Regulation of diaphragmatic nitric oxide synthase expression during hypobaric hypoxia

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The diaphragm is a skeletal muscle that is exposed to loads with magnitudes that depend on the mechanical characteristics of the respiratory system and timing, which are related to breathing frequency. As with other skeletal muscles, it has been well established that both nNOS and eNOS isoforms are expressed in the diaphragm of various species (15, 16, 19). More recent studies by our group demonstrated that the rate of NO synthesis in skeletal muscle fibers is a dynamic process that undergoes specific alterations in response to changes in muscle metabolic demands. For instance, an increase in muscle activity over a relatively short period (3 h of resistive loading) initiates a significant reduction of muscle NO synthesis (12). Muscle NO synthesis and eNOS and nNOS expression are also elevated during embryonic and postnatal development (10).

Hypobaric hypoxia is associated with numerous humoral, nutritional, and respiratory changes such as increased ventilatory muscle demands, which result in significant biochemical and histological adaptations in the ventilatory muscles in general and in the diaphragm in particular. For instance, diaphragmatic myosin heavy chain isoforms change significantly in chronically hypoxic rats as indicated by a decrease in type I isoform expression and an increase in expression of the type II isoform (23). Similar alterations were detected in the quadriceps of chronic obstructive lung disease patients (14).

Many investigators recently have addressed the influence of relatively short periods of hypoxia on NO synthesis and have reported specific changes in NOS expression, which depend on the NOS isoform and the type of cell that is involved. There is no information regarding the influence of hypobaric hypoxia on the expression of various NOS isoforms inside the ventilatory muscles. We hypothesized that moderate durations of hypobaric hypoxia (60 days) lead to a significant upregulation of both nNOS and eNOS expression in the ventilatory muscles. This hypothesis is based on the following observations. First, hypobaric hypoxia promotes a shift toward type II fibers that are rich in

NITRIC OXIDE (NO), a secondary messenger with diverse biological functions, is normally synthesized in skeletal muscles by neuronal (nNOS) and endothelial (eNOS) nitric oxide synthases. Recent studies have confirmed that while the nNOS isoform is localized at the sarcolemma of type II fibers and closely associates with the dystrophin complex (9), the eNOS isoform is mainly localized in the endothelium of skeletal muscle vasculature. The functional significance of both nNOS and eNOS isoforms in regulating skeletal muscle function is under investigation. However, increasing evidence suggests that many processes inside skeletal muscle fibers such as blood flow, glucose uptake, myoblast fusion, and excitation-contraction coupling are influenced by endogenous NO synthesis (1, 6, 18, 30, 31, 35).
the nNOS isoform (17). Second, hypobaric hypoxia is associated with increased ventilatory muscle activity due to augmentation of increased ventilation, which results in increased ventilatory muscle metabolic demands (23). Previous studies indicate that prolonged periods of increased skeletal muscle activity leads to a substantial rise in muscle nNOS expression (28). Third, hypobaric hypoxia causes vascular remodeling (smooth muscle and endothelial cell replication and extracellular matrix accumulation), which is mediated by vascular cell mitogens and growth factors such as vascular endothelial cell growth factor (VEGF), endothelin-1, and platelet-derived growth factor. These mitogens, particularly VEGF, are known to stimulate eNOS expression in the endothelial cells (20). On the other hand, we propose that prolonged (over several months) hypobaric hypoxia will be associated with downregulation of both nNOS and eNOS isoforms in the ventilatory muscles because of various nutritional and hormonal alterations associated with prolonged hypoxia. These alterations include a decline in thyroid function. Recent studies indicate that NOS expression is positively modulated by the thyroid hormone (34).

The main objective of this study, therefore, was to test whether moderate (60 days) and prolonged (9 mo) periods of hypobaric hypoxia elicit differential effects on constitutive NOS isoform expression in the diaphragm and whether hypobaric hypoxia induces the expression of the inducible NOS (iNOS). Finally, we evaluated the influence of hypobaric hypoxia on the expression of two enzymes involved in the recycling of L-citrulline to L-arginine [argininosuccinate synthase (AS) and argininosuccinate lyase (AL)].

