Insulin-stimulated plasma membrane association and activation of Akt2, aPKC ζ and aPKC λ in high fat fed rodent skeletal muscle

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Several recent reports using cell lines have suggested that both Akt and atypical protein kinase C (aPKC) ζ/λ are translocated to the plasma membrane (PM) in response to insulin. However, it has yet to be determined in skeletal muscle whether: (1) insulin increases PM-associated Akt2, aPKC ζ and/or λ protein concentration, (2) the activity of these kinases is altered by insulin at the PM, and (3) high fat feeding alters the insulin-stimulated PM concentration and/or activity of Akt2 and aPKC ζ/λ. Sprague-Dawley rats were randomly assigned to either normal ($n = 16$) or high fat ($n = 16$) dietary groups. Following a 12 week dietary period, animals were subjected to hind limb perfusions in the presence ($n = 8$ per group) or absence ($n = 8$ per group) of insulin. In normal skeletal muscle, total PI3-kinase, Akt2 and aPKC ζ/λ activities were increased by insulin. PM-associated aPKC ζ and λ, and aPKC ζ/λ activity, but not Akt2 or Akt2 activity, were increased by insulin in normal muscle. High fat feeding did not alter total skeletal muscle Akt2, aPKC ζ or aPKC λ protein concentration. Insulin-stimulated total PI3-kinase, Akt2 and aPKC ζ/λ activities were reduced in the high fat fed animals. Insulin-stimulated PM aPKC ζ, aPKC λ, aPKC ζ/λ activity and GLUT4 protein concentration were also reduced in high fat fed animals. These findings suggest that in skeletal muscle, insulin stimulates translocation of aPKC ζ and λ, but not Akt2, to the PM. In addition, high fat feeding impairs insulin-stimulated activation of total aPKC ζ/λ and Akt2, as well as PM association and activation of aPKC ζ and λ.

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Insulin stimulation increases phosphoinositide 3-kinase (PI3-kinase) activity, resulting in phosphorylation and activation of Akt and atypical protein kinase C (aPKC) ζ/λ (Ducluzeau *et al.* 2002; Chen *et al.* 2003; Katome *et al.* 2003). Akt2 appears to have a primary role in the regulation of insulin-stimulated glucose transport (Cho *et al.* 2001*a*; Jiang *et al.* 2003) while Akt1 and Akt3 may contribute to the regulation of glucose transport in a secondary role (Walker *et al.* 1998; Cho *et al.* 2001*b*; Brozinick *et al.* 2003). However, insulin-stimulated activation of Akt2 does not appear to fully account for GLUT4 translocation (Cong *et al.* 1997; Kotani *et al.* 1998) as it has been observed that dominant-negative Akt expression reduces, but does not prevent, GLUT4 translocation (Cong *et al.* 1997; Bandyopadhyay *et al.* 1999*b*; Ducluzeau *et al.* 2002). Rather, it appears that aPKC ζ/λ functions in parallel with Akt to facilitate insulin-stimulated GLUT4 translocation (Bandyopadhyay *et al.* 1999*a*; Standaert *et al.* 1999).

In response to insulin stimulation, both Akt2 (Andjelkovic *et al.* 1997; Goransson *et al.* 1998; Chen *et al.* 2003; Sasaoka *et al.* 2004) and aPKC ζ/λ (Standaert *et al.* 1999; Braiman *et al.* 2001) are translocated to the plasma membrane, where it is believed that they are activated (Galetic *et al.* 1999; Kanzaki *et al.* 2004). Insulin stimulation not only results in the translocation of aPKC ζ/λ to the plasma membrane in muscle cell cultures, but it is associated with GLUT4 vesicles (Braiman *et al.* 2001). It should be noted though that insulin-stimulated Akt and aPKC ζ/λ translocation to the plasma membrane has only been assessed in cell lines and adipocytes. However, it has been reported that exercise increases the activation and plasma membrane association of aPKC ζ/λ in human skeletal muscle (Nielsen *et al.* 2003; Perrini *et al.* 2004; Rose *et al.* 2004). Therefore, the initial aim of this investigation was to assess whether insulin increases the plasma membrane concentration and activation of Akt and/or aPKC ζ/λ in skeletal muscle.

It has been reported that a high-fat diet impairs carbohydrate metabolism in rodent skeletal muscle by reducing insulin-stimulated IRS-1-associated PI3-kinase,

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Akt2, and aPKC ζ/λ activities (Tremblay *et al.* 2001; Singh *et al.* 2003; Krisan *et al.* 2004; Yaspelkis *et al.* 2004) and may account for a reduced plasma membrane GLUT4 protein concentration (Yaspelkis *et al.* 2001; Singh *et al.* 2003). Thus, the second aim of this investigation was to assess whether insulin-stimulated compartmentalization and/or activation of aPKC ζ/λ and Akt2 is altered by chronic high fat feeding and related to reduced GLUT4 translocation in skeletal muscle.

Methods

Experimental design

Thirty-two male Sprague-Dawley rats approximately 6 weeks old ranging in weight from 185 to 220 g (Harlan, San Diego, CA, USA) were placed randomly into one of two groups: normal diet ($n = 16$) or high-fat diet ($n = 16$). The normal diet (no. 112386, Dyets Inc., Bethlehem, PA, USA) consisted of 63% carbohydrates, 17% fat, and 15% protein. The high fat diet (no. 112387, Dyets Inc.) contained 26% carbohydrates, 59% fat, and 15% protein. The vitamin, mineral, and fibre content were the same for both groups. The animals were on their respective diets for 12 weeks and allowed to feed *ad libitum*. This high fat diet has previously been shown to induce skeletal muscle insulin resistance in male Sprague-Dawley rats (Singh *et al.* 2003; Krisan *et al.* 2004; Yaspelkis *et al.* 2004). Rats were housed two per cage in a temperature controlled room (21°C) with an artificial light cycle 12 : 12 light–dark cycle. After 12 weeks of feeding, animals were subdivided into four groups: normal diet insulin ($n = 8$), normal diet basal ($n = 8$), high fat diet insulin ($n = 8$), high fat diet basal ($n = 8$), and subjected to hind limb perfusion.

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 Health and Human Resources.

Hind limb perfusions

Animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (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). 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% O_2–5% CO_2 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 or absence of 500 μU ml^{-1} insulin. The basic perfusate medium consisted of 30% washed time-expired human erythrocytes (Ogden Medical Center, Ogden, UT, USA), KHB, 4% dialysed bovine serum albumin (Fisher Scientific, Fairlawn, 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-[^{3}H]MG mM^{-1}, PerkinElmer Life Sciences, Boston, MA, USA) and 2 mM mannitol (60 μCi-[1-^{14}C]mannitol mM^{-1}, PerkinElmer Life Sciences). Immediately after the transport period, portions of the red quadricep (RQ) were excised from both hind limbs, blotted on gauze dampened with cold KHB, freeze clamped in liquid N_2 and stored at −80°C for later analysis.

