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ORIGINAL ARTICLE

The effect of 12 weeks endurance training at 2 different intensities on GLUT4 mRNA expression of soleus and gastrocnemius muscles in obese mice

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Abstract The aim of this study was to investigate the expression of GLUT4 mRNA in soleus and gastrocnemius muscles in obese mice in response to endurance training. Forty male C57BL/6 mice were used in this study. Eight mice (Normal Base [NB]) served as non-obese non-trained controls, and 32 mice were put on a high fat diet (HFD) regimen (60% kcal fat) for 12 weeks. At week 16, the obese mice were randomized into the following treatment groups ($n = 8$ each group): Obese Base [OB]; Low Intensity [LI]; High Intensity [HI]; or Obese Control [OC] groups. LI and HI trained for 5 days/week for 12 weeks on a motorized treadmill at 15 m/min on a 5% slope (for LI), and/or at 22 m/min on a 5% slope (for HI). OC mice were kept sedentarily in the cage during the training program. GLUT4 mRNA expression was measured in gastrocnemius and soleus muscles using a Real Time-PCR method. GLUT4 mRNA expression of soleus muscle in LI group increased about 2.2 fold, against about 1.6 fold for gastrocnemius ($p < .05$). In addition, GLUT4 mRNA expression of soleus and gastrocnemius muscles in LI and HI groups were significantly higher than OB and OC groups ($p < .05$). It can be concluded that any disturbance in body energy balance, especially by exercise training and/or high fat diet can influence these molecular and cellular mechanisms that act to establish a stable homeostasis.

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PALABRAS CLAVE
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El efecto de 12 semanas de entrenamiento de resistencia con 2 intensidades diferentes en la expresión de GLUT4 mARN en los músculos sóleo y gastrocnemio en ratones obesos

Resumen El objetivo de este estudio fue investigar la expresión de mARN de GLUT4 en los músculos sóleo y gastrocnemio en ratones obesos en respuesta al entrenamiento de resistencia. Cuarenta machos C57BL/6 ratones fueron utilizados en este estudio. Ocho ratones (normal base [NB]) sirvieron como no obesos controles no entrenados, y 32 ratones fueron puestos en una dieta alta en grasa (HFD), régimen que siguieron (60% de grasa, kcal) durante 12 semanas. En la semana 16 los ratones obesos fueron distribuidos aleatoriamente en los siguientes grupos de tratamiento (n = 8 cada grupo): base obesos (OB); baja intensidad (LI); alta intensidad (HI); o grupos de control (OC) obesos. LI y HI fueron entrenados durante 5 días/semana durante 12 semanas en una cinta rodante motorizada a 15 m/min en una pendiente del 5% (para LI) y/o en 22 m/min en una pendiente 5% (para HI). Los ratones OCse mantuvieron sedentariamente en la jaula durante el programa de formación. GLUT4 expresión de mRNA se midió en los músculos gastrocnemio y sóleo, utilizando el método en *real time*-PCR. La expresión de GLUT4 del mARN del músculo sóleo en el grupo LI aumentó aproximadamente ~2,2 veces, frente a ~1,6 veces para los gemelos ($p \leq 0,05$). Además, la expresión de GLUT4 mARN en los músculos sóleo y gastrocnemio en los grupos LI y HI fue significativamente mayor que en OB y en los grupos OC ($p \leq 0,05$). Se puede concluir que cualquier alteración en el equilibrio energético del cuerpo, especialmente por la práctica de ejercicio y/o dieta alta en grasas puede influir en esos mecanismos moleculares y celulares que actúan para establecer una homeostasis estable.

Introduction

It is generally accepted that obesity is predominantly associated with an impaired insulin-stimulated glucose uptake rate in skeletal muscle, which has been attributed to insulin resistance. Many studies have focused on the glucose transporter system as part of the underlying mechanisms. Glucose transport into the skeletal muscle cell mediated by the glucose transporter proteins GLUT1 and GLUT4.[1] The GLUT1 glucose transporter isoform is thought to support basal glucose transport,[2,3] while the GLUT4 isoform increases glucose transport in response to insulin and contraction. Insulin and contractions translocate the GLUT4 from the intracellular pool to the plasma membrane and to the T-tubules.[4,5]

