Chronic hypoxia increases insulin-stimulated glucose uptake in mouse soleus muscle

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Gamboa JL, Garcia-Cazarin ML, Andrade FH. Chronic hypoxia increases insulin-stimulated glucose uptake in mouse soleus muscle. Am J Physiol Regul Integr Comp Physiol 300: R85–R91, 2011. First published October 20, 2010; doi:10.1152/ajpregu.00078.2010.—People living at high altitude appear to have lower blood glucose levels and decreased incidence of diabetes. Faster glucose uptake and increased insulin sensitivity are likely explanations for these findings: skeletal muscle is the largest glucose sink in the body, and its adaptation to the hypoxia of altitude may influence glucose uptake and insulin sensitivity. This study tested the hypothesis that chronic normobaric hypoxia increases insulin-stimulated glucose uptake in soleus muscles and decreases plasma glucose levels. Adult male C57BL/6J mice were kept in normoxia [fraction of inspired O2 = 21% (Control)] or normobaric hypoxia [fraction of inspired O2 = 10% (Hypoxia)] for 4 wk. Then blood glucose and insulin levels, in vitro muscle glucose uptake, and indexes of insulin signaling were measured. Chronic hypoxia lowered blood glucose and plasma insulin [glucose: 14.3 ± 0.65 mM in Control vs. 9.9 ± 0.83 mM in Hypoxia (P < 0.001); insulin: 1.2 ± 0.2 ng/ml in Control vs. 0.7 ± 0.1 ng/ml in Hypoxia (P < 0.05)] and increased insulin sensitivity determined by homeostatic model assessment 2 ([21.5 ± 3.8 in Control vs. 39.3 ± 5.7 in Hypoxia (P < 0.03)]. There was no significant difference in basal glucose uptake in vitro in soleus muscle (1.59 ± 0.24 and 1.71 ± 0.15 µmol·g⁻¹·h⁻¹ in Control and Hypoxia, respectively). However, insulin-stimulated glucose uptake was 30% higher in the soleus after 4 wk of hypoxia than Control (6.24 ± 0.23 vs. 4.87 ± 0.37 µmol·g⁻¹·h⁻¹, P < 0.02). Muscle glycogen content was not significantly different between the two groups. Levels of glucose transporters 4 and 1, phosphoinositide 3-kinase, and AMP-activated protein kinase were not affected by chronic hypoxia. Akt phosphorylation following insulin stimulation in soleus muscle was significantly (25%) higher in Hypoxia than Control (P < 0.05). Neither glycogen synthase kinase nor AMP-activated protein kinase phosphorylation changed after 4 wk of hypoxia. These results demonstrate that the adaptation of skeletal muscles to chronic hypoxia increases insulin-stimulated glucose uptake.

metabolism; high-altitude medicine; insulin signaling; carbohydrate metabolism

APPROXIMATELY 140 million people live at high altitude (29), defined as >2,500 m above sea level, where low ambient PO₂ is a natural consequence. It has been known for some time that high-altitude populations have lower blood glucose levels and, perhaps, even lower incidence of type 2 diabetes mellitus (32, 36, 41). For example, Picon-Reategui (32) showed a quicker glucose clearance from blood in high-altitude populations. Others demonstrated that high-altitude natives present a lower glucose profile through 12 h of continuous blood glucose monitoring (6). Two additional studies found lower blood insulin and glucose in two different high-altitude populations (2, 25). These reports also showed high insulin sensitivity by the homeostatic model assessment (HOMA) method. Furthermore, increased reliance on glucose as substrate has been reported in lowlanders acclimatized to altitude (4, 34). However, a recent study showed insulin resistance after 2 days of altitude (4,559 m) that reversed partially after 7 days (23). This result seems contradictory, but it is important to mention that this is a short acclimatization period; full acclimatization requires ≥3 wk (3). Largely on the basis of these findings, it has been proposed that high-altitude natives have increased glucose extraction from peripheral tissues, in particular in skeletal muscles. Because skeletal muscle is the largest glucose sink in the body, chronic hypoxia of altitude may exert its effect on glucose metabolism by increasing muscle glucose uptake. Therefore, the objective of this study was to evaluate glucose uptake in skeletal muscles of mice kept in hypoxia for 4 wk. We hypothesized that chronic hypoxia increases insulin-stimulated glucose transport in soleus muscle and decreases plasma glucose levels.