3 MG transport

Rates of insulin-stimulated skeletal muscle 3-MG transport were calculated as previously described (Yaspelkis *et al.* 2001, 2004; Krisan *et al.* 2004).

Muscle homoginization for Western blotting

Portions were cut from the RQ, weighed frozen and homogenized in an ice-cold homogenization buffer (HB) 1 : 10 wt/vol containing 50.0 mM Hepes (pH 7.6), 150 mM NaCl, 200 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 20 mM NaF, 2 mM orthovanadate, 20 mM EDTA, 10% IGEPAL, 10% glycerol, 20 mM phenylmethylsufonylflouride, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 μg ml^{-1} leupeptin and 10 μg ml^{-1} aprotinin. The homogenate was then transferred to a microcentrifuge tube and centrifuged (19 600 *g*, 4°C) in a refrigerated microcentrifuge (Micromax RF, International Equipment Co., Needham Heights, MA, USA) for 15 min. The supernatant was collected, labelled as lysate and assayed for protein concentration using the Bradford method (Bradford, 1976) adapted for use with a Benchmark microplate reader (Bio-Rad, Richmond, CA, USA).

Plasma membrane fractionation

Plasma membrane fractions were prepared as we (Yaspelkis *et al.* 2001, 2002; Singh *et al.* 2003) and others (Ahmed *et al.* 1990; Turcotte *et al.* 1997, 1999) have previously described. Briefly, insulin-stimulated RQ samples were

Table 1. Membrane fractionation characterization

	Basal		Insulin	
	Normal Diet	High fat diet	Normal diet	High fat diet
5′-Nucleotidase activity (nmol min^{-1} mg $protein^{-1}$)				
Plasma membrane	330.22 ± 57.3	405.72 ± 72.2	314.35 ± 66.7	354.22 ± 68.8
Crude homogenate	57.72 ± 11.0	50.33 ± 9.9	57.00 ± 11.4	48.36 ± 14.0
Fold Increase	7.2 ± 2.1	8.2 ± 2.8	8.9 ± 2.6	10.8 ± 2.4
Percentage yield	13.5 ± 5.2	16.6 ± 7.2	16.2 ± 7.4	22.4 ± 4.8

Values are means ± S.E.M.

minced, diluted 1 : 7 in a 10 mM Tris−15% sucrose solution (pH 7.5) with 0.1 mM phenylmethylsufonyl fluoride, 10 mM EGTA, 10 mg ml^{-1} trypsin inhibitor and homogenized with a PT 2100 Polytron homogenizer (Kinemaica, Littau/Luzern, Switzerland). The homogenate was filtered and centrifuged at 100 000 *g* for 1 h at 4°C in a Sorvall TFT 50.38 fixed angle rotor (Kendro Laboratory Products, Newton, CT, USA). The pellet was resuspended in 6 ml of 1 mM Tris−15% sucrose buffer, and 50 μl of crude homogenate was collected and retained for later analysis. The remaining homogenate suspension was layered on continuous sucrose gradients (35% to 70%) and centrifuged at 120 000 *g* for 2 h at 4°C using a Sorvall Sure Spin 630 swinging bucket rotor (Kendro Laboratory Products). The plasma membrane layer was collected, washed in 10 mM Tris buffer, and centrifuged at 100 000 *g* for 1 h at 4°C using a Sorvall TFT 50.38 fixed angle rotor. The final plasma membrane pellet was resuspended in 200 μl of HB per gram of original tissue weight, frozen in liquid nitrogen and stored at −80°C for later analysis. The activity of the plasma membrane marker enzyme 5′-nucleotidase was assessed as previously described (Singh *et al.* 2003) in plasma membrane fractions and compared with the activity in crude homogenate fractions to insure that purified plasma membrane fractions were being used for analysis. Characterization of the membrane fractions obtained is presented in Table 1.

Western blotting

Lysate samples (140 μg protein for aPKC ζ and λ, 35 μg protein for Akt2, 70 μg of protein for GLUT4) and plasma membrane samples from the RQ (75 μg of protein for aPKC ζ and λ, 35 μg of protein for Akt2, 50 μg of protein for GLUT4) were added to Laemmli buffer (Laemmli, 1970). Sample proteins were subjected to SDS-PAGE run under reducing conditions on 7.5% (GLUT4) or 10% (Akt2, aPKC ζ and λ) resolving gels in a MiniProtean 3 dual-slab cell (Bio-Rad). Resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes using a semidry transfer unit (10 V for 30 min). Membranes were then blocked in 5% non-fat dry milk/Tris–Tween-buffered saline and incubated in affinity-purified rabbit polyclonal anti-Akt2 (cat no. 07-372, Upstate Biotechnology (UBT) Charlottesville, VA, USA), anti-aPKC ζ/λ (sc-216, Santa Cruz Biotechnology (SCBT) Santa Cruz, CA, USA) or GLUT4 (donated by Dr Samuel W. Cushman, National Institute of Diabetes and Digestive and Kidney Disease, Bethesda, MD, USA) followed by incubation with goat anti-rabbit IgG conjugated horseradish peroxidase (sc-2004, SCBT). Antibody binding was visualized by enhanced chemiluminescence (ECL) in accordance with the manufacturer's instructions (West Femto, Pierce Chemical Company, Rockford, IL, USA). Images were captured using a ChemiDoc system (Bio-Rad) equipped with a CCD camera and saved to a Macintosh G4 computer. Protein bands were quantified as a percentage of a muscle sample standard run on each gel using Quantity One analysis software (Bio-Rad).

IRS-1-associated PI3-kinase activity

IRS-1-associated PI3-kinase activity was determined as described elsewhere (Singh *et al.* 2003). Approximately 150 μg of RQ protein lysate was immunoprecipitated with 4 μg of anti-IRS-1 antibody (cat. no. 06-248, UBT), and HB for 2 h at 4°C. Following 1 h of separation the TLC plate was dried, exposed to a storage phosphor screen (Eastman Kodak Company, Rochester, NY, USA) and scanned with a phosphor imager (Personal Molecular Imager FX System, Bio-Rad). The image was imported into a Macintosh G4 computer and quantified using Quantity One analysis software (Bio-Rad). Kinase activity was calculated as a percentage of an insulin-stimulated muscle standard run on each TLC plate.

Akt2 kinase activity

Two hundred and fifty micrograms of either RQ lysate or plasma membrane protein were combined with 4 μg of anti-Akt2 (UBT) and incubated overnight at 4°C. One hundred microlitres of a slurry containing Pro-A beads was added to each immunoprecipitate and incubated with rotation at 4°C for 1.5 h. After incubation, the samples were centrifuged (18 300 *g*, 4°C) for 10 min, and the immunocomplex was washed with the same protocol as described for PI3-kinase activity (Singh *et al.* 2003).

