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# Effect of Mild Intermittent Hypoxia on Glucose Tolerance, Muscle Morphology and AMPK-PGC-1α Signaling

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## Abstract

**The main goal of this study was to investigate the long-term effect of daily 8-hour mild intermittent hypoxia (14-15% $O_2$) on glucose tolerance and muscle morphology of Sprague-Dawley rats. The involvement of AMPK-PGC-1α-VEGF signaling pathways in the skeletal muscle was also determined during the first 8 hours of hypoxia. We found that mRNA levels of VEGF and PGC-1α were significantly increased above control after 8-h mild hypoxia without a change in AMPK phosphorylation. After 8 weeks of mild intermittent hypoxia treatment, plasma glucose and insulin levels in oral glucose tolerance test (OGTT), epididymal fat mass, and body weight were significantly lower compared to the control group. While soleus muscle weight was not changed, capillary and fiber densities in the hypoxia group were 33% and 35% above the control suggesting reorganization of muscle fibers. In conclusion, our data provide strong evidence that long-term mild intermittent hypoxia decreases the diffusion distance of glucose and insulin across muscle fibers, and decreases adiposity in rats. These changes may account for the improved glucose tolerance observed following the 8-week hypoxia treatment, and provides grounds for investigating the development of a mild non-pharmacological intervention in the treatment of obesity and type 2 diabetes.**

**Key Words: hypoxia, obesity, insulin resistance, AMPK, angiogenesis**

## Introduction

Insulin resistance is characterized by an elevated fasting plasma insulin and exaggerated insulin response to an oral glucose challenge, which is causally linked to accumulation of visceral fat (23, 25, 27). Previous human studies have shown that short-term living at a moderate altitude (2,400 m and 1,700 m) can improve glycemic control (29, 39). However, exposure to higher altitude (approximately 4,559 m) causes an opposing effect (28). It appears that only moderate altitudes can confer the beneficial effect on

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improving insulin sensitivity and glucose tolerance.

Hypoxia application can be classified into two modes including intermittent and continuous modes (18). The major differences between both modes are the time of recovery and convenience in clinical application. The majority of systemic hypoxia studies related to glucose metabolism were performed under continuous mode with oxygen levels lower than 12% (8, 15, 35). A death rate of approximately 5% (2 among 40 rats) was found for a 12-h hypoxic condition of 12% oxygen in our preliminary experiment, which reflects risks in clinical application. However, using a mild intermittent hypoxia mode of 14-15% oxygen, 12 h a day for 4 weeks, we found significantly improved insulin sensitivity, and all rats became well-acclimated to the environment (unpublished data).

It is not surprising that hypoxia can alter whole-body glucose metabolism since fatty acid oxidation is compromised and, therefore, reliance on carbohydrate for energy must be increased. This is supported by the observation that hypoxia accelerates glucose uptake in isolated skeletal muscle (42). Since the skeletal muscle is the most important site for postprandial glucose disposal (11), changes in the metabolic properties of the skeletal muscle should affect whole-body insulin sensitivity.

Skeletal muscle glucose transporter 4 (GLUT4) concentration and capillary density are two key factors that can influence whole-body insulin sensitivity and adapt to compensate a chronic hypoxic stress (6, 38). GLUT4 is the main glucose transporter isoform expressed in the skeletal muscle (22) which has been found to increase during the early phase of hypoxia exposure (13). Dill *et al*. (2001) reported that simulated altitude hypoxia increased muscle GLUT4 expression by day 7, but this response was diminished towards baseline by day 28. Since the hypoxia stress was continuous, alternative long-term compensatory mechanism against hypoxia must have been established. One possibility could be an increase in the muscle capillary network. It is well known that hypoxia significantly elevates vascular endothelial growth factor (VEGF) expression. This is a major regulator of angiogenesis which can improve skeletal muscle oxygenation and delivery of glucose and insulin (6, 9).

Recent studies have suggested that both angiogenesis and GLUT4 expression are regulated by the AMPK-PGC-1α (AMP activated kinase) signaling pathway (17). AMPK can be phosphorylated at threonine 172 by muscle contraction and hypoxia. Increasing AMPK phosporylation has been found to elevate expression of PGC-1α (peroxysome-proliferator-activated receptor-γ coactivator-1α), a downstream target gene that plays a major role in the regulation of glucose metabolism (32). However, most of the aforementioned hypoxia studies on rat skeletal muscle were performed under extremely low oxygen concentration (< 2%) which is lethal and impractical during clinical application. In view of this limitation, the present study was designed to investigate the effects of an 8-week mild intermittent hypoxia treatment at 14-15% oxygen on glucose tolerance and several factors determining insulin sensitivity, including muscle GLUT4 protein, capillary density and epididymal fat accumulation. Involvement of the AMPK-PGC-1α-VEGF signaling pathway was also assessed in the skeletal muscle.

## Materials and Methods

### *Animal Care*

Twenty-eight male Sprague-Dawley rats, 250 g of body weight, age of two months, were housed individually and were provided normal rat chow (PMI Nutrition International, Brentwood, Mo., USA) and water *ad libitum* in the animal housing facility. All rats had one week to familiarize the new environment. The animal room temperature was maintained at 21 ± 1°C with a 12-h light-dark cycle. All procedures were approved by the TPEC Animal Care and Use Committee and conformed to the Guidelines for the Use of Research Animals published by the Council of Agriculture, Executive Yuan, Taiwan.

### *Experimental Procedure*

After allowing housing acclimatization, the rats were matched for weight and assigned to control (Control, n = 7) and hypoxia (Hypoxia, n =7) groups. The hypoxia treatment was in an intermittent mode (14-15% $O_2$, 8 h a day) for 8 weeks, 6 days per week. To evaluate the acute effects of 8-h hypoxia treatment on AMPK-PGC-1α-VEGF signaling pathway in the skeletal muscle, 14 additional rats (7 for each group) were placed in the same hypoxia chamber as mentioned for the long-term treated rats (14-15% $O_2$, 8 h) for one time. Hypoxia was generated by placing the rats in a 4-room isobaric chamber (L = 56 cm; W = 43 cm; H = 39 cm) with a constant fraction of inspired oxygen of 14-15% (GAO2 Altitude, Australia), and the oxygen concentration was monitored by an alarm oxygen sensor (Handi Oxygen Sensor, GAO2 Altitude, Australia). To determine the acute effect of hypoxia on intracellular signaling, a group of rats (N = 7) was anesthetized with pentobarbital sodium at 65.0 mg/kg body weight with the same number of control rats immediately after the first 8 hours of hypoxia exposure. Once anesthetized, the red and white quadriceps were removed from both hind-limbs and stored at -80°C until analysis. These rats were then euthanized by cardiac injection of pentobarbital sodium. To determine the long-term

effect of hypoxia, after the last hypoxia session, rats were allowed to recover for 5 h with normal feeding and then fasting for 8 h. They then received an oral glucose tolerance test (OGTT). Following the OGTT, rats were anesthetized as before and the red and white portions of the quadriceps and soleus muscle were excised. Epididymal fat tissue was also isolated and weighed on an AAA-160L balance (Adam Equipment, Danbury, CT, USA). Quadriceps muscle was used to analyze for GLUT4 expression, Thr-172 phospho-AMPK, AMPK, VEGF and PGC1-α, and the soleus for fiber density and capillary density.

