Mitochondrial content and distribution changes specific to mouse diaphragm after chronic normobaric hypoxia

Jorge L. Gamboa and Francisco H. Andrade
Department of Physiology, University of Kentucky, Lexington Kentucky

Submitted 9 June 2009; accepted in final form 5 December 2009

Gamboa JL, Andrade FH. Mitochondrial content and distribution changes specific to mouse diaphragm after chronic normobaric hypoxia. Am J Physiol Regul Integr Comp Physiol 298: R575–R583, 2010. First published December 9, 2009; doi:10.1152/ajpregu.00320.2009.—Chronic hypoxia reduces aerobic capacity (mitochondrial content) in limb skeletal muscles, and one of the causes seems to be decreased physical activity. Diaphragm and other respiratory muscles, however, may have a different pattern of adaptation as hypoxia increases the work of breathing. Thus, we hypothesized that chronic hypoxia would not reduce mitochondrial content in mouse diaphragm. Adult male C57BL/6J mice were kept in normoxia (FIO2 = 21%, control) or normobaric hypoxia (FIO2 = 10%, hypoxia) for 1, 2, and 4 wk. Mice were then killed, and the diaphragm and gastrocnemius muscles collected for analysis. In the diaphragm, cytochrome c oxidase histochemistry showed less intense staining in the hypoxia group. The total content of subunits from the electron transport chain, pyruvate dehydrogenase kinase 1 (PDK1), and voltage-dependent anion channel 1 (VDAC1) was evaluated by Western blot. These proteins decreased by 25–30% after 4 wk of hypoxia (P < 0.05 vs. control for all comparisons), matching a comparable decrease in diaphragmatic mitochondrial volume density (control 33.6 ± 5.5% vs. hypoxia 26.8 ± 6.7%, P = 0.013). Mitochondrial volume density or protein content did not change in gastrocnemius after hypoxia. Hypoxia decreased the content of peroxisome proliferator-activated receptor gamma (PPARγ) and PPARγ cofactor 1-alpha (PGC-1α) in diaphragm but not in gastrocnemius. PGC-1α mRNA levels in diaphragm were also reduced with hypoxia. BCL2/adenovirus E1B interacting protein 3 (BNIP-3) mRNA levels were upregulated after 1 and 2 wk of hypoxia and in gastrocnemius, respectively; BNIP-3 protein content increased only in the diaphragm after 4 wk of hypoxia. Contrary to our hypothesis, these results show that chronic hypoxia decreases mitochondrial content in mouse diaphragm, despite the increase in workload. A combination of reduced mitochondrial biogenesis and increased mitophagy seems to be responsible for the decrease in mitochondrial content in the mouse diaphragm after hypoxia.

Methods

Animals. All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee. Adult C57BL/6J mice were kept in normoxia (FIO2 = 21%, control) or normobaric hypoxia (FIO2 = 10%, hypoxia) for 1, 2, and 4 wk. Mice were then killed, and the diaphragm and gastrocnemius muscles collected for analysis. The diaphragm, cytochrome c oxidase histochemistry showed less intense staining in the hypoxia group. The total content of subunits from the electron transport chain, pyruvate dehydrogenase kinase 1 (PDK1), and voltage-dependent anion channel 1 (VDAC1) was evaluated by Western blot. These proteins decreased by 25–30% after 4 wk of hypoxia (P < 0.05 vs. control for all comparisons), matching a comparable decrease in diaphragmatic mitochondrial volume density (control 33.6 ± 5.5% vs. hypoxia 26.8 ± 6.7%, P = 0.013). Mitochondrial volume density or content did not change in gastrocnemius after hypoxia. Hypoxia decreased the content of peroxisome proliferator-activated receptor gamma (PPARγ) and PPARγ cofactor 1-alpha (PGC-1α) in diaphragm but not in gastrocnemius. PGC-1α mRNA levels in diaphragm were also reduced with hypoxia. BCL2/adenovirus E1B interacting protein 3 (BNIP-3) mRNA levels were upregulated after 1 and 2 wk of hypoxia and in gastrocnemius, respectively; BNIP-3 protein content increased only in the diaphragm after 4 wk of hypoxia. Contrary to our hypothesis, these results show that chronic hypoxia decreases mitochondrial content in mouse diaphragm, despite the increase in workload. A combination of reduced mitochondrial biogenesis and increased mitophagy seems to be responsible for the decrease in mitochondrial content in the mouse diaphragm after hypoxia.
C57BL/6J male mice (12 wk old) were exposed to normobaric hypoxia (10% O₂) for 2 or 4 wk. A total of 70 mice were used in the study: 22 control mice exposed to normoxia, 8 mice exposed for hypoxia for 1 wk and used only for RNA extraction, 18 mice exposed to hypoxia for 2 wk and 22 mice exposed to hypoxia for 4 wk. Mice were used because others have shown that they tolerate hypoxia well, and their adaptation to it is similar to that of humans (24). Hypoxia was induced using a Plexiglas chamber that was continuously flushed with nitrogen (Scott Gross, Lexington, KY) to maintain a FiO₂ of 10%. Control mice remained in normoxic conditions. Mice were then euthanized, and the diaphragm and gastrocnemius—the hind limb muscle chosen for comparison—were removed. For all of the experiments, costal diaphragm was dissected and removed after performing a median thoracotomy. Both gastrocnemius muscles were removed and randomly assigned for RNA extraction, histology, or biochemical analysis.