After the wash protocol, the samples were centrifuged (18 300 *g*, 4°C) for 10 min and the supernatant was removed. Ten microlitres of assay dilution buffer (cat. no. 20-108, UBT) was added to the immunocomplex in addition to PKA inhibitor peptide (cat. no. 12-151, UBT) and 10 μCi [γ-^{32}P]ATP (PerkinElmer Life Sciences). Kinase reactions were initiated by addition of the Crosstide substrate oligopeptide (cat. no. 12-331, UBT), and warmed to 37°C with constant mixing for 10 min. Reactions were halted by addition of Laemmli buffer (1 : 1). For analysis, 15 μl of the sample/Laemmli buffer were loaded onto a 20% Tris–tricine polyacrylamide gel in duplicate and electrophoresed at 100 V for 130 min using a MiniProtean 3 electrophoresis system (Bio-Rad). After electrophoresis, gels were wrapped in plastic wrap and exposed to a phosphor screen for 8 h. Images were captured and quantified as described above.

aPKC ζ/λ activity

Five hundred micrograms of either RQ lysate or plasma membrane proteins were added to 4 μg of anti-aPKC ζ/λ (sc-216, SCBT) and incubated overnight at 4°C. One hundred microlitres of a slurry containing Protein A–Sepharose (Pro-A) beads were combined with each of the samples and incubated with rotation at 4°C for 1.5 h. After incubation, the samples were centrifuged (18 300 *g*, 4°C) for 10 min and the immunocomplex was washed by using the same protocol as described for PI3-kinase activity (Singh *et al.* 2003). After the wash protocol, samples were centrifuged (18 300 *g*, 4°C) for 10 min, and the supernatant was discarded. The Pro-A beads were then resuspended in 75 μl of kinase buffer. The kinase reaction was started by the combining 5 μCi [γ-^{32}P]ATP (PerkinElmer Life Sciences), 40 μM ATP and 5 μl myelin basic protein (M8-184, Sigma-Aldrich, St Louis, MO, USA). The samples were vortexed periodically while incubating for 12 min at 37°C. Reactions were stopped by addition of Laemmli buffer (1 : 1). For analysis 15 μl of the sample/Laemmli buffer and subjected to SDS-PAGE run under reducing conditions on a 20% Tris-tricine resolving gel. After electrophoresis the gels were wrapped in plastic wrap and exposed to a phosphor screen for 8 h. Images were captured and quantified as described above.

Statistical analysis

A one-way analysis of variance (ANOVA) was used on all variables to determine whether significant differences exist 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 were expressed as means ± S.E.M.

Results

Body and epididymal fat pad mass

At the onset of the dietary period, the body mass of normal diet basal (202.1 ± 6.1 g), normal diet insulin (198.1 ± 3.0 g), high fat diet basal (204.2 ± 3.3 g), and high fat diet insulin (203.0 ± 3.5 g) animals were similar. Although the body mass of all animals increased throughout the study, no significant differences in body mass were observed among normal diet basal (472.8 ± 10.3 g), normal diet insulin (477.3 ± 12.3 g), high fat diet basal (488.1 ± 11.3 g), and high fat diet insulin (491.7 ± 11.9 g) animals at the end of the 12 week dietary period. At the end of the dietary period, the epididymal fat pad mass of the high fat diet basal (11.6 ± 1.0 g), and high fat diet insulin (12.3 ± 1.1 g) animals exhibited a trend ($P = 0.09$) to be heavier than the normal diet basal (10.0 ± 1.2 g) and normal diet insulin (9.0 ± 0.5 g) animals.

Glucose transport

Rates of 3-MG transport were similar between the normal diet (3.92 ± 0.17 μmol h^{-1} g^{-1}) and high fat fed (3.40 ± 0.24 μmol h^{-1} g^{-1}) animals in the absence of insulin. In the presence of insulin, rates of 3-MG transport were increased above basal levels. However, rates of insulin-stimulated 3-MG transport in the high fat fed animals (5.07 ± 0.28 μmol h^{-1} g^{-1}) were significantly less than ($P < 0.05$) that of the normal diet animals (7.04 ± 0.40 μmol h^{-1} g^{-1}).

IRS-1-associated PI3-kinase activity

In the absence of insulin, IRS-1-associated PI3-kinase activity was similar between the normal diet (20.87 ± 3.9%standard) and high fat fed (16.88 ± 2.7%standard) animals. While insulin increased PI3-kinase activity above basal levels in both the normal diet and high fat diet groups, insulin-stimulated PI3-kinase activity was greater in the normal diet animals (70.85 ± 4.3%standard) compared to high fat diet animals (39.72 ± 2.1%standard).

Akt2 protein concentration

Total Akt2 protein concentration was not different in the absence (141.3 ± 19.1 *versus* 154.45 ± 12.1%standard) or presence (147.30 ± 4.8 *versus* 151.82 ± 10.3%standard) of insulin between the normal diet and high fat diet groups, respectively. In addition, plasma membrane Akt2 protein concentration (Fig. 1*A*) was not different among the normal diet and high fat diet groups either in the absence or presence of insulin.

Akt2 activity

In the absence of insulin, total Akt2 activity was not different between the normal diet and high fat diet groups (Fig. 2*A*). In the presence of insulin, total Akt2 activity was increased above basal levels for both the normal diet and high fat diet animals. However, total insulin-stimulated Akt2 activity of the high fat diet animals was less than that of the normal diet animals. Although the high fat diet and insulin affected total Akt2 activity, plasma membrane Akt2 activity (Fig. 2*B*) was not different among the experimental groups.

aPKC ζ and λ protein concentration

Total aPKC ζ protein concentration was similar in the normal diet and high fat diet groups in the absence (50.36 ± 9.5 *versus* 55.33 ± 12.1%standard) or presence of insulin (56.35 ± 12.2 *versus* 43.76 ± 6.1%standard),