*Oral Glucose Tolerance Test (OGTT)*

At the end of the 8-week hypoxia intervention after an 8-h fast, an OGTT was performed. A 50% glucose (w/v) solution was orally delivered with a stomach tube to the rats during the OGTT. Blood samples were taken from the tail at 0 (fasting sample) and at 30, 50 and 80 min after the oral glucose load (1 g/kg body wt) for the determination of blood glucose and insulin. About 200 µl whole blood samples from the tail were transferred to microcentrifuge tubes and then centrifuged at 3,500 rpm for 10 min to obtain serum samples for the insulin assays. A glucose analyzer (Lifescan, Milpitas, CA, USA) was used for glucose determination. Insulin levels were measured by enzyme-linked immunosorbent assay (ELISA) with an anti-insulin monoclonal antibody. The serum sample was quantified on an ELISA analyzer (Tecan Genios, Salzburg, Austria) with the use of commercially available ELISA kits (Diagnostic Systems Laboratories, Webster, TX, USA) according to the manufacturer's procedures.

*Western Blotting Analysis*

About 75 mg of skeletal muscle from the red and white quadriceps were homogenized (1:20) in 20 mM ice-cold HEPES, 1 mM EDTA and 250 mM sucrose buffer (HES buffer, pH 7.4) with a Polytron (Brinkmann Instrument, Switzerland). The protein concentration of the homogenate was determined using a BioRad protein assay reagent (Richmond, CA, USA) according to the manufacturer's instructions. Sample homogenates and standards were diluted 1:1 with Laemmli sample buffer (125 mM Tris, 20% glycerol, 2% SDS, 0.008% bromophenol blue, pH 6.8). 75 µg of protein from sample homogenates were subjected to a polyvinylidene fluoride membrane (PVDF) as previously described (26). GLUT4, phospho-AMPK (Thr 172), AMPK, VEGF and PGC-1α antibodies were purchased from Santa Cruz Biothchnology, Inc. (Santa Cruz, CA, USA) and Cell Signaling Technology, Inc. (Temecula, CA, USA). Proteins were visualized by using an ECL Western Blot Detection Kit containing a secondary antibody against the rabbit antibody (Amersham, Arlington Heights, IL, USA) on Kodak film according to the manufacturer's instructions.

*Real-Time PCR*

RNA extraction from red and white quadriceps muscles was performed with TRIzol (Life Technologies) according to the manufacturer's direction. The tissue (50-100 mg) was dissolved in 1 ml of TRIzol and homogenized (20 s for 6 times) with a Polytron on a setting of 30. The lower aqueous phase was then transferred to a fresh tube and 100 µl of chloroform-isoamyl alcohol (24:1) was added and vigorously shaken. Samples were allowed to sit for 5 min and spun at 12,000 × g for 15 min at 4°C after which the upper aqueous phase was transferred to a new tube. The aqueous phase was mixed with 0.5 ml of isopropanol and the samples were placed in the freezer for 1 h. Samples were centrifuged at 12,000 × g for 15 min at 4°C, and the resulting pellets were washed with 0.5 ml of 75% ethanol in diethylpyrocarbonate (DEPC)-treated water. After centrifugation at 7,500 × g for 10 min, pellets were re-dissolved in 15 µl DEPC-treated water and allowed to dissolve on ice after which samples were ready for reverse transcription. For reverse transcription, 1 µg of total RNA was reverse transcribed using the BioRad Taqman RT-Kit. The resulting cDNA was stored at -80°C.

Quantitative real-time PCR was carried out on 18S rRNA (internal standard), VEGF, PGC-1α, and GLUT4 in 96-well optical reaction plates. The sequence-specific primers are listed in Table 1. All primers and TaqMan probes were designed using Beacon Designer 6.0 (Premier Biosoft, Palo Alto, CA, USA). The TaqMan probe was synthesized and tagged at the 5'-end with FAM as the reporter and TAMRA at the 3'-end as the quencher. The 25-µl PCR mixture consisted of 5 µl of reverse-transcribed cDNA, primers at a final concentration of 300 nM, TaqMan probe at a final concentration of 200 nM, and 12.5 µl TaqMan Universal Master Mix (BioRad Laboratories, Hercules, CA, USA). The PCR reaction was performed and analyzed using the BioRad MyIQTM PCR detection system and software. The reaction conditions were one cycle at 95°C for 3 min followed by a two-step PCR procedure consisting of 15 s at 95°C and 45 s at 60°C for 40 cycles. All standard dilutions, controls and experimental samples were run in triplicates. A standard curve was accepted as linear when the slope fell between -3.0 and -3.4 and the coefficient of correlation was above 0.99-1.00. The RNA levels of VEGF, PGC-1α and GLUT4 were normalized against 18S rRNA and the fold change was calculated by

**Table 1. Primers used for quantitative real-time polymerase chain reaction**

| Gene | Sequences (5' - 3') | Accession No. |
|---|---|---|
| GLUT4 | | NM_012751 |
| Forward | CGTTGGTCTCGGTGCTCTTAG | |
| Reverse | GACACATAACTCATGGATGGAACC | |
| Probe | CCAGCAGCAGCAGAGCCACCGTC | |
| VEGF | | NM_031836 |
| Forward | AACGAAAGCGCAAGAAATCCC | |
| Reverse | GTCTGCGGATCTTGGACAAAC | |
| Probe | TCCTGGAGCGTTCACTGTGAGCCT | |
| PGC-1α | | NM_031347 |
| Forward | GCCACTCCACCAAGAAAGGG | |
| Reverse | CCGTAGACTGGGCCGTTTAG | |
| Probe | TCCTCGTGTCCTCGGCTGAGCACT | |
| 18S rRNA | | X01117 |
| Forward | ACTGCGAATGGCTCATTAAATCAG | |
| Reverse | CTGATAAATGCACGCGTTCCC | |
| Probe | TGGTTCCTTTGGTCGCTCGCTCCT | |

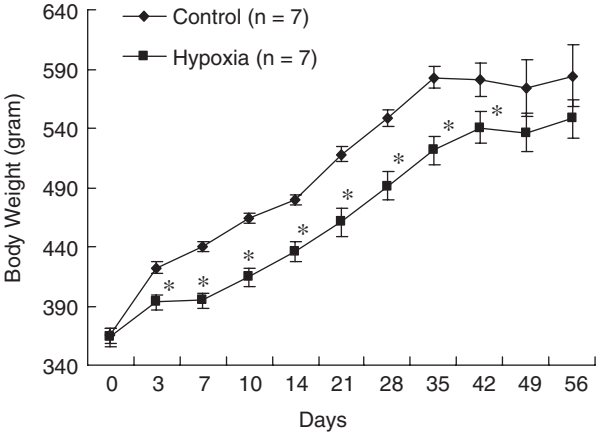


Fig. 1. Effects of chronic hypoxia exposure on body weight. *, significantly difference from the control, $P < 0.05$.

comparing the normalized RNA levels.

