Mechanisms of aortic smooth muscle hyporeactivity after prolonged hypoxia in rats

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Mechanisms of aortic smooth muscle hyporeactivity after prolonged hypoxia in rats. J Appl Physiol 92: 2625–2632, 2002; 10.1152/japplphysiol.00818.2001.—The aim of this study was to determine whether the effects of hypoxia on aortic contractility reflect a decrease in smooth muscle activation [phosphorylation of the 20-kDa myosin regulatory light chain (LC20)], the capacity for myofibrillar ATP hydrolysis (mATPase activity), or both. Our results indicate that, in endothelium-denuded aortic rings from rats exposed to hypoxia for 48 h (inspired \( \text{O}_2 \) concentration = 10%), contractions to phenylephrine and potassium chloride (KCl) are impaired compared with rings from normoxic rats. The proportion of phosphorylated to total LC20 during aortic contraction induced by \( 10^{-5} \) M phenylephrine was reduced after hypoxia (51.4 \( \pm \) 5.4% in normoxic control rats vs. 32.5 \( \pm \) 4.7% in hypoxic rats, \( P < 0.01 \)). Aortic mATPase activity was also decreased (maximum ATPase rate = 29.6 \( \pm \) 3.4 and 20.7 \( \pm \) 3.7 mmol-min \(^{-1}\)-mg protein\(^{-1}\) in control and hypoxic rats, respectively, \( P < 0.05 \)). Neither proliferation nor dedifferentiation of aortic smooth muscle was evident in this model; immunostaining for smooth muscle expression of the proliferating cell nuclear antigen was negative and smooth muscle-specific isoforms of myosin heavy chains, h-caldesmon, and calponin were increased, not decreased, after hypoxic exposure. Decreased aortic reactivity after hypoxia is associated with both impairment of smooth muscle activation and diminished capacity of the actomyosin complex, once activated, to hydrolyze ATP. These changes cannot be attributed to smooth muscle dedifferentiation or to reduced contractile protein expression.

systemic circulation; vascular smooth muscle; contractile protein expression; blood flow regulation; oxygen delivery.

THE VASCULAR REFLEXES THAT redistribute blood flow toward vital organs and enhance the capacity for oxygen extraction during acute hypoxia (minutes) are mediated by the sympathetic nervous system (4, 5, 9, 21). If hypoxia is prolonged (12–48 h), however, the reactivity of systemic arterial and arteriolar smooth muscle is decreased (1, 2, 10, 30) and sympathetic responses are impaired (16). This will limit the ability to maintain adequate vital organ oxygen supply if substrate supply must be increased to meet an increase in metabolic demand in the event of superimposed hypotension or if hypoxia acutely becomes more severe. Despite its clinical and physiological relevance, the mechanisms that mediate the effect of hypoxia on smooth muscle contractility are unknown.

Vascular smooth muscle contraction depends on sliding of myosin thick filaments over thin filaments composed of actin polymer and the regulatory proteins calponin, caldesmon, and tropomyosin. Initiation of contraction is regulated by phosphorylation of the 20-kDa myosin light chain (LC20; Refs. 19, 20), which permits actin activation of ATPase activity in the head of the myosin heavy chain (MHC) to provide the energy for cross-bridge cycling. We, therefore, hypothesized that vascular hyporeactivity after hypoxia may result from impairment of activation (decreased LC20 phosphorylation), diminished capacity of the actomyosin complex, once activated, to hydrolyze ATP, or both. Accordingly, the current study was carried out to determine the effect of hypoxic exposure on the level of LC20 phosphorylation during \( \alpha \)-agonist-induced contraction and on myofibrillar ATPase activity in systemic vascular smooth muscle. Hypoxia has previously been shown to induce proliferation and/or modulation from a contractile to a proliferative phenotype in smooth muscle and mesangial cells (7, 8, 27). An additional goal, therefore, was to determine whether the biochemical and functional changes that occur in the systemic vascular smooth muscle after hypoxia are associated with a shift to a more proliferative state with a corresponding decrease in contractile protein expression (12, 13, 14, 26, 28).