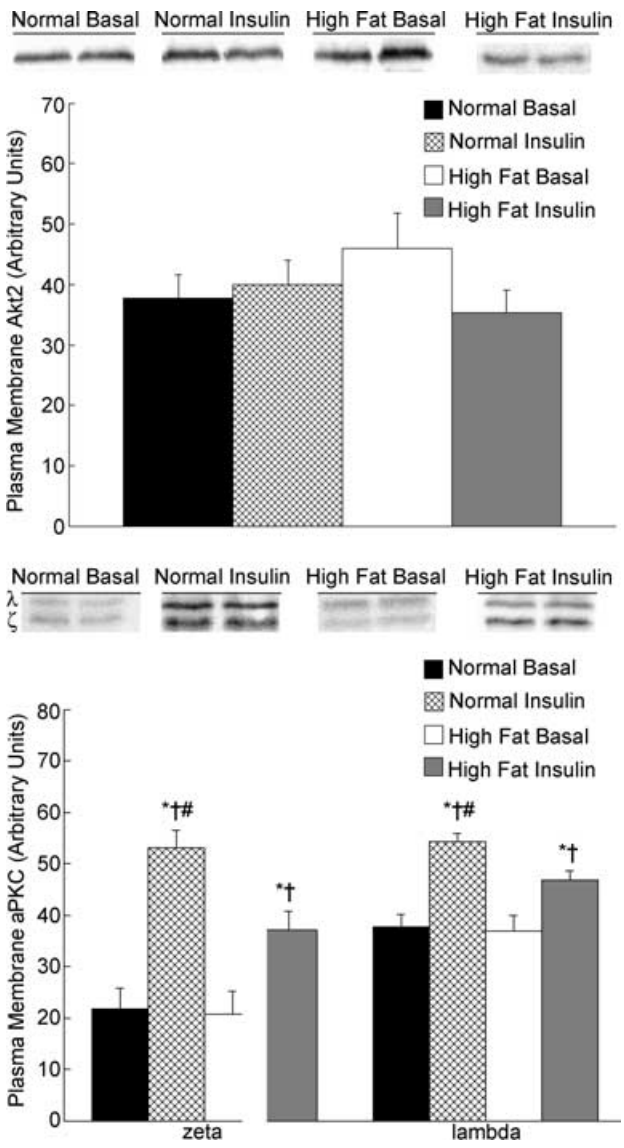

Figure 1. Red quadriceps total plasma membrane Akt2 (*A*) and plasma membrane aPKC ζ and λ (*B*) protein concentration from normal diet basal, normal diet insulin stimulated, high fat diet basal and high fat diet insulin stimulated animals
*Significantly different from normal diet basal ($P < 0.05$).
†Significantly different from high fat diet basal ($P < 0.05$).
#Significantly different from high fat diet insulin stimulated ($P < 0.05$).
Values are means ± S.E.M.

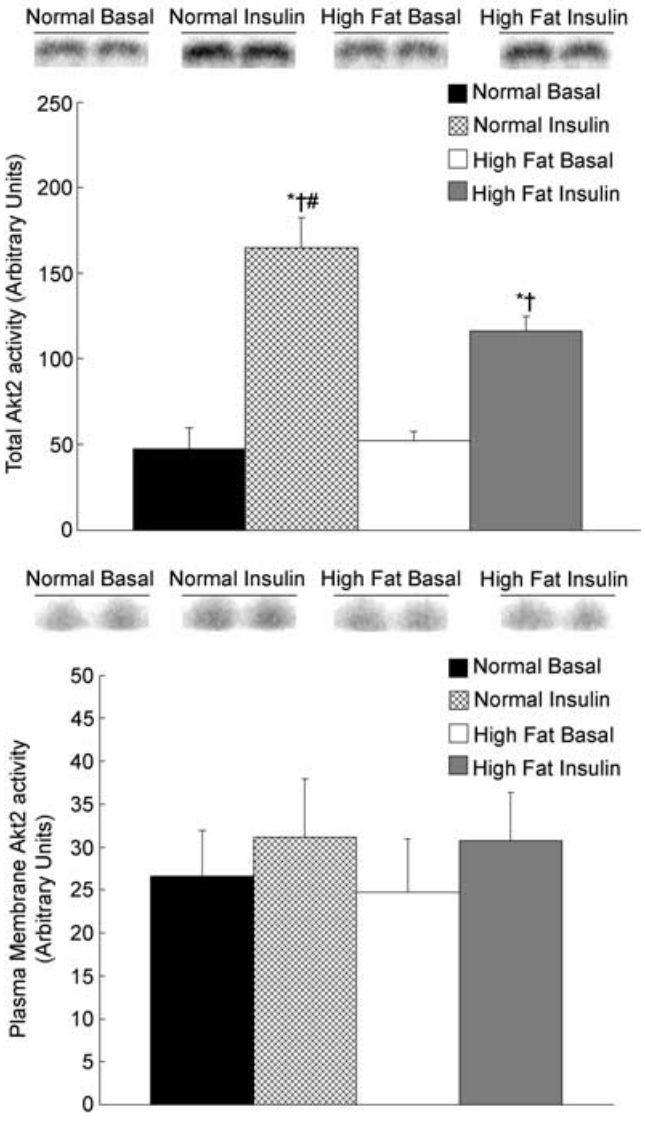

Figure 2. Red quadriceps total Akt2 protein activity (*A*) and plasma membrane Akt2 activity (*B*) from normal diet basal, normal diet insulin stimulated, high fat diet basal and high fat diet insulin stimulated animals
*Significantly different from normal diet basal ($P < 0.05$).
†Significantly different from high fat diet basal ($P < 0.05$).
#Significantly different from high fat diet insulin stimulated ($P < 0.05$).
Values are means ± S.E.M.

respectively. Similarly, total PKC λ protein concentration was not different in the normal diet and high fat diet groups in the absence (84.44 ± 15.0 *versus* 74.66 ± 13.3%standard) or presence (89.67 ± 21.3 *versus* 86.78 ± 17.3%standard) of insulin, respectively. In the absence of insulin, plasma membrane-associated aPKC ζ and λ (Fig. 1*B*) protein concentration were similar in the normal diet and high fat diet animals. In the presence of insulin, plasma membrane-associated aPKC ζ and λ were increased above basal levels in both dietary groups. However, insulin-stimulated plasma membrane-associated aPKC ζ and λ were reduced in the high fat diet animals compared to the normal diet animals.

