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Metabolic effects of intermittent hypoxia in mice: steady versus high-frequency applied hypoxia daily during the rest period

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Carreras A, Kayali F, Zhang J, Hirotsu C, Wang Y, Gozal D. Metabolic effects of intermittent hypoxia in mice: steady versus high-frequency applied hypoxia daily during the rest period. Am J Physiol Regul Integr Comp Physiol 303: R700–R709, 2012. First published August 15, 2012; doi:10.1152/ajpregu.00258.2012.—Intermittent hypoxia (IH) is a frequent occurrence in sleep and respiratory disorders. Both human and murine studies show that IH may be implicated in metabolic dysfunction. Although the effects of nocturnal low-frequency intermittent hypoxia (IHl) have not been extensively examined, it would appear that IHl and high-frequency intermittent hypoxia (IHf) may elicit distinct metabolic adaptations. To this effect, C57BL/6J mice were randomly assigned to IHf (cycles of 90 s 6.4% O2 and 90 s 21% O2 during daylight hours), IHl (8% O2 during daylight hours), or control (CTL) for 5 wk. At the end of exposures, some of the mice were subjected to a glucose tolerance test (GTT; after intraperitoneal injection of 2 mg glucose/g body wt), and others were subjected to an insulin tolerance test (ITT; 0.25 units Humulin/kg intraperitoneal injection of 2 mg glucose/g body wt), with plasma leptin and insulin levels being measured in fasting conditions. Skeletal muscles were harvested for GLUT4 and body wt), with plasma leptin and insulin levels being measured in fasting conditions. Skeletal muscles were harvested for GLUT4 and proliferator-activated receptor gamma coactivator 1- (PGC1-α) expression. Both IHf and IHl displayed reduced body weight increases compared with CTL. CTL mice had higher basal glycemic levels, but GTT kinetics revealed marked differences between IHl and IHf, with IHf manifesting the lowest insulin sensitivity with either IHf or CTL, and such findings were further confirmed by ITT. No differences emerged in PGC1-α expression across the three experimental groups. However, while cytosolic GLUT4 protein expression remained similar in IHl, IHf, and CTL, significant decreases in GLUT4 membrane fraction occurred in hypoxia and were most pronounced in IHl-exposed mice. Thus IHf and IHl elicit differential glucose homeostatic responses despite similar cumulative hypoxic profiles.

high-frequency intermittent hypoxia; low-frequency intermittent hypoxia; insulin resistance

SLEEP APNEA is the most common form of sleep disordered breathing. It is highly prevalent (2–10% of the population across the age spectrum), and it is associated with multiple and important morbid consequences including metabolic disturbances (21, 50, 52, 78). One of the major perturbations of sleep apnea is the occurrence of recurrent hypoxic events, i.e., high-frequency intermittent hypoxia (IHf), resulting from periodic collapse of the upper airway during sleep. These events are often terminated by arousal, and therefore elicit fragmentation of normal sleep architecture. Epidemiological studies have shown a disruption of metabolic pathways in sleep disordered breathing, with an association between indices of insulin resistance and the degree of hypoxemia as a consequence of obstructive sleep apnea (OSA) being identified (27, 53, 55–56). However, a detailed analysis of the metabolic adaptations to long-term IH has not been extensively pursued to date.

It is now well established that low-frequency IH (IHl) as seen in multiple cardiopulmonary disorders include alteration of cellular and systemic physiology, polycythemia, pulmonary hypertension, and weight loss (25, 48–49, 79). Previous studies have also shown effects of IHl on energy metabolism via regulation of genes encoding for glucose transporters and glycolytic enzymes (29, 61, 63), primarily through modulation of hypoxia-inducible factor 1-α (HIF-1α) subunit expression and transcriptional activity. Indeed, HIF-1α was reciprocally modulated by insulin and insulin-like growth factor pathways (1, 32, 69, 72, 80).