MATERIALS AND METHODS

Studies were carried out in male Sprague-Dawley rats (200–250 g). All protocols were in accordance with standards set by the Canadian Council on Animal Care and were approved by the institutional animal care committee. As described previously (2), rats were placed in a Plexiglas chamber (30 \( \times \) 18 \( \times \) 15 cm) into which the flow of air and nitrogen was controlled independently and from which gas...
outflow was through an underwater seal. Gas samples were drawn periodically from the chamber for analysis (model 995, AVL Instruments, Graz, Austria) to ensure that the appropriate ambient PO2 was maintained. Animals exposed to hypoxia breathed a mixture containing 10% oxygen, whereas control animals breathed air only under otherwise identical conditions.

Standard chemicals were purchased from Sigma Chemical (St. Louis, MO). Electrophoresis reagents were from Bio-Rad (Mississauga, Ontario), and enhanced chemiluminescence reagents and film were from Amersham (Oakville, Ontario). Antibodies from Sigma Chemical included primary mouse monoclonal antibody to smooth muscle-specific α-actin, polyclonal antisera to tropomyosin, SM1/S2 smooth muscle MHC, smooth muscle LC20, and secondary goat anti-mouse-HRP adsorbed against rat serum proteins. Rabbit anti-calponin and rabbit anti-h-caldesmon were kindly donated by Dr. M Walsh, University of Calgary. Donkey anti-rabbit-HRP from Amersham, and Ki67 antibody specific for proliferating cell nuclear antigen was purchased from DAKO (Mississauga, Ontario).

Functional studies. Thoracic aortas from normoxic rats and rats exposed to hypoxia were excised immediately after decapitation, cleaned, and sectioned into 4-mm rings. The endothelium was removed by gentle abrasion of the luminal surface, and rings were mounted in jacketed organ baths containing 95% O2-5% CO2-aerated Krebs-Henseleit solution (composition in mM: 120 NaCl, 25 NaHCO3, 11.1 glucose, 4.76 KCl, 1.18 MgSO4, 7H2O, 1.18 KH2PO4, and 2.5 CaCl2) maintained at 37°C. During the 60-min equilibration, the bathing medium was changed periodically and the rings stabilized at a baseline tension of 2 g. The absence of a functional endothelium was tested by ascertaining whether acetylcholine (10–6 M) was effective at relaxing aortic rings precontracted by phenylephrine (10–4 M). Failure of acetylcholine to elicit relaxation was taken as evidence of successful endothelial ablation (2). Phenylephrine and acetylcholine to elicit relaxation was taken as evidence of success-

LC20 phosphorylation. Thoracic aortic segments (4 mm) from normoxic rats and from rats exposed to hypoxia were denuded of endothelium (see Functional studies) and cut into helical strips. Strips were mounted in organ bath myographs that contained Krebs solution, bubbled with 95% O2-5% CO2, and maintained at 37°C. Baseline tension was adjusted to 1.5 g, the optimum tension for maximal contractile responses under our experimental conditions, and equilibrated for 1 h. From each animal, one strip remained under control condi-

Fig. 1. A: representative blot carried out on 20-kDa myosin regulatory light chain (LC20) extracted from aortic strips from a normoxic rat and from a rat exposed to hypoxia for 48 h stimulated with 10–6 M phenylephrine (PE) for 1 min. Bands corresponding to nonphosphorylated (non P), monophosphorylated (mono P), and diphosphorylated (di P) LC20 are illustrated. B: histogram illustrates mean levels of LC20 phosphorylation (percent total LC20) after 1 min of stimulation with 10–6 M PE in normoxic and hypoxia-exposed rats. Bars are means with lines denoting SE (n = 5 per group). *Significantly different from the normoxic control group (P < 0.05).
mM Na$_3$CN, 10 g/ml leupeptin, 0.25 mM phenylmethylsulfonyl fluoride, and 1 mM DTT at a ratio of 100 μg/mg original tissue weight. An aliquot of the lysates was removed, and protein concentration was determined by the Bradford assay. From this, the total amount of protein in the sample was calculated to express ATPase activity in moles of P$_i$ released per minute per milligrams of protein.