METHODS

Animals. This study was approved by the Institutional Animal Care and Use Committee at the University of Kentucky. Adult male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were used for the experiments. Mice, much as humans, adapt readily to chronic hypoxia. Other rodents (e.g., rats) are not found at ≥3,000 m, while small rodents, such as mice (Mus musculus) and deer mice (Peromyscus maniculatus), live as high as 4,500 m above sea level (24, 37). Mice were kept in normoxia (fraction of inspired O₂ = 21%) or normobaric hypoxia (fraction of inspired O₂ = 10%) in a transparent Lexan plastic chamber (BioSpherix, Redfield, NY). The chamber was opened once a week for <5 min for cleaning and addition of food and water. At the end of the study protocol (4 wk of hypoxia or normoxia), mice were euthanized by CO₂ asphyxia followed by pneumothorax and exsanguination after a median thoracotomy.

Glucose and insulin measurements. Blood was collected by cardiac puncture and centrifuged to obtain plasma. Glucose concentration was measured using a commercial kit (GAHK-20, Sigma, St. Louis, MO) based on coupled reactions catalyzed by hexokinase and glucose-6-phosphate dehydrogenase, where the concentration of NADH at 340 nm is proportional to plasma glucose concentration. Plasma insulin levels were measured by a rodent sandwich-type ELISA (EZRMI-13K, Linco Research, St. Charles, MO) using monoclonal antibodies against insulin coating the microtiter plate. HOMA2 was used to calculate the sensitivity (HOMA2%S) index with the HOMA2 calculator version 2.2 (Diabetes Trial Unit, University of Oxford). HOMA2%S indexes were calculated from mice in which we measured plasma insulin and blood glucose.

In vitro glucose uptake. After overnight fasting, mice were euthanized, and both soleus muscles were removed. The muscles were placed in organ baths and incubated in buffers gassed with 95% O₂-5% CO₂. Muscles were first incubated with Krebs-Ringer bicar-
bonate buffer (in mM: 117 NaCl, 4.7 KCl, 24.6 NaHCO₃, 1.2 KH₂PO₄, 1.2 CaCl₂, and 2.5 MgSO₄) in the presence of 2 mM pyruvate for 30 min at 37°C. Soleus muscles were then rinsed and incubated with Krebs buffer containing 1 mM 2-deoxy-[3H]glucose (2-([3H]glucose, 1.5 mCi/ml) and 7 mM D-[14C]mannitol (0.45 mCi/ml) with or without insulin (100 nM) for 30 min. Finally, the muscles were blotted dry with filter paper and digested with 250 µl of 1 N NaOH at 80°C for 10 min. After they were neutralized with 250 µl of 1 N HCl, the samples were centrifuged, and 350 µl of 1 N NaOH at 80°C for further use. Sections were stained against glucose transporters 4 and 1 (GLUT4 and GLUT1) and the p85 α-subunit of phosphoinositide-3-kinase (PI3K; Abcam, Cambridge, MA); phosphorylated Akt (p-Akt), Akt, phosphorylated AMP-activated protein kinase (p-AMPK), AMPK, and phosphorylated glycolgen synthase kinase 3 (p-GSK-3; Cell Signaling, Danvers, MA); and GSK3β (BD Bioscience, San Jose CA). After the membranes were washed with PBS and 0.1% Tween, they were incubated with Alexa Fluor 680-conjugated goat anti-rabbit secondary antibody (1:7,500 dilution; Invitrogen) and washed again with PBS and 0.1% Tween. Membranes were finally rinsed with PBS and scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Density of resulting bands was quantified using National Institutes of Health ImageJ software. GLUT4, GLUT1, and PI3K contents were evaluated in the basal, non-insulin-stimulated condition. Akt, p-Akt, GSK-3, p-GSK-3, AMPK, and p-AMPK were evaluated under basal conditions and after insulin stimulation by the same protocol used for glucose uptake, but without the radioactive tracers.