Other studies exploring the effects of moderate IH on body weight and blood sugar in mice showed lower body weight and glycemic levels than those in normoxic mice after 40 days of IH (40). In addition, previous studies conducted at altitude or in patients with OSA have shown that glucose homeostasis is influenced by hypoxia, both acutely, and also after more prolonged exposures, suggesting that insulin resistance and glucose intolerance are positively associated with the severity of IH (27, 56, 68, 73). Liyori and colleagues (26) showed an acute decrease in whole body insulin sensitivity in animals exposed to IH mimicking the severe hypoxic stress of OSA suggesting a causal relationship between IH exposures and the development of insulin resistance in healthy animals (26). Furthermore, 2–3 days of continuous hypoxia elicited insulin resistance (7, 35), whereas more than 6 wk of continuous hypoxia reduced fasting blood glucose levels but did not affect insulin resistance or glucose tolerance (9, 15, 35).

One of the most widely physiological tests used to identify impaired glucose tolerance is the glucose tolerance test (GTT). In mouse models, GTT is carried out following a period of fasting and glucose administration, both optimized in a relevant study performed by Andrikopoulos and colleagues (3). Besides GTT, insulin tolerance test (ITT) is another widely used assay in humans and animal models, and therefore these two tests provide the opportunity to examine aspects of insulin sensitivity. However, cellular glucose uptake is facilitated through transport mediated by the hexose transporter or translocator family of membrane proteins (GLUT), specifically
GLUT1 and GLUT4. GLUT4 function is highly regulated through not only changes in expression, but also by alterations in the topographic cellular distribution of GLUT4, such that increases in the GLUT4 within the plasma membrane (PM) will facilitate the reduction of plasma glucose levels (8). Overall, around 50% of intracellular GLUT4 is translocated to the cell surface upon insulin activation (44). Apart from GLUT isoforms, transcriptional coactivators can be also important targets for physiological regulation of glucose. For instance, peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) has been described as a transcriptional coactivator that operates as a key regulator of the activation a broad range of transcription factors for downstream regulation of genes encoding mitochondrial proteins and GLUT4 (10, 37, 39).

Another metabolic pathway potentially affected by hypoxia could involve leptin, the adipocyte-derived adipokine product of the ob gene. It has been shown that this hormone induces a negative energy balance by reducing appetite and increasing energy expenditure (20), which is found in circulation at proportional levels to the OSA severity (13, 28, 73). Presence of low levels of leptin or leptin resistance via its cognate receptor have been associated with high levels of insulin resistance (18, 47, 57).

Based on aforementioned considerations, we hypothesized that high- and low-frequency intermittent hypoxia would elicit differential metabolic effects on glycemic control in mice.

**MATERIALS AND METHODS**

**Animals**

Adult male C57BL/6J mice (7 wk old, 20–25 g) were 1) purchased from Jackson Laboratories (Bar Harbor, ME), 2) allowed to acclimate to their surroundings for at least 1 wk, and 3) always housed in groups of five in standard clear polycarbonate cages. Mice were maintained in a 12-h light/dark cycle (light on 7:00 AM to 7:00 PM) at a constant temperature (26 ± 1°C) and were allowed access to food and water ad libitum. At 8 wk of age, mice were randomly assigned into three hypoxic/normoxic group exposures (26 animals per group): control, IHH, and IHL for a period of 5 wk (wk). At the end of the experimental procedures, mice were euthanized by cervical dislocation, and all testing was conducted during the light phase. Animal experiments were performed according to protocols approved by IACUC of the University of Chicago and are in close agreement with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals.” All efforts were made to minimize animal suffering and to reduce the number of animals used.