In rodents, it is well known that the glucose uptake capacity is greater in red oxidative muscles than in white glycolytic muscles.[6–10] One underlying mechanism seems to be a greater level of GLUT4 expression, both intracellularly[6,7] and at the plasma membrane.[8] In human skeletal muscle, glucose uptake was positively associated with the proportion of type I fibers and negatively associated with the proportion of type IIb fibers.[11] These results were supported in the in vitro study by Zierath et al.[12] that reported the insulin-stimulated increase in glucose uptake over basal is strongly correlated, both positively with the percentage of type I muscle fibers and negatively with the percentage of type IIa fibers. However, unconvincing results regarding the relationship between fiber type distribution and GLUT4 content in human muscle have been reported.[13–15] Andersen et al.[13] found no correlation between fiber type and GLUT4 content, whereas Houmard and colleagues[15] showed a weak correlation between fiber type composition and GLUT4 content.

It has been shown that different muscles exhibit large differences in their GLUT4 content, and this variation is often associated with differences in insulin-stimulated glucose uptake.[16,17] As different muscles are composed of a mixture of several different muscle fiber types,[18] it is possible that a significant difference exists in GLUT4 content between muscles.

Possibly, the differences in GLUT4 content and insulin-stimulated glucose uptake are more related to training status. Changes in the skeletal muscle activity level is a key regulator of GLUT4 content in rats.[19,20] In humans, athletes have more GLUT4 than untrained age-matched control subjects,[21,22] and in both normal healthy control subjects and individuals with diminished insulin-stimulated glucose uptake, exercise training has been shown to increase GLUT4 content.[20,23,24] Additionally, a decrease in activity level will decrease GLUT4 content.[20,25] Finally, changes in physical activity and GLUT4 content have been shown to be connected with changes in insulin-stimulated glucose uptake.[20]

The main objective of our work was to investigate the GLUT4 mRNA expression in soleus (a predominantly slow-twitch muscle) and gastrocnemius (a predominantly fast-twitch muscle) in obese mice in response to endurance training.

Subjects and methods

Animals

Forty male C57BL/6 mice (4 weeks age) were used in this study. Eight non-obese mice (Normal Base [NB] group;

Standard diet fed ad libitum) served as non-obese non-trained controls (NB group was considered as a baseline value in calculating gene expression in Real-Time that is expressed as relative values) and 32 mice were put on a high fat diet (HFD) regimen for 12 wk consisting of ad libitum access to a 60% kcal fat diet (High-Fat Diet, Razi Vaccine & Serum Research Institute, Iran). At week 16, the obese mice were randomized into the following treatment groups ($n = 8$ each group): (1) Obese Base [OB] group; (2) Low Intensity [LI] group; (3) High Intensity [HI] group; or (4) Obese Control [OC] group. OB mice were killed before the training program. LI and HI trained for 5 days/wk for 12 wk on a motorized treadmill. OC served as non-trained controls. All mice were housed binary in cages and the temperature of the animal room was maintained at 22 °C, and an artificial 12:12-h light–dark cycle was set. A familiarization period of two weeks in which mice ran 7–10 m/min for 10–15 min on a 5% slope was applied. Thereafter, training was continued in next 12 wk for 60 min continuously at 15 m/min on a 5% slope (for LI) and/or for 41 min continuously at 22 m/min on a 5% slope (for HI). Total daily work was matched for both groups and set at 900 m distance running. OC mice were kept sedentarily in the cage during the training program. Weight was recorded weekly. LI, HI and OC were killed at end of training program. Food was withdrawn 12–14 h before killing. Experiments were approved by the Research Ethics Committee.

Muscle preparation

Mice were anesthetized intraperitoneally with a mixture of Ketamine (30–50 mg/kg bw) and Xylazine (3–5 mg/kg bw) and were killed via direct heart blood withdrawal for muscle sampling 48 h after last exercise session, and the soleus and gastrocnemius muscles were dissected. Muscle samples were quickly frozen into liquid nitrogen and stored at −80 °C. Tissue preparation and total RNA extraction procedures are described elsewhere in details.[26]

Real-time PCR

RNA was reverse-transcribed with reverse transcriptase and random hexamer primers according to the manufacturer's instructions (AccuPower Green Star qPCR PreMix, BiONEER, Daejeon, Korea). Then, PCR-mastermix containing the specific primers, Hot Star Taq DNA polymerase and SYBR-Green PCR buffer were added. All the samples were determined as duplicates, and for a negative control the same set-up was used except for the addition of reverse transcriptase. No PCR product was detected under these latter conditions.