Cytochrome c oxidase histochemistry. Diaphragm and gastrocnemius were covered with OCT-embedding medium and frozen in isopentane cooled to its freezing point in liquid nitrogen. Cross sections (10-μm thickness) were collected and used for cytochrome c oxidase histochemistry. Slides were incubated in phosphate buffer (pH 7.4) containing cytochrome c (1 mg/ml), 4,3,3'-diaminobenzidine (0.5 mg/ml), and catalase (0.02 mg/ml). Sections were then dehydrated with ethanol and mounted in Permount. Images were obtained using a light microscope (model E600; Nikon, Melville, NY) attached to a computer-controlled digital camera (Spot RT; Diagnostic Instruments, Sterling Heights, MI).

Western blot analyses. Muscle homogenates (30 μg of protein) were resolved electrophoretically in 10–20% SDS-polyacrylamide gels (Bio-Rad, Hercules CA) and transferred to polyvinylidene difluoride membranes (Immobilon-FL, Millipore, Billerica, MA). Equal protein loading was confirmed by Ponceau S staining. Membranes were blocked for 1 h with 1:1 dilution of Odyssey blocking buffer PBS at room temperature. Membranes were then incubated using the same buffer containing 0.2% Tween and primary antibodies at the concentration recommended by manufacturers. The monoclonal antibodies against subunits of mitochondrial respiratory complexes (all from Invitrogen, Carlsbad, CA) were a 30-kDa subunit from complex I, VB subunit from complex IV, and a subunit from complex V. Other primary antibodies were against pyruvate dehydrogenase kinase 1 (PDH-1; Abcam, Cambridge MA) and voltage-dependent anion channel 1 (VDAC-1; Abcam), peroxisome proliferator-activated receptor gamma (PPARγ; Abcam), PGC-1α (Novus Biological, Littleton, CO), and BNIP-3 (Abcam). After washing the membranes with PBS and 0.1% Tween, we incubated them with Alexa Fluor 680-conjugated goat anti-mouse secondary antibody (1:7,500; Invitrogen) and then rewashed them 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). The density of resulting bands was quantified using National Institutes of Health Image J software.

Mitochondrial volume density. Eight mice (4 control and 4 mice exposed to hypoxia for 4 wk) were perfused with 4% glutaraldehyde and 2% paraformaldehyde for electron microscopy. Gastrocnemius and diaphragm were then dissected and cut into pieces and fixed in glutaraldehyde (4%) and paraformaldehyde (2%) for 2 h at 4 C. Samples were then postfixed in osmium tetroxide (1%) for 1 h, dehydrated, and embedded for further sectioning. To measure mitochondrial volume density, thin (80 nm) fiber transverse sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope. Mitochondrial volume density (% of muscle fiber volume occupied by mitochondria) was determined using a standard point counting method (144 point grid) (19).