**Exposures To High- Or Low-Frequency Intermittent Hypoxia**

Mice were placed in identical commercially designed chambers (30 × 20 × 20 inches; Oxycycler A44XO, BioSpherix, Redfield, NY) (Fig. 1A) operated under a 12:12 h light-dark cycle (7:00 AM to 7:00 PM) for a period of 5 wk before any assay (either GTT or ITT testing or skeletal muscle harvesting). Programmed gas concentrations were circulated into each chamber, and an internal O2 analyzer measured the O2 concentration continuously. Deviations from the fixed concentrations were automatically corrected by a computerized system of

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Fig. 1. Schematics of the hypoxic chambers. Commercially available ventilated cages that mimic usual housing conditions are placed in computerized hypoxic chambers to achieve intermittent hypoxia (IH) exposures in mice (A). Hypoxia results from targeted nitrogen enrichment of the air, followed by reoxygenation using oxygen. The two different IH patterns are shown on right based on O2 sensor-acquired data within the experimental hypoxic chambers. IH cycles correspond to the different hypoxic patterns where the fraction of inspired oxygen (FiO2) oscillates from 21% to 6.4% during the 12-h daylight in the high-frequency IH (IHH) pattern (B) and 8% of inspired oxygen during the 12-h daylight in the low-frequency IH (IHL) pattern (C).
solenoid valves controlling gas outlets adding either N2 or O2. Ambient CO2 in the chamber was maintained at less than 0.01%, and humidity was also maintained at 40–50% by circulating the gas through a freezer and silica gel. Ambient temperature was kept at 26°C as described previously by Gozal and colleagues (22). Two episodic hypoxia profiles were used in the study, IHH and IH4, as shown in Fig. 1, B and C, respectively. Low-frequency intermittent hypoxia-exposed mice were subjected for 12 h during daylight to a continuous FIO2 of 8% (Fig. 1C), a level that is associated with reproducible nadir of oxyhemoglobin saturations in the 75–80% range. For the rest of the 12-h lights-off period, normoxic (FIO2, 21%) conditions were applied. IHH-exposed mice were subjected for 12 h during daylight to intermittent hypoxia/normoxia cycles of 3-min duration [Hypoxia, nadir of FIO2; 6.4% for 90 s alternating with normoxia (FIO2, 21%) for 90 s; Fig. 1B]. This IH profile is associated with reproducible nadir of oxyhemoglobin saturations in the 65–72% range. Normoxic (FIO2, 21%) conditions were used during the 12-h lights-off period. Control animals were exposed to circulating room air (FIO2, 21%) during daylight and lights-off period. The nadir of FIO2 and the cycle duration were designed to obtain similar cumulative hypoxic profiles between IHH and IH4 during the 12-h daylight time period. Immediately after the 5 wk exposures, 30 animals (10 per condition) were used for GTT, 30 animals (10 per condition) were used for ITT, and 18 mice (6 per condition) were euthanized, and skeletal muscles (quadriceps) were quickly removed, frozen in liquid nitrogen, and kept at −80°C until analysis.

**Body Weight**

Body weight was checked weekly for a period of 5 wk always at the same time of the day (middle of the light cycle period). Body weight gain was determined by subtracting the body weight on first day of hypoxia exposure from the body weight on subsequent days.

**GTT and ITT**

Both tests were performed following 5 wk of either control IHH or IH4 conditions. In both tests, animals were fasted for 3 h with water available ad libitum. An intraperitoneal injection (26 gauge 3/8” needle) of sterile glucose (2 mg/g of body wt for GTT) or an intraperitoneal injection of sterile humulin (0.25 U/kg of body wt for ITT) was administered. At the beginning of both tests, the tip of the tail was nicked using a sterile surgical blade. Blood recovered from the tip of the tail at different time points (for GTT: 0, 4, 15, 30, 60, 90, 120 min after injection; ITT: 0, 4, 15, 30, 60, 75, 90, 105, 120 min after injection) was tested using an OneTouch Ultra2 glucometer (Life Scan; Milpitas, CA). At the indicated time points, venous blood samples were collected in heparin-coated capillary tubes from the tail vein. Insulin and leptin assays were carried out on selected time points using enzyme-linked immunosorbent assay (ELISA) kits (Millipore; St. Charles, MO) according to the manufacturer’s protocol. The linear range of the insulin assay was 0.2–10 ng/ml, with the limit of sensitivity at 0.2 ng/ml (35 pM), and intra- and interindividual coefficients of variation up to 8.37% and 17.9% respectively at lower concentrations (0.32 ng/ml) of this analyte. Similarly for the leptin assay, the linear range was 0.2 up to 30 ng/ml with the sensitivity threshold at 0.05 ng/ml (~3.13 pM). The intra-assay variation coefficient was up to 1.76% at high concentrations of leptin (17.60 ng/ml), and 4.59% of interindividual coefficient of variation at low concentrations (1.66 ng/ml) of this analyte.