Samples were incubated with shaking at 4°C for 1 h before measurement of myosin ATPase activity by a previously described colorimetric assay (22, 28). Briefly, particulate matter in the lysates was sedimented by using a microcentrifuge, and the supernatant was aliquoted and warmed to room temperature. Myosin ATPase activity was initiated by adding MgATP and CaCl$_2$ simultaneously to final concentrations of 1 mM and 100 μM, respectively. Reactions were terminated at time points up to 3 min thereafter by adding ice-cold TCA to a final concentration of 10%. Precipitated protein was then resolubilized in urea loading buffer (see above) and used to determine the amount of phosphorylated to unphosphorylated LC20, as described above. The concentration was determined by using 4--methylsulfonyl fluoride, 1 mM DTT, and 1 ml of color reagent (a 1:1 mixture of 0.074% vanillin, 1% polyvinyl alcohol and 2.88 M H$_2$SO$_4$ 3.7% ammonium molybdate). After 60 s, 215 μl of 1 M citric acid was added to stabilize the developed color. Optical density was measured 15 min later at 620 nm. A P$_i$ standard was prepared by using KH$_2$PO$_4$/10% TCA. All P$_i$ measurements were made in triplicate.

Protein extraction and electrophoresis. Levels of MHCs, α-actin, tropomyosin, caldesmon, and calponin were measured in the thoracic aortas from normoxic rats and rats exposed to hypoxia by Western analysis. Aortas from these animals were excised, frozen in liquid nitrogen, then ground with lysis buffer (0.25 M sucrose, 40 mM MOPS-NaOH, pH 7.4, 1 mM EDTA, 2 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor plus 0.5% Nonidet P-40) in a prefrozen mortar and pestle. Lysates were centrifuged, and supernatants were stored at −70°C for protein analysis. Electrophoresis was carried out by using 4--12% gradient SDS-PAGE minigels (Helixx). In addition, 4% SDS-PAGE gels were used for separation of MHC isoforms. Protein concentration was determined by Bradford assay, and appropriate volumes of extraction buffer to produce constant protein loading in each separation of MHC isoforms. Protein concentration was determined by the Bradford assay. From this, the total amount of protein in the sample was calculated to express ATPase activity in moles of P$_i$ released per minute per milligrams of protein.

Immunoblotting. Immunoblotting was the same for SDS-PAGE and urea/glycerol preparations, except that the latter were thoroughly air dried before blocking to fix the protein onto the membrane. Membranes were blocked for 2 h at room temperature with 5% dried milk in Tween 20 Tris-base solution (TTBS). All antibodies were diluted in TTBS with 1% dried milk, with incubations for 1 h (secondary antibody) or 1.5 h (primary antibody) at room temperature. Concentrations varied among primary antibodies and were individually optimized in pilot experiments; secondary antibodies were diluted 1:1,000. Nonspecific binding of the secondary antibody was absent under these conditions. Blots were developed by using enhanced chemiluminescence and were routinely in the linear range of optical density, as determined in pilot studies or by internal standards. Signals were digitized by using a Hewlett-Packard scanner, analyzed with commercially available software (31), and expressed as optical density in arbitrary units.

Proliferating cell nuclear antigen immunohistochemistry. Thoracic aortas from normoxic rats and from rats exposed to hypoxia for 48 h were removed and fixed in 4% parafomaldehyde. After soaking in 30% sucrose solution and paraffin mounting, tissues were cut into 7-mm sections. Sections were immunostained with the proliferating cell nuclear antigen (PCNA)-specific antiserum, Ki67, by using an avidin-biotin-peroxidase method. Briefly, sections were dewaxed in toluene and dehydrated in ethanol, then immersed in 2% hydrogen peroxide to block endogenous peroxidase activity. Sections were permeabilized with 0.2% Triton X-100 and incubated with 10% normal goat serum to reduce nonspecific binding of the antiserum. The serum was drained, and sections were incubated with Ki67 overnight at 4°C. Sections were then washed three times with PBS and incubated with biotin-conjugated anti-mouse IgG for 60 min. After further washing with PBS, samples were incubated with avidin-biotin-peroxidase complex (Vectastain Elite kit, Vector Laboratories, Burlingame, CA) for 60 min at room temperature. Immunoreactive sites were visualized with diaminobenzine and hydrogen peroxide. Preparations were then counterstained with hematoxylin, dehydrated, cleared, and covered with glass coverslips. For negative controls, sections were incubated with antisera that had been neutralized by preincubation with ligand.