RNA isolation and RT-PCR. Muscles were removed, and RNA was isolated using TRIZol (Invitrogen), according to the manufacturer’s instructions. RNA was then subjected to DNase treatment using a Turbo DNA-free kit (Applied Biosystems, Foster City, CA). Reverse transcription was performed using Superscript II RNAse H-reverse transcriptase. Primers were designed using PrimerExpress (Applied Biosystems). The evaluated genes include PPARγ coactivator 1-alpha (PGC-1α), nuclear respiratory factors (NRF) 1 and 2, mitochondrial transcription factor A (TFAM), autophagy gene 8 (ATG-8 or microtubular-associated protein 1 light chain 3α), 5 (ATG-5) and 7 (ATG-7), and BNIP-3 and BNIP-3-like protein (BNIP-3L). Analyses were performed in triplicate, using SYBR Green and 18S rRNA as the calibrator housekeeping gene. Fluorescence signal was detected using ABI PRISM 7500 Sequence Detection System. Fold-change expression related to control group was expressed using the threshold cycle method. Primer sequences are shown in table 1.

Statistical analysis. Data were analyzed using t-tests when comparing only two groups: control and 4 wk of hypoxia. One-way ANOVA was used to test for differences between multiple groups. Tukey’s post hoc test was used to identify pair-wise differences. Significance was considered when P ≤ 0.05.

RESULTS

All of the mice in the hypoxia group survived and completed their stay in the hypoxic environment. After 4 wk of hypoxia, mice had higher hematocrit (control, 35.0 ± 2.9% vs. hypoxia, 55.0 ± 5.3%, P = 0.006, n = 10 mice sampled per group) and lower body weight (control, 28.6 ± 1.8 g vs. hypoxia, 25.0 ± 1.5 g, P < 0.001), similar to changes described by others (6, 7). Further analysis showed that most of the body weight was lost during the 1st wk of hypoxia; afterward, both groups gained weight at the same rate (0.52 vs. 0.48 g/wk, control and hypoxia groups, respectively). In addition, gastrocnemius wet weight did not change after 4 wk of hypoxia (336.0 ± 17.8 mg and 331.8 ± 8.9 mg, control and hypoxia, respectively, P = 0.84, n = 6 muscles sampled in each group).

Hypoxia decreases mitochondrial content in the diaphragm. We used cytochrome c oxidase histochemistry as an initial approach to document changes in muscle mitochondrial content in response to hypoxia. After 4 wk of hypoxia, there was less intense staining in the diaphragm, which was not noted after 2 wk of hypoxia. This is suggestive of decreased mitochondrial content. There was no detectable change in the gastrocnemius at either time point (Fig. 1).

We then used Western blot analyses of whole diaphragm and gastrocnemius muscle homogenates to evaluate whether mitochondrial protein content changed after hypoxia. There was a significant decrease in the content of proteins involved in oxidative phosphorylation after 4 wk of hypoxia, but not after 2 wk (Fig. 2A). To examine whether the reduction was restricted to the respiratory complexes, other mitochondrial proteins were measured. PDK-1 and VDAC-1 contents were also reduced after 4 wk of hypoxia. No changes were noted in the gastrocnemius at either time point (data not shown).

