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ORIGINAL PAPER

Perturbations of the stress-induced GLUT4 localization pathway in slow-twitch muscles of obese Zucker rats

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Abstract Past studies have suggested that the stress-induced GLUT4 localization pathway is damaged in fast-twitch muscles (white muscles) of obese subjects. In this study, we used obese rodents in an attempt to determine whether the stress-induced GLUT4 localization pathway is abnormal in slow-twitch muscles (red muscles), which are responsible for most daily activities. Protein expression levels of the intracellular stress sensor AMP-activated protein kinase (AMPK), its upstream kinase LKB1, its downstream protein AS160 and the glucose transporter protein 4 (GLUT4) in the red gastrocnemius muscle were measured under either resting or stress conditions (1 h of swimming or 14% hypoxia) in both lean and obese Zucker rats (n= 7 for each group). At rest, obese rats displayed higher fasting plasma insulin levels and increased muscle AMPK and AS160 phosphorylation levels compared with lean controls. No significant difference was found in the protein levels of LKB1, total GLUT4, or membrane GLUT4 between the obese and lean control groups. After one hour of swimming, AMPK and AS160 phosphorylation levels and the amount of GLUT4 translocated to the plasma membrane were significantly elevated in lean rats but remained unchanged in obese rats relative to their resting conditions. One hour 14% hypoxia did not cause significant changes in the LKB1-AMPK-AS160-GLUT4 pathway in either lean or obese rats. This study demonstrated that the AMPK-AS160-GLUT4 pathway was altered at basal levels and after exercise stimulation in the slow-twitch muscle of obese Zucker rats.

Keywords Obese · Exercise · Hypoxia · Slow-twitch muscle · Glucose uptake · AMPK

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Introduction

Obesity is a serious health problem worldwide and may predispose sufferers to glucose intolerance and

the development of metabolism-related syndromes such as type 2 diabetes mellitus and cardiovascular disease [6]. However, the mechanisms causing glucose intolerance in obese subjects, which may burden them with metabolic morbidities, remain unclear.

Skeletal muscle is the major site of postprandial glucose disposal [4]. In obese subjects, it has been demonstrated that damage to the skeletal muscle insulin signaling pathway is related to peripheral glucose intolerance [1, 11, 14]. In addition to inducing glucose uptake through the action of insulin, skeletal muscles also absorb large amounts of glucose under stress conditions, such as exercise or hypoxia. Stress-induced skeletal muscle glucose uptake is mediated by the AMP-activated protein kinase (AMPK)-related signals [7, 8, 13, 16].

AMPK is an intracellular energy sensor that increases skeletal muscle glucose transport after stimulation by stress [8–10, 16]. Cells produce a large amount of AMP in response to sudden physiological challenges, such as exercise or hypoxia. An increased AMP-to-ATP ratio generates a conformational change in AMPK. AMPK then becomes more easily phosphorylated by its upstream kinase, LKB1, a constitutively active enzyme that phosphorylates AMPK at position Thr^{172}. It is known that LKB1 can be phosphorylated by a number of protein kinases, but the role of such phosphorylation in the activity of LKB1 is still unclear [25]. Phosphorylated AMPK can phosphorylate and activate AS160 (Akt substrate 160). AS160 is a newly identified potential trigger factor involved in the regulation of intracellular glucose transporter protein 4 (GLUT4) translocation to the plasma membrane from the intracellular pool [12]. GLUT4 is the major glucose transporter expressed in skeletal muscle [18]. Stimulation of the LKB1-AMPK-AS160-GLUT4 pathway can help muscles quickly absorb large amounts of glucose.