Glycogen content. Soleus muscles were dissected and placed in 30% sucrose in PBS for cryoprotection. For 24 h, muscles were embedded in optimal cutting temperature compound (OCT, Tissue-Tek) and frozen in 2-methylbutane cooled to its freezing point in liquid nitrogen. Serial sections (10 µm) were cut in a cryostat, air-dried, and stored at −80°C for further use. Sections were stained with periodic acid-Schiff (PAS) for visualization of intracellular glycogen content. Glycogen was also quantified with a commercial kit (BioVision). Briefly, muscles were dissected, weighed, digested, and hydrolyzed with 2 N HCl. Samples were then centrifuged (10,000 g for 5 min, and the supernatant was used for glycogen determination by fluorescence spectroscopy (Spectra Max M2, Molecular Devices, Sunnyvale, CA).

Statistics. The means of the groups were compared using Student’s t-test. Two-way ANOVA was used to evaluate the effects of insulin and hypoxia. Significance level was considered at P < 0.05. SPSS, SAS, and GraphPad software programs were used for the analysis.

RESULTS

Chronic hypoxia increases hematocrit and decreases whole body weight. Chronic hypoxia results in increased hematocrit (37.7 ± 1.4% and 56.0 ± 1.5% in Control and Hypoxia, respectively, P < 0.001) and decreased body weight (26.4 ± 0.6 and 24.3 ± 0.7 g in Control and Hypoxia, respectively, P < 0.05), as we and others described previously (5, 8, 14).

Chronic hypoxia decreases circulating glucose and insulin. We measured blood levels of glucose and insulin to determine whether 4 wk of normobaric hypoxia had an effect on total body glucose metabolism. Blood glucose was significantly (30%) lower in the Hypoxia than the Control group (P < 0.001; Fig. 1A), and this was not due to hyperinsulinemia. Insulin concentration was 40% lower in Hypoxia than the Control group (P < 0.05; Fig. 1B). The combination of low blood glucose and low insulin suggests that chronic hypoxia increases insulin sensitivity. Indeed, the HOMA2%S index was significantly higher in the Hypoxia group (P < 0.03; Fig. 1C).

Fig. 1. Plasma glucose and insulin after 4 wk of hypoxia. A: plasma glucose levels were significantly lower in Hypoxia than Control group: 9.9 ± 0.8 (n = 7 mice) and 14.3 ± 0.7 mM (n = 9 mice), respectively (P < 0.002 vs. Control). B: insulin levels were also lower in Hypoxia group: 1.2 ± 0.1 ng/ml (n = 12 mice) vs. 0.7 ± 0.1 ng/ml (n = 9 mice). *P < 0.03 vs. Control. Values are means ± SE. C: homeostatic model assessment 2 (HOMA2) sensitivity (HOMA2%S) index was significantly higher in Hypoxia than Control group. *P < 0.03 vs. Control.
Chronic hypoxia increases insulin-stimulated glucose uptake in soleus muscle. Glucose uptake into soleus muscles was measured by the incorporation of 2-3H-glucose under resting conditions (basal) and after addition of insulin (insulin-stimulated). There was no difference in the basal glucose uptake (non-insulin-stimulated) between the Control and Hypoxia groups (Fig. 2A). On the other hand, insulin-stimulated glucose uptake was significantly (30%) higher in the Hypoxia than the Control group ($P < 0.05$; Fig. 2A). Since GLUT4 mediates insulin-stimulated glucose uptake in skeletal muscle, we examined its content by Western blotting. Chronic hypoxia did not change GLUT4 content in soleus muscles compared with the Control group (Fig. 2B). The content of GLUT1, the other glucose transporter in skeletal muscles, was also similar between Control and Hypoxia soleus muscles (Fig. 2B). These data point to increased insulin sensitivity in muscles of the Hypoxia group, leading to enhanced GLUT4 translocation as the likely mechanism behind the higher glucose uptake.

Increased insulin-mediated Akt phosphorylation after chronic hypoxia. Total Akt, PI3K, and GSK3 protein content was not significantly different in the Hypoxia group (Fig. 3A). Furthermore, p-Akt and p-GSK3 levels were similar between the two groups in the absence of insulin (Basal, Fig. 3B). As expected, insulin increased the ratio of p-Akt to total Akt in all muscles, but the magnitude of the effect was 27% greater in the Hypoxia group ($P < 0.05$; Fig. 3B). Furthermore, the ratio of insulin to basal p-Akt was 60% higher in the Hypoxia than the Control group ($P < 0.05$; Fig. 3B). Insulin also increased the phosphorylation of GSK3 in muscle; however, there was no significant difference in the p-GSK3-to-total GSK3 ratio between the groups ($P = 0.3$; Fig. 3B). We also measured AMPK and p-AMPK in the basal condition and after insulin stimulation. Neither total AMPK nor p-AMPK was different between the Control and Hypoxia groups (Fig. 4).

