Pdk-1/Hif-1α Ratios Define Geniohyoid Muscle Fiber Phenotypes

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Running Title: Pdk-1 Levels in Partial Hif-1 KO Muscles

Abstract: Background: The relationships between muscle fiber-type expression and the ratio between pyruvate dehydrogenase kinase (Pdk)-1 and hypoxia inducible factor (Hif)-1α were investigated using Hif-1α heterozygous knockout (“Het”) mice. Because Pdk-1/Hif-1α ratio changes could alter skeletal muscle phenotypes following brief sustained hypoxia as demonstrated earlier, we aimed to examine if the genetically down-regulated Hif-1α level influences skeletal muscle phenotypes in an intermittent hypoxic (IH) condition. This query is important because upper airway dilating muscles in patients with obstructive sleep apnea who frequently exposed to IH conditions tend to have an increased Type II fibers with increased Hif-1α levels.

Methods: Here, we examined whether Pdk-1/Hif-1 ratios play a role in Het Hif-1α KO mice when exposed to brief IH (altering ambient oxygen levels between 10.3% and 20.8% every 240s). We performed single fiber analysis and Western blots on the harvested geniohyoid (GH) muscle after IH treatment for 5h.

Results: Wild-type (WT) GH muscles in mice contain muscle fibers composed of myosin heavy chain (MyHC) IIA and MyHC IIB/IIB proteins, as opposed to the GH muscle in Het mice expressing MyHC IIA, IIA/IIB as well as IIB (p<0.001). Fiber composition in WT GH showed no significant changes under IH. In Het mice, a higher proportion of MyHC IIB fibers were expressed, as the number of IIA fibers decreased (p<0.05) in IH. Pdk-1/Hif-1α ratios in the Het-GH muscle did not alter significantly after 5h IH exposure.

Conclusion: The increased numbers of glycolytic fibers with high Pdk-1/Hif-1 ratios resulted in Het-GH muscles being able to avoid excessive “oxidative stress” under IH. However, Het-GH muscles might have become more fatigable after IH exposure, compared to WT mice, since Het-GH muscles after IH contain higher numbers of MyHC IIB-containing glycolytic fibers that are more fatigable in nature than WT-GH muscles.

Keywords: Fatigability, Geniohyoid Muscle, Hif-1α, Hypoxia, Myosin Heavy Chains, Pdk-1

Introduction

Healthy normal subjects experience spontaneous periodic breathing and central apnea during sleep at high (≥ 4000 m) altitude [1] and the upper airway (UA) muscles are exposed to intermittent hypoxic (IH) conditions during sleep. Thus, abrupt changes in sleep architecture and physiology in patients with obstructive sleep apnea (OSA) travelling at high altitude can be life threatening [2]. Because UA dilators, such as the geniohyoid (GH) muscle are more fatigable in OSA patients [3], guarding UA patency in such patients at high altitude is a concern [4]. Although retro-glossal airway expanders such as genioglossus (GG) and GH muscles are fatigue-resistant in normal conditions, they quickly become fatigable after short-term intermittent hypoxic (IH) challenges, as observed in healthy rodents [5] and humans [6]. Excessively fatigued tongue protruding muscles in patients with OSA at high altitude can life-threat during sleep, otherwise could be mild apneic at sea level.

Previously, we demonstrated altered GH muscle fiber-type compositions in rodents from mainly IIA myosin heavy chains (MyHC) to IIA and IIA/IIB combinations following 5h post IH-challenges [5]. The findings suggest that these polymorphic changes begin immediately after IH, and continue until the GH muscle is sufficiently adapted to functional demands by virtue of increased numbers of glycolytic MyHC IIB-containing fibers. We suspected that ‘polymorphism’ in skeletal muscle fibers is a primary tactic for phenotypic adaptation to hypoxia. More recently, we investigated potential roles of the ratio between pyruvate dehydrogenase kinase (Pdk)-1 and hypoxia-inducible factor-1α (Hif-1α, a transcription factor primarily detecting oxygen levels in animal cells) during muscle fiber type conversion. We suggested that each muscle constitutively maintains a
specific ratio of Pdk-1/Hif-1α with respect to its preset functions [7].