**PGC1-α and GLUT4 Protein Expression**

Total cellular protein or cytosolic and membrane protein fractions were extracted from frozen skeletal muscle tissue (quadriceps) following a slightly modified protocol, as previously described by Wang and colleagues (74). Frozen tissues were homogenized using a BBX24 Bullet Blender homogenizer (Next Advance, Averill Park, NY) such that 100 mg of tissue and a volume of beads (0.5 mm zirconium oxide beads-ZSB05) equal to the mass of tissue were added to the sample. Two volumes of buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 10 mM benzamidine, 50 μg/ml phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10% glycerol (vol/vol) were also added before placing the sample into the homogenizer. At the end of the homogenization process, the homogenate was incubated on a rocking platform in the presence of 20 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) at 4°C for 2 h. After centrifugation at 14,000 g for 30 min at 4°C, the supernatant was collected as total cellular protein. In another set of skeletal muscle samples, the homogenate was centrifuged without CHAPS incubation at 14,000 g for 30 min at 4°C, and the supernatant was collected as the cytosolic fraction. Next, the pellet was washed once with the previous buffer, resuspended in the sample buffer with 20 mM CHAPS, sonicated using a F60 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA), and incubated on a rocking platform at 4°C for 2 h. Finally, after a centrifugation at 14,000 g for 30 min at 4°C, the supernatant was collected as the membrane fraction. For all samples, protein concentration was determined using the modified Bradford method-Dc Protein Assay (Bio-Rad Laboratories, Hercules, CA) using Protein Assay Standard II (bovine serum albumin) (Bio-Rad Laboratories) as standard.

Protein samples were electrophoresed under reducing denaturing conditions in 8% polyacrylamide-SDS gels and transferred by electroblotting onto a nitrocellulose membrane. Equal loading and transfer efficiency of total cellular protein for PGC1-α test or either cytosolic or membraneous protein for GLUT4 test were carefully documented using membrane reversible Ponceau staining and anti-β-tubulin antibody (Upstate-Millipore; St. Charles, MO). After being blocked in 5% albumin solution from bovine serum (Sigma; St. Louis, MO) for 1 h, membranes were incubated with either an anti-PGC1-α monoclonal antibody (Calbiochem-EMD Millipore, St. Charles, MO) or an anti-GLUT4 polyclonal antibody (Santa Cruz; Santa Cruz, CA), followed by incubation with horse-radish peroxidase-conjugated secondary antibodies (Santa Cruz). Signal detection was facilitated with Immun-Star WesternC Membrane Reversal Kit (Bio-Rad Laboratories). PGC1-α, GLUT4, and β-tubulin signals were quantitated using a Molecular Image ChemiDoc XR5+ Imaging System (Bio-Rad Laboratories). PGC1-α and GLUT4 expression were normalized to the β-tubulin.

**Data Analysis**

Body weight matching was not pursued among both IH conditions and CT group, as will be discussed below. Slope A obtained from the GTT (Fig. 3B) was calculated using the glucose levels measured at times 0–15 min after glucose injection. Slope B from the same test (Fig. 3C) was computed between the peak serum glucose levels (15 min) and 120 min after glucose injection. In contrast, only slope A was calculated for the ITT (Fig. 3H), and included glucose levels measured at time 4 min till nadir of glucose levels (60 min) after humulin injection. In addition, area under the curve (AUCg) analyses were calculated for each of the three conditions during GTT. Furthermore, the homeostatic model of insulin resistance (HOMA) was calculated using baseline glucose and insulin concentrations using the following equation: fasting glucose (mg/dl) × fasting insulin (µU/ml)/405. All data are reported as means ± SE. Comparison within glucose, hormones (insulin and leptin), and protein expression levels of GLUT4 and PGC1-α between hypoxic and normoxic conditions were assessed by one-way ANOVA test followed by unpaired Student’s t-test with Bonferroni posttests. A P value of <0.05 was defined as significant.
RESULTS

