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Jayne C. Carberry
University College Dublin

Clodagh McMorrow
University College Dublin

Aidan Bradford
Royal College of Surgeons in Ireland, abradford@rcsi.ie

James FX Jones
University College Dublin

Ken d. O'Halloran
University College Cork

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Effects of sustained hypoxia on sternohyoid and diaphragm muscle during development

J. Carberry[1,], C. McMorrow[1], A. Bradford[2], J.F.X. Jones[1], K.D. O'Halloran[3].

[1]UCD School of Medicine and Medical Science, University College Dublin, Dublin 4, Ireland, [2]Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin 2, Ireland, [3]Department of Physiology, School of Medicine, University College Cork, Cork, Ireland.

Address for correspondence:

Jayne C. Carberry

Neuroscience Research Australia

PO Box 1165

Sydney

NSW 2031

Australia

Tel:+61-2-9399 1834

Email: j.carberry@neura.edu.au

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Abstract

Sustained hypoxia (SH) is a dominant feature of respiratory disease. Despite the clinical significance, the effects of SH on respiratory muscle form and function during development are relatively under-explored.

Wistar rats were exposed to 1 week of SH (ambient pressure = 450mmHg) or normoxia at various time-points during development. Sternohyoid and diaphragm muscle contractile and endurance properties were assessed *in vitro*. Muscle SDH and MHC composition were determined. The role of reactive oxygen species in hypoxia-induced muscle remodelling was assessed.

SH increased sternohyoid muscle force and fatigue in early but not late development – effects that persisted after return to normoxia. Hypoxia-induced sternohyoid muscle fatigue was not attributable to fibre type transitions or a decrease in oxidative capacity. Chronic supplementation with the superoxide scavenger – Tempol did not prevent hypoxia-induced sternohyoid muscle fatigue, suggesting that mechanisms unrelated to oxidative stress underpin hypoxia-induced maladaptation in sternohyoid muscle. SH had no effect diaphragm muscle fatigue.

We conclude that there are critical windows during development for hypoxia-induced airway dilator muscle maladaptation. SH-induced impairment of upper airway muscle endurance may persist into later life. Upper airway muscle dysfunction could have deleterious consequences for the control of pharyngeal airway calibre *in vivo*.

Introduction

The effector organs of the respiratory control system – the striated muscles of breathing – play a critical role in oxygen and acid-base homeostasis. Respiratory muscle remodelling may have adaptive or maladaptive consequences for respiratory control. SH is commonly encountered in healthy individuals at high altitude and in patients with various respiratory diseases (e.g. COPD).

Skeletal muscle has the capacity to adapt to SH by way of modulation of skeletal muscle vasculature [1, 2], muscle enzyme activities [3-6], muscle fibre size and distribution [7-10] and contractile performance [11-13]. Moreover, SH has been shown to elicit functional plasticity in respiratory muscles [9, 10] in a manner that differs to the phenotypic changes occurring in limb muscles [10, 14, 15].

Hypoxia is common in premature babies and in infants with congenital heart disease or bronchopulmonary dysplasia [16, 17]. SH exposure during development elicits plasticity in respiratory control [18, 19] and was shown to decrease diaphragm muscle specific tension [20]. Moreover, diaphragm muscles of SIDS infants have reportedly fewer type I fatigue resistant fibres than healthy infants [21]. In general however, there is a great paucity of information concerning the effects of SH on respiratory muscle during development, despite the obvious clinical relevance.

We therefore sought to determine the effects of exposure to SH at different stages of early development on rat diaphragm and pharyngeal dilator (sternohyoid) muscle contractile and endurance properties, oxidative capacity, myosin heavy chain (MHC) fibre distribution and cross-sectional area. In addition, we sought to examine the

putative role of reactive oxygen species (ROS) in SH-induced muscle plasticity. Hypoxia has been shown to increase free-radical species in muscle [22, 23]. Redox signalling can influence transcriptional activators leading to altered muscle phenotype [24]. Furthermore, muscle-derived ROS and NO target contractile proteins, ryanodine receptor calcium channels, SERCA and Na^+-K^+ pumps [9, 25-27] thus altering muscle contractile and endurance properties. The superoxide scavenger Tempol recovered chronic intermittent hypoxia-induced upper airway muscle impairment in a rat model of sleep apnoea [28, 29]. This suggests a role for superoxide ($O_2^{\bullet -}$) or downstream oxidants in hypoxia-induced muscle dysfunction. Therefore, given the potential clinical application, we tested the hypothesis that antioxidant treatment would prevent SH-induced aberrant functional remodelling in neonatal rat respiratory muscles. Part of this study was published in short form following presentation at the XIth Oxford Conference, Nara, Japan, 2009 [30].

Methods

A detailed description of the methods is provided in the online supplement.

Animals

Experiments were performed on Wistar rats of mixed sex. For SH studies, litters (with respective dams) were placed in a hypobaric chamber at an ambient pressure of 450 mmHg for 7 days starting at postnatal day (P)1 (n=7), P6 (n=8), or P11 (n=8); muscle function studies were conducted at P19. In separate litters, hypoxic exposure began in weaned animals starting at P21 or P31 and experiments were performed at P29 (n=8) and P39 (n=7) respectively. Age-matched controls (P19, n=9; P29, n=11; P39, n=8) were maintained under normobaric conditions (~760 mmHg) in parallel. One additional litter was exposed to SH (starting at P11) and received daily oral administration of Tempol (100mg/kg, n=7) - a superoxide scavenger, starting 3 days prior to hypoxic exposure and continuing until P19. The n values shown above represent the total number of animals used for each group. For some protocols, we did not obtain data from all animals due to technical difficulties and for those data sets the adjusted n value is reported in the figure legend.