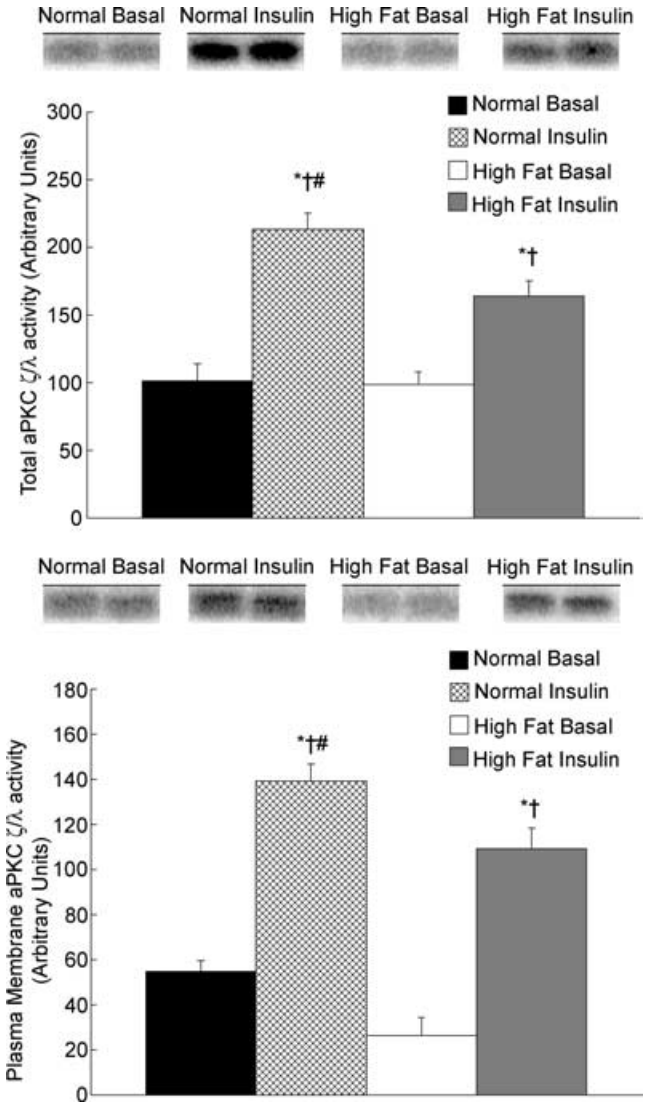

Figure 3. Red quadriceps total aPKC ζ/λ protein activity (*A*) and plasma membrane total aPKC ζ/λ activity (*B*) from normal diet basal, normal diet insulin stimulated, high fat diet basal and high fat diet insulin stimulated animals

*Significantly different from normal diet basal ($P < 0.05$).
†Significantly different from high fat diet basal ($P < 0.05$).
#Significantly different from high fat diet insulin stimulated ($P < 0.05$).
Values are means ± S.E.M.

aPKC ζ/λ activity

Total aPKC ζ/λ activity under basal conditions was similar between the normal diet and high fat diet animals. Insulin stimulation increased total aPKC ζ/λ kinase activity above basal levels in both dietary groups (Fig. 3*A*). However, insulin-stimulated total aPKC ζ/λ kinase activity from the high fat diet animals was significantly lower when compared with the normal diet animals. Similar observations were made for aPKC ζ/λ activity in the plasma membrane (Fig. 3*B*). Specifically, in the absence of insulin no differences existed between normal diet and high fat diet animals; insulin increased plasma membrane aPKC ζ/λ activity above basal levels in both dietary groups; and insulin-stimulated aPKC ζ/λ activity was lower in the high fat diet animals when compared to normal diet animals (Fig. 3*B*).

GLUT4 protein concentration

Total GLUT4 protein concentration was similar in the normal diet basal and normal diet insulin-stimulated animals, and was significantly greater than that of both the high fat diet basal and high fat diet insulin-stimulated animals (Fig. 4*A*). In the absence of insulin, plasma membrane GLUT4 protein concentration was similar in the normal diet and high fat diet animals (Fig. 4*B*). Insulin increased the plasma membrane GLUT4 protein concentration above basal levels in both dietary groups. However, insulin-stimulated plasma membrane GLUT4 protein concentration was significantly lower in the high fat diet animals compared to the normal diet animals.

Discussion

In normal rodent skeletal muscle we observed that insulin stimulation increased rates of skeletal muscle glucose transport above basal levels and appeared to be a result of the plasma membrane GLUT4 protein concentration being increased, which is consistent with previous investigations that report a relationship existing between GLUT4 and rates of 3-MG transport (Friedman *et al.* 1990; Banks *et al.* 1992). We also observed that insulin increased the activities of PI3-kinase, Akt2 and aPKC ζ/λ above basal levels in the skeletal muscle lysate from the normal diet animals, which is also consistent with a number of previous investigations (Krisan *et al.* 2004; Yaspelkis *et al.* 2004). Having established that insulin activated components of the insulin signalling cascade, we turned our focus towards identifying whether insulin

altered the plasma membrane content and/or activities of Akt2 and aPKC ζ/λ in the skeletal muscle of normal rodents.

Three isoforms of Akt have been identified in rodent skeletal muscle and it has been shown that the activation of Akt2 (Cho *et al.* 2001*a*), but not Akt1 (Cho *et al.* 2001*b*), is involved in insulin-stimulated glucose transport. It has also been reported in 3T3-L1 adipocytes that if Akt2 is deleted then GLUT4 translocation is reduced (Bae *et al.* 2003), and in response to insulin stimulation Akt2 becomes associated with the plasma membrane (Calera *et al.* 1998; Mitsuuchi *et al.* 1998; Bae *et al.* 2003). Thus, we chose to narrow our focus to Akt2 for this investigation. In response to insulin we did not observe plasma membrane Akt2 protein concentration or plasma membrane Akt2 activity to be increased above basal levels in normal rodent skeletal muscle. We are unaware of any other investigations that have evaluated the effects of insulin on plasma membrane-associated Akt2 protein concentration or activity in skeletal muscle. In contrast to our findings it has been observed that insulin increases plasma membrane-associated Akt in human ovarian carcinoma cells (Mitsuuchi *et al.* 1998), human embryonic kidney cells (Andjelkovic *et al.* 1997), rat adipocytes (Goransson *et al.* 1998; Bae *et al.* 2003; Chen *et al.* 2003) and 3T3-L1 adipocytes (Hill *et al.* 1999; Ducluzeau *et al.* 2002; Sasaoka *et al.* 2004). The differences existing among tissues are not readily reconcilable, but a plausible explanation may be that skeletal muscle has the capacity for glycogen synthesis whereas the previously investigated cell lines do not store glycogen. Specifically, insulin-stimulated activation of Akt causes serine phosphorylation of glycogen synthase kinase 3β (GSK3β), which causes deactivation of GSK3 and in turn allows for the activation of glycogen synthase and the subsequent storage of glycogen (Frame & Cohen, 2001). As glycogen storage occurs primarily in the sarcoplasm of skeletal muscle it may be of greater metabolic relevance that Akt does not increase its concentration and activity in the plasma membrane of skeletal muscle, unlike cell lines or other tissues. However, this does not minimize the fact that activation of Akt2 is clearly necessary in order for glucose transport to occur in skeletal muscle (Cho *et al.* 2001*a*).

In contrast to Akt2, we observed that insulin increased aPKC ζ protein concentration, aPKC λ protein concentration and aPKC ζ/λ activity in plasma membrane prepared from normal rodent skeletal muscle. This finding is consistent with previous reports that have shown insulin increases plasma membrane associated aPKC ζ/λ in myotubes (Braiman *et al.* 2001), rat adipocytes (Standaert *et al.* 1999), and 3T3-L1 adipocytes (Bandyopadhyay *et al.* 1997; Standaert *et al.* 1999; Kanzaki *et al.* 2004). The physiological significance of plasma membrane aPKC ζ and λ protein concentration, and aPKC ζ/λ activity being increased by insulin has not been fully elucidated, but it may contribute to facilitating GLUT4 translocation as it has been reported that aPKC ζ/λ directly interacts with GLUT4 containing vesicles (Standaert *et al.* 1999; Braiman *et al.* 2001; Kanzaki *et al.* 2004).