Effect of Chronic IH_H and IH_L Exposures on Body Weight Gain

Mice exposed either to IH_H or IH_L gained less weight over 5 wk (2.82 ± 0.22 g and 3.67 ± 0.24 g on week 5 after exposure, respectively; n = 26 per group) compared with the normoxic controls (n = 26; 5.45 ± 0.22 g at the end of the exposure; P < 0.05) with IH_H being lower than IH_L. This difference reached statistical significance after the first week of exposure (Fig. 2; P < 0.05).

Effect of IH_H and IH_L on Glucose and Insulin Sensitivity

Glucose tolerance test. As shown in Fig. 3A, IH_H and IH_L had lower fasting glucose levels (138.3 and 65.6 mg/dl, respectively; n = 10 per group) compared with IH_H (198.3 mg/dl; n = 10). HOMA values were higher in IH_H compared with CTL (Fig. 3E; IH_H vs. IH_L, P < 0.05; IH_H and IH_L vs. CTL: P < 0.05). The complex relationships between basal levels of plasma glucose and insulin administration are shown in Fig. 4A. At T_0, both IH_H- and IH_L-exposed mice were associated with increased insulin plasma concentrations, particularly among IH_H-exposed animals (0.27 ± 0.03 ng/ml, means ± SE) compared with CTL group (0.10 ± 0.04 ng/ml, means ± SE; P < 0.05). In contrast, insulin levels at T_120 were lower in both IH_H groups (0.78 ± 0.04 ng/ml, means ± SE; CTL: 0.41 ± 0.005 ng/ml, means ± SE, IH_H: 0.40 ± 0.05 ng/ml, means ± SE, IH_L: n = 10 per group; P < 0.05).

Leptin levels were significantly lower in animals exposed to IH (0.55 ± 0.07 ng/ml, means ± SE, IH_H: 0.49 ± 0.005 ng/ml, means ± SE, IH_L: P NS) compared with CTL (0.95 ± 0.19 ng/ml, means ± SE; n = 20 per group; P = 0.241; Fig. 4B).

Effect of IH_H and IH_L on PGC1-α and GLUT4 Protein Expression in Skeletal Muscle

PGC1-α expression. Compared with CTL mice, PGC1-α protein expression was unaltered by either IH_H or IH_L exposures (Fig. 5A).

GLUT4 expression. Different trends in GLUT4 protein expression emerged in the cytosolic and membrane fractions (Fig. 5, B and C). GLUT4 cytosolic protein expression was increased in skeletal muscle of IH_L-exposed mice compared with either CTL or IH_H conditions (n = 6 per group; 1.71 ± 0.54 intensity units (IU), means ± SE, IH_L: 1.68 ± 0.54 IU, means ± SE, IH_H: 1.39 ± 0.45 IU, means ± SE, CTL; P NS) (Fig. 5B). In contrast, expression of GLUT4 in the membrane fraction was significantly lower in IH_H (0.62 ± 0.03 IU, means ± SE) and IH_L (0.39 ± 0.06 IU, means ± SE) groups compared with CTL mice (0.74 ± 0.14 IU, means ± SE; P < 0.05), with IH_L group being the lowest (Fig. 5C). Accordingly, GLUT4 membrane-to-cytosolic protein ratios, which are indicative of translocated GLUT4 protein (i.e., biologically active), were markedly lower in hypoxic mice compared with controls (Fig. 5D), and a significant correlation between GLUT4 (Membr.Prot./Cyto.Prot) and slope A from ITT was apparent (Fig. 5E).