*Histochemical Staining*

Several sections of each soleus muscle were cut at 10-μm thick at -22°C and were stained for alkaline phosphatase activity to identify all capillaries and for succinate dehydrogenase (SDH) and myofibrillar-ATPase with pre-incubation at pH 4.55 to distinguish the three main muscle fiber types. For classification of the fibers, fiber density, capillary density and capillary-to-fiber ratio, histochemical sections containing > 300 fibers were counted. For the measurement of fiber cross-sectional area, 20-40 of each fiber type was assessed from each muscle.

**Table 2. Effects of chronic hypoxia exposure on epididymal fat and soleus muscle weights**

| | Epididymal fat mass (g) | Soleus weight (g) |
|---|---|---|
| Control | 7.03 ± 0.98 | 0.21 ± 0.01 |
| Hypoxia | 4.41 ± 0.48* | 0.20 ± 0.01 |

*, significance against the control group, $P < 0.05$.

*Statistical Analysis*

A student *t* test was used to compare the mean difference between the control and the hypoxia groups on all variables tested. A level of $P < 0.05$ was set for significance for all tests. Data are presented as means ± SE.

## Results

The mean body weight for both groups was initially similar before treatment. After 8 weeks of mild intermittent hypoxia, we observed differences in the body weight of the hypoxia group after one week and this weight change continued up to six weeks beyond which there was no change in body weight (Fig. 1). In addition, the weight of the epididymal fat was significantly lower than the control after 8 weeks of mild intermittent hypoxia treatment. The epididymal fat and soleus muscle weights are shown in Table 2. The soleus muscle weight was not significantly different between the control and the hypoxia groups.

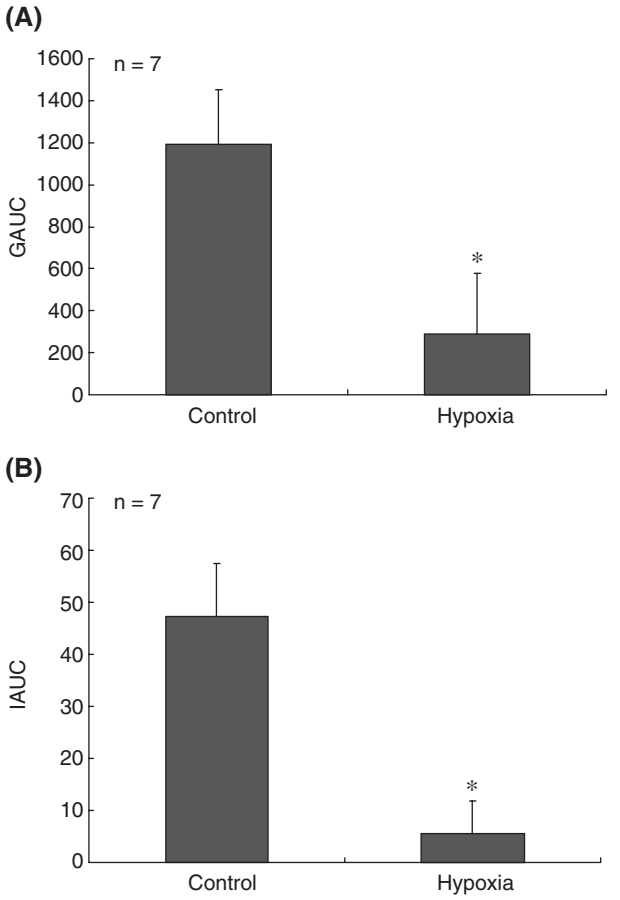


Fig. 2. Effects of chronic hypoxia exposure on glucose tolerance (A) and insulinemia (B). * , significance against the age-matched control group, $P < 0.05$.

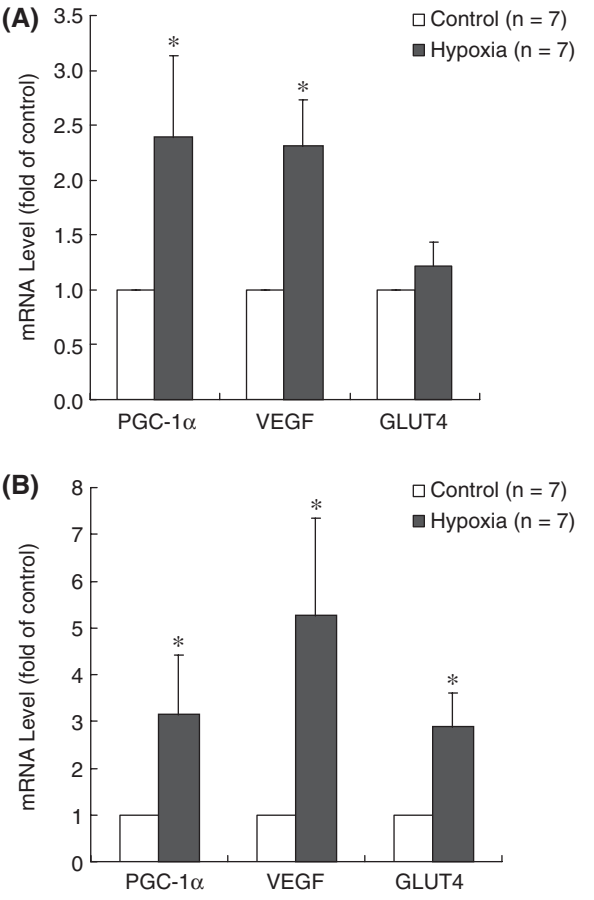


Fig. 3. Acute effects of hypoxia exposure on PGC-1α, VEGF and GLUT4 mRNA levels in the red (A) and white (B) quadriceps muscles. *, significantly difference from the control, $P < 0.05$.

The areas under the curve for glucose (GAUC) and insulin (IAUC) during OGTT are shown in Fig. 2. Rats subjected to the long-term intermittent hypoxia exposure had a significantly lower GAUC and IAUC than the control group.