Data analysis. Differences among multiple means were analyzed by two-way ANOVA. If ANOVA revealed significant differences, post hoc analysis of differences between individual means was carried out by using the Student-Newman-Keuls procedure. Unless otherwise stated, data are presented as means ± SE in n number of animals, with P < 0.05 representing statistical significance.

RESULTS

Functional studies. Maximum tensions recorded during both phenylephrine- and KCl-induced contractions were reduced in endothelium-denuded aortic rings from rats exposed to hypoxia compared with the normoxic control group (Fig. 2, Table 1).

Phosphorylation of LC20. Stimulation with 10−5 M phenylephrine caused an increase in LC20 phosphorylation in aortic strips from both normoxic rats and rats exposed to hypoxia for 48 h. Hypoxic exposure significantly decreased phosphorylation of LC20 in response to phenylephrine stimulation (Fig. 1). LC20 phosphorylation in unstimulated strips from control and hypoxia-exposed rats did not differ (4.5 ± 2.3 vs. 3.5 ± 1.9% for normoxic and hypoxia-exposed groups, respectively, P = 0.45 for difference).

mATPase activity. The rate of liberation of P$_i$ in the in vitro assay was diminished in aortas from rats exposed to hypoxia for 48 h compared with the norm-
moxic controls (Fig. 3, A and B). LC20 phosphorylation in the in vitro assay after direct activation of myosin light chain kinase by the addition of Ca$^{2+}$/H11001 did not differ between the two groups (Fig. 3 C), indicating that the difference in ATPase activity is not due to a difference in vitro activation of actomyosin ATPase in the reaction mixture.

**Contractile protein expression.** Exposure to hypoxia for 48 h had no effect on the expression of smooth muscle-specific α-actin (Fig. 4, A and B) or tropomyosin (Fig. 4, C and D). In contrast, levels of calponin (Fig. 5, A and B) and caldesmon (Fig. 5, C and D) were increased in aortas from hypoxic rats compared with the normoxic group. Caldesmon expression was approximately doubled after hypoxia ($P < 0.01$), and calponin was increased by more than sevenfold ($P < 0.01$).

Hypoxia also increased expression of the smooth muscle MHC ($P < 0.05$) (Fig. 6). Protein separation using 4% SDS-PAGE gels, followed by electrotransfer and then either nonspecific staining of the membrane (India ink) or SM-1/SM-2 immunoblotting, revealed no differences between hypoxic and normoxic samples in the molecular weight of MHC, suggesting that MHC isoform expression was not altered by hypoxia (data not shown).

**Ki67 immunohistochemistry.** Immunohistochemical staining of aortic tissue slices by using Ki67 monoclonal antibody, specific for PCNA, failed to detect this

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**Table 1. Maximum tensions and −log EC$ _{50} $ values for phenylephrine- and KCl-induced contractions in endothelium-denuded aortic rings**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Maximum Tension, g/mg dry wt</th>
<th>−Log EC$ _{50} $</th>
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<tbody>
<tr>
<td></td>
<td>Normoxia 48-h Hypoxia</td>
<td></td>
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<tr>
<td>Phenylephrine (n = 12 per group)</td>
<td>1.94 ± 0.04</td>
<td>7.86 ± 0.04 M</td>
</tr>
<tr>
<td>KCl (n = 5 per group)</td>
<td>1.24 ± 0.18</td>
<td>14.14 ± 3.30 mM</td>
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Values are means ± SE and were obtained from normoxic rats and rats exposed to hypoxia for 48 h. $^*$P < 0.05 for difference between normoxic and hypoxia-exposed groups.
Fig. 4. Expression of the thin filament proteins α-actin (A and B) and tropomyosin (C and D) is unchanged by hypoxia (A and C: representative blots; B and D: histograms illustrate mean ± SE aortic levels of α-actin and tropomyosin in normoxic and hypoxia-exposed rats; n = 5 per group). P > 0.05 for differences between normoxic and hypoxia-exposed groups.