In recent years, the roles of AMPK-related signals in the development of metabolic syndromes have been reported [15, 23]. These studies have focused on whether obese subjects have abnormal AMPK expression in fast-twitch muscles (white muscles). One study using obese Zucker rats revealed abnormalities in AMPK activity in the epitrochlearis muscle before and after electronic stimulation [2]. People with body mass indexes (BMIs)>30 also show blunted AMPK and AS160 activation after moderate exercise of the basal vastus lateralis muscle [20]. However, it has been determined that slow-twitch muscles (red muscles) are used for most activities of daily life, such as maintaining posture, walking and low-intensity endurance running [17]. To the best of our knowledge, there are no studies examining whether AMPK-related GLUT4 localization pathway is altered in the red muscles of obese subjects.

Thus, the aim of this study was to determine whether red muscle of obese rats have abnormal LKB1-AMPK-AS160-GLUT4 expression under basal (resting) condition or stress conditions, such as exercise or hypoxia. Our study utilized obese Zucker rats as an animal model for obesity and lean Zucker rats as controls. One hour of acute swimming or 14% hypoxia, which is within the acceptable physiological range of the animals [3], was used to stimulate the experimental animals. The LKB1, AMPK, AS160, and GLUT4 protein expression levels in the red gastrocnemius muscle were measured in both lean and obese Zucker rats under resting conditions, after 1 h swimming, or after 1 h 14% hypoxia.

Materials and methods

Experimental animals Male lean (*Fa/fa, Fa/Fa*) and obese (*fa/fa*) Zucker rats ($n=7$ for each group) were housed in a temperature-controlled room (21±1°C) with a photoperiod of 12 h light and 12 h darkness. All rats were provided with unlimited water and chow (PMI Nutrition International, Brentwood, MO, USA). This study was approved by the Animal Management Committee of Taipei Veterans General Hospital and conformed to the guidelines for the use of laboratory animals published by the ROC Department of Agriculture.

Experimental design and training protocols Both obese and lean Zucker rats (20 weeks of age) were randomly assigned into one of three groups: control, acute exercise, and acute hypoxia ($n=7$ for each group). This study was carried out at sea level. The exercise and hypoxia protocols were carried out once for 1 h as described previously [3]. During the 1-h treatment, rats in the control group were prevented from eating, but water was freely available. After the treatment, all rats were anesthetized immediately for muscle collection.

The exercise protocol consisted of swimming in several specially designed plastic containers (water depth, 70 cm; water temperature, 29±1°C). Two days before the test, all rats (including controls) were placed into swimming containers for 10–15 min once a day to acclimate to the water. During the 1-h swimming test, the rats swam with 2–5% of their body weight loaded onto their tails to prevent floating.

Hypoxia was generated by placing rats in a four-room isobaric chamber (56 cm long, 43 cm wide and 39 cm tall) with constant 14% inspired oxygen maintained by a hypoxic air machine (GAO_2 Altitude, Australia). The oxygen concentration in the isobaric chamber was monitored with an oxygen sensor equipped with an alarm (GAO_2 Altitude, Australia) throughout the experimental period. Water was freely available throughout the duration of hypoxic treatment.

All of the rats were anesthetized immediately after 1 h of treatment with an intraperitoneal injection of pentobarbital sodium (65.0 mg/kg body weight). The red gastrocnemius muscles were then excised as soon as possible and frozen in liquid N_2. These muscle sections were later used for glycogen assays and western blots.

Fasting glucose and insulin Measurements of fasting glucose and insulin levels were performed in both obese and lean rats at 19 weeks of age. Prior to the assay, rats were fasted for 12 h during the light cycle (sleep phase). Blood samples (0.6 ml) were drawn from the abdominal vena cava and collected to measure whole blood glucose content and serum insulin content. Whole blood glucose content was measured using a one-touch digital sugar analyzer (Roche, Precision Sof-Tact™). Serum insulin levels were determined using a commercially available kit (Mercodia, Rat Insulin ELISA 10-1250-01).