Our next line of inquiry was to evaluate high fat feeding effects on skeletal muscle Akt2 and aPKC ζ/λ protein concentration, plasma membrane association and activation. Confirming that the high fat diet impaired insulin-stimulated skeletal muscle carbohydrate

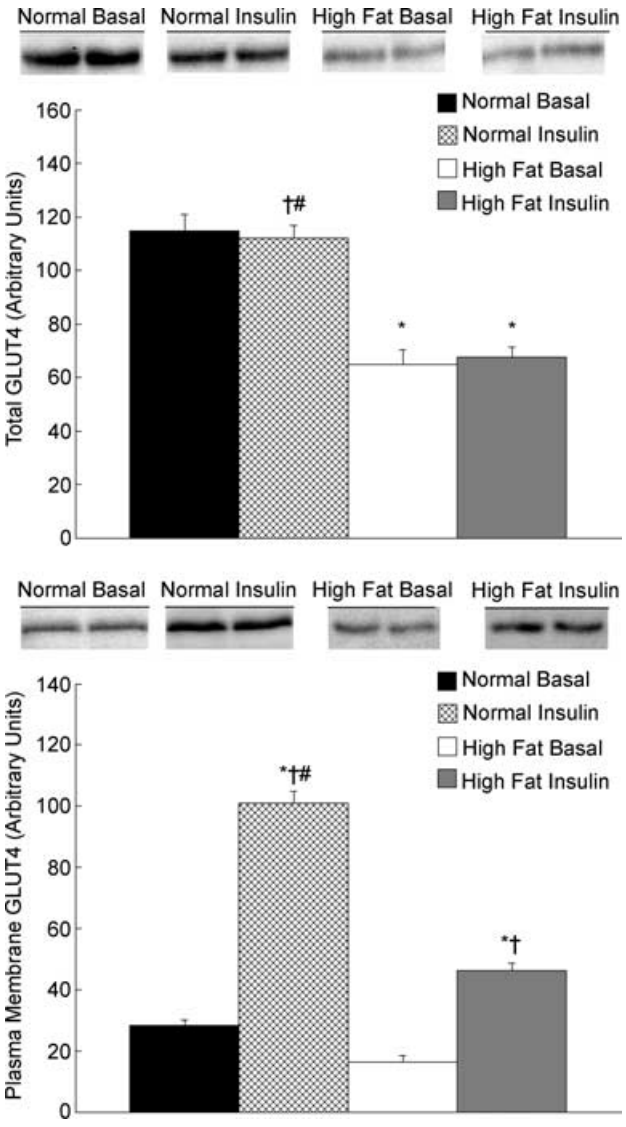

Figure 4. Red quadriceps total GLUT4 protein concentration (*A*) and plasma membrane GLUT4 protein concentration (*B*) from normal diet basal, normal diet insulin stimulated, high fat diet basal and high fat diet insulin stimulated animals

*Significantly different from normal diet basal ($P < 0.05$).
†Significantly different from high fat diet basal ($P < 0.05$).
#Significantly different from high fat diet insulin stimulated ($P < 0.05$).
Values are means ± S.E.M.

metabolism we found that rates of hind limb 3-MG transport, PI3-kinase activity and plasma membrane GLUT4 protein concentration were significantly reduced in the high fat fed animals compared to the normal diet animals. These observations are in agreement with a number of investigations that have also shown a high fat diet to decrease insulin-stimulated 3-MG transport (Kraegen *et al.* 1986; Barnard *et al.* 1998; Hansen *et al.* 1998; Wilkes *et al.* 1998; Buettner *et al.* 2000; Halseth *et al.* 2000; Yaspelkis *et al.* 2001; Singh *et al.* 2003; Krisan *et al.* 2004), PI3-kinase activity (Tremblay *et al.* 2001; Singh *et al.* 2003; Krisan *et al.* 2004; Yaspelkis *et al.* 2004) and plasma membrane GLUT4 protein concentration (Hansen *et al.* 1998; Tremblay *et al.* 2001; Yaspelkis *et al.* 2001; Singh *et al.* 2003; Yaspelkis *et al.* 2004) in rodent skeletal muscle. Additionally, we found that total skeletal muscle insulin-stimulated Akt2 and aPKC ζ/λ activities were reduced in the high fat fed animals even though total skeletal muscle Akt2, aPKC ζ and λ protein concentrations were unaltered. These observations are not entirely unexpected since they are consistent with those of several recent investigations that have also reported Akt2 and aPKC ζ/λ activities to be reduced in the absence of alterations in total protein concentration and phosphorylation (Tremblay *et al.* 2001; Kanoh *et al.* 2003; Krisan *et al.* 2004). However, to the best of our knowledge, it has not previously been addressed whether insulin-stimulated Akt2, aPKC ζ and λ protein concentration and/or activation of these kinases are altered in plasma membrane prepared from high fat fed rodent skeletal muscle.

Similar to what we observed in the plasma membranes of the normal diet animals, we did not find that insulin increased either the plasma membrane associated Akt2 protein concentration or activity above basal levels. Nevertheless, despite a lack of effect at the plasma membrane, the high fat diet did alter total Akt2 kinase activity. While the mechanism by which high fat feeding results in a decrease total Akt2 activity has not been fully resolved, it is likely to be due to insulin-stimulated PI3-kinase activity being reduced.

With respect to the plasma membrane protein concentration and activation of aPKC ζ and λ protein concentration in the high fat fed skeletal muscle, we found that they were increased above basal levels in response to insulin. However, the insulin-stimulated increase in plasma membrane associated aPKC ζ and λ, and aPKC ζ/λ activities in the skeletal muscle obtained from high fat fed animals was less than that of the normal diet animals. While this observation has not been previously reported in rodent skeletal muscle, it is likely that the reduced plasma membrane protein concentration and activation of aPKC ζ and λ is a direct result of insulin-stimulated PI3-kinase activity being reduced in the high fat fed animals. It is also possible that alterations in the CAP/Cbl insulin signalling cascade (novel insulin signalling cascade) may contribute to the concentration and activation of aPKC ζ/λ being reduced in the plasma membranes obtained from the high fat fed rodent skeletal muscle. Kanzaki *et al.* (2004) have recently reported that insulin stimulates the recruitment of aPKC ζ/λ to the plasma membrane of 3T3L1 adipocytes through the activation of the CAP/Cbl insulin signalling cascade rather than through the PI3-kinase-dependent (classical) insulin signalling cascade, and that the CAP/Cbl cascade converges with the PI3-kinase-dependent pathway via the interaction of TC10 with aPKC ζ/λ at the plasma membrane. Of interest, we have recently observed that insulin-stimulated activation of the CAP/Cbl insulin signalling cascade in skeletal muscle is improved by aerobic exercise training (Bernard *et al.* 2005). However, additional investigation of the CAP/Cbl insulin signalling cascade is warranted to elucidate the existence and functional significance of the interaction of TC10 with aPKC ζ/λ, and if these contribute to GLUT4 translocation in skeletal muscle.

