

# Tempol Ameliorates Pharyngeal Dilator Muscle Dysfunction in a Rodent Model of Chronic Intermittent Hypoxia

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Respiratory muscle dysfunction is implicated in the pathophysiology of obstructive sleep apnea syndrome (OSAS), an oxidative stress disorder prevalent in men. Pharmacotherapy for OSAS is an attractive option, and antioxidant treatments may prove beneficial. We examined the effects of chronic intermittent hypoxia (CIH) on breathing and pharyngeal dilator muscle structure and function in male and female rats. Additionally, we tested the efficacy of antioxidant treatment in preventing (chronic administration) or reversing (acute administration) CIH-induced effects in male rats. Adult male and female Wistar rats were exposed to alternating cycles of normoxia and hypoxia (90 s each;  $FiO_2 = 5\%$   $O_2$  at nadir;  $SaO_2 \sim 80\%$ ) or sham treatment for 8 h/d for 9 days. Tempol (1 mM, superoxide dismutase mimetic) was administered to subgroups of sham- and CIH-treated animals. Breathing was assessed by whole-body plethysmography. Sternohyoid muscle contractile and endurance properties were examined *in vitro*. Muscle fiber type and cross-sectional area and the activity of key metabolic enzymes were determined. CIH decreased sternohyoid muscle force in male rats only. This was not attributable to fiber transitions or alterations in oxidative or glycolytic enzyme activity. Muscle weakness after CIH was prevented by chronic Tempol supplementation and was reversed by acute antioxidant treatment *in vitro*. CIH increased normoxic ventilation in male rats only. Sex differences exist in the effects of CIH on the respiratory system, which may contribute to the higher prevalence of OSAS in male subjects. Antioxidant treatment may be beneficial as an adjunct OSAS therapy.

**Keywords:** hypoxic ventilatory response; myosin; obstructive sleep apnea syndrome; oxidative stress; plethysmography

Obstructive sleep apnea syndrome (OSAS) is a common respiratory disorder that is characterized by repeated occlusions of the upper airway during sleep, resulting in arterial hypoxemia and sleep fragmentation (1). Disorders of breathing during sleep are associated with significant cardiovascular and neurocognitive morbidities in patients and are a major public health concern (1). The striated muscles of the pharynx are accessory muscles of breathing that play a pivotal role in the control and maintenance of upper airway patency. Dysfunction of the pharyngeal dilator muscles increases susceptibility to airway collapse and may trigger a vicious cycle perpetuating obstructive airway conditions (2, 3). Upper airway muscle remodeling

## CLINICAL RELEVANCE

In an animal model of sleep-disordered breathing, we demonstrated that chronic intermittent hypoxia causes upper airway muscle weakness in male but not female rats. A superoxide scavenger was effective in preventing or reversing pharyngeal dilator muscle dysfunction, suggesting that antioxidant strategies may have application in the treatment of human obstructive sleep apnea syndrome.

occurs in patients with OSAS (4–7) and in animal models of the disorder (2, 8–10). Chronic intermittent hypoxia (CIH), a dominant feature of OSAS due to recurrent apnea, impairs upper airway muscle function (8, 11–14) and motor control of the upper airway (15–17). Several studies have also demonstrated that episodic hypoxia affects breathing (18, 19), with potentially “adaptive” and “maladaptive” consequences for cardiorespiratory homeostasis (20). Male sex is an independent risk factor for the development of OSAS. Sex differences in the control of breathing (21–23), including ventilatory responses to acute (24) and chronic (25) intermittent hypoxia, have been described. Moreover, skeletal muscle responses to chronic hypoxia can differ in male and female subjects (26). We speculated that sex differences exist in the effects of CIH on breathing and respiratory muscle function that manifest as a relative protection from CIH-induced respiratory plasticity in female subjects. The first aim of this study was to compare the effects of CIH on breathing and sternohyoid muscle function in adult male and female rats. We sought to test the following hypotheses: (1) CIH-induced plasticity in the control of breathing shows significant sex differences and (2) CIH-induced deficits in sternohyoid muscle function are greater in male rats.

Pharmacotherapy for OSAS is considered a viable and attractive clinical option (27) especially in light of poor patient compliance with continuous positive airway pressure, which is the gold standard treatment for OSAS. Antioxidant treatment may prove beneficial in this regard, particularly because OSAS is recognized as an oxidative stress disorder (28). Reactive oxygen species (ROS) are implicated in skeletal muscle function in health and disease (29, 30), and antioxidants have been shown to improve respiratory muscle performance (31, 32). Therefore, in view of the findings from the first objective, the second aim of this study was to determine if tempol, a superoxide dismutase mimetic, could prevent or reverse CIH-induced impairment of upper airway muscle function in male rats. Ventilatory adaptation to CIH has been described in rodents (33, 34), and some forms of respiratory plasticity resulting from episodic hypoxia exposure are known to be ROS dependent (35–37). The final aim of our study was to determine if chronic tempol supplementation ameliorates or prevents CIH-induced plasticity in breathing. Some aspects of the study have appeared in preliminary reports (38–42).