Glycogen assay Approximately 50 mg of red gastrocnemius muscle was dissolved in 1 M KOH at 70°C for 30 min. The dissolved homogenate was neutralized with glacial acetic acid and incubated overnight in acetate buffer (0.3 M sodium acetate, pH 4.8) containing amyloglucosidase (Boehringer Mannheim, Indianapolis, IN, USA). The reaction mixture was neutralized with 1 M NaOH. Samples were then analyzed by measuring glucosyl units with the Trinder reaction (Sigma, St. Louis, MO, USA).

Plasma membrane extraction A crude membrane fraction was prepared using the method of Wu et al. [26] with modifications. Approximately 50 mg of red gastrocnemius muscle was homogenized (1:20 *w*/*v*) in 20 mM ice-cold HEPES with 1 mM EDTA and 250 mM sucrose (HES buffer, pH 7.4). The homogenate was centrifuged at 100×*g* for 10 min at 4°C, and then the supernatant was aspirated and centrifuged at 25,000×*g* for 1 h at 4°C. The pellet, which contained the total fraction of plasma membranes, was resuspended in a buffer containing 50 mM Tris-HCl, 120 mM NaCl, and 6 mM $MgCl_2$ 6 H_2O. The protein concentration of the plasma membrane fraction was determined using the Bio-Rad Protein Assay reagent (Bio-Rad, Richmond, CA, USA) in accordance with the manufacturer's instructions. Protein from the plasma membrane fraction (50 μg) was used in a GLUT4 western blotting assay.

Western blotting Approximately 50 mg of skeletal muscle was homogenized (1:20 *w*/*v*) in HES buffer with 1% protease inhibitor cocktail (Sigma, P2714) and 1% phosphatase inhibitor cocktail (Sigma, P2850) using a Polytron system (Brinkmann Instruments, Littau, Switzerland). Homogenates were centrifuged at 100×*g* for 10 min. The supernatants were collected and protein concentration was determined using the Bio-Rad Protein Assay reagent (Bio-Rad, Richmond, CA, USA) in accordance with the manufacturer's instructions. Proteins from sample homogenates (50 μg) and standards were diluted 1:1 with Laemmli sample buffer (125 mM Tris, 20% glycerol, 2% SDS and 0.008% bromophenol blue, pH 6.8) and separated on a 10% SDS-polyacrylamide gel by electrophoresis at 120 V for 90 min. The fractionated proteins from the sample homogenates were transferred to polyvinylidene fluoride membranes. The membranes were blocked for 60 min in Tris-buffered saline with 0.16% Tween 20 (TTBS) containing 7% non-fat milk. Western blots were performed after incubation with the following primary antibodies overnight at 4°C: anti-LKB1 (Cell Signaling, Beverly, MA, USA), anti-AMPKα (Cell Signaling), anti-pAMPK^{Thr172} (Cell Signaling), anti-AS160 (Upstate Biotechnology, Lake Placid, NY, USA), anti-pAS160 (Upstate Biotechnology), anti-GLUT4 (Chemicon, Temecula, CA, USA), anti-Na-K ATPase (Cell Signaling) and anti-β-actin (Cell Signaling). We used Na-K ATPase and β-actin as internal controls for

membrane proteins and cytoplasmic proteins, respectively. The membranes were incubated with the appropriate anti-rabbit secondary antibody diluted in TTBS. All muscle proteins were visualized using the ECL Western Blot Detection Kit (Amersham, Arlington Heights, IL, USA) on Kodak film in accordance with the manufacturer's instructions. The intensity of western blot bands was quantified using Quantity One™ Software (Bio-Rad, Richmond, CA, USA).

Statistical analysis Two-way analysis of variance was used to analyze all variables across the experimental groups. Fisher's protected least significant difference test, which holds the value of a type I error constant for each test, was then utilized to distinguish significant differences between pairs of groups. A level of $P<0.05$ was set for significance in all tests. Data have been expressed as means±SEM.