GLUT4 mRNA and β-actin, a house-keeping gene, levels were determined by quantitative reverse transcription–PCR of total cell RNA by using the forward primer 5′ CCG CGG CCT CCT ATG AGA TAC T3′ and the reverse primer 5′ AGG CAC CCC GAA GAT GAG T3′ for amplification of GLUT4 mRNA and the forward primer 5′ ACA ATG AGC TGC GTG TGG CC 3′ and the reverse primer 5′ CCT CGT AGA TGG GCA CAG TG 3′ for amplification of β-actin mRNA. Amplification products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

Real-time quantitation of GLUT4 to β-actin mRNA was performed using a SYBR-Green PCR assay (Rotor-gene 6000, Corbett). GLUT4 mRNA and β-actin mRNA were amplified in separate tubes and the thermal cycling protocol for 40 cycles was denaturation at 95 °C for 20 s, annealing at 60 °C for 60 s, extension at 72 °C for 30 s that started with initial denaturation at 95 °C for 15 min and completed with final extension at 72 °C for 10 min. During the extension step, increase in fluorescence was measured in real time. Data were obtained as CT values (threshold cycle). Relative gene expression was calculated using the Pfaffl formula[27]:

$$\text{Ratio} = \frac{(E_{target})^{\Delta CT_{target}^{(control-sample)}}}{(E_{ref})^{\Delta CT_{ref}^{(control-sample)}}}$$

Data analysis

Values are presented as mean ± SD. One-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences test were used to assess the effects of training on relative mRNA expression of GLUT4 in different groups. Paired *t*-test, also, was used to assess the differences between soleus and gastrocnemius muscle GLUT4 mRNA in each group. For all tests SPSS 21.0 software was used (SPSS Inc., Chicago, IL, USA), and $p \leq 0.05$ was considered statistically significant.

Results

Mean weight values of mice in different groups during HFD feeding and training phases are presented in Fig. 1. Weight in those groups who consumed HFD (i.e. OB, OC, LI and HI) have significantly increased compared to NB from the 5th week during HFD feeding phase which stayed higher until the 12th week ($p \leq 0.05$). The weight gain in all 4 treatment groups was 18% in average compared to NB that reached 32% at the 12th week (Fig. 1).

During the training phase, only the training groups (LI & HI) and OC were studied and weight loss in training groups started to differentiate from OC group from the 6th week which became significant from the 8th week ($p \leq 0.05$). So that, weight loss in training groups (compared to OC group) was 5% and 7% in the 6th and 12th weeks of training phase, respectively. Interestingly, the difference of weight loss between LI and HI appeared from the 9th week and became statistically different in the 11th and 12th weeks ($p \leq 0.05$). In fact, the rate of weight loss was higher in HI than LI, so that weight changes percentage at the end of 12 weeks training in HI group was 8% vs. 5.5% in LI group.

GLUT4 mRNA expression of soleus muscle in the mice who engaged in LI group increased about 2.2 fold, against ∼1.6 fold for gastrocnemius, relative to NB ($p \leq 0.05$); similarly, relative GLUT4 mRNA expression increased, albeit statistically nonsignificant, by high intensity exercise training in soleus and gastrocnemius muscles by ∼2.1 and ∼1.8 fold, respectively ($p > 0.05$). In addition, GLUT4 mRNA expression of soleus and gastrocnemius muscles in LI and HI groups were significantly higher than OB and OC groups ($p \leq 0.05$), which have slightly down-regulated in latter groups (Fig. 2).