Muscle glycogen content does not change after chronic hypoxia. A histological examination using glycogen staining with PAS showed more glycogen in muscles in the Control than the Hypoxia group: PAS staining was intense in some muscle fibers in the Control group, while staining was fairly uniform in all fibers in the Hypoxia group (Fig. 5A). A quantitative biochemical glycogen assay did not find a significant difference in glycogen content between the Hypoxia and Control soleus muscles ($P = 0.08$; Fig. 5B).

DISCUSSION

Our main finding is that insulin sensitivity was increased by 4 wk of normobaric hypoxia. The results support the initial hypothesis: in the Hypoxia group, blood glucose was lower and insulin-stimulated glucose transport into soleus muscles and phosphorylation of Akt were increased after insulin stimulation. In addition, we found that plasma insulin concentration decreases following chronic hypoxia, a likely consequence of greater insulin sensitivity.

Chronic hypoxia and glucose metabolism. This study was based on the observation that high-altitude populations have lower basal glucose and insulin levels (2, 25, 32), suggesting that the adaptive response to low ambient PO2 includes changes in insulin sensitivity and glucose metabolism. After 4 wk of hypoxia, we found lower blood glucose and insulin levels, higher insulin sensitivity (HOMA2%S) index, and higher insulin-stimulated glucose uptake in isolated soleus muscles. Our results are consistent with studies showing improved glucose tolerance tests and preferential use of carbohydrates as metabolic substrates after adaptation to chronic hypoxia (4, 9, 18). This change in substrate preference after adaptation to hypoxia reflects the fact that more ATP per mole of O2 is produced by carbohydrate than by fat oxidation (18). It is also instructive to contrast the effects of chronic hypoxia presented in this study.
Fig. 3. Insulin signaling after 4 wk of hypoxia. A: Western blot analysis of phosphoinositide-3-kinase (PI3K), Akt, and glycogen synthase kinase 3 (GSK3) content in soleus muscles from Control and Hypoxia groups. No significant differences were found. B: representative Western blots of phosphorylated Akt (p-Akt), total Akt, phosphorylated GSK3 (p-GSK3), and total GSK3 (top) show no evidence of differences in total Akt or GSK3 between groups (shown also in A) or in response to insulin. A greater increase of p-Akt was observed in Hypoxia group after insulin stimulation. Middle: phosphorylated-to-total protein ratios for Akt and GSK3. There were no group differences in ratios under basal conditions (without insulin); however, there was a significantly greater increase in p-Akt-to-total Akt ratio after insulin stimulation in Hypoxia group. Values are means ± SE (n = 6 mice in each group). *P < 0.05, Control vs. Hypoxia. Bottom: insulin-to-basal ratio of p-GSK3 and p-Akt. Note significantly greater insulin effect in Hypoxia group. Values are means ± SE (n = 6 mice in each group). *P < 0.02, Control vs. Hypoxia. No difference was noted between Control and Hypoxia groups in insulin-to-basal ratio of p-GSK3.
to the adverse consequences of multiple short episodes (seconds to minutes in duration) of hypoxia used to mimic sleep apnea: mice exposed to this type of intermittent hypoxia develop many features of metabolic syndrome, including insulin resistance (11, 19, 33).