The results of quantitative PCR are shown in Fig. 3. PGC-1α and VEGF mRNA expressions in both of the red and white quadriceps muscles were significantly elevated above the control level following an acute 8-h hypoxia treatment (Figs. 3A and 3B). GLUT4 mRNA increase was observed only in the white quadriceps muscle (Fig. 3B).

Acute and chronic effects of mild intermittent hypoxia on AMPK phosphorylation (threonine 172) are shown in Fig. 4. AMPK and phospho-AMPK in both of the red and white quadriceps muscles were not significantly different among the groups after acute and chronic hypoxia treatments (Fig. 4). No significant difference in the GLUT4 protein level was observed in the red and white quadriceps muscles between the control and hypoxia groups following acute and chronic hypoxia treatment (Fig. 5). PGC-1α protein level was not different between the control and the chronic hypoxia groups in both the red and white quadriceps muscles (Fig. 6). In addition, no significant difference in the VEGF protein level was observed in the red and white quadriceps muscles following acute and chronic hypoxia treatment (Fig. 7).

The fiber density (FD) and capillary density (CD) of the soleus muscle are shown in Table 3. The eight-week hypoxia treatment significantly increased FD and CD above the control level. No significant difference was observed in the ratio of C/F (capillaries/fibers) between the control and the hypoxia groups.

## Discussion

Visceral fat content is closely associated with the degree of insulin sensitivity and glucose tolerance. In an early study, Fushiki *et al*. (1992) demonstrated a significant fat-reducing effect using a hypoxia protocol with ~11% oxygen in a continuous mode. However, in clinical practice, placing individuals under such a severe level of hypoxia for 24 h a day would not be

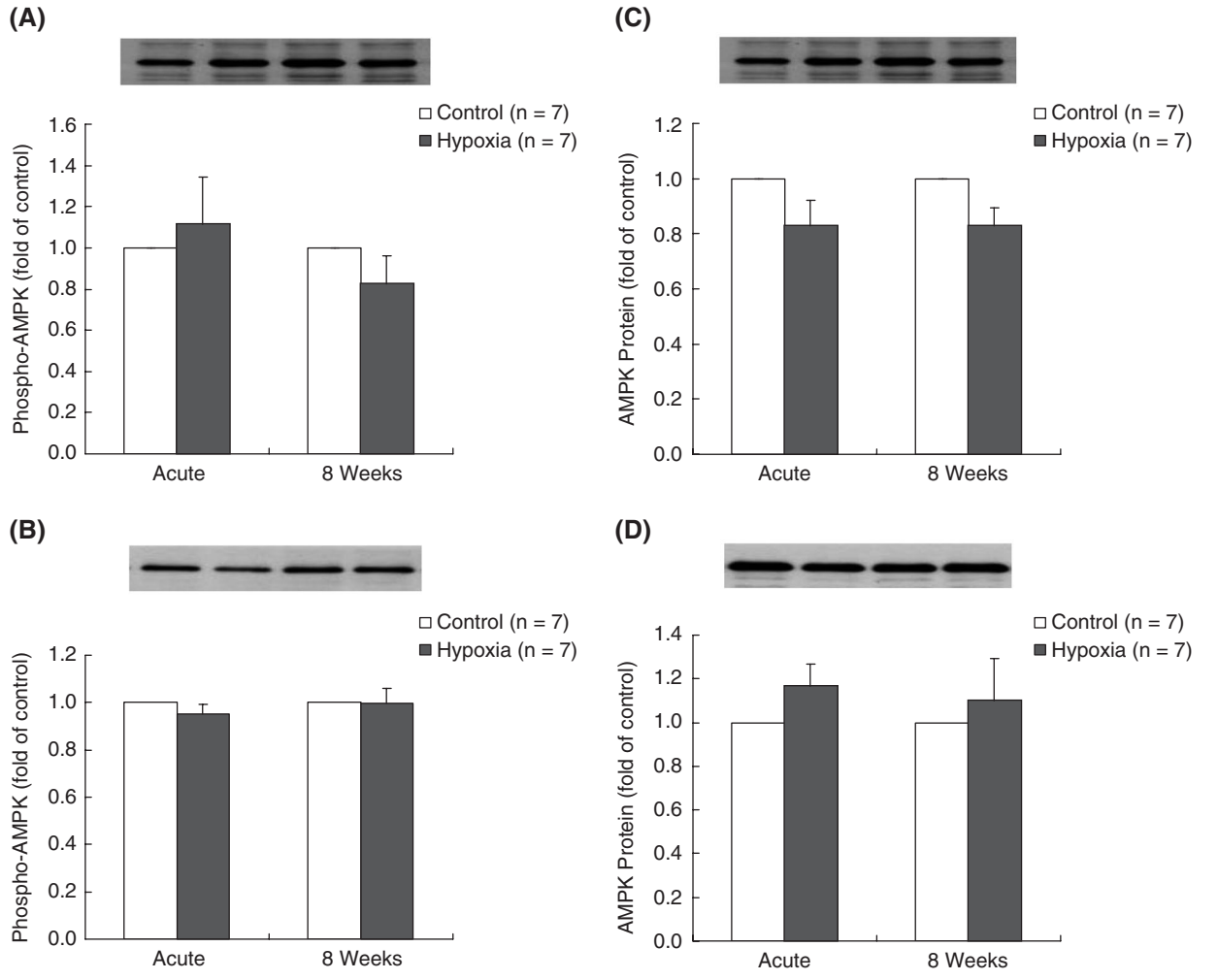


Fig. 4. Acute and chronic effects of hypoxia exposure on AMPK phosphorylation (Thr 172). No difference in threonine 172 phosphorylation of AMPK was observed between the control and hypoxia group in the red (A) and white (B) quadriceps muscles. Total AMPK protein expression is shown in (C) and (D).