DISCUSSION

In the present study, we aimed to assess the impact of different cycling frequencies in the application of the IH stimulus, a key clinical manifestation of OSA, on glucose
regulation. Despite similar cumulative hypoxic profiles, presentation of IH using different cycling frequencies was associated with distinct glycemic homeostatic responses. From the present study, it becomes apparent that IH induces reductions in body weight gain, lowers fasting glucose levels, and also reduces the uptake of glucose after in vivo glucose administration, as well as insulin receptor sensitivity. Furthermore, lower levels of translocated GLUT4 to the plasma membrane in skeletal muscle are apparent. All of these phenomena are exacerbated in the context of IHL compared with high-frequency hypoxic oscillations, the latter differing from control conditions. Of note, although the concept that sustained and intermittent chronic hypoxic exposures impose divergent effects on metabolic regulation is not novel and has been explored in several studies in both human and animals using exercise procedures (14, 66) or hypobaric hypoxia (46), we are unaware of any specific studies addressing the issue of hypoxia cycle duration on metabolic outcomes. In the discussion that follows, we will further explore the relationships and putative pathways linking IH frequency to insulin resistance. However, before we address these issues, several technical points appear worthy of mention. Indeed, the IH profile used herein is markedly similar to those reported by previous investigators, and as such our current findings can be potentially compared with such earlier studies (53, 58 –59). Second, body weight matching was not pursued among both IH-exposed mice and CTL group. Particularly, underfeeding CTL mice or overfeeding IH-exposed mice would be necessary to achieve weight matching (30, 58) between groups. Such interventions may however alter glycemic homeostasis per se and therefore mask the effects of IH explored in the present study. We reasoned that if an association between insulin resistance and IH emerged, the present findings would be all the more compelling considering the reductions in weight anticipated in the context of IH exposures. Third, GTT is routinely performed in mice following an overnight fast, with the food being removed...
around 4 PM, and the glucose load being administered via IP injection the following morning. However, it is well known that mice are nocturnal feeders, with \( \sim 70\% \) of their daily caloric intake occurring during the dark cycle (6), and that their metabolic rate is much higher than humans. Therefore, an overnight fast represents a comparatively longer fasting time compared with humans and is more akin to starvation. In addition, there is no consistency in the amount of glucose administered with studies using 1 g/kg (4, 5, 54) or 2 g/kg (5, 24, 31, 43, 81) or both doses to determine glucose tolerance (23, 62). In 2008, GTT was reevaluated in mice to determine the most appropriate fasting duration, route of glucose administration, and the ideal amount of glucose (3). Based on such considerations, we fasted mice for 3 h in both GTT and ITT and administered IP 2 mg glucose/g body wt during GTT.

Low- and High-Frequency IH and Body Weight Gain

Factors contributing to hypoxia-induced weight loss are complex and incompletely understood. Several investigators have demonstrated that body weight will continue to increase in mice submitted to chronic IH (58), whereas other laboratories have reported decreases in body weight accrual velocity in mice subjected to IH compared with controls (12, 17, 71, 76). Row et al. in 2002, measured body weights in postnatal rats and reported significantly lower body weight gain in rat pups exposed to IH than age-match controls housed in room air (60). In a recent study carried out in male lean mice exposed to different hypoxic patterns (normoxia, IH 12 times/h, IH 60 times/h or sustained hypoxia), body weights were similar among all hypoxic mice independent of the hypoxic regimen used but gained less weight than control animals (58). Consistent with such findings, we also found lower body weight gain in both hypoxic groups compared with room air-exposed adult mice independent from the IH frequency pattern for the first 7 days of exposure. However, lower body weight increases emerged in IH_H compared with IH_L, starting as of 14 days of hypoxic exposures. Consequently, body weight gain appears to be influenced by the IH frequency.