Using skeletal muscle-specific Hif-1α null mice, Mason and Johnson [8] reported that muscle oxidative capacity improved as Pdk-1 level decreased in Hif-1α null mice, a finding corroborated by studies on human elite athletes [9], which suggests that acute Hif-1α responses to hypoxia in skeletal muscle are blunted during long-term endurance training. The same group reported an increased capillary ratio and elevated levels of oxidative enzymes improves muscle endurance as well. This outcome may be similar to hypoxia-coping muscle responses that are often observed during long-term stays at high altitude, a consequence of vascular endothelial growth factor (VEGF)-induced effects [10]. However, Himalayan Sherpas who are highly adapted to altitude show a lower muscle mitochondrial volume density, low citrate synthase levels, and high lactate production as well as a 48% higher lactate dehydrogenase level compared to control Lowlanders [11]. Yet, Sherpas still appear to be protected to oxidative stress.

As Hif-1 levels influence skeletal muscle adaptation to hypoxic conditions [12], we suspect that another course of skeletal muscle adaptation to hypoxia is immediate glycolytic transformation of muscle fibers via PDK-1 [13]. However, a question remains: what would occur in the GH muscle exposed to hypoxic conditions when Hif-1α level in the muscle is controlled low genetically? This query is important, because the GH muscle is a constantly used upper airway dilator, enlarging the pharyngeal lumen in rodents and humans, easing airflow in both species [14,15]. If GH muscle endurance in partial Hif-1 knockout (KO) mice can be improved in response to an IH challenge, as suggested [8], we could assist breathing in OSA patients travelling a high altitude using a comparable method. For doing so, first, we examined muscle phenotypes of the GH muscle in wild-type mice vs. those in partial Hif-1 KO mice. Second, we investigated fiber type changes in GH muscles in response to a short-term IH challenge to understand what might occur in muscles of OSA patients during a short (5h in this experiment) sleep. The findings would have provide insights into preconditioning of upper airway muscles in OSA patients, and improving airflow.

Materials and Methods

Ethical Approval

The experimental protocols were approved by the Institutional Animal Review Committee at UCLA (ARC #2003-125-12). The set of protocols were in accordance with the National Institute of Health guide for the care and use of laboratory animals. All studies, including the in vivo studies, were performed at University of California at Los Angeles.

Partial Hif-1 deficient mice

We used heterozygous knock-out for a null allele at Hif-1α locus (Het-KO, C57/BL6 x SV129 outbred) young male mice (4 or 5 weeks of age) and their wild-type littermates gifted from the Semenza Lab [16]. Eight wild-type (WT) and 7 Het mice survived transport. Hif-1αKO embryos manifested neural tube defects along with a marked decrease of vascular endothelial growth factor (Vegf) mRNA expression at an early developmental age, and these defects are lethal [16]. The Het mice (Hif-1α+/−) develop normally, appear indistinguishable from their WT (Hif-1α+/+) littermates, yet show impaired responses to oxygen. Of these, 4 WT and 5 Het were treated with intermittent hypoxia. Four WT and 2 Het mice were untreated controls.

Experimental Conditioning of Animals

Protocols for the experimental conditioning were described earlier [5]. In brief, animals were housed in a commercially-designed chamber (76 cm × 51 cm × 51 cm; Proox model 110, BioSpherix Instruments, Redfield, NY), with a modified control system and a temperature range from 22 to 24°C. The chamber operated under a 12 h light/dark cycle (light phase from 6:00 a.m. to 6:00 p.m.) with food and water continuously available. O2 concentration was controlled by a cycle timer (model 4608, Artisan, Randolph, NJ), which regulated N2 delivery from tanks. Moment-to-moment levels of O2 concentration were quantified and adjusted by an O2 sensor system (Proox 110, BioSpherix, Redfield, NY). Carbon dioxide concentration was maintained between 0 and 0.1%. Animals were exposed to normobaric intermittent hypoxic conditions for 5h, where intermittent hypoxia (IH) alternating between 20.8 and 10.3% oxygen every 4 min, as previously outlined [5].

Tissue Harvest

After hypoxic conditioning, both control and experimental animals were euthanized by overdose of pentobarbital (100 mg/kg, i.p.). The muscles analyzed were the geniohyoid (GH, the muscle of interest) and tibialis anterior (TA, for reference). The TA and GH muscles were removed and snap-frozen in isopentane and cooled by liquid nitrogen at −70°C. Muscle samples were preserved for single fiber gel electrophoresis and Western blot assays.