To directly quantify the effect of hypoxia on mitochondrial content, we used electron microscopy and stereology to determine mitochondrial volume density (% of muscle fiber volume occupied by mitochondria). As shown in Fig. 3, 4 wk of hypoxia decreased the mitochondrial volume density in diaphragm muscle fibers (20.5% reduction after hypoxia compared with controls, P = 0.013), but not in gastrocnemius. Interestingly, we observed that mitochondria clustered preferentially underneath the sarcolemma in diaphragm muscle fibers, in particular, around the capillaries (Fig. 4). This phenomenon was not noticed in the gastrocnemius.
Hypoxia decreases mitochondrial biogenesis and triggers autophagy. Decreased mitochondrial biogenesis could explain the loss of mitochondrial volume density in the diaphragm after chronic hypoxia. We screened the expression of mitochondrial biogenic factors by quantitative RT-PCR. PGC-1α mRNA abundance was decreased in diaphragm starting at 1 wk of hypoxia compared with the control group (Fig. 5A, P values of 0.001, 0.003, and 0.001 after 1, 2, and 4 wk of hypoxia, respectively). PGC-1α protein content in the diaphragm was also reduced from 2 wk of hypoxia (Fig. 5B, P < 0.04). There was no change in either mRNA abundance or protein content of PGC-1α in gastrocnemius. We also measured PPARγ since it influences the expression of PGC-1α. PPARγ protein content decreased in the diaphragm after 2 and 4 wk of hypoxia compared with control (30.1% and 36.4% respectively, Fig. 5C) and did not change in gastrocnemius. Another cellular process that could explain the loss of mitochondria in the diaphragm during chronic hypoxia is autophagy. We measured the expression of several mitochondrial autophagic factors in diaphragm and gastrocnemius samples at different time points from the start of hypoxia. As shown in Fig. 6A, BNIP-3 mRNA abundance increased after 1 wk of hypoxia in the diaphragm (P = 0.001) and after 2 wk in gastrocnemius (P = 0.02). Western blot analysis showed increased BNIP-3 content in diaphragm, but not in gastrocnemius, after 4 wk of hypoxia (Fig. 6B, P < 0.001). BNIP-3 migrates oddly in SDS PAGE, showing bands at 30 kDa and at 60 kDa that correspond to monomers and dimers, respectively (8). The 30-kDa band is presented in Fig. 6B. The presence of this marker for autophagy correlated with the observation of mitophagic vesicles by electron microscopy in diaphragm fibers from the hypoxia group (Fig. 6C). These structures were rarely observed in gastrocnemius from the hypoxia group and were completely absent in either muscle from the control group.

**DISCUSSION**

The goal of this study was to contrast the adaptive responses of limb and respiratory muscles to chronic hypoxia. Our hypothesis was that chronic hypoxia would not change mitochondrial volume density and function in the mouse diaphragm.

### Table 1. List of primers used for quantitative PCR

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Accession Number</th>
<th>Sequence</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGC-1α</td>
<td>AF049330.1</td>
<td>Forward</td>
<td>AGACGGATTTGCCCTGATTTG</td>
</tr>
<tr>
<td>TFAM</td>
<td>NM_009360.4</td>
<td>Forward</td>
<td>CGACCCTGAGAAGCTGCAA</td>
</tr>
<tr>
<td>NRF-1</td>
<td>NM_010938.3</td>
<td>Forward</td>
<td>TTTCAAGCTCTCTCTGAGACAATA</td>
</tr>
<tr>
<td>NRF-2</td>
<td>NM_010902.3</td>
<td>Forward</td>
<td>CAGGTCGGTGAATCTGGAAGAT</td>
</tr>
<tr>
<td>C-Myc</td>
<td>NM_010849.4</td>
<td>Forward</td>
<td>AGGTCCCTTCTGAGACAGAATGA</td>
</tr>
<tr>
<td>PPARγ</td>
<td>NM_011146.3</td>
<td>Forward</td>
<td>TGAGATGGAGGCTCTGTTTATTC</td>
</tr>
<tr>
<td>BNIP-3</td>
<td>NM_009760</td>
<td>Forward</td>
<td>GCACCCGAGGACACCCACCTT</td>
</tr>
<tr>
<td>BNIP-3L</td>
<td>NM_009761.3</td>
<td>Forward</td>
<td>GCAATGCGAGCAGGAAAATTAA</td>
</tr>
<tr>
<td>ATG-5</td>
<td>NM_053069.5</td>
<td>Forward</td>
<td>ACTGCTTCTCGTGGAGACACAGA</td>
</tr>
<tr>
<td>ATG-7</td>
<td>NM_028835.3</td>
<td>Forward</td>
<td>CCGCAAGATCTCTCATGCGAAA</td>
</tr>
<tr>
<td>LC-3</td>
<td>NM_026160.4</td>
<td>Forward</td>
<td>GACGCGTCTCTGACAGATTTT</td>
</tr>
<tr>
<td>Housekeeping RNA</td>
<td>18S</td>
<td>Forward</td>
<td>AGTCCCGGCGCTTTGTAGACA</td>
</tr>
</tbody>
</table>