Fig. 5. Aortic levels of h-caldesmon (A and B) and calponin (C and D) are increased after exposure to hypoxia. A and C: representative blots. B and D: histograms illustrate mean ± SE aortic levels of h-caldesmon and calponin in normoxic and hypoxia-exposed rats (n = 5 per group). **P < 0.01 for difference from the normoxic control group.
antigen in aortic smooth muscle cells in either normoxic rats or in rats exposed to hypoxia for 48 h. In aortas from hypoxic rats, however, the endothelium did show positive staining with this antibody, providing a positive control for the preparation (Fig. 7).

**DISCUSSION**

The results of this study demonstrate that, after exposure to hypoxia for 48 h, the agonist-induced contractions of rat aorta are reduced due to impaired smooth muscle contractility. This decrease in vasoreactivity is associated with a reduction in phosphorylation of LC20 during stimulation with $\alpha$-agonist and a decrease in aortic myofibrillar ATPase activity. Although previous studies have suggested that hypoxia elicits a shift to a proliferative phenotype in vascular smooth muscle cells, we found no evidence of aortic smooth muscle proliferation in this model.

Previous studies of the effects of chronic hypoxia (weeks) on in vivo systemic vasoreactivity have yielded conflicting results. Augmented (1), impaired (10, 17), and unchanged (15) responses to agonist infusions have been reported in various models. Depression of in vivo pressor responses after 4 wk of hypoxia appeared to be mediated by a direct effect on the blood vessels because it was associated with decreased in vitro reactivity of aortic segments (10). Similarly, exposure to prolonged hypoxia of a shorter duration (12–48 h) has been shown to impair reflex pressor responsiveness (16) and in vitro reactivity of arterial segments (2). The mechanisms that mediate the effects of hypoxia on vascular reactivity are as yet unclear and appear to differ among acute (minutes), prolonged (hours to days), and chronic (weeks) durations of exposure (2, 10, 16).

In the present study, we have evaluated the effects of exposure to hypoxia in vivo on the in vitro reactivity of aortic segments. The study does not include experiments designed to dissociate the direct effects of hypoxia from those of changes in flow or neurohumoral mediators. Nevertheless, these factors comprise part of the response to systemic hypoxia, and the hypoxic exposures presented in this study simulate a clinically and physiologically relevant condition. Our results indicate that prolonged hypoxia depresses both phenylephrine- and KCl-induced contraction in endothelium-denuded aortic rings. This finding is consistent with previous results (2) and confirms that, in contrast to
the response to acute hypoxia that elicits systemic arterial relaxation through endothelial release of nitric oxide (3), prostaglandins (25, 32), or hyperpolarizing factor(s) (18, 23), the effect of prolonged hypoxia is due to a change in aortic smooth muscle function. The magnitude of the decrease in the response to phenylephrine in the present study is comparable to the reduction in contraction to KCl (37 and 30% reductions in maximum tensions, respectively). This and the lack of effect of hypoxia on the EC_{50} for phenylephrine argue against mediation of the effect by mechanisms selectively affecting adrenoreceptor-mediated responses. Because the response to maximum depolarization is reduced, this effect cannot be attributed to smooth muscle membrane hyperpolarization.