Low- and High-Frequency IH and Insulin Resistance

The selected IH profiles aimed to reproduce the overall cumulative hourly oxygen desaturations patterns routinely observed in moderate to severe OSA patients with oxyhemoglobin saturation levels ranging from 65 to 80% (11, 19, 22, 67). A large body of evidence derived from epidemiological cohorts and clinical populations indicates that OSA may also contribute to the development of metabolic disorders including glycemic regulation impairments (27, 55–56, 65). Furthermore, in healthy humans, acute IH induced decreased insulin sensitivity and inadequate increases in pancreatic insulin secretion (42). Iiyori and colleagues (26) used a euglycemic hyperinsulinemic clamp in mice and showed that acute IH (60 times/h over 9 h) induced insulin resistance. We now show that chronic IH will lead to lower fasting glycemic levels and relatively higher levels of insulin concentrations, specifically in IH_L-exposed mice and not as much in IH_H-exposed mice, yet suggesting the presence of insulin resistance state after both types of IH exposures. There were however discrepant findings between HOMA and either AUC_e or slope analyses. Indeed, HOMA results suggested that IH_H would be worse affected. This is not the case for the other two analytical approaches, which suggest that IH_L is most severely affected. An important caveat for HOMA validity is that it imputes a dynamic \( \beta \)-cell function (i.e., glucose-stimulated insulin secretion) from fasting steady-state data. Therefore, based on the concordance between the two dynamic assessments of glucose and insulin relationships (i.e., AUC_e and slope analyses), we would postulate that the \( \text{IH}_L > \text{IH}_H > \text{CTL} \) effect is most likely reflective of the true changes in glucose homeostasis. In addition, as can be seen from Fig. 3F, there were complex changes in both the baseline glycemic and insulin levels with each type of exposure, potentially reflecting underlying changes in the "homeostat" level of plasma glucose after prolonged hypoxic exposures, as well as changes in either basal secretion of insulin or in insulin sensitivity under those conditions. Reineke and collaborators (58) showed that even infrequent episodic hypoxic events (12 times/h) were sufficient to disturb insulin and glucose regulation in lean mice. We should note that in the present study we...
Fig. 5. Effect of IH on glucose transporter 4 (GLUT4) and proliferator-activated receptor gamma coactivator-1α (PGC-1α) expression in skeletal muscle. Effect of 5-wk IHH, IHL, and CTL on PGC-1α and GLUT4 protein content in skeletal muscle. Representative Western blots of PGC-1α protein expression (A), GLUT4 cytosolic protein expression (B), and GLUT4 membrane protein expression (C) are shown. Samples on the autoradiographs (Western blot images panels) are expressed as percentage of the CTL group. Ratio between GLUT4 membrane protein expression and GLUT4 cytosolic protein expression were represented in D. Intensity units results are means ± SE and *P < 0.05 significantly different from the CTL group (n = 6 per group). Correlation between GLUT4 (Membr.Prot./Cyto.Prot) and slope A from ITT is shown (E).
did not explore the presence of hypoxic injury to pancreatic β-cells during IH, and therefore the contribution of such previously reported effects of IH may have contributed to the kinetics of glycemia and insulin levels following IP glucose injection (75, 77). Another important comment pertains to the marked differences in peak glycemic levels that occurred after IP glucose injection. Notwithstanding the differences in body weight, this glucose dosage has been previously shown to reliably reflect existing differences in insulin sensitivity (3). However, it will be important in future studies to compare whether the oral glucose administration route will provide further information on the deregulation of overall glycemic homeostatic processes. Furthermore, the recent development of frequent sampling approaches in the mouse may provide improved resolution on the regulation of various glucose disposition compartments (2).

In the present study, we also show that after glucose injection, the resultant circulating glucose levels require longer times to be cleared from the circulation and to reach preinjection levels in both hypoxic groups, with the kinetics of glycemic decay in IH-exposed mice being the most affected. Although the oral route of exogenous glucose administration is more physiological, we used an intraperitoneal administration to reduce the variability associated with inconsistent rates of gastric emptying, which can complicate data interpretation. On the other hand, the ITT was developed as a simple way to evaluate insulin action in vivo in humans. In mice, this test was modified, where a larger bolus of insulin is given after a fast of variable duration and the glucose concentration is monitored for a longer duration (~90–120 min). For this test, the fall in blood glucose is used as a reflection of insulin action, such as a fall by ~50% of glucose concentrations in wild-type mice on a chow diet and in normoxia (45). For both GTT and ITT, we opted to display the data as the kinetic slope from the baseline point to the highest or lowest glucose levels, respectively, thereby normalizing across potential confounders. Thus both GTT and ITT findings coincide in their findings and clearly establish a consistent perturbation of insulin sensitivity in the context of IH that is further modulated by the IH stimulus presentation frequency.