**Fig. 1.** Cytochrome c oxidase histochemistry shows less intense staining in hypoxic diaphragm [hypoxia, 4 wk (H-4W) and hypoxia, 2 wk (H-2W)] compared with the normoxic controls (n = 4 in each group). No differences were observed after 2 wk of hypoxia in diaphragm or in gastrocnemius (scale bars = 50 μm).
compared with limb muscles. Instead, the results demonstrate that mitochondrial content, measured histochemically, by protein content and stereology, actually decreases in the diaphragm, while the gastrocnemius remains relatively unaffected. It was particularly surprising that 4 wk of hypoxia did not increase the mitochondrial content in the mouse diaphragm, despite the increased work of breathing. We did find that the mitochondria shifted position and localized preferentially in the subsarcolemmal region, especially where the diaphragm muscle fibers were closer to capillaries. The results also suggest that the change in mitochondrial volume density in the mouse diaphragm is due to a combination of decreased biogenesis and increased autophagy.

Mitochondrial biogenesis and hypoxia. Mitochondrial biogenesis (and the resulting increase in aerobic capacity) is initiated by different pathways, many of which rely on PGC-1α. This transcription cofactor is highly expressed in tissues with elevated metabolic rate such as brain, kidney, heart, and skeletal muscle (28). Increased PGC-1α stimulates the transcription of NRFs, followed by TFAM and nuclear-encoded mitochondrial proteins of the oxidative phosphorylation (42). On the other hand, downregulation of PGC-1α expression is associated with diminished mitochondrial biogenesis (25). In the particular case of chronic hypoxia, PGC-1α expression may be altered by a number of potential mechanisms. c-Myc is a transcription factor involved in mitochondrial biogenesis (26); it also activates PGC-1β, a member of the PGC-1 family that shares functions with PGC-1α (49). The inhibition of c-myc activity by hypoxia-inducible factor (HIF) decreases mitochondrial biogenesis in cancer cells and could play a role in chronic hypoxia as well (49). We found no change in c-myc expression after 4 wk of hypoxia in either diaphragm or gastrocnemius (data not shown). Hypoxia could also reduce the expression of PPARγ, the binding partner of PGC-1α (27, 48, 51). In turn, decreased PPARγ would also diminish PGC-1α expression (17). In view of that, we evaluated PPARγ protein content and found that it was decreased after 2 and 4 wk of hypoxia in diaphragm. Changes in PGC-1α and PPARγ content follow the same pattern (reduced after 2 and 4 wk of hypoxia), suggesting that PPARγ may have a role in PGC-1α expression under hypoxic conditions. The reduction in PGC-1α mRNA and protein content may be sufficient to decrease mitochondria biogenesis in diaphragm. It should be noted that other investigators have shown increased PGC-1α expression in mouse brain after 6 and 24 h of hypoxia (15) and in sheep fetal adipose tissue after 3 mo of high-altitude hypoxia (37). The apparent discrepancy with our findings may be due to effects of acute hypoxia (up to 24 h) and tissue specificity on PGC-1α expression. Thus, in the mouse brain study, the increase in PGC-1α expression after 48 h of hypoxia is not different from the control group. Acute hypoxia could result in a decrease in food intake (44), and this would be the stimulus to increase PGC-1α expression, because fasting increases the expression of PGC-1 (23). On the other hand, the increased PGC-1α expression in hypoxic fetal sheep adipocytes may be tissue specific and related to role of the nonshivering thermogenesis of brown fat in newborns (38). Considering the changes in expression of mitochondrial biogenic factors and the reduction in mitochondrial content, it seems that the active phase of diaphragm remodeling occurs somewhere between 2 and 4 wk of hypoxia exposure. The decrease in mitochondrial volume density in the diaphragm following hypoxia was accompanied by a redistribution of the mitochondria to the subsarcolemmal compartment, in particular, close to capillaries (Fig. 4). This seems an adaptive response intended to decrease the diffusion distance from capillary to mitochondria and increase the efficiency of oxygen transfer, as previously proposed (30).