Results

Body weight, fasting insulin, and fasting glucose

At 20 weeks of age, obese Zucker rats were significantly heavier than lean rats (Table 1). In addition, obese rats were characterized by a fivefold increase in fasting insulin level compared to that of lean rats (4.65±0.49 vs. 0.87±0.02 ng/mL, respectively; $P<0.05$), but there was no significant difference in fasting glucose (109.33±7.24 vs. 103.12±4.35 mg/dL, respectively).

Glycogen content before and after treatment

In the control groups, the glycogen content was similar in obese and lean Zucker rats (Fig. 1). For both obese and lean rats, acute 1-h hypoxia did not alter skeletal muscle glycogen levels. In contrast, acute 1-h exercise significantly decreased glycogen levels for both lean and obese Zucker rats comparably (lean rats, 18.7% vs. obese rats, 17.1%), which indicates that the swimming exercise intensity was similar for lean and obese rats.

Table 1 The body weight, fasting insulin and fasting glucose content in obese and lean Zucker rats at 19 weeks of age

	Lean (n=7)	Obese (n=7)
Body weight (g)	319.22±10.45	450.22±18.28*
Fasting insulin (ng/mL)	0.87±0.02	4.65±0.49*
Fasting glucose (mg/dL)	103.12±4.35	109.33±7.24

Data are mean±SEM

*$P<0.05$ vs. the lean rats

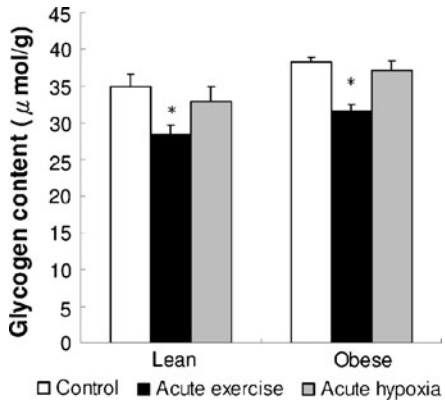

Fig. 1 Muscle glycogen content in control, acute exercise, and acute hypoxia groups of both lean and obese Zucker rats. Each *bar* represents a mean±SEM. *$P<0.05$ vs. the respective control

Basal protein profiles

Under basal conditions, the levels of LKB1 (AMPK kinase), AMPK, AS160, total GLUT4 and membrane GLUT4 in skeletal muscle were not significantly different between the lean and obese Zucker rats (Figs. 2, 3b, 4b, 5, and 6). However, the pAMPK level, pAMPK-to-AMPK ratio, pAS160 level, and pAS160-to-AS160 ratio were significantly higher in the obese Zucker rats than in the lean rats (Figs. 3a,c, 4a,c).

Protein profiles after acute treatments

The LKB1, AMPK, AS160, and total GLUT4 protein levels in both the acute exercise and hypoxia groups were not significantly different from those in the control group for either lean or obese Zucker rats (Figs. 2, 3b, 4b and 5). The pAMPK level, pAMPK-to-AMPK ratio, pAS160 level, pAS160-to-AS160 ratio, and plasma membrane GLUT4 protein level were significantly higher in the lean acute exercise group compared with the lean control group. However, these parameters did not change significantly in obese Zucker rats with acute exercise treatment.