In summary, we observed in normal skeletal muscle that insulin increased total PI3-kinase, Akt2 and aPKC ζ/λ activities above basal levels. In response to insulin, plasma membrane-associated aPKC ζ, aPKC λ and GLUT4, but not Akt2, were increased, and that only aPKC ζ/λ activity was increased at the plasma membrane. High fat feeding did not alter total Akt2 or aPKC ζ and λ protein concentrations. However, insulin-stimulated total PI3-kinase, Akt2 and aPKC ζ/λ activities were reduced in the skeletal muscle of the high fat fed animals. Insulin-stimulated plasma membrane-associated aPKC ζ and λ protein concentration, aPKC ζ/λ activity and GLUT4 protein concentration were also reduced in the high fat fed animals. These findings suggest that in skeletal muscle, insulin stimulates translocation of aPKC ζ and λ to the plasma membrane, whereas Akt2 is undetected at the plasma membrane. In addition, high fat feeding impairs insulin-stimulated translocation and activation of aPKC ζ/λ, which may contribute to decreasing insulin-stimulated plasma membrane GLUT4 protein concentration and rates of insulin-stimulated glucose transport in the skeletal muscle of the high fat fed animals.

References

Ahmed A, Taylor PM & Rennie MJ (1990). Characteristic of glutamate transport in sarcolemmal vesicles from rat skeletal muscle. *Am J Physiol Endocrinol Metabol* **259**, E284–E291.

Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, Frech M *et al.* (1997). Role of translocation in the activation and function of protein kinase B. *J Biol Chem* **272**, 31515–31524.

Bae SS, Cho H, Mu J & Birnbaum MJ (2003). Isoform-specific regulation of insulin-dependent glucose uptake by Akt/protein kinase B. *J Biol Chem* **278**, 49530–49536.

Bandyopadhyay G, Standaert ML, Kikkawa U, Ono Y, Moscat J & Farese RV (1999*a*). Effects of transiently expressed atypical (ζ,λ), conventional (α,β), and novel (δ,ε) protein kinase C isoforms on insulin-stimulated translocation of epitope-tagged GLUT4 glucose transport in rat adipocytes: specific interchangeable effects of protein kinase C-ζ and C-1. *Biochem J* **337**, 461–470.

Bandyopadhyay G, Standaert ML, Sajan MP, Karnitz LM, Cong L, Quon MJ *et al.* (1999*b*). Dependence of insulin-stimulated glucose transporter 4 translocation on 3-phosphoinositide-dependent protein kinase-1 and its target threonine-410 in the activation loop of protein kinase C-ζ. *Mol Endocrinol* **13**, 1767–1772.

Bandyopadhyay G, Standaert ML, Zhou LYuB, Avignon A, Galloway G *et al.* (1997). Activation of protein kinase C (alpha, beta, and zeta) by insulin in 3T3-L1 cells. Transfection studies suggest a role for PKC-zeta in glucose transport. *J Biol Chem* **272**, 2551–2558.

Banks EA, Brozinick JT Jr, Yaspelkis BB III, Kang HY & Ivy JL (1992). Muscle glucose transport, GLUT4 content and degree of exercise training in obese Zucker rats. *Am J Physiol Endocrinol Metab* **263**, E1010–E1015.

Barnard RJ, Roberts CK, Varon SM & Berger JJ (1998). Diet-induced insulin resistance precedes other aspects of the metabolic syndrome. *J Appl Physiol* **84**, 1311–1315.

Bernard JR, Crain AM, Rivas DA, Herr HJ, Reeder DW & Yaspelkis BB III (2005). Chronic aerobic exercise enhances classical and novel insulin signaling in Sprague Dawley rat skeletal muscle. *Acta Physiol Scand* **184**, 357–366.

Bradford MM (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.

Braiman L, Alt A, Kuroki T, Ohba M, Bak A, Tennenbaum T *et al.* (2001). Activation of protein kinase C ζ induces serine phosphorylation of VAMP2 in the GLUT4 compartment and increases glucose transport in skeletal muscle. *Mol Cell Biol* **21**, 7852–7861.

Brozinick JT Jr, Roberts BR & Dohm GL (2003). Defective signaling through Akt-2 and -3 but not Akt-1 in insulin-resistant human skeletal muscle. *Diabetes* **52**, 935–941.

Buettner R, Newgard CB, Rhodes CJ & O'Doherty RM (2000). Correction of diet-induced hyperglycemia, hyperinsulinemia, and skeletal muscle insulin resistance by moderate hyperleptinemia. *Am J Physiol Endocrinol Metabol* **278**, E563–E569.

Calera MR, Martinez C, Hongzhi L, Jack AK, Birnbaum MJ & Pilch PF (1998). Insulin increases the association of Akt-2 with Glut4-containing vesicles. *J Biol Chem* **273**, 7201–7204.

Chen X, Al-Hasani H, Olausson T, Wenthzel AM, Smith U & Cushman SW (2003). Activity, phosphorylation state and subcellular distribution of GLUT4-targeted Akt2 in rat adipose cells. *J Cell Sci* **116**, 3511–3518.

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

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

Cong L, Chen H, Li Y, Zhou L, McGibbon MA, Tayor SI *et al.* (1997). Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. *Mol Endo* **11**, 1881–1890.

Ducluzeau PH, Fletcher LM, Welsh GI & Tavare JM (2002). Functional consequence of targeting protein kinase B/Akt to GLUT4 vesicles. *J Cell Sci* **115**, 2857–2866.

Frame S & Cohen P (2001). GSK3 takes centre stage more than 20 years after its discovery. *Biochem J* **359**, 1–16.

Friedman JE, Sherman WM, Reed MJ, Elton CW & Dohm GL (1990). Exercise training increases glucose transporter GLUT-4 in skeletal muscle of obese Zucker (fa/fa) rats. *FEBS Lett* **268**, 13–16.

Galetic I, Andjelkovic M, Meier R, Brodbeck D, Park J & Hemmings BA (1999). Mechanism of protein kinase B activation by insulin/insulin-like growth factor-1 revealed by specific inhibitors of phosphoinositide 3-kinase-significance for diabetes and cancer. *Pharmacol Ther* **82**, 409–425.