Mitochondrial autophagy and hypoxia. The half-life of mitochondria is about 2 wk (35) and depends on the balance between biogenesis and degradation (autophagy). Autophagy, or “mitophagy” when dealing specifically with mitochondria, is a process where cytoplasm and organelles are encapsulated in a double-membrane vesicle. Then, the outer vesicular membrane fuses with lysosomes, and its contents are degraded and recycled (33). Eleven autophagic genes (ATG genes) respon-
sible for the induction, nucleation, expansion, and fusion of autophagic vesicles have been identified in mammals (46). The ATG gene beclin-1 (or ATG-6) is a BH3-only protein and essential in the early steps of autophagy. BH3-only proteins are a subgroup of the BCL-2 family and are called that because they share with BCL-2 proteins a short domain (9 to 16 amino acids the BH3 domain); this domain can subsequently bind to a specific pocket of the BCL-2 proteins (21). Beclin-1 can be inhibited by the interaction of the BH-3 domain with BCL-2 or BCL-XL (32). BNIP-3, another BH3-only protein, also binds BCL-2 and prevents its interaction with beclin-1 (31). Thus, beclin-1 is no longer inactive and induces autophagy. BNIP-3 is a hypoxia-inducible gene (45) and is preferentially translocated to the mitochondria inducing mitophagy in cultured fibroblast and cardiac myocytes (16, 50). In rodent skeletal muscle, BNIP-3 is upregulated after 44 h of hypoxia (2). In this

Fig. 3. A: mitochondria volume density decreased in the diaphragm but not in gastrocnemius after 4 wk of hypoxia. Representative electron micrographs of diaphragm (top: scale bar = 1 μm) and gastrocnemius (bottom: scale bar = 2 μm). Some of the mitochondria are labeled with white arrows. B: there is a noticeable reduction in mitochondrial density after 4 wk of hypoxia in diaphragm (*P = 0.013, n = 4 in each group), but no change in the gastrocnemius muscle. Values are given as means ± SE.

Fig. 4. Electron micrographs showing abundant subsarcolemmal mitochondria in diaphragm muscle fibers after hypoxia (arrows, scale bar = 2 μm). Top: typical distribution of mitochondria and capillaries (c) in control mice. After 4 wk of hypoxia, large clusters of mitochondria (arrows) are observed under the sarcolemma that projects and encircles adjacent capillaries (bottom).
study, BNIP-3 mRNA content increased after 1 and 2 wk of hypoxia in diaphragm and gastrocnemius respectively, and BNIP-3 protein content increased only in diaphragm after 4 wk of hypoxia. Interestingly, BNIP-3L had the same pattern of expression in diaphragm and gastrocnemius: mRNA levels were higher in both muscles after 1 and 2 wk of hypoxia, respectively. BNIP-3L is also a hypoxia-inducible gene and have been recently identified as an autophagic factor (3). The presence of increased hypoxia-induced mitophagy in diaphragm is also demonstrated by the presence of many autophagic vesicles in the hypoxic diaphragm (Fig. 6C).

Increased mitophagy and reduced mitochondrial biogenesis have been suggested as adaptive mechanisms to acute hypoxia. Cell culture experiments have shown that up to 48 h of hypoxia can reduce mitochondrial respiration and mitochondrial mass, the later because of increased mitophagy (50). Acute hypoxia also affects mitochondrial biogenesis in renal cancer cells (49). We found that chronic hypoxia, at least in the diaphragm, affects mitochondrial biogenesis and autophagy. PGC-1α downregulation would probably decrease biogenesis, whereas upregulation of BNIP-3 would account for increased mitophagy. Interestingly, no changes in mitochondrial mass were observed in gastrocnemius. This difference between the diaphragm and gastrocnemius may reflect their workloads. The diaphragm increases its activity during hypoxia. However, its mitochondrial content decreases (Fig. 2), a seemingly paradoxical change that is accompanied by a redistribution of mitochondria to the periphery. These consequences combine to reduce the diffusion distance from the vascular compartment to the mitochondria. Another adaptive mechanism would be to increase the capillary to fiber interface, which has been considered a critical factor for oxygen diffusion (34). Gastrocnemius, on the other hand, does not need all these adaptations given that these muscles are less active during the exposure to hypoxia.