Our data reveal that LC_{20} phosphorylation during stimulation with phenylephrine is reduced in aortic strips from rats exposed to hypoxia, which indicates that the decrease in contractility is, at least in part, due to a failure of activation of contraction. Although this pertains to \( \alpha \)-agonist-induced contraction in our present study, the impairment of contraction also affects the response to depolarization and so is likely mediated by mechanisms downstream of the cell membrane. Altered expression of myosin light chain kinase or the capacity for its activation by \( \mathrm{Ca}^{2+} \)-calmodulin (19, 20) and/or changes in the activity of the opposing myosin light chain phosphatase (11) are mechanisms that would affect activation pathways common to both stimuli. Further studies are now needed to assess the mechanisms’ roles in the reduced reactivity of systemic vascular smooth muscle after hypoxia.

Because the ATPase activity of the myosin molecule provides the chemical energy for cross-bridge cycling and contraction, we examined this activity in aortas from normoxic rats and rats exposed to hypoxia. Aortic mATPase activity was decreased in the hypoxia-exposed group, suggesting that hypoxia leads to changes that limit cross-bridge cycling energetics and contribute to the impairment of vasoreactivity. mATPase activity may be affected by changes in the protein composition of either thin or thick myofilaments. We found that the thin filament proteins actin and tropomyosin were unchanged by hypoxia, whereas the smooth muscle-specific isoforms of calponin and caldesmon were increased. Smooth muscle caldesmon binds to actin, tropomyosin, and myosin, and its interaction with these proteins potently inhibits actin-activated myosin ATPase activity (24). Similarly, calponin binds with high affinity to actin and inhibits actin-activated ATPase activity of smooth muscle myosin (the movement of actin over myosin in vitro) and force development in permeabilized smooth muscle strips and single smooth muscle cells (34–36). Therefore, enhanced expression of these two regulatory proteins suggests a possible explanation for the reduction in mATPase activity, which merits further evaluation.

Hypoxia has been shown to induce proliferation and/or modulation from a contractile to a proliferative phenotype in mesangial and smooth muscle cells (7, 8, 27, 37). Reduced content of smooth muscle-specific isoforms of thick- and thin-filament proteins and the appearance of immature or nonmuscle isoforms are characteristic of this phenotypic change (6, 13, 38). Furthermore, in pulmonary arterial smooth muscle, a shift in MHC expression in favor of lower molecular mass (196–198 kDa) nonmuscle isoforms has been reported after chronic hypoxia (14 days) and correlated with the development of impaired reactivity of these vessels to agonists (26). Accordingly, we proposed that transition to the proliferative phenotype with a corresponding reduction in smooth muscle-specific contractile-protein expression could account for the functional and biochemical changes that we observed in aortic smooth muscle after prolonged hypoxia. In contrast to our hypothesis, however, we found that levels of smooth muscle \( \alpha \)-actin are unchanged, and smooth muscle myosin, caldesmon, and calponin are increased in aortas from hypoxic compared with normoxic animals. Finally, aortic smooth muscle cells did not express the proliferative marker PCNA under the conditions that produced contractile impairment. Importantly, the PCNA-specific antibody did label endothelial cells, providing a positive control for these preparations. Neither proliferation nor reduction in smooth muscle-specific contractile protein expression could, therefore, be substantiated.

In summary, our results indicate that hypoxia attenuates the contractile responses of aortic smooth muscle, decreases LC_{20} phosphorylation after a contractile stimulus, and decreases mATPase activity. Reflex sympathetic augmentation of systemic vascular tone is essential to maintaining arterial blood pressure and vital organ perfusion during hemorrhage and other hypotensive stresses. Adrenoreceptor-mediated adjustment of systemic arterial and arteriolar tone is also important in the adaptations that redistribute blood flow toward vital organs and augment oxygen extraction during reductions in oxygen delivery (5, 9). Impairment of the contractile capacity of the systemic vascular smooth muscle will handicap these responses and compromise the ability to defend vital organ oxygen supply. Smooth muscle contraction relies on LC_{20} phosphorylation for activation and on mATPase for energy. This study demonstrates that these two crucial processes are impaired by prolonged hypoxia (48 h). These changes may, therefore, play a pathophysiological role in events that evolve over this time frame, such as the organ system dysfunction that occurs during reductions in oxygen delivery due to shock and cardiopulmonary diseases.

REFERENCES