The study was conducted with a model that was used previously to address changes in diaphragmatic fiber-type expression in response to chronic hypoxia (23). Conscious rats were exposed to a moderate level of hypobaric hypoxia for 60 days or 9 mo after birth. With the use of immunoblotting, we examined the relative changes in diaphragmatic eNOS, nNOS, iNOS, AS, and AL protein expression. We also probed diaphragmatic muscle samples with an antibody selective to manganese superoxide dismutase (Mn SOD) to evaluate the possibility that changes in NOS isoform expression in hypoxic rats represent a general adaptive behavior involving not only NO synthesis but also antioxidant enzymes such as Mn SOD.

**MATERIALS AND METHODS**

**Animal preparation.** All experiments were approved by the appropriate Animal Ethics Committees of McGill University. Experiments were performed on two groups of adult Sprague-Dawley rats, normoxic and chronic hypoxic. Animals were studied either at 60 days of age (n = 8 per group) or after 9–11 mo of age (n = 6 per group). The hypoxic group was continuously exposed to hypobaric hypoxia from the first day after birth. Hypobaric hypoxia (barometric pressure of ~505 mmHg, which corresponds to Po2 of ~95 mmHg) was established in chambers that were opened twice a week for 20 min to clean and replenish food and water. The animals were weaned (days 22–23) and were then housed in separate cages containing one or more animals and allowed free access to food and water. The animals were exposed to a daily 12:12-h light-dark cycle and kept under ambient temperature and humidity. The normoxic group was housed in similar chambers at barometric pressure of 760 mmHg (Po2 150 mmHg) and were maintained under conditions similar to those of the hypoxic group.

**Sample preparation.** The animals were anesthetized with 30 mg/kg pentobarbital sodium. Bleeding was then initiated by severing the carotid arteries and jugular veins. The abdomen was opened, and the costal diaphragm was removed, blotted on absorbing paper, weighed, and stored under ~80°C. About 100 mg of muscle sample were homogenized by hand in a glass tissue grinder with ice-cold homogenization buffer containing 0.2% sodium dodecyl sulfate, 0.6 M β-mercaptoethanol, 28 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 22 mM Tris base, 0.002% leupeptin, 250 mM phenylmethylsulfonyl fluoride, and 0.5% trypsin inhibitor. The sample was then heated at 95°C for 12 min, placed on ice for 30 min, and centrifuged (10,000 g), and the supernatant was then collected. The resultant pellet was resuspended in a homogenization buffer, and the procedure was repeated three to four times until the protein concentration of the resuspended pellet was ~5% of the protein concentration of the total supernatant. The procedure was used to extract as much protein as possible from muscle samples. Supernatants from repeated centrifugation were pooled, and protein concentration was measured with the Bradford technique (Bio-Rad).

**Immunoblotting.** Crude muscle homogenate proteins (80 μg; see above) were heated for 15 min at 90°C and then loaded on gradient (4–12%) sodium dodecyl sulfate-Tris glycine polyacrylamide gels. The proteins were transferred electrotherically onto polyvinylidene difluoride membranes and were blocked with 5% nonfat dry milk and subsequently incubated overnight at 4°C with primary monoclonal antibodies (all obtained from Transduction Laboratories, Lexington, KY). Lysates of cytokine-activated macrophages, endothelial cells, and pituitary cells were used as positive controls for iNOS, eNOS, and nNOS proteins, respectively (provided by Transduction Laboratories). AS and AL proteins (1:1,000) were detected with polyclonal antibodies that were raised in rabbits against recombinant proteins and were used previously to detect these proteins in the diaphragm and other rat tissues (24). A polyclonal antibody raised against Mn SOD (1:500) was used to detect diaphragmatic Mn SOD expression (33). Specific proteins were detected with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies and enhanced chemiluminescence reagents (Amersham). Predetermined molecular weight standards (Novex) were used as markers. The membranes were stained with silver stain and scanned to verify that equal amounts of proteins were loaded on different lanes.