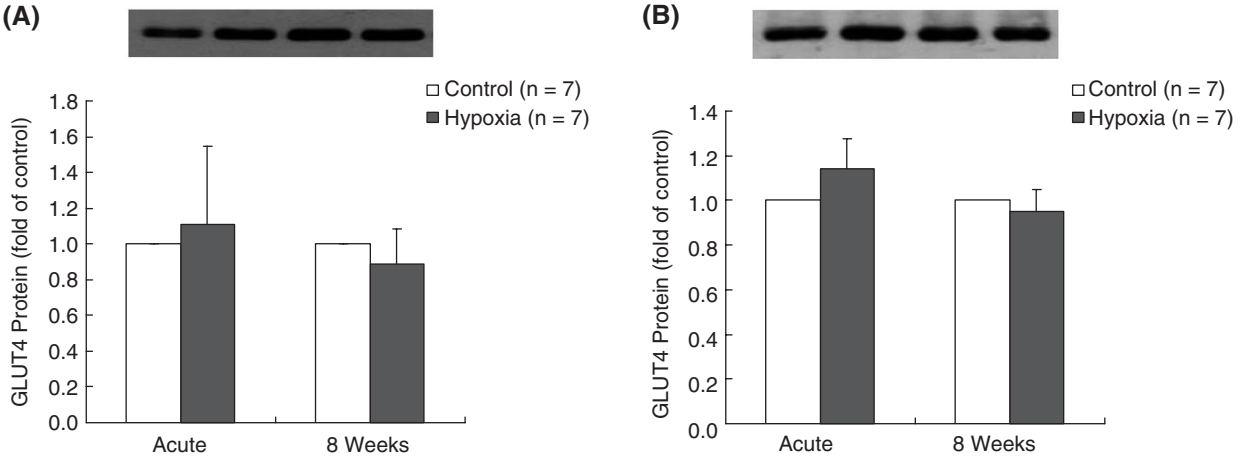


Fig. 5. Acute and chronic effects of hypoxia exposure on GLUT4 protein levels. The GLUT4 protein levels for the red (A) and white (B) quadriceps muscles were not significantly changed.

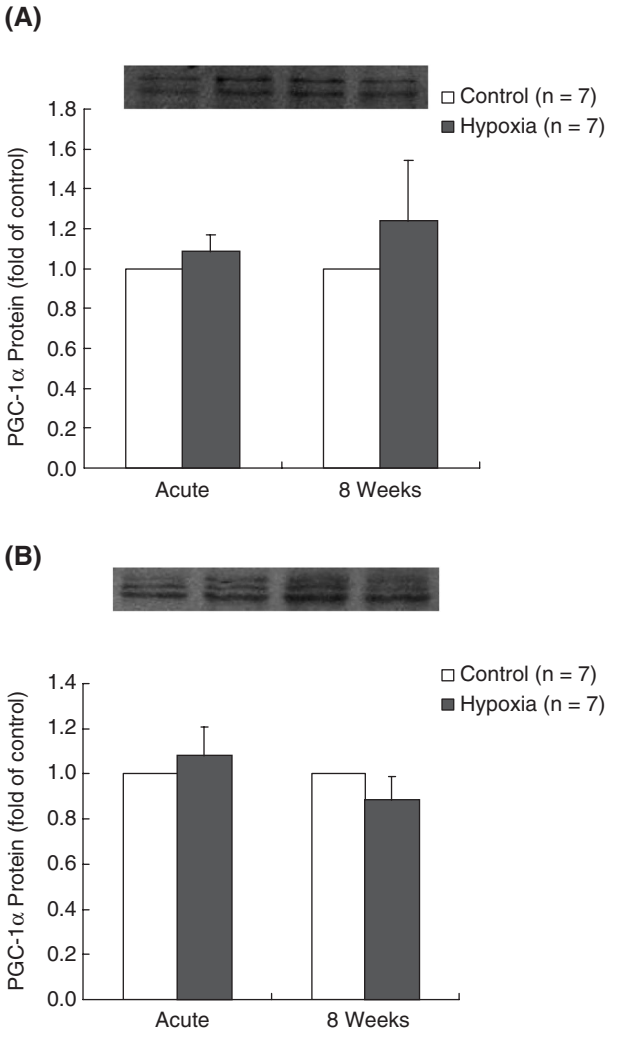


Fig. 6. Acute and chronic effects of hypoxia exposure on PGC-1α protein levels. The PGC-1α protein levels for the red (A) and white (B) quadriceps muscles were not significantly changed.

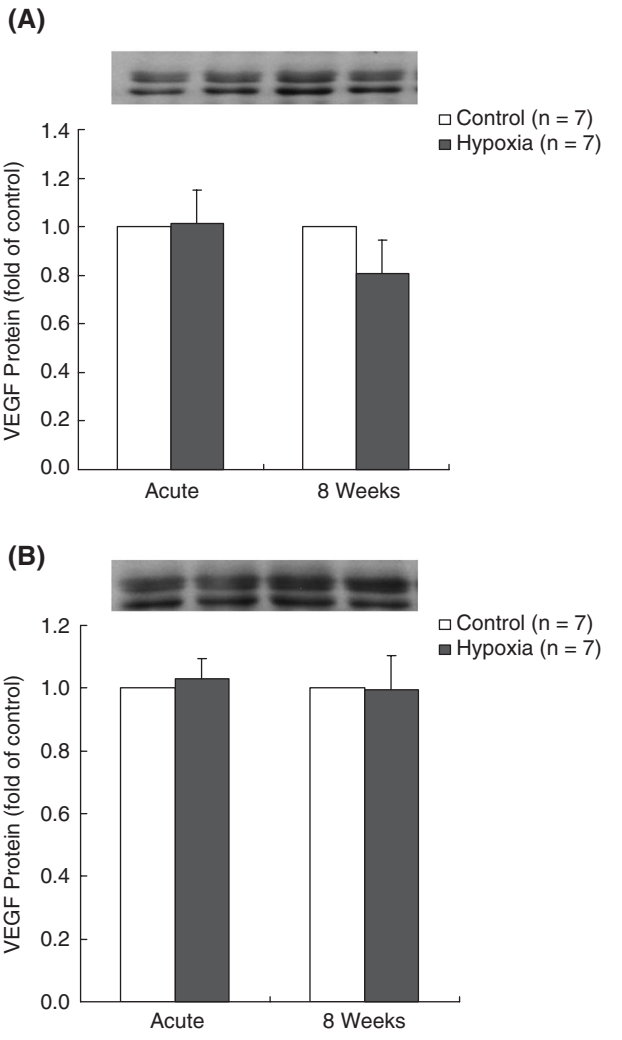


Fig. 7. Acute and chronic effects of hypoxia exposure on VEGF protein levels. The VEGF protein levels for the red (A) and white (B) quadriceps muscles were not significantly changed.

practical. In this study, we demonstrated a significant decrease in epididymal fat storage and increase in insulin sensitivity with changes in muscle morphology after 8 weeks of a mild intermittent hypoxia protocol. The daily 8-h treatment period approximates the average sleeping duration for humans. In contrast to most previous hypoxia studies, the present hypoxia protocol is mild (14-15% oxygen, 8 h a day) and would be more clinically applicable for improving metabolic variables associated with insulin resistance.

The duration of the intermittent hypoxia protocol appears to be crucial for its body weight-reducing effect. Unlike our study, Panisello *et al*. (2008) was unable to demonstrate a similar result with 4-h hypoxia per day with 11 % $O_2$ (33). Taken together, duration seems to be more important than intensity of hypoxia with regard to weight-reducing effects.