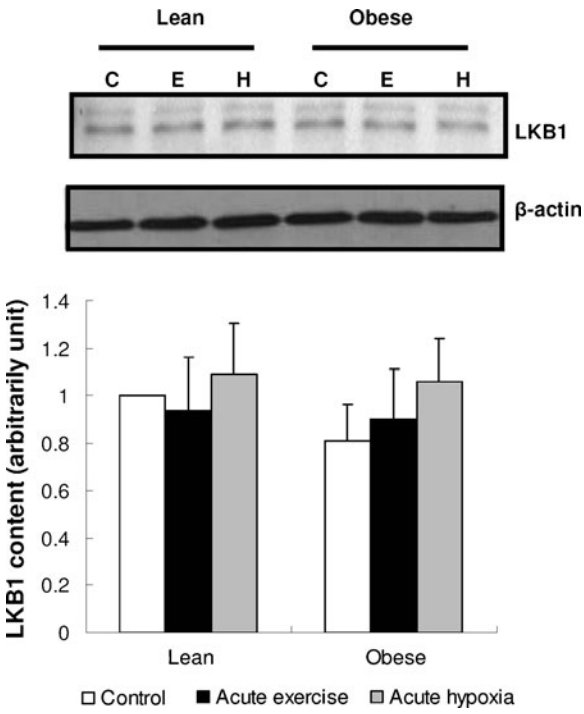

Fig. 2 LKB1 protein content in control, acute exercise, and acute hypoxia groups of both lean and obese Zucker rats. Each *bar* represents a mean±SEM

Acute hypoxia had no significant effect on the pAMPK-to-AMPK ratio, pAS160-to-AS160 ratio or plasma membrane GLUT4 level in either lean or obese Zucker rats (Figs. 3a,c, 4a,c, and 6).

Discussion

In this study, we examined a stress-induced pathway consisting of LKB1, AMPK, AS160 and GLUT4 under conditions of exercise or hypoxia in slow-twitch muscle (red gastrocnemius muscle) in both obese and lean Zucker rats. We discovered that obese rats exhibit abnormalities in this pathway that have not been described previously. In obese rats, basal AMPK and AS160 phosphorylation levels were increased relative to those of lean rats, but basal levels of membrane GLUT4 were similar to those of lean rats. These results lead us to speculate that there may be a block in signaling between AS160 and GLUT4 in obese rats. Moreover, exercise-induced AMPK and AS160 phosphorylation and GLUT4 localization were attenuated in obese rats. Such