**NOS activity.** Diaphragmatic NOS activity was measured with the l-citrulline assay (10). Frozen tissues were homogenized in 6 volumes (wt/vol) of homogenization buffer (pH 7.4, 10 mM HEPES buffer, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mg/ml phenylmethylsulfonyl fluoride, 0.32 mM sucrose, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin A). The crude homogenates were centrifuged at 4°C for 15 min at 10,000 rpm. The supernatant (50 μl) was added to 10-ml prewarmed (37°C) tubes containing 100 μl of reaction buffer of the following composition: 50 mM KH2PO4, 60 mM valine, 1.5 mM NADPH, 10 mM flavine adenine dinucleotide, 1.2 mM MgCl2, 2 mM CaCl2, 1 mg/ml BSA, 1 μg/ml calmodulin, 10 μM tetrahydrobiopterin, and 25 μl of 120 μM stock l-[2,3-3H]arginine (150–200 counts·min–1·pM–1). The
samples were incubated for 30 min at 37°C, and the reaction was terminated by the addition of cold (4°C) stop buffer (pH 5.5, 100 mM HEPES and 12 mM EDTA). To obtain free L-[3H]citrulline for the determination of enzyme activity, 2 ml of Dowex 50 W resin (8% cross-linked, Na\(^+\) form) were added to eliminate excess L-[2,3-3H]arginine. The supernatant was assayed for L-[3H]citrulline by using liquid scintillation counting. Enzyme activity is expressed in picomoles of L-citrulline produced per minute per milligram of total protein.

NOS activity was also measured in the presence of 1 mM N\(^\text{G}\)-nitro-L-arginine methyl ester (L-NAME; NOS inhibitor). Total NOS activity was calculated as the difference between that measured in the absence and presence of L-NAME.

**RESULTS**

**Sixty days.** Chronic hypoxia for 60 days had no effect on body weight or diaphragmatic mass (as percentage of body mass; Table 1). Hematocrit, however, increased significantly in the hypoxic group compared with that in the normoxic group (Table 1).

**Table 1.** Age, body weight, Hct, and diaphragmatic mass in 4 groups of animals

<table>
<thead>
<tr>
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<th>n</th>
<th>Age, mo</th>
<th>Body Weight, g</th>
<th>Hct, %</th>
<th>Diaphragmatic Mass, %body mass</th>
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<tr>
<td>60 days</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Normoxia</td>
<td>8</td>
<td>2</td>
<td>245 ± 19</td>
<td>53 ± 1</td>
<td>34 ± 1</td>
</tr>
<tr>
<td>Hypoxia</td>
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<td>2</td>
<td>205 ± 18</td>
<td>73 ± 3*</td>
<td>33 ± 2</td>
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<tr>
<td>9–11 mo</td>
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<tr>
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<td>6</td>
<td>9</td>
<td>355 ± 21</td>
<td>54 ± 2</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>6</td>
<td>11</td>
<td>364 ± 18</td>
<td>79 ± 2*</td>
<td>31 ± 2</td>
</tr>
</tbody>
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Values are means ± SE; n, no. of rats studied. Hct, hematocrit. *P < 0.001 compared with hypoxia.

**Data analysis.** Protein band intensities were quantified by scanning blots containing six samples per given animal group. Scanning was performed with an imaging densitometer (model GS700, Bio-Rad, 12-bit precision and 42-μm resolution). Optical densities of the protein bands were quantified with SigmaGel software (Jandel Scientific, San Rafael, CA). Densities and NOS activity values were compared between normoxic and hypoxic groups with two-way analysis of variance. P < 0.05 was considered significant.

**Immunoblotting of diaphragmatic proteins with anti-nNOS antibody detected a prominent band at an apparent mass of 166 kDa (Fig. 1). At 60 days of age, hypoxia was associated with more than ninefold induction of nNOS protein (Fig. 1). Anti-eNOS antibody detected a single protein band at 130 kDa, which was upregulated by an approximate twofold in response to 60 days of hypoxia (Fig. 2). iNOS protein was not detected in the normoxic and hypoxic groups (Fig. 3). Furthermore, 60 days of hypoxia elicited no significant changes in AS, AL, and Mn SOD protein expression. Figure 4 illustrates the mean values of optical densities of eight different diaphragmatic samples in each group of animals. Hypoxia was associated with an approximate two- and ninefold rise in the expression of eNOS and nNOS, respectively, whereas no significant changes were detected in AS, AL, and Mn SOD expressions. Figure 5 illustrates the changes in diaphragmatic NOS activity. Hypobaric hypoxia for 60 days resulted in a significant increase in diaphragmatic NOS activity (Fig. 5).
Nine to eleven months. Although body weight and diaphragmatic mass were not different among the two groups, hematocrit rose significantly in the hypoxic group compared with that in the normoxic group (Table 1). Prolonged (9–11 mo) periods of hypoxia elicit (qualitatively and quantitatively) different changes in diaphragmatic NOS expression than those associated with shorter periods of hypoxia (60 days). Prolonged hypoxia reduced nNOS protein expression, which averaged about 20% of that observed in the normoxic group (Fig. 6). Similarly, diaphragmatic eNOS expression was lower in the hypoxic group and averaged about 15% of that observed in the normoxic group (Fig. 6). Expression of AS, AL, and Mn SOD was not altered by prolonged hypoxia. Figure 7 shows the mean values of optical densities of six different diaphragmatic samples in each group of animals. Prolonged hypobaric hypoxia (9 mo) resulted in a significant attenuation of diaphragmatic NOS activity compared with that in normoxic animals (Fig. 5).