Single Fiber Gel Electrophoresis

We analyzed single fibers harvested from a muscle to examine muscle fiber types. The techniques used were introduced previously [7]. Briefly, fibers were mechanically isolated from the non-tendinous muscle portion under a microscope at 10×, and placed directly into 12.5 ul of SDS sample buffer (100 mM Tris Base, 100 mM Tris (pH 6.8), 5% glycerol, 4% SDS, 0.05% Bromophenol Blue, 5% B
Western blot Assays for Hif-1α and Pdk-1 Proteins

Muscle tissue harvested from 2 animals per group was pooled for Western blot assays as previously described [7]. Briefly, harvested tissues of approximately 150 mg were equally partitioned and ground in a 1.5 ml Eppendorf tube. Ten volumes of RIPA extraction buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) was added to the tissue. Following homogenization, samples were centrifuged for 10 min at 13,000 rpm at 4°C. The collected supernatant was subjected to Bio-Rad DC protein assay using bovine serum albumin (BSA) as a standard. For Western blotting, 50 μg of total proteins were heated at 95°C for 5 min and separated by 4–12% gradient SDS-PAGE gel for Hif-1α and Pdk-1 proteins. The proteins were transferred to 0.45 μm polyvinylidene difluoride membranes, which were stained with Ponceau red. Then, the membranes were blocked with 5% weight-to-volume (w/v) non-fat dry milk in 1 × PBS with 0.1% Tween-20 for 1 h at room temperature (RT). The membranes were incubated for 2 h at RT with anti-Hif-1α polyclonal antibody (1:500; Cell Signaling, Cat. #3716) and anti-Pdk-1 polyclonal antibody (1:500, Assay Designs, Cat. # KAP-PK112). Horseradish peroxide-conjugated goat-anti-rabbit antibody (1: 2000; Santa Cruz Biotechnology, Santa Cruz, CA) was used as a secondary antibody. For loading-control, β-Actin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) was used. Bands were visualized by incubating blots in SuperSignal West Femto solution (Pierce, Cat. # 34094) for 15 min at RT, and the images were obtained in Bio-Rad ChemiDoc viewer (Bio-Rad, Hercules, CA). Density measurements on 3 gels were averaged and expressed in folds.

Fatigability Test on Geniohyoid Muscles using In Situ setup

Two WT and 2 KO mice treated in IH conditions were maintained under deep anesthesia by intraperitoneal injection of pentobarbital sodium (80 mg/kg) for muscle fatigue tests as previously detailed [7]. After infra-hyoid muscles were severed, the GH muscle was exposed and isolated. By doing so, the GG and GH muscles only are attached to the hyoid bone. The lateral and medial branches of the hypoglossal nerve were transected at the distal portion of the last ramified branches, leaving a collateral of the medial branch intact in the proximal side of the amputation site. This collateral branch (indicated by asterisks in Fig. 1A) solely innervates the GH muscle in mice; thus, the GH muscle only (excluding the GG) contracts when the nerve trunk was stimulated. The mid-sagittal part of the hyoid bone was tied to a force transducer (model FT03, Astro-Med, West Warwick, RI) by using 3-0 silk suture as the GH muscle was suspended between the mandible symphysis and the force transducer as shown in Fig. 1B. A skin pouch, filled with mineral oil, was made to provide baths for isolation of the nerve trunks; the hypoglossal trunks were stimulated with a pair of wire electrodes. For in situ data collection, the GH muscles were stimulated by using a Grass S48 stimulator (Astro-Med Inc., West Warwick, RI), and forces were assessed using a force transducer and 15LT Bipolar Amplifier System (Astro-Med). After the muscle length was adjusted (L₀) in order to obtain a consistent tension-output using the baseline voltage, fatigue was induced by stimulation of the hypoglossal nerve at 30 Hz with 300-ms trains every second. After the first force output (tension) at L₀ was determined, force-outputs were measured and normalized by expressing the force generated at 30, 60, 90, and 120 s as a percentage of the initial tension measurement at the first pulse. Data acquisition and analyses were performed by a Polyview system (Astro-Med) and LabView (National Instruments, Austin, TX).
**Statistical Methods**
To compare fiber-type compositions between WT- vs. Het-KO mice and between control vs. IH conditions, we used χ² tests on 3×2 contingency tables (MyHC types (IIa, IIa/IIb, IIb) × control vs. IH). One-way ANOVA examined differences in the ratios between the Pdk-1 and Hif-1α protein density measured on Western blots. Sidak’s multiple comparisons test (GraphPad Prizm v. 7) was used for post hoc comparisons. All tests were two-sided, and a P<0.05 was considered statistically significant. We did not perform inference tests on changes of GH muscle fatigability.