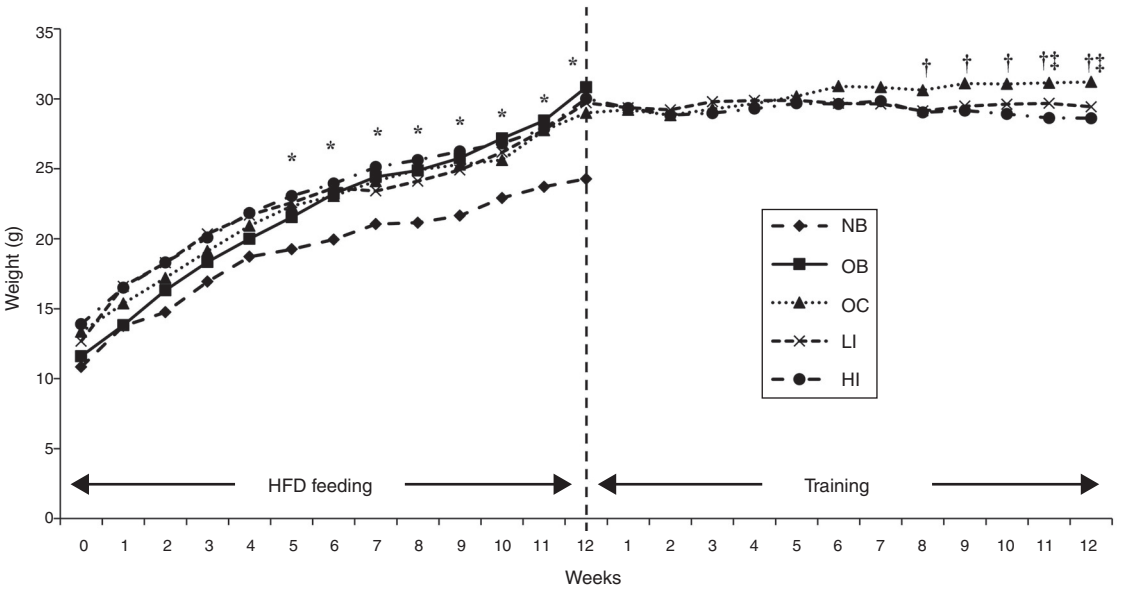

Figure 1 Weight changes in mice during HFD feeding and training phases. *, significant difference between obese and NB groups; †, significant difference between training groups and OB; ‡, significant difference between HI and LI groups. NB = Normal Base, OB = Obese Base, OC = Obese Control, LI = Low Intensity training, HI = High Intensity training.

Discussion

Ploug et al.[28] found that GLUT4 in nonstimulated (by insulin) fibers is distributed along all the muscle fibers, and is present both at the surface (68% of total GLUT4) and in the core (32% of total GLUT4) of the fibers. They also found that the nuclei are displaced by and aligned with the blood vessels that course along the fiber surface, thereby placing a large fraction of GLUT4 close to the source of glucose and obviating the need for diffusion over long distances.[28] It is well documented that in the normal state, GLUT4 cycles slowly between the plasma membrane and one or more intracellular compartments, with the vast majority of the transporter residing in vesicular compartments within the cell interior.[28,29] Insulin stimulated accumulation of GLUT4 protein at the cell surface can be caused by 10–20 fold increase in the rate of exocytosis with a smaller decrease (2–3 fold) in the rate of GLUT4 endocytosis.[5,10,30]

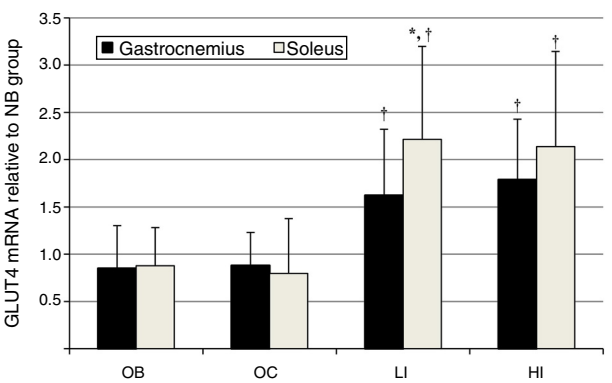

Figure 2 GLUT4 mRNA content of experimental groups' gastrocnemius and soleus muscle relative to matched muscle in NB group. *, significant increase relative to NB ($p \leq 0.05$); †, significant difference in compare to OB and OC ($p \leq 0.05$).

Insulin stimulates glucose transport through GLUT4 translocation from the intracellular storage pool to the plasma membrane. Insulin resistance caused by insulin deficiency or abnormal insulin signaling results in a decrease in GLUT4 expression and translocation, and then causes hyperglycemia and diabetes.[30–32] Muscle contraction has also been shown to increase GLUT4 content in the cell membrane.[29,30] Many studies suggest that exercise training cause GLUT4 protein expression and translocation to plasma membrane by a distinct mechanism from insulin signaling.[5,33] Most studies on rat skeletal muscle have indicated that more GLUT4 is present in the type I fibers compared with the type II fibers,[10,34] as we found in mice, too. In this study, we used only the soleus muscle, generally known as a type I muscle.