DISCUSSION

The main finding of this study is that chronic hypobaric hypoxia elicits selective changes in diaphragm...
matic NOS expression and activity and that those changes are related to the duration of hypoxic exposure. Sixty days of hypobaric hypoxia in newborn rats was associated with upregulation of eNOS and nNOS protein levels and an increase in diaphragmatic NOS activity, whereas 9 mo of hypoxia reduced the expression of both enzymes and attenuated NOS activity. The changes in diaphragmatic NOS expression in response to hypoxia are limited to the constitutive isoforms because iNOS was not induced by exposure to hypobaric hypoxia. In addition, hypoxia had no effects on diaphragmatic expressions of AS, AL, and Mn SOD proteins.

Regulation of eNOS and nNOS expression by hypoxia. Studies on the influence of relatively short periods (a few hours to 3 wk) of hypoxia on constitutive NOS isoform expression revealed conflicting results depending on the animal species, the duration of hypoxia, and the cell and organ involved. For example, 12–48 h of in vitro hypoxia elicited a decline in pulmonary endothelial cell eNOS expression, whereas eNOS expression in aortic endothelial cells rose significantly (3, 21). Similarly, fetal pulmonary eNOS expression declines significantly in response to 48 h of in vitro hypoxia, whereas bronchiolar epithelial NO production remains independent of PO₂ levels (25). Contrasting results also have been reported regarding the influence of in vivo hypoxia on lung eNOS expression. Although Xue et al. (37) demonstrated a substantial upregulation of eNOS protein expression in pulmonary endothelial cells and de novo induction of NOS expression in vascular smooth muscles in response to 2–4 wk of hypoxia in rats, whole lung eNOS protein expression after 10–12 days of hypoxia in newborn piglets was only about 40% of that observed in normoxic animals (11). The reasons behind these contradictory results regarding eNOS expression are not clear. It is likely that a combination of a direct influence of hypoxia on an eNOS promoter and/or secondary changes in shear stress and hormonal levels may result in the tailoring of local NO production to meet altered NO requirements during hypoxia.

Unlike the eNOS isoform, the influence of hypoxia on the expression of the nNOS isoform has been shown

![Fig. 6. The influence of 9 mo of hypoxia on diaphragmatic nNOS and eNOS protein expression. Note that the levels of both proteins declined significantly in the hypoxic group compared with those in the normoxic group.](image-url)

![Fig. 7. Mean values of optical densities of various proteins in response to 9 mo of hypoxia. **P < 0.01 compared with the normoxic group.](image-url)
consistently to be stimulative, especially in the central and peripheral nervous system. For instance, subacute hypoxia (12–48 h) in conscious rats results in significant upregulation of both nNOS mRNA and protein expression in the cerebellum (13). Similarly, Prabhakar et al. (27) reported approximately 10- and 2-fold increases in nNOS mRNA in nodose ganglia and cerebellums after 12 h of hypoxia in conscious rats, respectively.