Effects of SH on respiratory muscle force and fatigue

The sternohyoid and diaphragm muscles from normoxic and SH rats were excised and contractile and endurance properties were determined *in vitro*. Muscles were snap frozen in isopentane cooled by liquid nitrogen. The samples were stored at -80°C for later use.

Effects of SH on respiratory muscle MHC fibre type and oxidative enzyme activity

MHC Immunocytochemistry

Myosin heavy chain (MHC) fibre types were determined by indirect immunofluoresence with monoclonal antibodies on serial transverse sections of muscle from normoxic and SH groups. MHC isoforms were detected using a triple-labelling technique employing a cocktail of antibodies as previously described [9]. This enabled the detection of MHC I, IIA and IIB on the same tissue section. *Pure* IIX fibres were identified on separate sections using an indirect approach [9].

Succinate dehydrogenase

Serial 10μm transverse sections of normoxic and SH respiratory muscles were cut at -22°C on a cryostat. The activity of the mitochondrial enzyme succinate dehydrogenase (SDH) was determined as an index of the oxidative capacity of the muscles. Muscle samples from all of the experimental groups were processed together under identical conditions. Control (blank) reactions were performed with omission of the primary substrate.

Data Analysis

Specific force was calculated in N/cm^2 of muscle cross-sectional area (CSA). Non-linear regression (curve-fit) analysis was employed (Graph Pad Prism) for force-frequency relationship, allowing us to determine EF_{50} values (*i.e.* stimulus frequency producing 50% of peak force). For the fatigue trials, muscle forces were measured at time zero (initial force) and at 1 min intervals and a fatigue index (*i.e.* ratio of force at 5 min of fatigue to initial force) was determined. Scion Image™ software was used to

determine optical density as a measure of SDH activity. We quantified muscle MHC fibre types by calculating their relative contribution to muscle CSA (i.e. areal density) using Cell A™ software using 4 square test frames (200 x 200 μm each) per muscle section. The test frames were randomly placed over muscle sections. All data are expressed as mean ± SEM.

Statistical Analysis

Statistical comparisons were made between normoxic and SH groups using one-way ANOVA (body mass, contractile kinetics, peak tetanic force and fatigue index) with a Newman-Keuls post-hoc test (comparing all groups) and Dunnett's post-hoc test (comparing all groups to control) where appropriate. A two-tailed unpaired Student's *t* test was used to compare data sets in P29 and P39 animals (normoxia vs. sustained hypoxia). $P<0.05$ was the criterion for significance in all tests.

Results

Effects of SH on body mass, haematocrit and cardiac mass

Values are shown in Table E1. Exposure to SH in early life (pre-weaning groups) had no significant effect on body mass (Table E1). SH caused a significant decrease in body mass in P29 and P39 groups. SH increased haematocrit and right ventricle mass in all groups (Table E1).

Effects of SH on respiratory muscle force and fatigue

Sternohyoid

The effects of SH on sternohyoid muscle twitch force and contractile kinetics are shown in Table E2. SH increased sternohyoid force-frequency relationship (Fig. E2) and peak force (Fig. 1B) if exposed to hypoxia during the first month of life. SH had no effect on peak force when SH exposure began in late development (P39; Fig 1. C). SH did not affect sternohyoid muscle EF_{50} in any age group (data not shown). SH significantly increased sternohyoid muscle fatigue when exposure began pre-weaning (Fig. 2A) but not post-weaning (Figs. 2B and 2C).

Diaphragm

The effects of SH on diaphragm muscle twitch force and contractile kinetics are shown in Table E3 (online supplement). SH significantly increased diaphragm peak isometric force in rats exposed to hypoxia beginning P1 (Fig. 3A). SH had no effect on peak force in all other groups (Fig. 3). SH had no effect on diaphragm EF_{50} values (data not shown). SH had no significant effect on diaphragm fatigue index (Fig. 4).

Effects of SH on respiratory muscle MHC fibre type and oxidative enzyme activity

Sternohyoid

SH had no significant effect on sternohyoid muscle SDH activity at any age during development (data not shown). Representative immunofluorescent images are shown in Fig. 5. Areal density values and fibre CSAs are shown in Table 1. SH in early life (pre-weaning groups) had no effect on areal density values of MHC type I, IIA and IIB isoforms (Table 1). No *pure* IIx fibres were detected in P19 sternohyoid. SH exposure was associated with significant fibre hypertrophy for some exposure periods during early development (Table 1).

Diaphragm

SH had no significant effect on diaphragm muscle SDH activity (data not shown). Representative immunofluorescent images are shown in Fig. 5. Areal density values and fibre CSAs are shown in Table 2. SH in early life (pre-weaning groups) had no major effect on areal density values and CSAs of MHC fibre types (Table 2).

Effects of antioxidant treatment on SH-induced increase in sternohyoid muscle force and fatigue

Chronic administration of Tempol (100 mg/kg p.o.) had no effect on SH-induced increase in sternohyoid muscle peak force and fatigue (Fig. E1).