Adipose tissue produces a myriad of hormones, peptides (adipokines) and molecules that have been implicated in the impairment of insulin sensitivity and related to metabolic syndromes associated disorders (19, 21). For example, TNF-α (20, 34) and fatty acid (7, 21, 30) are two factors which increase their release into circulation during development of adiposity and causes insulin resistance. In the present study, systemic hypoxia treatment was found to improve oral glucose tolerance and elevate insulin sensitivity with decreased fat mass. This beneficial effect of the new hypoxia protocol appears to be partly associated with possible changes in the adipokine levels, secondary to reduced adiposity.

The skeletal muscle is the largest tissue responsible for the insulin-stimulated disposal of glucose (11, 31). Thus, changes in the metabolic property of the skeletal muscle should affect the whole-body insulin sensitivity. In this study, acute 8-h mild

**Table 3. Effects of chronic hypoxia exposure on fiber density (FD) and capillary density (CD) in soleus muscle**

| | CD (capillaries/mm$^{-2}$) | FD (fibers/mm$^{-2}$) | C/F (capillaries/fibers) |
|---|---|---|---|
| Control | 396 ± 35 | 219.8 ± 12 | 1.8 ± 0.1 |
| Hypoxia | 529 ± 56* | 296.7 ± 20.1* | 1.8 ± 0.2 |

*, significance against the control group, $P < 0.05$.

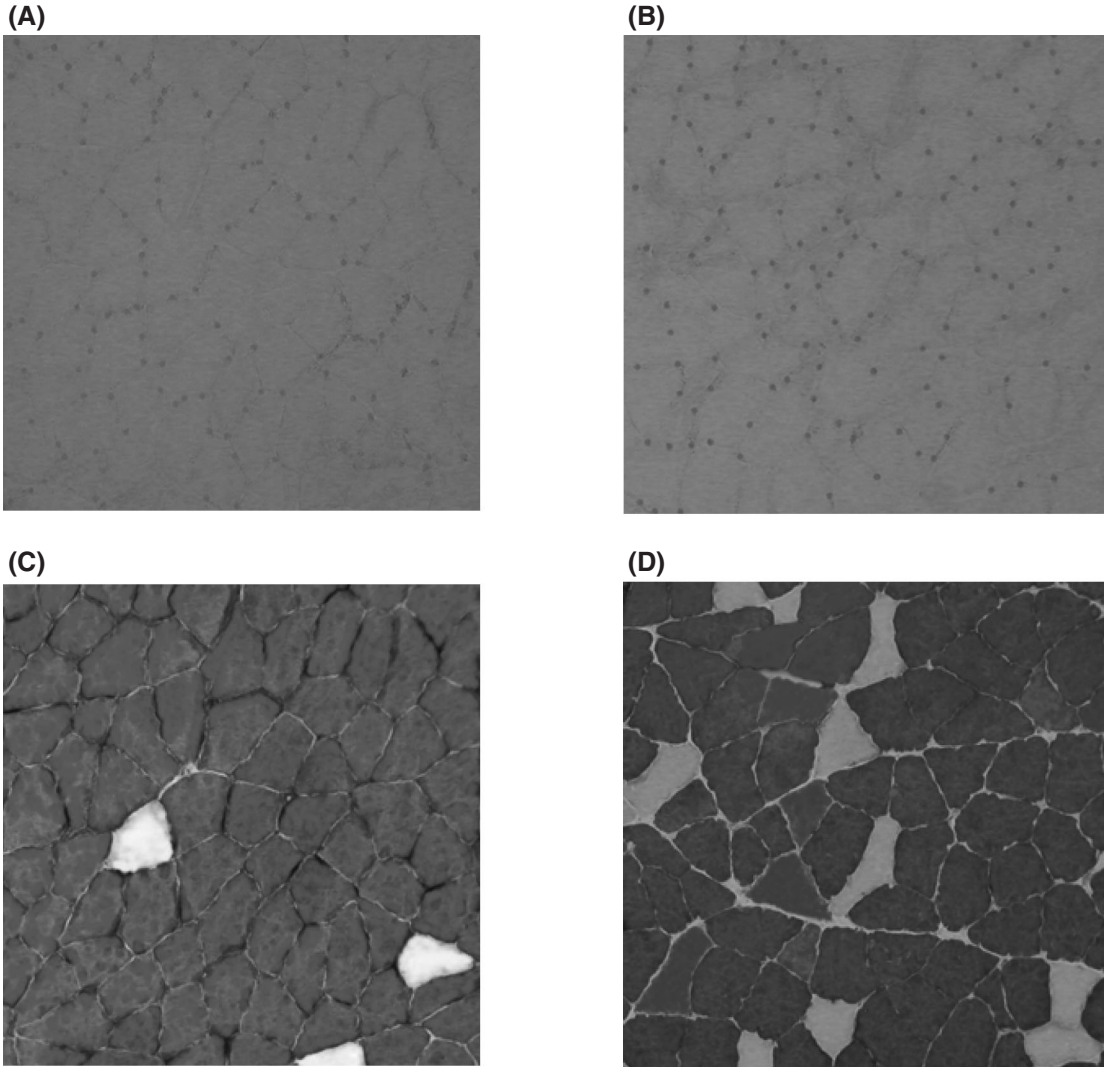


Fig. 8. Representative autoradiographs for the chronic hypoxia effect on soleus muscle capillary density and muscle fiber density (A, B, C, and D).

hypoxia increased the VEGF mRNA level in the quadriceps muscles suggesting that this treatment may be sufficient to induce angiogenesis in the skeletal muscle. In agreement with this result, we found that long-term mild intermittent hypoxia significantly increased muscle capillary density. Since increasing perfusion of insulin and glucose to skeletal muscle has been found to elevate the rate of insulin-mediated glucose uptake (6), our result suggests that the improvements in glucose tolerance and insulin sensitivity by hypoxia were associated, at least in part, with increased capillary density in the skeletal muscle. To the best of our knowledge, this is the most moderate hypoxia protocol that has been demonstrated to increase capillary density in the skeletal muscle.

GLUT4 protein, also known as insulin responsive glucose transporter, plays a key role in postprandial glucose transport in the skeletal muscle. In the current study, we found a significant increase in the GLUT4 mRNA level after acute 8-h mild hypoxia

in the white but not the red quadriceps. GLUT4 protein level, however, was not elevated following 8 weeks of chronic hypoxia in either muscle type. This result is in conflict with the results from previous reports using shorter periods of hypoxia (10, 13, 43). Two possibilities that could explain this discrepancy are the differences in the methods of hypoxia administration and the time of observation. Xia *et al*. (1997) reported an increased GLUT4 protein levels after 1-month of continuous hypoxia at 9% oxygen (proximate to the altitude of Mt. Everest). This is a very stringent hypoxic condition which might result in a greater magnitude of adaptive response than in the present study. Furthermore, Dill *et al*. (2001) have shown increases in muscle GLUT4 mRNA and protein levels after 7 days of hypobaric hypoxia corresponding to an altitude of 4,878 m, but this increase diminished towards baseline by day 28. Hypoxia is known to stimulate many physiological adaptations aimed to enhance peripheral oxygen supply such as increase in capillary density (5, 12, 37). Thus, it is possible that increased GLUT4 expression might occur during the early phase of hypoxia exposure. However, once a greater vascular network is established, enhancement in the expression of the glucose transporter would not be required for greater demand in anaerobic glycolysis.