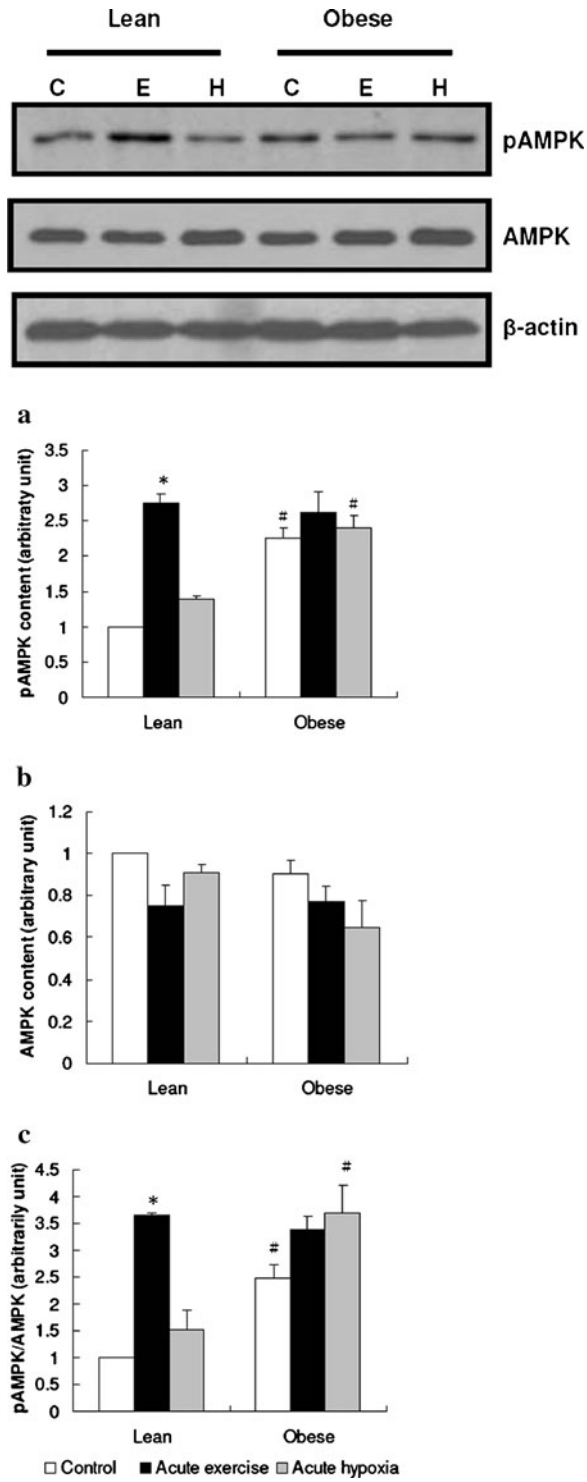

Fig. 3 pAMPK protein content (**a**), AMPK protein content (**b**), and pAMPK/AMPK ratio in control, acute exercise, and acute hypoxia groups of both lean and obese Zucker rats. Each *bar* represents a mean±SEM. *$P<0.05$ vs. the respective control. $^{\#}P<0.05$ lean vs. obese

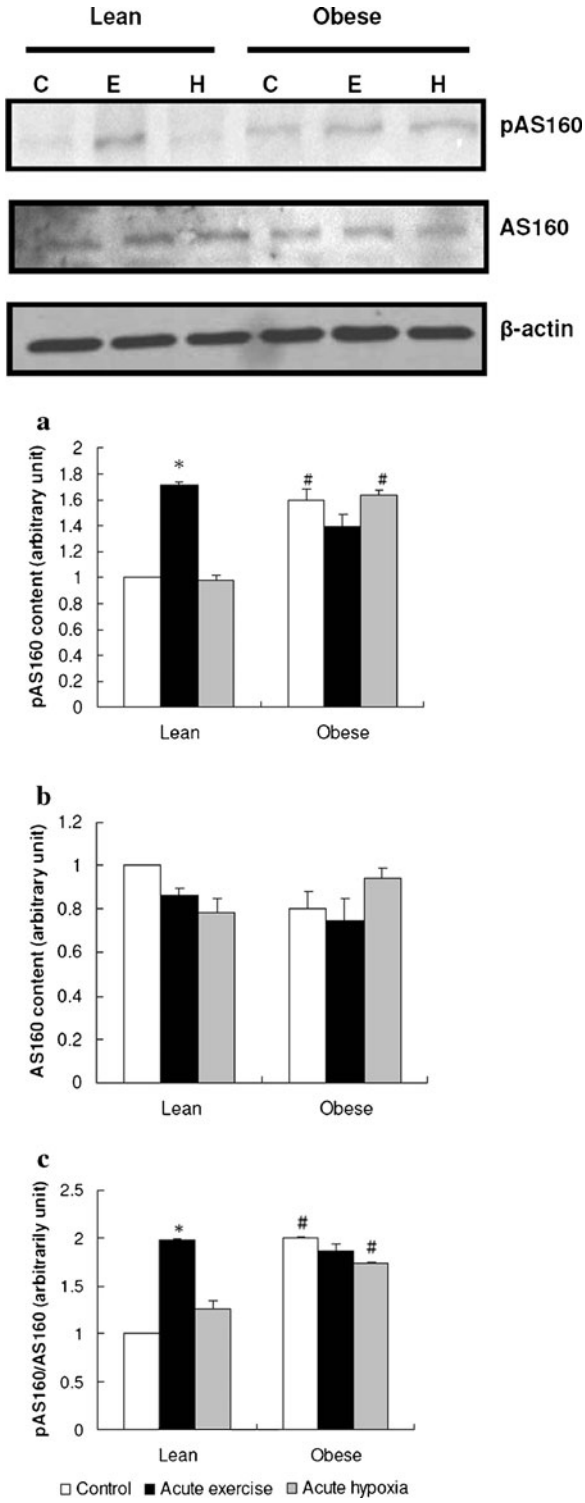

Fig. 4 pAS160 protein content (**a**), AS160 protein content (**b**), and pAS160/AS160 ratio in control, acute exercise, and acute hypoxia groups of both lean and obese Zucker rats. Each *bar* represents a mean±SEM. *$P<0.05$ vs. the respective control. $^{\#}P<0.05$ lean vs. obese

perturbations of the stress-induced GLUT4 translocation pathway in slow-twitch muscle may be among the factors that cause peripheral glucose intolerance in patients with metabolic syndrome.