Goransson O, Wijkander J, Manganiello V & Degerman E (1998). Insulin-induced translocation of protein kinase B to the plasma membrane in rat adipocytes. *Biochem Biophys Res Commun* **246**, 249–254.

Halseth AE, Bracy DP & Wasserman DH (2000). Limitation to basal and insulin-stimulated skeletal muscle glucose uptake in the high-fat-fed rat. *Am J Physiol Endocrinol Metab* **279**, E1064–E1071.

Hansen PA, Han DH, Marshall BA, Nolte LA, Chen MM, Mueckler M *et al.* (1998). A high fat diet impairs stimulation of glucose transport in muscle. Functional evaluation of potential mechanisms. *J Biol Chem* **273**, 26157–26163.

Hill MM, Clark SF, Tucker DF, Birnbaum MJ, James DE & Macaulay SL (1999). A role for protein kinase Bβ/Akt2 in insulin-stimulated GLUT4 translocation in adipocytes. *Mol Cell Biol* **19**, 7771–7781.

Ivy JL, Brozinick JT Jr, Torgan CE & Kastello GM (1989). Skeletal muscle glucose transport in obese Zucker rats after exercise training. *J Appl Physiol* **66**, 2635–2641.

Jiang ZY, Zhou QL, Coleman KA, Chouinard M, Boese Q & Czech MP (2003). Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. *Proc Natl Acad Sci U S A* **100**, 7569–7574.

Kanoh Y, Sajan MP, Bandyopadhyay G, Miura A, Standaert ML & Farese RV (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. *Endocrinol* **144**, 947–954.

Kanzaki M, Mora S, Hwang J, Saltiel AR & Pessin JE (2004). Atypical protein kinase C (PKC ζ/λ) is a convergent downstream target of the insulin stimulated phosphatidylinositol 3-kinase and TC10 signaling pathway. *J Cell Biol* **164**, 279–290.

Katome T, Obata T, Matsushima R, Masuyama N, Cantley LC, Gotoh Y *et al.* (2003). Use of RNA interference-mediated gene silencing and adenoviral overexpression to elucidate the roles of Akt/protein kinase B isoforms in insulin actions. *J Biol Chem* **278**, 28312–28323.

Kotani K, Ogawa W, Matsumoto M, Kitamura T, Sakaue H, Hino Y, *et al.* (1998). Requirement of atypical protein kinase Cλ for insulin stimulation of glucose uptake but not for Akt activation in 3T3/L1 adipocytes. *Mol Cell Biol* **18**, 6971–6981.

Kraegen EW, James DE, Storlein LH, Burleigh KM & Chisholm DJ (1986). In vivo insulin resistance in individual peripheral tissues of the high fat fed rat: assessment by euglycaemic clamp plus deoxyglucose administration. *Diabetologia* **29**, 192–198.

Krisan AD, Collins DE, Crain AM, Kwong CC, Singh MK, Bernard JR *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.

Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Mitsuuchi Y, Johnson SW, Moonblatt S & Testa JR (1998). Translocation and activation of AKT2 in response to stimulation by insulin. *J Cell Biochem* **70**, 433–441.

Nielsen JN, Frosig C, Sajan MP, Miura A, Standaert ML, Graham DA *et al.* (2003). Increased atypical PKC activity in endurance-trained human skeletal muscle. *Biochem Biophys Res Commun* **312**, 1147–1153.

Perrini S, Henriksson J, Zierath JR & Widegren U (2004). Exercise-induced protein kinase C isoform-specific activation in human skeletal muscle. *Diabetes* **53**, 21–24.

Rose AJ, Michell BJ, Kemp BE & Hargreaves M (2004). Effect of exercise on protein kinase C activity and localization in human skeletal muscle. *J Physiol* **561**, 861–870.

Ruderman NB, Houghton CRS & Helms R (1971). Evaluation of the isolated perfused rat hindquarter for the study of muscle metabolism. *Biochem J* **124**, 639–651.

Sasaoka T, Wada T, Fukui K, Murakami S, Ishihara H, Suzuki R *et al.* (2004). SH2-containing inositol phosphatase 2 predominantly regulates Akt2, and not Akt1, phosphorylation at the plasma membrane in response to insulin in 3T3-L1 adipocytes. *J Biol Chem* **279**, 14835–14843.

Singh MK, Krisan AD, Crain AM, Collins DE & Yaspelkis BB III (2003). High-fat diet and leptin treatment alter skeletal muscle insulin-stimulated phosphatidylinositol 3-kinase activity and glucose transport. *Metabol* **52**, 1196–1205.

Standaert ML, Bandyopadhyay G, Galloway L, Poklepovic A, Sajan MP, Cenni V *et al.* (1999). Insulin activates protein kinases C-zeta and C-lambda by an autophosphorylation-dependent mechanism and stimulates their translocation to the plasma membrane in rat adipocytes. *J Biol Chem* **274**, 25308–25316.

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.

Turcotte LP, Srivastava AK & Chiasson J-L (1997). Fasting increases plasma membrane fatty acid-binding protein ($FAMP_{PM}$) in red skeletal muscle. *Mol Cell Biochem* **166**, 153–158.

Turcotte LP, Swenberger JR, Tucker MZ & Yee AJ (1999). Training-induced elevation in $FABP_{pm}$ is associated with increased palmitate use in contracting muscle. *J Appl Physiol* **87**, 285–293.

Walker KS, Deak M, Paterson A, Hudson K, Cohen P & Alessi DR (1998). Activation of protein kinase B beta and gamma isoforms by in vivo and by 3-phosphoinositide-dependent protein kinase-1 in vitro: comparison with protein kinase B alpha. *Biochem J* **331**, 299–308.

Wilkes JJ, Bonen A & Bell RC (1998). A modified high-fat diet induces insulin resistance in rat skeletal muscle but not adipocytes. *Am J Physiol Endocrinol Metab* **275**, E679–E686.

Yaspelkis BB III, Davis JR, Saberi M, Smith TL, Jazayeri R, Singh M *et al.* (2001). Leptin administration improves skeletal muscle insulin responsiveness in diet-induced insulin-resistant rats. *Am J Physiol Endocrinol Metab* **280**, E130–E142.

Yaspelkis BB III, Saberi M, Singh MK, Trevino B & Smith TL (2002). Chronic leptin treatment normalizes basal glucose transport in a fiber type-specific manner in high-fat-fed rats. *Metabol* **51**, 859–863.

Yaspelkis BB III, Singh MK, Krisan AD, Collins DE, Kwong CC, Bernard JR *et al.* (2004). Chronic leptin treatment enhances insulin-stimulated glucose disposal in skeletal muscle of high-fat fed rodents. *Life Sci* **74**, 1801–1816.

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