No information is available yet regarding the influence of hypobaric hypoxia on skeletal muscle NOS expression. Our results indicate for the first time that prolonged periods of hypobaric hypoxia (60 days to 9 mo) elicit differential changes in constitutive NOS isoform expression, which are related to the duration of hypoxia. After 60 days of hypoxia in newborn rats, diaphragmatic eNOS and nNOS expression rose significantly, whereas 9 mo of hypoxia elicited an opposite response. The exact molecular mechanisms responsible for changes in diaphragmatic NOS expression in our study remain unclear. We speculate that changes in protein levels of diaphragmatic eNOS and nNOS isoforms are the results of transcriptional and/or post-transcriptional mechanisms. One of these mechanisms is likely to be a change in NOS mRNA stability. Indeed, hypoxia evokes significant shortening of eNOS mRNA half-life in pulmonary endothelial cells (21). By comparison, a decline in eNOS transcription was the only alteration noted in response to severe hypoxia in aortic endothelial cells, whereas eNOS mRNA stability remained unchanged (3). Our results do not exclude changes in diaphragmatic eNOS and nNOS transcription and/or alterations in mRNA stability of these isoforms in response to 60 days or 9 mo of hypoxia.

Another issue that should be addressed is whether the aging process is involved in the differential alterations of diaphragmatic NOS expression in response to 60 days or 9 mo of hypobaric hypoxia. Previous studies revealed that age-related changes in NOS expression differ among various organs. In mouse hearts, eNOS and nNOS activities increase by 120 and 47%, respectively, between 2 and 6 mo of age (5). By comparison, no significant changes were detected in nNOS expression in the human brain during aging (7). With respect to skeletal muscles, Richards et al. (29) compared NOS activity in various muscles of 8- and 24-mo-old rats and reported a significant reduction in NOS activity with aging. We measured NOS activity and eNOS and nNOS expression during early postnatal development in rats and found a significant decline in these parameters in adult animals compared with those in newborn animals (10). To assess the influence of aging on differential NOS expression in our experiment, we performed immunoblotting on five animals in each of the normoxic groups (60 days and 9 mo old) using various antibodies listed in MATERIALS AND METHODS. Diaphragmatic eNOS and nNOS optical densities of normoxic 9-mo-old diaphragms averaged about 120 ± 10 and 107 ± 5%, respectively, of those of 60-day-old normoxic diaphragms (both values are not statistically significant). Similarly, no significant age-related changes in diaphragmatic AS, AL, and Mn SOD expression were detected among the two normoxic groups. We should emphasize that diaphragmatic NOS activities were not different among the normoxic groups (Fig. 5). These results indicate that aging does not explain the differences in the responses of diaphragmatic NOS expression and activity to moderate and prolonged periods of hypobaric hypoxia. Clearly, more studies are needed to elucidate the molecular mechanisms behind the changes in diaphragmatic NOS isoforms in response to hypobaric hypoxia.

An interesting observation in our study is that hypoxia-induced changes in diaphragmatic NOS activity were relatively smaller than those of diaphragmatic NOS protein expression. For instance, whereas diaphragmatic NOS activity rose by about 50% in response to 60 days of hypobaric hypoxia, diaphragmatic eNOS and nNOS expression increased by about 2.5- and 9-fold, respectively. The reasons behind this observation are not yet clear; however, it has been established that NOS activity is influenced not only by the level of NOS proteins but by the availability of cofactors such as tetrahydrobiopterin and the presence of endogenous inhibitors such as N^G,N^G-dimethyl-L-arginine, protein inhibitor of nNOS and caveolin 3. We speculate that the disproportionate changes in muscle NOS activity compared with NOS protein expression in this study are due to hypoxia-induced alterations in the levels of NOS cofactors and endogenous inhibitors. Clearly, more studies are needed to elucidate the direction and the mechanisms through which hypoxia may influence the availability and the expression of NOS cofactors and endogenous inhibitors.

It should be emphasized that one should be cautious in extrapolating the observed changes in diaphragmatic constitutive NOS expression in response to hypobaric hypoxia to other skeletal muscle because only the diaphragm was examined in the current study. We speculate, however, that similar changes in NOS expression are likely to develop in the intercostal muscles in response to hypobaric hypoxia. Our previous studies in spontaneously breathing animals and in animals exposed to inspiratory resistive loading or sepsis indicate that NOS expression and activity in the intercostal muscles were similar to those of the diaphragm (15, 16).