Discussion

The major findings of this study are: 1) SH increases sternohyoid (pharyngeal dilator) muscle force frequency and fatigue, effects that persist during neonatal development; 2) SH-induced respiratory muscle plasticity is age-dependent and differentially expressed in airway dilator and thoracic pump muscles; 3) SH-induced fatigue in sternohyoid muscle is not related to changes in SDH activity or MHC isoform composition; 4) Chronic administration of the superoxide scavenger – Tempol does not prevent SH-induced sternohyoid muscle fatigue.

Effects of SH on respiratory muscle force and fatigue

There is a general paucity of information concerning the effects of SH on respiratory muscle, which is quite surprising, given the clinical significance. Respiratory muscle remodelling following SH was reported in adult rats and differential effects of SH on airway dilator and thoracic pump muscles have been noted [9, 12]. Of interest, diaphragm muscle is either resistant [10-12] or even shows improved endurance [9] following SH. A recent study by Gamboa and Andrade [10] highlights the potential importance of a down-regulation in uncoupling protein 3 (UCP-3) in hypoxic adaptation in respiratory muscle. This adaptation may be unique to the diaphragm since SH causes limb muscle fatigue [10, 14, 15] and fatigue is also reported for the adult sternohyoid muscle in one [12] but not another [9] study. The present study extends these observations showing that one week of SH is sufficient to cause sternohyoid muscle dysfunction in neonatal rats if the exposure occurs early in life. It is also apparent that there are differential effects of SH within respiratory muscles (sternohyoid vs. diaphragm), which is consistent with previous studies on limb muscles [11, 13, 14]. Whether the functional effects of SH are dependent upon the

structural makeup and metabolic profiles of various muscles remains to be determined. Slow twitch and fast twitch fibres differ in ATP utilisation, oxidative capacity and blood flow distribution, thus it is plausible that decreased oxygen availability might have diverse consequences for muscle physiology. Interestingly, Howlett and Hogan [31] showed that muscle composed of glycolytic fibres is more susceptible to decreased oxygen supply *in vivo* compared to muscle composed of oxidative fibres.

In light of this, differences in structural phenotype may explain the observation of age-dependent plasticity following SH. The structural makeup of skeletal muscle undergoes developmental changes towards the adult phenotype from birth. Respiratory muscles in rat undergo significant changes in fibre type profile during the first month of life [32, 33]. Kass and Bazzy [20] found that impairment of diaphragm muscle force following SH decreased with age. We also predicted age-dependent muscle plasticity. In our study, SH exposure in early developing animals (1st month of life) resulted in alterations in sternohyoid muscle function and these effects persisted for several days upon return to normoxia. Conversely, when animals were exposed to the same hypoxic stimulus at an older age no functional effects were observed. Thus, there would appear to be critical periods during development when the sternohyoid muscle is most vulnerable to hypoxia-induced increased fatigue. Moreover, it appears that SH-induced functional effects persist for at least as long as the initial SH exposure. Whether the functional changes reported herein for the sternohyoid, namely increased force and fatigue, represents adaptive or maladaptive remodelling is less clear but these changes may have consequences for upper airway control in later life. Our results suggest the intriguing possibility that early life hypoxia may predispose to

upper airway instability in later life, including increased propensity for obstructive apnoea [34, 35]

Effects of SH on respiratory muscle MHC fibre type and oxidative enzyme activity

The mechanism for the SH-induced decrease in sternohyoid endurance is unknown. Changes in skeletal muscle endurance are often associated with altered oxidative enzyme activities [36-38]. In humans, reductions in oxidative capacity following hypoxia have been reported [4, 7, 39, 40]. However, SH-induced changes in sternohyoid muscle endurance were not attributable to changes in SDH activity. This contrasts with other animal studies where SH decreased oxidative enzyme activity [5, 6, 41]. However, in these studies, hypoxic exposures ranged from 2-10 weeks. We acknowledge that only one oxidative enzyme was assessed in our study and it is possible that the activity of others (e.g. citrate synthase, cytochrome c oxidase) was decreased. Other studies have reported reductions in oxidative enzyme activity in limb muscles concomitant with increases in glycolytic enzyme activity [4, 41] following SH. In our study, we did not measure glycolytic enzyme activity. Recently, our laboratory has shown that sternohyoid muscle fatigue during development correlates with age-related increases in glycerol phosphate dehydrogenase (GPDH) activity [42]. Increased GPDH activity while SDH activity remains unchanged alters the oxidative/glycolytic ratio. Thus, it appears that a decrease in the ratio of oxidative-to-glycolytic enzymes results in a more fatiguable muscle during development [42]. It is possible that glycolytic enzyme activity is enhanced during SH. Further investigation of respiratory muscle glycolytic enzyme activity following SH may yield clues as to the mechanisms underpinning hypoxia-induced sternohyoid muscle dysfunction.

We postulated that changes in MHC isoform expression were responsible for the changes in muscle contractility. A slow-to-fast fibre transition would be consistent with the increases in sternohyoid isometric force and fatigue that we observed. However, SH did not affect MHC areal density in the sternohyoid, thus a slow-to-fast phenotypic transition does not underlie the hypoxia-induced alterations in muscle contractility and endurance. Reports in the literature of the effects of SH on skeletal muscle phenotype are conflicting. Human studies have reported increased proportions of slow fibres in high altitude natives compared to sea-level dwellers [43, 44]. However, in simulated altitude studies no change in fibre type profile was found [39]. In limb muscles of COPD patients, there is evidence of a reduced compliment of slow fibres with shifts towards higher proportions of fast fibres [45-48]. In the diaphragm of COPD patients, it is thought that the reverse happens i.e. increases in slow and decreases in IIa fibres [49, 50], but whilst COPD is characterised by chronic hypoxia the disease is complex and many other factors contribute to respiratory muscle remodelling.