**Results**
**Immediate response of the GH muscle to hypoxic exposure in Het mice**
In total, 228 single fibers harvested from at least 3 GH muscles were counted on gels after electrophoresis (Fig. 2A) and statistically analyzed (Fig. 2B). Wild-GH muscles differed from Het-KO GH muscles in the control condition (χ² = 25.01, P <0.001) and after IH exposure (χ² = 27.37, P <0.001). Wild-GH muscles consisted of fibers containing MyHC IIa (54%) and MyHC IIa/IIb (46%), with no fibers containing MyHC IIb; whereas, GH muscles from Het KO mice contained MyHC IIa (65%), MyHC IIa/IIb (25%) and IIb (15%). When the mice were exposed to IH, wild-mice GH changed to MyHC IIa (41%), MyHC IIa/IIb (59%), and no IIb-containing fibers, while Het-KO mice showed MyHC IIa (40%), MyHC IIa/IIb (30%) and IIb (30%). No fibers contained MyHC I or MyHC IIx. As shown, no fibers contained MyHC IIb; yet, IIa/IIb fibers increased slightly in response to IH. GH muscles in Het-KO mice contained MyHC IIb, and instead, the proportion of fibers containing IIa/IIb declined. Under IH conditioning, MyHC IIb fibers increased as IIa fibers decreased.
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A.  

Single fiber gel electrophoresis assays exhibit MyHC polymorphic alterations. **A.** Gels stained with Coomassie blue. The gel-a denotes all phenotypes of MyHC proteins produced in a lane dropped with multiple fibers of gastrocnemius muscle. We used this lane as a ladder. The gel-b is produced from Het-KO GH muscles. The gels-c are from Het-KO GH muscles after IH. Note that numbers of MyHC IIb containing fibers increased markedly after IH treatment. **B.** Graphical summary of the statistical analyses. The numbers in each horizontal bar indicate percentages of fibers expressing corresponding MyHC proteins. Chi-square tests were performed using actual frequency (not percentages) of fibers (228 fibers used for GH; 196 fibers used for TA). * indicates p<0.05, ** indicates p<0.01.

**Immediate response of the Tibialis Anterior (TA) muscle to hypoxic exposure**  
A total of 196 single fibers were harvested from TA muscles for reference (See Fig. 2B). Wild-TA muscles obtained from the mice consisted of fibers containing MyHC Ila (26%), MyHC Ila/IIb (53%) and IIb (21%); whereas, the TA muscles from Het-KO mice contain MyHC Ila (9%), MyHC Ila/IIb (20%) and IIb (71%). When the animals were exposed to IH, WT animals’ TA changed to MyHC Ila (6%), MyHC Ila/IIb (27%) and MyHC IIb (21%) (χ² = 41.56, P < 0.001); whereas, Het KO mice showed MyHC Ila (5%), MyHC Ila/IIb (9%) and IIb (86%), levels that did not statistically differ. Het-KO TA muscles had a significantly different (χ² = 24.47, P < 0.001) fiber composition from those of WT. No fibers contained MyHC I nor MyHC IIx.

**Immediate response of the GH muscle to hypoxic exposure and implications of the ratio Pdk-1/Hif-1α**  
We quantified the levels of Pdk-1 and Hif-1α using Western blot assays (Fig. 3A). Then, we calculated ratios between density levels of those proteins referenced to β-Actin in the GH muscle. As shown, the ratio Pdk-1/Hif-1α in GH muscles increased by 42% when wild-type animals were exposed to IH (Fig. 3B). We noted, here, that Pdk-1 expression was increased. In contrast, the ratio increased by 78% in the GH muscles of Het-KO mice because Hif-1 levels were genetically low (36% decreased in Het-KO mice compared to the wild-type), rather than significantly increased Pdk-1. In fact, Hif-1 levels remained the same in the GH muscle of the Het-KO mice after IH exposure. Hif-1α levels were 0.64 of the WT before IH, and 0.63 of the WT after IH exposure. Thus, the Pdk-1/Hif-1α ratio in Het-GH muscles did not change after IH exposure. Hif-1 levels in the GH muscle increased most in WT-IH, (i.e. 56% more than the baseline wild-type) after IH exposure. Increased Pdk-1/Hif-1α levels maintained the muscle fibers in a glycolytic state.