Compared with lean rats, obese rats were found to have higher baseline AMPK and AS160 phosphorylation levels but equivalent downstream basal GLUT4 membrane localization. The inconsistency between pAS160 and membrane-localized GLUT4 levels in obese rats implies that AS160 may abnormally regulate GLUT4 in the setting of obesity. This is the first study indicating the possibility of a perturbed signal between AS160 and GLUT4 in obese subjects. The higher baseline AMPK and AS160 phosphorylation levels that we observed in the skeletal muscle of obese rats is presumed to compensate for this

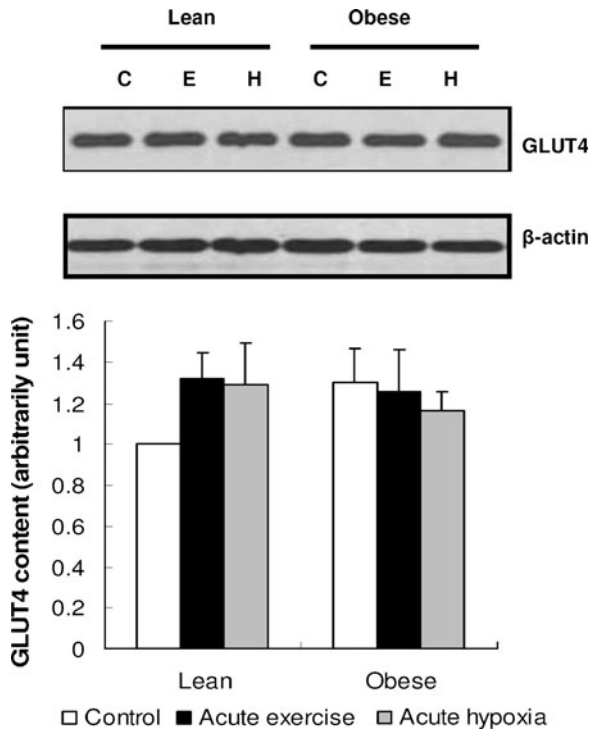

Fig. 5 Total GLUT4 protein content in control, acute exercise, and acute hypoxia groups of both lean and obese Zucker rats. Each *bar* represents a mean±SEM

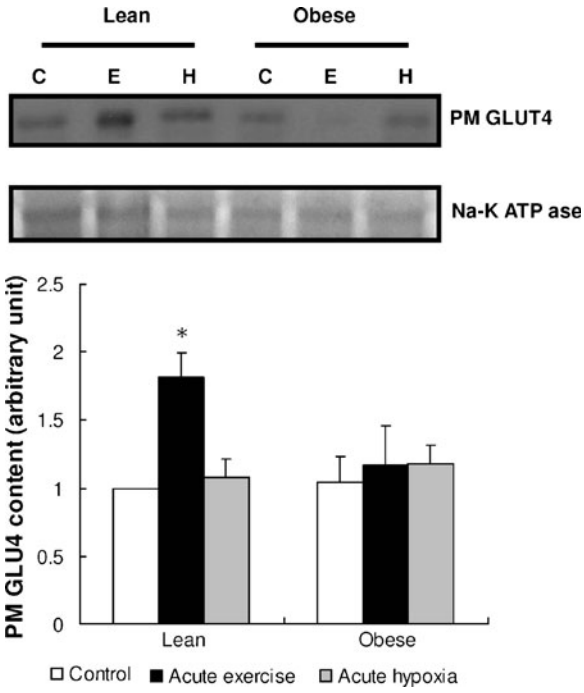

Fig. 6 Plasma membrane GLUT4 protein content in control, acute exercise, and acute hypoxia groups of both lean and obese Zucker rats. Each *bar* represents a mean±SEM. *$P<0.05$ vs. the respective control. *PM GLUT4* plasma membrane GLUT4

perturbation and maintain normal basal amounts of membrane-localized GLUT4.