It has been suggested that the adaptation of skeletal muscle to hypoxia includes changes in fiber type and size (12). Constantly active muscles (such as diaphragm) show increased capillary density after chronic hypoxia, compared with less active limb muscles (12). Our findings support the idea that adaptation to hypoxia differs depending on the activity level of the muscle under study. In other words, the combination of hypoxia and muscle activity may be required to reduce intramuscular Po2 to low enough levels to stabilize HIF-1α (13). HIF is a heterodimer composed of α- and β-subunits that induces the expression of a broad range of genes in response to low levels of oxygen (43). Thus, low oxygen tissue levels would activate HIF, leading to mitophagy and

Fig. 5. A: Quantitative RT-PCR of mitochondrial biogenic factors. There was a decreased expression of PGC-1α in diaphragm after 1, 2, and 4 wk of hypoxia (H) compared with the control normoxia levels. No change was observed in gastrocnemius. There was no difference in any of the other biogenic factors evaluated (i.e., TFAM, NRF1, or NRF2) in diaphragm or gastrocnemius (n = 6 in each group). Western blot analysis of PGC-1α (B) and PPARγ (C) revealed decreased protein contents in diaphragm after 2 and 4 wk of hypoxia (H-2W and H-4W respectively) compared with control (n = 6 in each group). Values are expressed as means ± SE; *P < 0.05 for indicated comparisons.
inhibition of mitochondrial biogenesis (13). This sequence of events does not discount the possibility that HIF-independent pathways, such as increased in AMP kinase activity, may also be important for skeletal muscle adaptation to chronic hypoxia (5).

While we have not seen any changes in gastrocnemius, some studies in humans have documented the decrease in mitochondrial oxidative capacity in leg muscles (14, 18, 20, 22). We have to be cautious when interpreting these findings since they were performed at extreme levels of hypoxia combined with exercise. The discrepancy between those results and ours may reside in the fact that we did not include a formal exercise training protocol. Therefore, it is possible that the combination of hypoxia with endurance training decreases mitochondrial mass in the limb muscles of mice.

Fig. 6. BNIP-3 expression was higher after 1 wk of hypoxia (H-1W) in diaphragm (A) and after 2 wk of hypoxia (H-2W) in gastrocnemius (B). LC-3, ATG-5, and ATG-7 mRNA levels were unchanged in either diaphragm or gastrocnemius. B: BNIP-3 protein content in diaphragm was increased after 4 wk of hypoxia of hypoxia (H-4W) compared with control (C) and 2 wk-hypoxia (H-2W) groups (*P < 0.05). No change in BNIP-3 protein content was observed in gastrocnemius. C: Electron micrographs of diaphragm fibers from mice after 4 wk of hypoxia. There were vesicles consistent with autophagosomes (top: arrows). Higher magnification views of these vesicles are shown in the lower panels (scale bar = 0.2 μm for all the images). Values are given as means ± SE (n = 6 in each group).
Perspectives and Significance

In summary, we found that chronic hypoxia is associated with a reduction in mitochondrial volume density in diaphragm. Lower levels of PGC-1α seem to be responsible for reduced mitochondrial biogenesis. Increased mitophagy may also occur because of hypoxia-induced BNIP-3 expression. Both mechanisms (reduced biogenesis and increased mitophagy) may combine to decrease mitochondrial mass in the mouse diaphragm after chronic hypoxia. It is still necessary to assess whether activity level or other unknown factors are responsible for the different pattern of adaptation between gastrocnemius and diaphragm. Nevertheless, these findings point out that skeletal muscle adaptation to hypoxia cannot be generalized when only one type of muscle is analyzed.

ACKNOWLEDGMENTS

We would like to acknowledge Miguel Andrade for his assistance with data collection.

REFERENCES