**Induction of iNOS, AS, and AL by hypoxia.** Under normal conditions, very weak iNOS expression is detectable in the ventilatory or limb muscles of various species. However, exposure to bacterial lipopolysaccharides induces significant iNOS expression in skeletal muscle fibers of various muscles (8). Induction of iNOS is usually accompanied by increased requirements for L-arginine, which is supplied through active transport from the extracellular space, protein degradation, and recycling of L-citrulline to L-arginine. This recycling occurs via a two-step enzymatic process that requires the activities of AS and AL enzymes. The expression of these enzymes has been widely reported to be induced along with iNOS in response to proinflammatory cytokines or lipopolysaccharides (24). Little is known about
the influence of hypoxia on iNOS, AS, and AL expression. In murine cell lines, iNOS is induced by hypoxia only when these cells were costimulated with interferon-γ (22). In the respiratory system, Palmer and colleagues (26) have reported recently that 3 wk of hypoxia in conscious rats is associated with the induction of iNOS mRNA in bronchial epithelial, pulmonary endothelial, and smooth muscle cells (26). Induction of iNOS by hypoxia appears to vary between different tissues. We reported recently that no significant iNOS induction occurs in the brain in response to 12–48 h of hypoxia in rats (13). Similarly, few studies have addressed the influence of hypoxia on AS and AL expression. Su and Block (32) reported that 24 h of in vitro hypoxia elicit a significant downregulation of both AS activity and expression in cultured pulmonary endothelial cells (32). These results contrast with our recent findings indicating no significant alterations in brain AS and AL expression in response to 12–48 h of in vivo hypoxia in rats (13).

Our current results indicate that prolonged periods of hypobaric hypoxia had no influence on iNOS induction in the diaphragm. These results indicate that despite the presence of a putative hypoxia-induced element in iNOS promoter, induction of the iNOS gene in response to in vivo hypoxia is regulated at the local level. The lack of induction of diaphragmatic AS and AL in the present study also suggests the link between iNOS induction and recycling of L-citrulline to L-arginine is particularly strong during in vivo hypoxia.

Implications. The changes in the expression of nNOS and eNOS isoforms described in this study are likely to have important impacts on the following processes in skeletal muscle fibers. 1) Glucose transport. Skeletal muscle fibers, particularly neonatal or immature fibers, rely to a greater extent on glycolysis and glucose uptake for ATP production during hypoxia rather than during normoxia. To achieve this goal, many steps involved in muscle glucose uptake are augmented, including the expression of glucose transporters GLUT-1 and GLUT-4 (36). Recent studies have confirmed that NO has an important role in muscle glucose uptake. Indeed, basal and insulin- and contract-stimulated glucose uptakes are enhanced by endogenous NO synthesis (4, 18). On the basis of the aforementioned studies, one can conclude that elevated nNOS and eNOS expression during 60 days of hypobaric hypoxia represent an adaptive response by neonatal muscles to cope with increased demands for glucose uptake. In addition, reduction of NOS activity as a result of down-regulation of both eNOS and nNOS in 9-mo-old hypoxic rats is likely to have adverse effects on glucose uptake of muscle fibers. 2) Muscle fiber maturation. Recent studies from our laboratories and others (6, 10) have illustrated that NO promotes myoblast fusion in skeletal muscles during embryonic and early postnatal development. Thus increased muscle NO synthesis during moderate periods of hypobaric hypoxia is likely to promote myoblast fusion and maturation of neonatal muscle fibers. 3) Reactive oxygen species (ROS). Although excessive NO release is damaging to tissues as a result of the formation of the free radical peroxynitrite, most investigators agree that basal NO production in skeletal muscles plays an important role as a ROS scavenger. Recent studies indicate that NO enhances excitation-contraction coupling by protecting sarcoplasmatic reticulum ryanodine receptors from ROS-mediated oxidative modifications, resulting eventually in activation of these receptors (1, 31). We speculate that a reduction in muscle NO production in response to hypobaric hypoxia will have adverse effects on muscle antioxidant capacity and excitation-contraction particularly in 9-mo-old rats because of the already depressed antioxidant enzyme activities in these rats compared with those in younger animals (2).

Muscle blood flow. The involvement of NO release from endothelial cells in the regulation of muscle blood flow is very well documented. We propose that the increase in eNOS expression in response to moderate periods of hypobaric hypoxia will promote vascular dilation and increased blood flow supply to the developing muscle fibers.

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