase: the key switch mechanism in insulin signaling. *Biochem J* **333**, 471–490.

Singh, M.K., Krisan, A.D., Crain, A.M., Collins, D.E. & Yaspelkis, B.B., III 2003. High-fat diet and leptin treatment alter skeletal muscle insulin-stimulated phosphatidylinositol 3-kinase activity and glucose transport. *Metabolism* **52**, 1196–1205.

Slentz, C.A., Gulve, E.A., Rodnick, K.J. *et al.* 1992. Glucose transporters and maximal transport are increased in endurance-trained rat soleus. *J Appl Physiol* **73**, 486–492.

Thirone, A.C., Carvalheira, J.B., Hirata, A.E., Velloso, L.A. & Saad, M.J. 2004. Regulation of Cbl-associated protein/Cbl pathway in muscle and adipose tissues of two animal models of insulin resistance. *Endocrinology* **145**, 281–293.

Tremblay, F., Lavigne, C., Jacques, H. & Marette, A. 2001. Defective insulin-induced GLUT4 translocation in skeletal muscle of high fat-fed rats is associated with alterations in both Akt/protein kinase B and atypical protein kinase C (ζ/λ) activities. *Diabetes* **50**, 1901–1910.

Vanhaesebroeck, B.D. & Alessi, R. 2000. The PI3K-PDK-1 connection: more than just a road to PKB. *Biochem J* **346**, 561–576.

Vollenweider, P., Menard, B. & Nicod, P. 2002. Insulin resistance, defective insulin receptor substrate 2-associated phosphatidylinositol 3′-kinase activation, and impaired atypical protein kinase C (zeta/lambda) activation in myotubes from obese patients with impaired glucose tolerance. *Diabetes* **51**, 1052–1059.

Wadley, G.D., Bruce, C.R., Konstantopoulos, N. *et al.* 2004. The effect of insulin and exercise on c-Cbl protein abundance and phosphorylation in insulin-resistant skeletal muscle in lean and obese Zucker rats. *Diabetologia* **47**, 412–419.

Yaspelkis, B.B. III, Singh, M.K., Trevino, B., Krisan, A.D. & Collins, D.E. 2002. Resistance training increases glucose uptake and transport in rat skeletal muscle. *Acta Physiol Scand* **175**, 315–323.

Zhou, Q. & Dohm, G.L. 1997. Treadmill running increases phosphatidylinositol 3-kinase activity in rat skeletal muscle. *Biochem Biophys Res Commun* **236**, 647–650.