Conflicting reports concerning hypoxia-related fibre type transitions extend to animal models of SH. Some studies report no significant change in fibre distribution following SH [9, 51, 52]. Mortola and Naso [53] showed that 9 months of hypoxia (~14% O_2), but not 60 days, induced slow-to-fast fibre type transitions in rodent limb and diaphragm muscle. Conversely, Bigard and colleagues [54] showed that 4 weeks of SH was sufficient to induce slow-to-fast fibre transitions in the soleus of the developing rat [54], although the hypoxic exposure was more severe (~10% O_2). Similar reports of a slow-to-fast fibre transition have been reported in adult rat limb muscles following 4 and 5 weeks of SH at 10% and ~15% O_2 respectively [14, 55].

Shiota and co-workers [11] reported a shift from fast-to-slow phenotype in the extensor digitorum longus (EDL) muscle, with no reported changes in the soleus muscle. A 10 week exposure to sustained hypoxia in developing rats (1 month old) caused a relative increase in the proportion of IIa fibres and decrease in the slow fibre distribution of the soleus muscle, whereas the EDL increased in IIa but decreased in IIb fibre proportions [13]. Thus, it appears that hypoxia-induced fibre type transitions depend on the structural makeup of the muscle [11, 13]. This supports the notion that fast twitch muscles are more susceptible to fatigue than slow twitch muscles during SH. It has also been shown that moderate systemic hypoxia can affect muscle fibre types in different regions within a muscle [56]. Thus, results may depend on the method of analysis (whole muscle vs. regional areas). Potential findings may be lost if regional data are grouped [56]. In our older animals, sternohyoid muscle was cut into longitudinal strips for structural and functional analysis. This may have influenced our findings if regional effects are a factor. Also it should be noted that structural and functional remodelling is likely dependent on the intensity and duration of hypoxic exposure making comparisons between different studies difficult to interpret.

Effects of antioxidant treatment on SH-induced increase in sternohyoid muscle force and fatigue

Tempol is a stable membrane permeable nitroxide compound that acts as a SOD mimetic and a superoxide scavenger [57]. Superoxide scavengers were shown to protect diaphragm maximum tetanic force in hypoxia *in vitro* [58]. Furthermore, Tempol was effective in the protection of $O_2^{\bullet-}$ - induced impairment in tetanic force and maximum Ca^{2+} - activated force due to heat stress [59] and hypoxia [28]. In adult

rats, Tempol administered *in vitro* improved upper airway muscle tension, an effect which persisted during repeated muscle activation [28]. Moreover, Tempol-incubated adult sternohyoid showed improved force and performance in the early phase of fatigue during hypoxia [28]. Recent studies in our laboratory have shown that daily administration of Tempol prevents chronic intermittent hypoxia-induced upper airway muscle dysfunction [29]. We speculated that oxidative stress was also implicated in SH-induced sternohyoid muscle fatigue. However, daily administration of Tempol to neonatal pups did not prevent SH-induced effects in the sternohyoid muscle. Our finding suggests that oxidative stress does not contribute to hypoxia-induced functional remodelling in sternohyoid muscle during development. In isolated perfused rat diaphragm, significant increases in $O_2^{\bullet-}$ during fatigue were determined by measuring the level of cytochrome c reduction but superoxide dismutase (SOD) did not ameliorate diaphragm fatigue [60]. As such, SOD-mimetics may not always be the best approach for protection of skeletal muscle function during oxidative stress (hypoxia). Alternatively, other free radicals such as nitric oxide (NO) may have a more important regulatory role in hypoxia-induced muscle plasticity. Indeed, recent findings from our laboratory have implicated NO in diaphragm muscle adaptation to sustained hypoxic stress. [9]. Additionally, we have shown that NOS inhibition *in vitro* has a positive inotropic effect on the sternohyoid at P19 (unpublished observations), consistent with reports that NO has an inhibitory action in skeletal muscle contractility [61]. SH may alter sternohyoid muscle NOS expression and activity and such changes may underpin the functional changes reported in this study.

Limits and strengths of the study

Our data is derived from isolated muscle function studies. This preparation offers many advantages allowing a study of the effects of SH on respiratory myocyte function under controlled conditions (stimulus, oxygen, temperature etc) such that changes in muscle contractile and endurance properties can be attributed to intrinsic changes at the level of the muscle itself. However, this approach also has significant limitations. It is difficult to translate the observation of sternohyoid muscle fatigue to the *in vivo* setting, where for example there may be sufficient or otherwise neural compensation for increased muscle fatigue. As such we do not know if the control of airway calibre is adversely affected in SH-treated rats. Moreover, our data are derived from an animal model and the reader should exercise caution in extrapolating the findings to human infants, though we argue that the findings may have relevance to neonatal hypoxia. However, it is not clear if SH during early life in humans is associated with airway dilator muscle dysfunction and studies addressing this gap in our knowledge are necessary. The group sizes in our study are relatively small and it is possible that some comparisons (particularly in the context of respiratory muscle structure) are underpowered for statistical purposes perhaps contributing to the apparent lack of effect for some parameters.