AMPK is a serine-threonine kinase that has been reported to regulate muscle angiogenesis and GLUT4 expression during hypoxia and exercise (2, 3, 36). AMPK mediates its effect *via* activation of PGC-1α, a transcriptional coactivator that plays a major role in cellular adaptation against energy deficiency (14). AMPK activation requires phosphorylation of threonine 172 within the catalytic subunit of AMPK. In the current study, PGC-1α and GLUT4 mRNA levels were significantly increased by acute 8-hour mild hypoxia, but threonine 172 phosphorylation of AMPK was unchanged 8 hours later. It should be noted that increasing PGC-1α and GLUT4 gene expression from the stimulation of AMPK activity may occur in different time frames. Thus, we cannot preclude the possibility that AMPK phosphorylation occurs earlier than increases in PGC-1α and GLUT4 gene expression, and was diminished by the end of the 8-hour hypoxia exposure.

According to most previous reports, chronic hypoxia causes a reduction of muscle fiber cross-section area which could be attributed to muscle atrophy (4, 40, 41). In the present study, we found a similar result in the muscle fiber cross-section area (CSA) with a milder degree of systemic hypoxia, but the muscle weight was not different between the control and the hypoxia groups. Our result is also similar to a previous study with continuous exposure to 12% oxygen, which showed that the muscle tissue was either unchanged or slightly increased in CSA after 3 weeks of systemic hypoxia (12). Thus, the best explanation for our result of reduced CSA with no change in muscle weight would be either an increase in muscle fiber splitting or muscle fiber replacement. Muscle ischemia or hypoxia can be generated during vigorous resistance exercise which has been shown to cause muscle fiber splitting concurrent with muscle hypertrophy (16). Hypoxia has been reported to increase the rate of skeletal muscle apoptosis (1) and to induce muscle fiber regeneration (24). Therefore, muscle weight could be maintained by paralleled increases in apoptosis of mature muscle fibers and regeneration of new muscle fibers during systemic hypoxia.

Insulin resistance has been considered as a common origin of several age-associated diseases including type 2 diabetes, coronary heart disease, stroke, hypertension and cancer (25). Therefore, developing practical methods to enhance insulin sensitivity is urgently needed in the prevention and the treatment of metabolic syndromes. The new intermittent hypoxia method developed in this study is by far the most gentle hypoxia intervention method to demonstrate a positive effective on insulin sensitivity and body fat mass. Whether this mild intermittent hypoxia method can produce the same beneficial effect in genetically obese animals and humans requires further investigation.

In conclusion, our hypoxia protocol significantly improved glucose tolerance and insulin sensitivity. These beneficial effects might be related to lower visceral fat and increased capillary density although no change was found in GLUT4, VEGF and PGC-1α protein expression levels with 8 weeks intermittent hypoxia. In our study, we also found reductions in muscle fiber cross sectional area without concurrent reduction in muscle weight. These results suggest that the mild intermittent hypoxia protocol may be used to develop as a therapeutic modality for the improvement of glucose tolerance and insulin sensitivity.

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## References

1. Aravindan, N., Aravindan, S., Shanmugasundaram, K. and Shaw, A. Periods of systemic partial hypoxia induces apoptosis and inflammation in rat skeletal muscle. *Mol. Cell. Biochem*. 302: 51-58, 2007.
2. Aschenbach, W., Sakamoto, K. and Goodyear, L. 5' adenosine monophosphate-activated protein kinase, metabolism and exercise. *Sports Med.* 34: 91-103, 2004.