According to our findings, obese Zucker rats have higher AMPK phosphorylation levels in red gastrocnemius muscle than lean rats under basal conditions. This result is inconsistent with the finding by Sriwijitkamol et al. [21] which showed that obese Zucker rats had lower AMPK phosphorylation levels in mixed gastrocnemius muscle than lean rats. However, Sriwijitkamol et al. carried out their study using fasting animals, while the rats in our study had access to chow. It has been reported that low glucose availability can increase AMPK phosphorylation in order to facilitate energy utilization [5]. Thus, we posit that the low pAMPK levels observed by Sriwijitkamol et al. are due to the inability of AMPK in the muscles of obese rats to be phosphorylated under conditions of hunger.

Our study is the first to demonstrate that AMPK and AS160 in the red gastrocnemius muscle of obese rats show a blunted response to physical exercise. This result is consistent with studies conducted on basal vastus lateralis muscles (white, fast-twitch muscles) of humans with BMIs >30. In the human study, AMPK and AS160 phosphorylation levels did not change in obese patients after 40 min of moderate exercise but increased several fold in lean subjects after the same amount of exercise [20]. Based on our results, we speculate that the blunted reaction of AMPK and AS160 in obese Zucker rats may stem from excessive levels of basal phosphorylation, which leads to a lack of sites for phosphorylation under stressful conditions.

Through the use of a muscle-specific LKB1 knockout mouse model, LKB1 has been established as the major kinase upstream of AMPK that responds to muscle contraction [19]. In our study, LKB1 levels were similar in each group, but the ratio of pAMPK to AMPK, molecules downstream of LKB1, was not. The pAMPK-to-AMPK ratio was higher in all obese groups and the lean exercise group compared with the lean control group. We speculate that the discrepancy between LKB1 levels and AMPK phosphorylation levels is based on inactivation of unknown LKB1 phosphatases.

In contrast with our results, Sriwijitkamol et al. [21] reported low LKB1 content in the mixed gastrocnemius muscle of obese Zucker rats. There are some major differences between Sriwijitkamol's study and ours: (1) the age of the animals, (2) the muscle type, and (3) the fasting time before muscle tissue collection. The most likely reason for the difference in results is muscle fiber composition. The gastrocnemius muscle contains a mixture of fiber types. The superficial portion of the muscle consists of white glycolytic tissue, which expresses much less LKB1 compared with the deep, red portion of the muscle [22]. It is currently unknown whether obesity can result in a selective influence on LKB1 expression among different types of muscle fiber.

Recent studies have found that intermittent hypoxia can achieve effects similar to exercise training. In human athletes, training under an oxygen concentration of 10–14% produces the best results [24]. However, our study showed that 14% hypoxia does not trigger an AMPK-related pathway in lean or obese Zucker rats. This difference may be due to the experimental species in question. We did not test these rats further under a lower oxygen concentration, which may be harmful to Zucker rats.

In summary, our results revealed several abnormalities in the red gastrocnemius muscle of obese Zucker rats: (1) the linkage between AS160 and GLUT4 may be damaged, (2) basal AMPK and AS160 phosphorylation levels were increased, which may blunt the response to exercise, and (3) the downstream translocation of GLUT4 to the plasma membrane after exercise was attenuated. These findings indicate that the AMPK-AS160-GLUT4 pathway in the slow-twitch muscle (red muscle) of obese Zucker rats was altered at both the basal level and after exercise stimulation. It also suggests that there may be a link between a dysfunction in the stress-induced GLUT4 localization pathway of the skeletal muscle and glucose intolerance in obese rats. These findings provide new information about the onset of metabolic syndrome.

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