Summary and Conclusion

In summary, we observed SH-induced functional effects in neonatal but not adolescent sternohyoid muscle. Conversely, the diaphragm muscle was resistant to hypoxia-induced functional change. Our findings are consistent with the view that there are critical 'windows' during development wherein hypoxia-induced maladaptation may occur with potentially long-lasting effects. Functional alterations

in the sternohyoid muscle were not attributable to fibre type transitions or decreased oxidative capacity. Antioxidant treatment did not prevent CH-induced sternohyoid muscle fatigue suggesting that oxidative stress is not the underlying mechanism driving hypoxic maladaptation in developing sternohyoid muscle. Respiratory muscle impairment is implicated in a range of respiratory disorders and dysfunction can ultimately lead to respiratory failure. Adjunct therapies aimed at improving airway dilator muscle performance during hypoxia may prove beneficial in the treatment of respiratory disorders. Thus, the molecular mechanism of hypoxia-induced muscle dysfunction warrants further investigation.

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Table 1. Sternohyoid Muscle Fibre Areal Density and Cross-Sectional Area in Normoxic and SH rats

	Normoxia	Hypoxia $_{1-8}$	Hypoxia $_{6-13}$	Hypoxia $_{11-18}$
Sternohyoid				
Areal Density (%)				
I	7 ± 1	10 ± 2	7 ± 1	9 ± 1
IIa	27 ± 2	32 ± 3	31 ± 3	30 ± 2
IIx	0 ± 0	0 ± 0	0 ± 0	0 ± 0
IIb	41 ± 2	36 ± 2	30 ± 4	40 ± 4
CSA (μm^2)				
I	379 ± 14	495 ± 20*	374 ± 25	477 ± 24*
IIa	334 ± 14	408 ± 31	339 ± 21	414 ± 39
IIx	-	-	-	-
IIb	746 ± 51	790 ± 41	651 ± 32	1017 ± 84*

Group data (mean ± SEM) for sternohyoid muscle areal density (%) and CSA (μm^2) of MHC I, IIa, IIx and IIb fibres. There were no *pure* IIx fibres in the P19 sternohyoid muscle. Statistical analysis was performed using Kruskal-Wallis non-parametric tests with Dunn's multiple comparison test or one-way ANOVA followed by Dunnett's post-hoc test where appropriate. * indicates significant difference from normoxia; $P<0.05$, n=5-9 for all groups.

Table 2. Diaphragm Muscle Fibre Areal Density and Cross-Sectional Area in Normoxic and SH rats

	Normoxia	Hypoxia $_{1-8}$	Hypoxia $_{6-13}$	Hypoxia $_{11-18}$
Diaphragm				
Areal Density (%)				
I	21 ± 2	22 ± 3	18 ± 5	20 ± 3
IIa	53 ± 4	60 ± 3	60 ± 1	40 ± 2*
IIx	0 ± 0	0 ± 0	0 ± 0	6 ± 3*
IIb	0 ± 0	1 ± 1	1 ± 1	3 ± 1*
CSA (μm^2)				
I	663 ± 53	712 ± 125	674 ± 86	712 ± 34
IIa	671 ± 49	621 ± 41	600 ± 45	688 ± 19
IIx	-	-	-	1089 ± 102 (4/7)
IIb	-	1351 (1/6)	730 (1/6)	1184 ± 180 (3/7)

Group data (mean ± SEM) for diaphragm muscle areal density (%) and CSA (μm^2) of MHC I, IIa, IIx and IIb fibres. There were no *pure* IIx and IIb fibres in the P19 normoxic diaphragm muscle. Statistical analysis was performed using Kruskal-Wallis non-parametric tests with Dunn's multiple comparison test or one-way ANOVA followed by Dunnett's post-hoc test where appropriate. * indicates significant difference from normoxia; $P<0.05$, n=6-9 for all groups. Numbers in parentheses indicate animals per group.

FIG. 1 Values (mean ± SEM) for sternohyoid peak isometric force (N/cm^2) in rats exposed to normoxia or sustained hypoxia (SH) for 7 days starting at P1, P6, P11, P21 or P31. Muscle studies were performed at P19 (A), P29 (B) and P39 (C). Statistical analysis was performed using (A) one-way ANOVA (P=0.14), see Fig. E2 for force-frequency relationship (P19); Student's unpaired *t* test for B (P=0.05) and C (P=0.27); n=6-8 for all groups.