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## MATERIALS AND METHODS

Full details of the methods are provided in the online supplement.

### Animal Model of CIH

Experiments were performed on 30 adult male ( $289 \pm 48$  g, mean  $\pm$  SD) and 16 adult female ( $197 \pm 9$  g) Wistar rats. CIH treatment consisted of alternating 90-second cycles of normoxia (21% O<sub>2</sub>) and hypoxia (reaching 5% O<sub>2</sub> at the nadir; SaO<sub>2</sub>  $\sim$  80%; see Figure E1 in the online supplement). Sham rats were exposed to alternating cycles of normoxia under identical experimental conditions. Exposures lasted 8 h/d for 9 treatment days. Subgroups of sham ( $n = 8$ ) and CIH-treated ( $n = 8$ ) male rats were given the superoxide dismutase mimetic tempol (1 mM) by mouth throughout the protocol.

### Whole-Body Plethysmography

On the day after gas treatments, respiratory parameters were measured by whole-body plethysmography in unrestrained rats during quiet rest. Breathing was assessed during normoxia, acute hypoxia (F<sub>I</sub>O<sub>2</sub> = 0.10, balance N<sub>2</sub> for 20 minutes), and acute hypercapnia (F<sub>I</sub>CO<sub>2</sub> = 0.05, balance O<sub>2</sub> for 10 minutes).

### Muscle Physiology

Longitudinal strips of sternohyoid muscle were excised and studied in Krebs solution (containing 25  $\mu$ M d-tubocurarine) at 35°C gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub> for control conditions or 95% N<sub>2</sub>/5% CO<sub>2</sub> to create tissue hypoxia. Sternohyoid muscle contractile and endurance properties were determined as previously described (32).

### Succinate Dehydrogenase, Glycerol-3-phosphate Dehydrogenase, and Nicotinamide Adenine Dinucleotide Phosphate Diaphorase Enzyme Histochemistry

Longitudinal muscle strips were snap frozen in isopentane, cooled in liquid nitrogen, and stored at  $-80^{\circ}$ C. Transverse sections (10  $\mu$ m) were mounted on polysine-coated glass slides. Succinate dehydrogenase (SDH), glycerol-3-phosphate dehydrogenase (GPDH), and reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase activities were determined.

### Immunocytochemistry

Indirect immunofluorescence for myosin heavy chain (MHC) isoform composition was performed on serial unfixed muscle sections. Sham- and CIH-treated muscles were incubated with a cocktail of primary antibodies that targeted MHC type 1, 2A, and 2B fibers or, in separate sections, a primary antibody that targeted all isoforms but MHC 2X,

allowing us to identify pure MHC 2X fibers. A rabbit antilaminin antibody was used to highlight the perimeter of muscle fibers.

### Data Analysis

For ventilatory measurements, normoxic epochs were pooled to generate one set of baseline data. Peak ventilatory responses to hypoxia and hypercapnia were determined. For isolated muscle studies, specific force was calculated in N/cm<sup>2</sup> of muscle cross-sectional area (CSA). Performance and fatigue indices were determined as previously described (32). To determine SDH, GPDH, and NADPH diaphorase activities, the optical density of muscle sections, normalized to total CSA, was calculated. Using immunofluorescent images, areal and numerical density and CSAs for each MHC fiber type were determined. All data per animal were averaged before computing group means; data are expressed as mean  $\pm$  SEM. A two-way ANOVA (CIH  $\times$  drug or CIH  $\times$  acute gas challenge) was used to compare ventilatory parameters (Table 1). A two-way ANOVA (CIH  $\times$  drug) was used to compare fiber type, CSA, and enzyme histochemistry (Table 2) as well as minimum and maximum force, half-maximal effective frequency (EF<sub>50</sub>), force potentiation, fatigue index, and performance index (Figures 1–5). A three-way ANOVA (CIH  $\times$  drug  $\times$  frequency or time) was used to compare the force–frequency relationship (Figures 1 and 2, *upper graphs*) and force versus time curves during repeated muscle stimulation (Figures 4 and 5, *upper graphs*). All other data sets were compared using a two-tailed unpaired Student's *t* test or a Mann-Whitney test, as appropriate. In all tests,  $P < 0.05$  was the criterion for statistical significance.

## RESULTS

### Body Mass, Hematocrit, and Cardiac Mass

Data are shown in Table E1 in the online supplement. CIH treatment significantly decreased age-related body mass gain in male ( $P = 0.0081$ , Student's unpaired *t* test) and female ( $P < 0.0001$ ) rats. Hematocrit was significantly increased in CIH-treated male ( $P < 0.0001$ ) and female ( $P < 0.0001$ ) rats compared with corresponding sham control rats. CIH significantly increased right ventricular mass ( $P < 0.0001$ ) and the right/left ventricular mass ratio ( $P = 0.0002$ ) in male but not female rats. Tempol treatment prevented CIH-induced right ventricular hypertrophy in male rats.