Glucose uptake after chronic hypoxia. Because of its total mass, skeletal muscle is an important sink of glucose, and changes in its glucose uptake capacity impact glucose homeostasis. For instance, in diabetic patients, glucose transport into skeletal muscles is impaired and synthesis of glycogen is decreased, apparently as a result of decreased activation of the PI3K/Akt pathway (10, 27). Cellular glucose uptake is facilitated transport mediated by the hexose transporter or translocator family of membrane proteins (GLUT). Two members of this family, GLUT1 and GLUT4, are present in skeletal muscle: GLUT1 is responsible for basal glucose uptake, and GLUT4 is needed for insulin- and activity-stimulated glucose transport (26, 40). We found that chronic hypoxia does not alter basal glucose uptake or GLUT1 content in soleus muscle. Instead, insulin-stimulated glucose uptake into muscle was significantly faster in the Hypoxia group, but there was no change in total GLUT4 content, similar to a report in rats after 4 wk of hypoxia (9). Thus a possible explanation for the increased glucose uptake in soleus muscle is enhanced translocation of available GLUT4 transporters to the sarcolemma in response to insulin. Future studies on the mechanisms by which chronic hypoxia may increase GLUT4 translocation are warranted. One consideration is whether the conditions of the glucose uptake method itself increase the production of reactive oxygen species (ROS) in the muscles under study. ROS increase glucose uptake (21). It is possible that the muscles from the Hypoxia group generate more ROS than control muscles in the hyperoxic (95% O₂) environment of the assay, resulting in faster glucose uptake. Endogenous and exogenous ROS increase glucose uptake by potentiating steps of insulin signaling leading to GLUT4 recruitment and also by activating the MAPK pathways (7, 16). Under some conditions, ROS may also decrease insulin-stimulated glucose uptake via MAPK signaling (1, 20). It is clear then that glucose uptake is quite sensitive to fluctuations in cellular ROS levels and that the effect may be bimodal. In our particular case of increased insulin sensitivity following chronic hypoxia, a more direct test of the potential influence of ROS on the process is needed to settle this issue definitively.

Chronic hypoxia augments insulin signaling. Insulin-dependent glucose uptake in skeletal muscle is mediated by the translocation of GLUT4 transporters following the activation of the PI3K/Akt pathway (35). Akt induces GLUT4 translocation to the sarcolemma through the 160-kDa Akt (AS160) (22). We found that chronic hypoxia does not change the content of PI3K or its downstream effector Akt, but it increases the fraction of p-Akt after insulin stimulation compared with normoxia, indicating enhanced activation of this pathway. Therefore, we propose that chronic hypoxia probably accelerates insulin-stimulated glucose uptake by increasing Akt phosphorylation and downstream recruitment of GLUT4 transporters. Usually, Akt activation leads to increased glycogen synthesis, secondary to GSK3 inhibition. That was not the case after chronic hypoxia: muscle glycogen content did not increase. Quite the contrary, there was a tendency for glycogen content to be lower in muscles from the Hypoxia group. Consistent with this result, previous studies reported a decrease or no change in glycogen content in chronic hypoxia (9, 15, 38, 39). Therefore, it is possible that chronic hypoxia induces
skeletal muscles to rely less on glycogen, as increased glucose transport may be sufficient to meet the metabolic requirements.

**Chronic hypoxia and AMPK in skeletal muscle.** Other possible mechanisms may explain the increased insulin sensitivity after chronic hypoxia. One of these mechanisms is AMPK, which is activated by phosphorylation when the AMP-to-ATP ratio increases (13). Thus this enzyme senses energy deficiency states and is activated by acute hypoxia (28). AMPK activation results in increased insulin-independent glucose transport to skeletal and heart muscle (13) but also may enhance insulin sensitivity (12). However, it is not clear whether chronic hypoxia increases AMPK activity. A recent study found that 8 wk of hypobaric hypoxia does not increase AMPK activation in mouse soleus and extensor digitorum longus muscles (30). We also evaluated changes in the ratio of p-AMPK to total AMPK in soleus muscles and found no difference between the Hypoxia and the Control group. Therefore, AMPK activation does not seem to be important after 4 wk of chronic hypoxia.

**Perspectives and Significance**

This study shows that 4 wk of normobaric hypoxia reduces blood levels of glucose and insulin and increases insulin-induced glucose uptake in mouse skeletal muscles. The increase in insulin sensitivity coincides with Akt phosphorylation, suggesting that this contributes to the increase in glucose uptake (31). The unexpected absence of changes in glycogen content and GSK3 phosphorylation suggests that chronic hypoxia has a more nuanced effect on carbohydrate metabolism than purely increasing insulin sensitivity. Nevertheless, these findings are significant, because they are relevant to insulin resistance, a condition that is present and precedes the development of type 2 diabetes mellitus. Future studies will characterize the molecular mechanism responsible for the increase in insulin sensitivity induced by chronic hypoxia, which may lead to the development of therapeutic targets and interventions for insulin resistance and diabetes.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

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