The changes in GLUT4 and PGC1-α were also examined in skeletal muscle. As mentioned, PGC1-α serves as a transcriptional coactivator that plays a key role in coordinating cellular metabolic flux in skeletal muscle, in which ~90% of insulin-stimulated glucose uptake occurs (33). GLUT4 delivery to the cell surface is coordinated by insulin signaling and requires GLUT4 mobilization from intracellular membrane compartments, recognition of GLUT4-containing vesicles at the PM, and finally fusion of these two membranes (38). Whereas total GLUT4 (data not shown) and PGC1-α remained unaltered by hypoxic exposures, we found that expression of GLUT4 in the membrane was significantly lower in IH than in controls, with no changes in GLUT4 expression in the cytosol. A potential explanation for such findings resides in the known activity of insulin. Indeed, insulin stimulates constitutive exocytosis (8) and particularly that of GLUT4 (38). Although the molecular mechanisms underlying the specificity of insulin action on GLUT4 vesicles remain to be established, insulin promotes tethering and fusion of GLUT4 vesicles to the plasma membrane (41), within the characteristic time frames of appearance of GLUT4 in the PM (16, 70). Interestingly, using a different IH profile, improved glucose tolerance in the absence of increased GLUT4 protein expression was previously reported (14). The unbalanced distribution of GLUT4, as clearly shown in Fig. 5D, may account for the IH⁺-animals inability to facilitate the transport of glucose to the intracellular space, as described in our experiments using both GTT and ITT (Fig. 5E). Thus the frequency of IH is critical for the development of insulin resistance and impaired glucose tolerance, thereby confirming Polotsky et al. previous findings (53).

**Low- and High-Frequency IH and Leptin**

Low fasting leptin levels probably play an important role in the alterations reported on insulin and glucose responses to IH. It has been shown that leptin can act at the level of pancreas, by downregulating insulin gene transcription and insulin secretion (34, 64). Conversely, leptin deficiency plays a key role in the acceleration of insulin resistance in mice exposed to IH, yet it remains unclear whether mice with intact leptin pathways are also susceptible to metabolic dysfunction after exposure to IH (53). We found lower baseline levels of leptin expression in both hypoxic groups with no significant differences between high- and low-frequency hypoxia. Therefore, hypoxia per se, rather than the frequency of IH, appears to be the determinant factor underlying the emergence of lower leptin levels.

We should emphasize that a significant limitation of our study included the absence of any assessments of differences in the levels of stress hormones among the three experimental groups. Indeed, a recent work by O’Donnell and colleagues (77) has postulated that the initial stress response, i.e., corticosterone levels, was a major determinant to the acute metabolic changes observed after short-lasting period of IH. This group of investigators has just published a study that emphasizes the differences between acute and chronic metabolic adaptations to IH, whereby longer durations of IH exposures appear to alleviate the magnitude of metabolic dysfunction induced by IH (36).

**Perspectives and Significance**

The results of the present study demonstrate that intermittent hypoxia, independently of the frequency, leads to impairments in glucose metabolism in mice. Indeed, IH is associated with reduced body weight, increase in insulin resistance, and low leptin levels. Furthermore, the alterations in GLUT4 cellular distribution in skeletal muscle are further supportive of a putative mechanism for the effects of IH on insulin sensitivity. These findings may have substantial implications for the understanding of metabolic regulation and deregulation in diseases associated with hypoxia, such as a sleep apnea and chronic obstructive lung disease.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: A.C., F.K., J.Z., and C.H. performed experiments; A.C., J.Z., and D.G. analyzed data; A.C. and D.G. interpreted results of
experiments: A.C. prepared figures; A.C. and D.G. drafted manuscript; A.C., F.K., J.Z., C.H., Y.W., and D.G. approved final version of manuscript; Y.W. and D.G. conception and design of research; D.G. edited and revised manuscript.

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