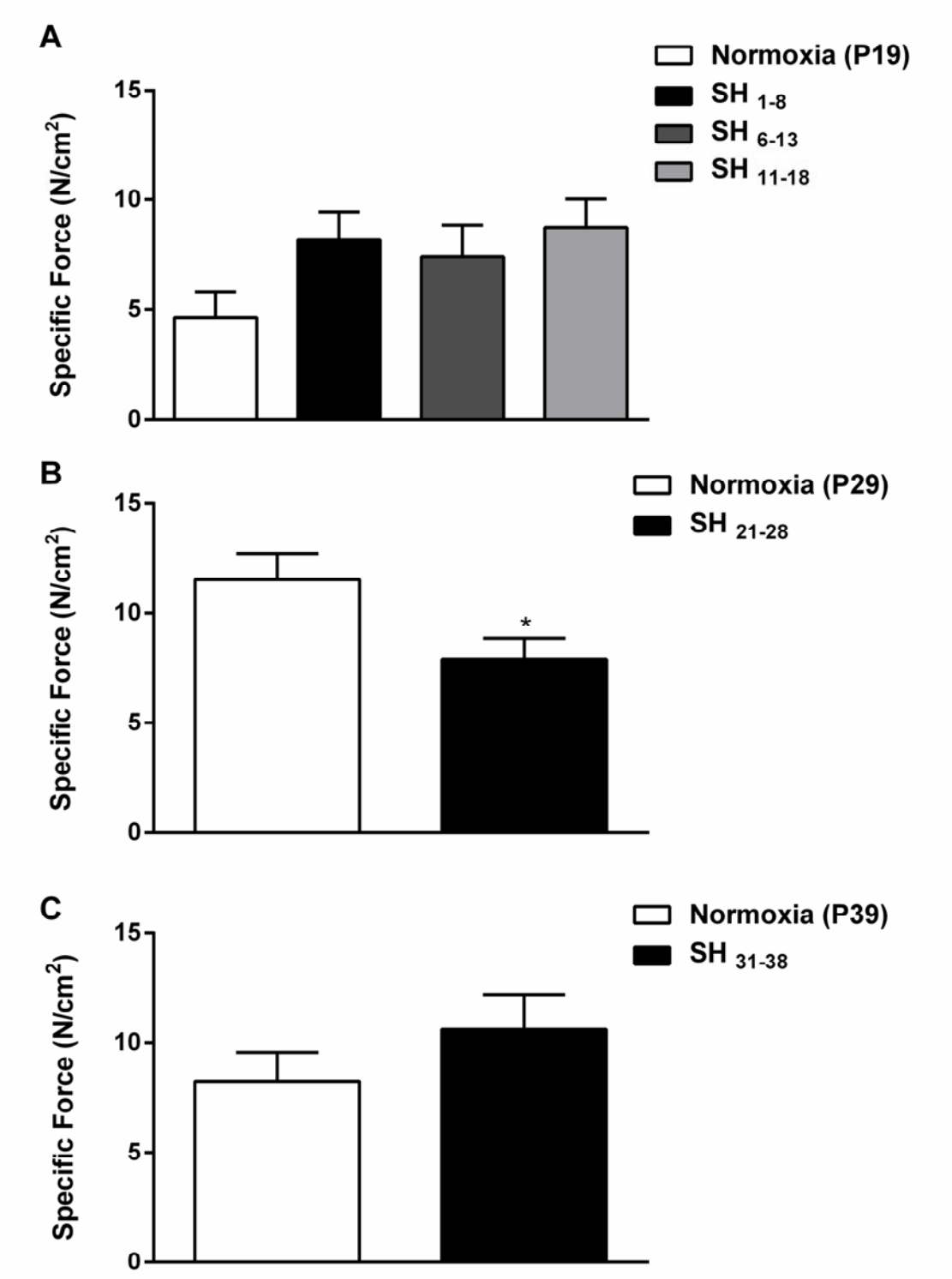

FIG. 2 Values (mean ± SEM) for sternohyoid fatigue index in rats exposed to normoxia or sustained hypoxia (SH) for 7 days starting at P1, P6, P11, P21 or P31. Muscle studies were performed at P19 (A), P29 (B) and P39 (C). * indicates significant difference from normoxia, one-way ANOVA (P=0.02) with Dunnett's post-hoc test, P<0.05 (A); Student's unpaired *t* test for B (P=0.26) and Mann Whitney *t* test C (P=0.28); n=6-8 for all groups.