3. Aschenbach, W.G., Hirshman, M.F., Fujii, N., Sakamoto, K., Howlett, K.F. and Goodyear, L.J. Effect of AICAR treatment on glycogen metabolism in skeletal muscle. *Diabetes* 51: 567-573, 2002.
4. Banchero, N., Gimenez, M., Rostami, A. and Eby, S. Effects of simulated altitude on $O_2$ transport in dogs. *Respir. Physiol*. 27: 305-321, 1976.
5. Baron, A., Steinberg, H., Chaker, H., Leaming, R., Johnson, A. and Brechtel, G. Insulin-mediated skeletal muscle vasodilation contributes to both insulin sensitivity and responsiveness in lean humans. *J. Clin. Invest*. 96: 786-792, 1995.
6. Baron, A.D., Tarshoby, M., Hook, G., Lazaridis, E.N., Cronin, J., Johnson, A. and Steinberg, H.O. Interaction between insulin sensitivity and muscle perfusion on glucose uptake in human skeletal muscle: evidence for capillary recruitment. *Diabetes* 49: 768-774, 2000.
7. Boden, G., Chen, X., Ruiz, J., White, J. and Rossetti, L. Mechanisms of fatty acid-induced inhibition of glucose uptake. *J. Clin. Invest*. 93: 2438-2446, 1994.
8. Boero, J.A., Ascher, J., Arregui, A., Rovainen, C. and Woolsey, T.A. Increased brain capillaries in chronic hypoxia. *J. Appl. Physiol*. 86: 1211-1219, 1999.
9. Breen, E., Tang, K., Olfert, M., Knapp, A. and Wagner, P. Skeletal muscle capillarity during hypoxia: VEGF and its activation. *High Alt. Med. Biol*. 9: 158-166, 2008.
10. Chou, S., Chiu, L., Cho, Y., Ho, H., Ivy, J., Ho, C. and Kuo, C. Effect of systemic hypoxia on GLUT4 protein expression in exercised rat heart. *Jpn. J. Physiol*. 54: 357-363, 2004.
11. DeFronzo, R., Jacot, E., Jequier, E., Maeder, E., Wahren, J. and Felber, J. The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes* 30: 1000-1007, 1981.
12. Deveci, D., Marshall, J.M. and Egginton, S. Relationship between capillary angiogenesis, fiber type, and fiber size in chronic systemic hypoxia. *Am. J. Physiol. Heart Circ. Physiol*. 281: H241-H252, 2001.
13. Dill, R.P., Chadan, S.G., Li, C. and Parkhouse, W.S. Aging and glucose transporter plasticity in response to hypobaric hypoxia. *Mech. Ageing Dev*. 122: 533-545, 2001.
14. Finck, B. and Kelly, D. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. *J. Clin. Invest.* 116: 615-622, 2006.
15. Fushiki, T., Kano, T., Ito, K., Hirofuji, C., Inoue, K., Moritani, T. and Sugimoto, E. Effects of chronic hypoxia on the whole-body insulin action in rats. *Can. J. Physiol. Pharmacol*. 70: 1522-1524, 1992.
16. Gonyea, W., Ericson, G.C. and Bonde-Petersen, F. Skeletal muscle fiber splitting induced by weight-lifting exercise in cats. *Acta Physiol. Scand*. 99: 105-109, 1977.
17. Hardie, D.G. and Sakamoto, K. AMPK: A key sensor of fuel and energy status in skeletal muscle. *Physiology* 21: 48-60, 2006.
18. Hoppeler, H. and Vogt, M. Muscle tissue adaptations to hypoxia. *J. Exp. Biol*. 204: 3133-3139, 2001.
19. Hosogai, N., Fukuhara, A., Oshima, K., Miyata, Y., Tanaka, S., Segawa, K., Furukawa, S., Tochino, Y., Komuro, R., Matsuda, M. and Shimomura, I. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes* 56: 901-911, 2007.
20. Hotamisligil, G. The role of TNFalpha and TNF receptors in obesity and insulin resistance. *J. Intern. Med*. 245: 621-625, 1999.
21. Kahn, B. and Flier, J. Obesity and insulin resistance. *J. Clin. Invest.* 106: 473-481, 2000.
22. Kern, M., Wells, J., Stephens, J., Elton, C., Friedman, J., Tapscott, E., Pekala, P. and Dohm, G. Insulin responsiveness in skeletal muscle is determined by glucose transporter (Glut4) protein level. *Biochem. J*. 270: 397-400, 1990.
23. Kolterman, O., Insel, J., Saekow, M. and Olefsky, J. Mechanisms of insulin resistance in human obesity: evidence for receptor and postreceptor defects. *J. Clin. Invest*. 65: 1272-1284, 1980.
24. Kook, S., Son, Y., Lee, K., Lee, H., Chung, W., Choi, K. and Lee, J. Hypoxia affects positively the proliferation of bovine satellite cells and their myogenic differentiation through up-regulation of MyoD. *Cell. Biol. Int*. 32: 871-878, 2008.
25. Kopelman, P. Obesity as a medical problem. *Nature* 404: 635-643, 2000.
26. Kuo, C.H., Browning, K. and Ivy, J. Regulation of GLUT4 protein expression and glycogen storage after prolonged exercise. *Acta Physiol. Scand.* 165: 193-201, 1999.
27. Lai, Y.C., Chen, C.Y. and Kuo, C.H. Exercise and type 2 diabetes. *Adapt. Med.* 1: 1-16, 2009.
28. Larsen, J.J., Hansen, J.M., Olsen, N.V., Galbo, H. and Dela, F. The effect of altitude hypoxia on glucose homeostasis in men. *J. Physiol*. 504: 241-249, 1997.
29. Lee, W., Chen, J., Ho, H., Hou, C., Liang, M., Shen, Y. and Kuo, C.H. Short-term altitude mountain living improves glycemic control. *High Alt. Med. Biol*. 4: 81-91, 2003.
30. Lewis, G.F., Carpentier, A., Adeli, K. and Giacca, A. Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. *Endocr. Rev*. 23: 201-229, 2002.
31. Liu, T.C., Kuo, C.H. and Wang, P.S. Exercise and testosterone. *Adapt. Med*. 1: 24-29, 2009.
32. Ojuka, E. Role of calcium and AMP kinase in the regulation of mitochondrial biogenesis and GLUT4 levels in muscle. *Proc. Nutr. Soc*. 63: 275-278, 2004.
33. Panisello, P., Torrella, J., Esteva, S., Pages, T. and Viscor, G. Capillary supply, fibre types and fibre morphometry in rat tibialis anterior and diaphragm muscles after intermittent exposure to hypobaric hypoxia. *Eur. J. Appl. Physiol*. 103: 203-213, 2008.
34. Peraldi, P. and Spiegelman, B. TNF-$\alpha$ and insulin resistance: summary and future prospects. *Mol. Cell. Biochem.* 182: 169-175, 1998.
35. Poole, D. and Mathieu-Costello, O. Skeletal muscle capillary geometry: adaptation to chronic hypoxia. *Respir. Physiol*. 77: 21-29, 1989.
36. Prior, B.M., Yang, H.T. and Terjung, R.L. What makes vessels grow with exercise training? *J. Appl. Physiol*. 97: 1119-1128, 2004.
37. Rattigan, S., Clark, M. and Barrett, E. Hemodynamic actions of insulin in rat skeletal muscle: evidence for capillary recruitment. *Diabetes* 46: 1381-1388, 1997.
38. Ryder, J., Yang, J., Galuska, D., Rincon, J., Bjornholm, M., Krook, A., Lund, S., Pedersen, O., Wallberg-Henriksson, H., Zierath, J. and Holman, G. Use of a novel impermeable biotinylated photolabeling reagent to assess insulin- and hypoxia-stimulated cell surface GLUT4 content in skeletal muscle from type 2 diabetic patients. *Diabetes* 49: 647-654, 2000.
39. Schobersberger, W., Schmid, P., Lechleitner, M., von Duvillard, S., Hortnagl, H., Gunga, H., Klingler, A., Fries, D., Kirsch, K., Spiesberger, R., Pokan, R., Hofmann, P., Hoppichler, F., Riedmann, G., Baumgartner, H. and Humpeler, E. Austrian Moderate Altitude Study 2000 (AMAS 2000). The effects of moderate altitude (1,700 m) on cardiovascular and metabolic variables in patients with metabolic syndrome. *Eur. J. Appl. Physiol*. 88: 506-514, 2003.
40. Sillau, A., Aquin, L., Bui, M. and Banchero, N. Chronic hypoxia does not affect guinea pig skeletal muscle capillarity. *Pflugers Arch.* 386: 39-45, 1980.
41. Snyder, G., Wilcox, E. and Burnham, E. Effects of hypoxia on muscle capillarity in rats. *Respir. Physiol*. 62: 135-140, 1985.
42. Wright, D.C., Geiger, P.C., Holloszy, J.O. and Han, D.-H. Contraction- and hypoxia-stimulated glucose transport is mediated by a $Ca^{2+}$-dependent mechanism in slow-twitch rat soleus muscle. *Am. J. Physiol. Endocrinol. Metab*. 288: E1062-E1066, 2005.
43. Xia, Y., Warshaw, J.B. and Haddad, G.G. Effect of chronic hypoxia on glucose transporters in heart and skeletal muscle of immature and adult rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 273: R1734-R1741, 1997.