Our study is in agreement with other studies[35–39] that indicate exercise trainings increase GLUT4 protein expression in diabetic subjects like as what occurred in our obese subjects.

We found that GLUT-4 mRNA expression is increased by 12 weeks endurance training, both in soleus (a predominantly slow-twitch muscle) and gastrocnemius (a predominantly fast-twitch muscle) in obese mice, with an intensity-depended pattern. This means that exercise intensity can affect the GLUT-4 gene expression (and, probably, protein) response to training which may result in significant metabolic changes especially in obese cases. In agreement with our findings, it has been shown that, GLUT-4 protein and glucose transport are markedly higher in red-oxidative (type I and IIa) muscle fibers than in white-glycolytic fibers (type IIb).[10,40] This might be an important factor in the adaptation to exercise, because endurance training results in a shift from type IIb to type IIa fibers.[41] However, in human skeletal muscles, there is a much smaller difference in the GLUT-4 expression in different muscle fiber types.[34,42]

Daugaard et al.[34] isolated individual muscle fibers and typed them according to myosin isoform. GLUT-4 was ~20% higher in fibers expressing myosin heavy chain I than in those expressing myosin heavy chain IIA or IIB. No difference could be detected between IIa and IIb fibers. After 2 weeks of exercise training, GLUT-4 was increased by ~23% in type I muscles, but there were no changes in type IIa or IIb. However, the low-intensity exercise that was used is primarily known to recruit type I fibers.

According to our findings, low intensity exercise training resulted in increased expression of GLUT4 mRNA in the oxidative muscle (i.e. soleus), probably due to cell requirements related to cellular oxidative capacity. On the other hand, high intensity exercise training did not result in increased expression of GLUT4 mRNA in the soleus, nor in the gastrocnemius muscles. As gastrocnemius muscle consists the mixture of red and white fibers, another explanation would be that exercise training with low intensity was not enough to exercise or even activate red fibers of the gastrocnemius muscle, and a nonsignificant increase in this muscle may be come from its white fibers. This scenario can be extended to what observed findings related to high intensity; i.e. a nonsignificant increase in this muscle by high intensity exercise may be come from its red fibers only.

Hormones also regulate muscle glucose transporter protein concentration. GLUT-4 expression was increased by insulin and thyroid hormones[20,43] and decreased by elevated cAMP.[44] Regulation of GLUT-4 expression by contractile activity is independent of hormonal regulation, because treadmill running increased GLUT-4 in diabetic rats.[45] The effects of insulin-deficiency and denervation on GLUT-4 concentrations were additive.[45] Physical training also increases muscle GLUT-4 protein and mRNA in patients with type 2 diabetes.[46] These results suggest that muscle contractile activity directly modulates muscle GLUT-4 expression, independent of insulin action. Histone deacetylase (HDAC5) is a critical mediator of changes to GLUT4 mRNA levels in response to exercise.[51] A series of histone modifications mediated by histone deacetylases and histone methyltransferases have been shown to climax in a metabolic knockdown of the GLUT4 gene in the skeletal muscle of rats.[52]

There is considerable evidence that GLUT4 mRNA levels in adipose and muscle tissue decrease with obesity,[47,48] and that increases in GLUT4 mRNA can alleviate or compensate insulin resistance.[49,50] Indeed, our finding that GLUT4 mRNA levels were significantly increased by exercise training, and this increase was associated with weight loss, supports this idea. Therefore, increased transcription of GLUT4 in muscle during weight loss may be a certain event in reversing insulin resistance.

Collectively, previous studies and our findings suggest that transcriptional regulation of the GLUT4 gene is responsive to body energy balance changes. Additionally, we found an intensity- and fiber type-dependent response of GLUT4 to exercise training that is not considered in previous studies.

It can be concluded that any disturbance in body energy balance especially by exercise training and/or high fat diet can influence such molecular and cellular mechanisms which act to establish a stable homeostasis. GLUT4 would decrease if energy intake is increased and, reversely, would increase if energy expenditure is increased. These changes are almost similar in slow- and fast-twitch muscles with slightly more responsiveness in slow-twitch muscles that are more engaged in glucose metabolism.

Conflicts of interest

The authors have no conflicts of interest to declare.

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