![Fig. 2](image_url)
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**Fig. 3** Western blots (A) and fold-expressions of Western blot assay results (B) on GH muscles. A. Comparisons of Hif-1α (detected at 120 kD) and Pdk-1 (detected at 48 kD) expression in GH muscles obtained from WT and Het KO mice in ambient air and after 5 h intermittent hypoxia (IH). B. Density levels of Hif-1α (gray bars) and Pdk-1 proteins measured on 3 gels (n=3) were first standardized to β-Actin, and then calculated relative to the control WT GH muscle. Ratios between Pdk-1 and Hif-1α protein concentrations (Pdk-1/Hif-1α in blank bars) in GH muscles are presented. The ratios Pdk-1/Hif-1α in hypoxic conditions are standardized to the ratios taken from WT control muscles; thus, each ratio measured in control state WT GH was used as a denominator. Note that the Pdk-1/Hif-1α ratio increased significantly in Het-KO muscles. Standard errors for each measurement are displayed using error bars.

**Fatigability of the GH muscle in partial Hif-1α KO mice increases after IH challenges**

Measurements were obtained from 4 IH-treated mice (2 Het and 2 WT) that successfully completed physiology studies (Fig. 4). We did not perform any inference tests on group differences due to insufficient sample size. As indicated, IH-treated Het mice became more fatigable (79% of the initial measurements) after 5h IH exposure and the control mice sustained (106% of the initial measurements) the tension for 120 seconds.

**Fig. 4** Fatigability measurements on the geniohyoid muscle after 5h intermittent hypoxic exposure. When muscle tensions produced by the GH muscles were measured from Wild-type vs. Het-KO mice, marked differences revealed
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Discussion

Tongue and hyoid muscles help maintain the oro-/hypopharyngeal airway, and are more fatigable in OSA patients [17,18]. This increased fatigability poses a danger for OSA patients, because apneic symptoms usually worsen when exposed to hypoxic atmosphere, even with short exposure [4]. The airway dilating muscles in OSA patients are more glycolytic [6], thus naturally more fatigable than those in normal subjects [6]. As such, when glycolytic muscles are exposed to intermittent hypoxia (as in OSA patients), the muscles less-likely acclimatize to the condition via the oxidative pathway through angiogenesis [8] for they are glycolytic. Thus, the more glycolytic the UA dilators are, the more difficult to overcome fatigability of the muscles as apneic symptoms worsen with altitude.

With normal oxygen levels, skeletal muscles catabolize glucose to pyruvate, and pyruvate is then transported into the tricarboxylic acid (TCA) cycle in the mitochondrion where the chain process of electrons produces ATP. Elevation of HIF-1α levels in hypoxia promotes ATP production through increased anabolic glycolysis; however, an elevated hypoxic condition causes oxidative stress from uncontrolled mitochondrial generation of reactive oxygen species (ROS). Under hypoxic conditions, leakage of electrons from the respiratory chain results in increased ROS. To attenuate the overproduced ROS, PDK-1, a direct downstream molecule of HIF-1, phosphorylates the pyruvate dehydrogenase (PDH) and then inactivates the PDH enzyme complex that converts pyruvate to acetyl-coenzyme A [13]. Therefore, as PDK-1 levels increase, ATP production increases via the ‘Pasteur effect’ (a glycolytic shunting of pyruvate that leads to reduction of pyruvate into lactate) as ROS production decreases.

Skeletal muscles in hypoxia may go through two different adaptive pathways; one, via shunting the TCA cycle to avoid ‘oxidative stress’, and two, a pathway led by vascular endothelial growth factor (VEGF). Glycolytic shunt due to hypoxia occurs promptly as a survival tactic. The VEGF-leading pathway is activated by HIF-1α also, yet favorite oxidative conditions, such as increased capillary vessels and up-regulation of oxidative enzymes follow later. This set of adaptations improve endurance of the muscles over time, as introduced by Mason et al [19,20] and others [9]. However, since this type of adaptation which uses metabolic shifts toward oxidation would not occur immediately; repeated exercise of Hif-1α KO mice led to extensive muscle damage [19] and increased fatigability due to IH as shown in Fig. 4. The Hif-1α KO mice introduced by Mason et al., exhibited a few other interesting metabolic characteristics, including increased activity of rate-limiting mitochondrial enzymes and increased fatty acid oxidation in the muscles, and decreased lactate amounts in the serum of their exercising Hif-1α null mice. They also reported that the average Hif-1α deletion level of gastrocnemius muscles confirmed by extracted DNA was 54.9%. When muscle fiber types based on histology were compared, a slight decrease of Type Ila (94.4% → 90.8%) fibers appeared, and an increase of IIb (5.6% → 9.2%) fibers in the gastrocnemius muscle found, despite elevated expressions of VEGF (73%) and GLUT (Glucose transporter) 4 (67%).