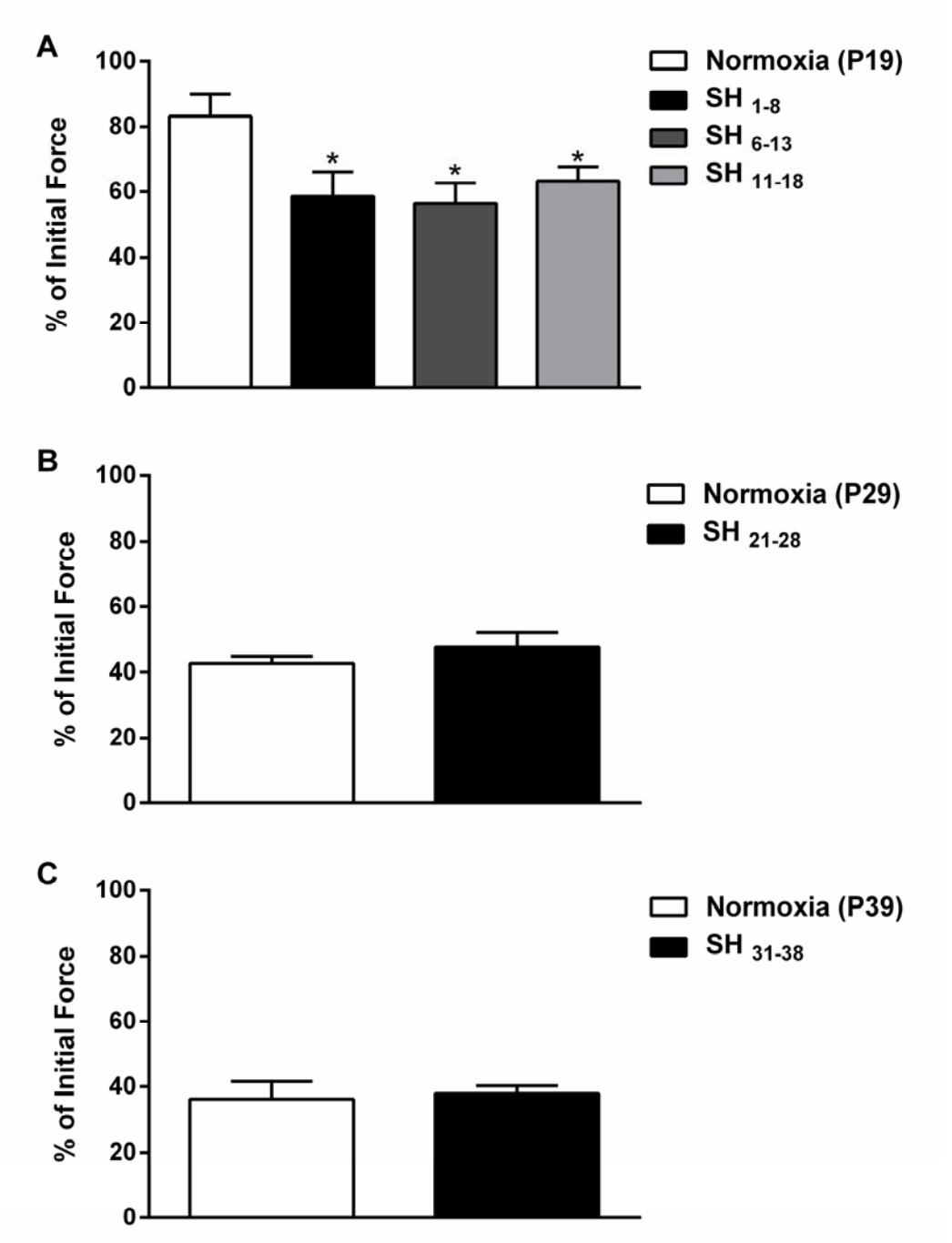

FIG. 3 Values (mean ± SEM) for diaphragm peak isometric force (N/cm^2) in rats exposed to normoxia or sustained hypoxia (SH) for 7 days starting at P1, P6, P11, P21 or P31. Muscle studies were performed at P19 (A), P29 (B) and P39 (C). * indicates significant difference from normoxia, one-way ANOVA (P=0.02) with Dunnett's post-hoc test (A), P<0.05; Student's unpaired *t* test for B (P=0.09) and C (P=0.99); n=5-8 for all groups.