On the other hand, the GH muscle in our Het-mice showed an increased proportion of Type Ila MyHC-containing fibers by 9% (54% → 65%), compared to that of control WT mice. However, when pre-exposed to intermittent hypoxic conditions, mimicking OSA, the GH muscles become more glycolytic to avoid further damage. Using Hif-1α null mice, Mason et al. [19] showed that endurance was already improved via the adaptive oxidative pathway. However, their data also demonstrated, muscle endurance deteriorated significantly in the last day of a 4 day running experiment compared with the performance of their WT controls. The Het-KO mice used in our experiments showed more glycolytic fibers in the GH muscle after IH exposure (15% → 30% P<0.05). Glycolytic changes in muscle fibers after hypoxic exposure appear to precede oxidative adaptation. Due to the increased numbers of glycolytic fibers with the high Pdk-1/Hif-1α ratio, the GH muscle in the Het-KO mice could avoid ‘oxidative stress’ under IH; however, the fatigue-prone GH muscles of the Het-KO mice turned into more-fatigable after IH exposure, compared with the fatigue-resistant GH muscles in the WT mice.

GH muscles in our WT mice do not contain fibers expressing MyHC IIb; yet, Het-KO mice do. The average Hif-1 protein level in GH muscles of Het-KO mice was 36% lower than that of Control WT (Fig. 3B). Then, the ratio Pdk-1/Hif-1 of Het-KO mice tends to stay elevated in hypoxia. Due to high Pdk-1/Hif-1 levels (78% higher compared to WT), pyruvate tends to shunt the oxidative process through the TCA cycle [13]. This condition favors the glycolytic pathway for conversion to glycolytic fibers with MyHC IIb. Skeletal muscles in Het-mice turned to more glycolytic forms in response to IH exposure, and thus, probably became more fatigable rather than fatigue-resistant.

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Skeletal muscles can readily alter their major contractile protein composition, as they can be polymorphic in phenotypes in accordance with function or the environment, such as oxygen levels, irrespective of the presence of innervation [12,21]. For such adaptation, metabolic conditions in the muscle proper are constantly monitored by a ubiquitous constitutional transcription factor, HIF-1α. However, maintaining homeostasis of PDK-1 levels may be equally important for adaptation of skeletal muscles to hypoxia. The PDK-1/HIF-1α ratio needs to be sustained at a homeostatic level in the fatigue-prone upper airway muscles, that are a special concern for OSA patients, because maladaptation of breathing muscles in patients travelling areas in high altitude could be life threatening.

Limitations of the study
The results are based on 15 animals in total. The small sample size limited the experimental assays, and precluded use of some inference tests. The loss of 3 animals during fatigability studies especially impacted these assessments.

Conclusions
We conclude that geniohyoid muscle fibers in heterozygous Hif-1α KO mice contains MyHC Ibβ protein. Muscle-type change may be dependent on the ratio PDK-1/HIF-1α. After a brief exposure to IH could make the geniohyoid muscle more fatigable in heterozygous Hif-1α KO mice. Patients with OSA in a hypoxic condition such as high altitude can experience ‘quickly’ deteriorated upper airway muscle function.

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Statement on the Welfare of Animals
All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Conflict of Interest
The authors declare that they have no conflict of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors’ Contributions
This study used the methods previously used by Pae et al. [5,7] previously. Thus, the methods description reproduce their wordings unintentionally. Substantial contributions to conception (RMH, EKP) and design (EKP), data acquisition (GL, DDN, EKP) or data analysis (GL, DDN, EKP) and interpretation (RMH, EKP); Drafting the article (EKP) or critically revising it (RMH) for important intellectual content; Final approval of the version to be published (All authors); Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved (GL, DDN, EKP).

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