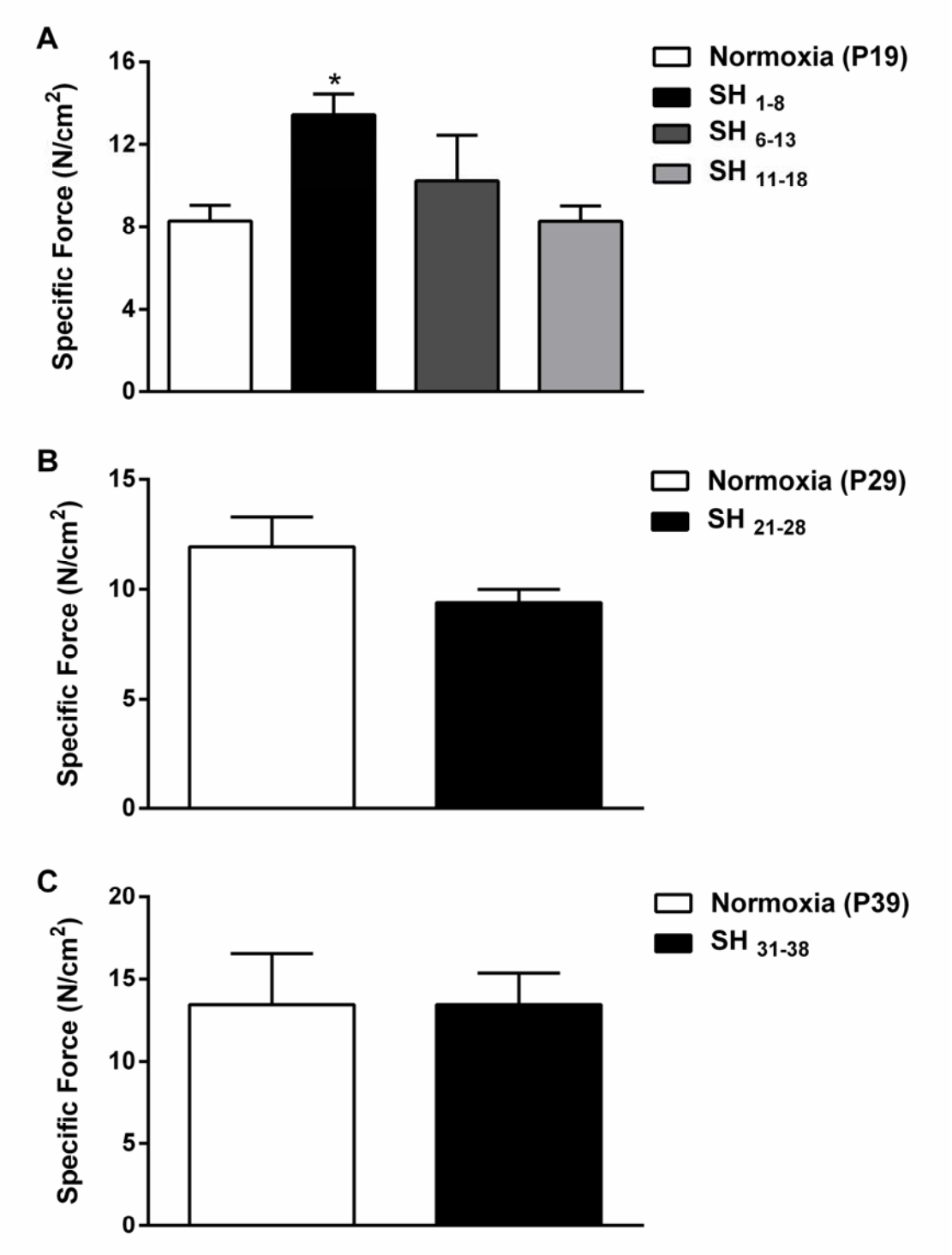

FIG. 4 Values (mean ± SEM) for diaphragm fatigue index in rats exposed to normoxia or sustained hypoxia (SH) for 7 days starting at P1, P6, P11, P21 or P31. Muscle studies were performed at P19 (A), P29 (B) and P39 (C). Statistical analysis was performed using one-way ANOVA (P=0.08) A, Student's unpaired *t* test for B (P=0.58) and C (P=0.76); n=5-8 for all groups.

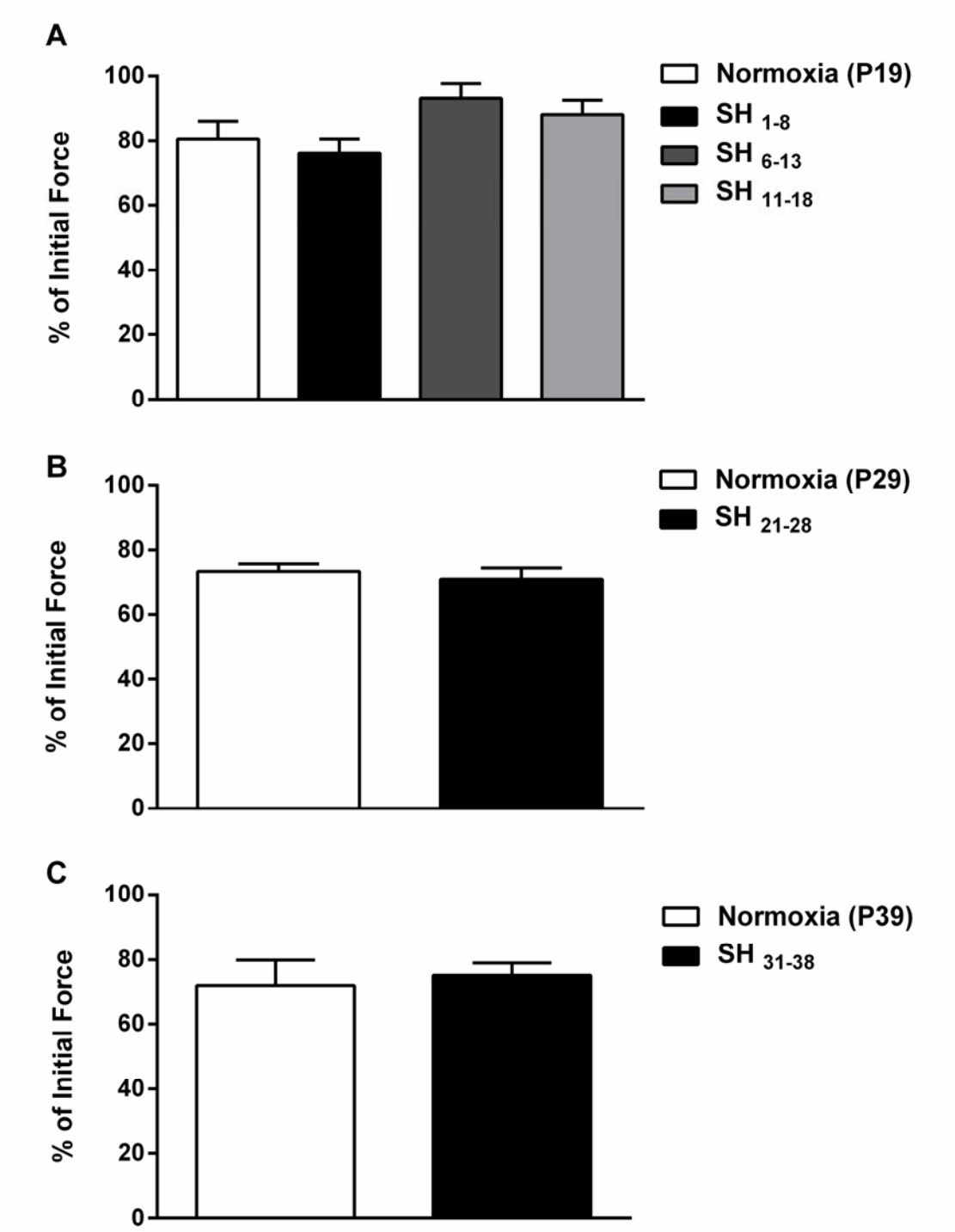

FIG. 5 Immunofluorescent images of P19 sternohyoid (STERNO) and diaphragm (DIA) muscle following incubation with antibodies against myosin heavy chain (MHC) I (blue), IIa (red) and IIb (green). The basement membrane protein - laminin is labelled (green) in the diaphragm image. Scale bar represents 200μm.