### Ventilatory Parameters

**Baseline normoxic breathing.** Ventilatory data breathing normoxic gas for all groups are shown in Table 1. CIH treatment

**TABLE 1. VENTILATORY DATA IN SHAM- AND CHRONIC INTERMITTENT HYPOXIA-TREATED RATS WITH AND WITHOUT CHRONIC TEMPOL ADMINISTRATION UNDER NORMOXIC, HYPOXIC, AND HYPERCAPNIC CONDITIONS\***

	Sham Male Rats ( $n = 7$ )	CIH Male Rats ( $n = 7$ )	Sham Male Rats + Tempol ( $n = 8$ )	CIH Male Rats + Tempol ( $n = 8$ )	Sham Female Rats ( $n = 8$ )	CIH Female Rats ( $n = 8$ )
<b>Normoxia</b>						
V <sub>E</sub> , ml/min/100 g	62.0 $\pm$ 3.6	71.2 $\pm$ 4.2	59.2 $\pm$ 4.3	64.8 $\pm$ 3.7	77.8 $\pm$ 4.7	82.3 $\pm$ 2.6
V <sub>T</sub> , ml/100 g	0.66 $\pm$ 0.02	0.71 $\pm$ 0.04	0.70 $\pm$ 0.05	0.75 $\pm$ 0.06	0.97 $\pm$ 0.06	1.00 $\pm$ 0.04
V <sub>T</sub> /T <sub>I</sub> , ml/s/100 g	2.4 $\pm$ 0.1	2.9 $\pm$ 0.2	2.5 $\pm$ 0.2	2.7 $\pm$ 0.2	3.3 $\pm$ 0.2	3.6 $\pm$ 0.1
f <sub>R</sub> , breaths/min	96.5 $\pm$ 3.6	108.9 $\pm$ 9.2	89.5 $\pm$ 1.8	92.6 $\pm$ 5.4	87.7 $\pm$ 7.2	85.3 $\pm$ 2.4
<b>Hypoxia (10%O<sub>2</sub>)</b>						
V <sub>E</sub> , ml/min/100 g	104.8 $\pm$ 8.0	122.5 $\pm$ 7.4	104.5 $\pm$ 10.6	132.0 $\pm$ 16.5	87.6 $\pm$ 4.6	98.3 $\pm$ 10.5
V <sub>T</sub> , ml/100 g	0.79 $\pm$ 0.04	0.93 $\pm$ 0.06	0.80 $\pm$ 0.09	0.89 $\pm$ 0.14	0.99 $\pm$ 0.06	1.05 $\pm$ 0.11
V <sub>T</sub> /T <sub>I</sub> , ml/s/100 g	4.1 $\pm$ 0.3	4.5 $\pm$ 0.3	4.2 $\pm$ 0.5	5.1 $\pm$ 0.6	3.9 $\pm$ 0.3	4.4 $\pm$ 0.6
f <sub>R</sub> , breaths/min	147.6 $\pm$ 16.0	141.7 $\pm$ 11.3	139.2 $\pm$ 11.4	167.3 $\pm$ 18.8	94.9 $\pm$ 4.8	102.6 $\pm$ 5.3
<b>Hypercapnia (5%CO<sub>2</sub>)</b>						
V <sub>E</sub> , ml/min/100 g	135.6 $\pm$ 17.6	158.6 $\pm$ 20.1	125.4 $\pm$ 17.3	145.6 $\pm$ 23.6	139.6 $\pm$ 13.9	136.9 $\pm$ 17.4
V <sub>T</sub> , ml/100 g	0.99 $\pm$ 0.03	1.12 $\pm$ 0.10	0.94 $\pm$ 0.09	1.08 $\pm$ 0.14	1.37 $\pm$ 0.08	1.26 $\pm$ 0.09
V <sub>T</sub> /T <sub>I</sub> , ml/s/100 g	4.9 $\pm$ 0.6	5.6 $\pm$ 0.8	4.6 $\pm$ 0.6	5.2 $\pm$ 0.7	5.6 $\pm$ 0.5	5.4 $\pm$ 0.7
f <sub>R</sub> , breaths/min	141.4 $\pm$ 17.8	148.9 $\pm$ 17.8	140.6 $\pm$ 12.1	151.6 $\pm$ 28.4	105.7 $\pm$ 8.5	117.5 $\pm$ 16.0

*Definition of abbreviations:* CIH = chronic intermittent hypoxia; f<sub>R</sub> = respiratory frequency; V<sub>E</sub>, minute ventilation; V<sub>T</sub>, tidal volume; V<sub>T</sub>/T<sub>I</sub>, mean inspiratory flow.

\*Data are shown as mean  $\pm$  SEM. Two-way ANOVA (CIH  $\times$  tempol or CIH  $\times$  acute gas challenge) was performed. Statistical commentary is provided in the RESULTS section.

**TABLE 2. VALUES FOR MUSCLE FIBER AREAL DENSITY, NUMERICAL DENSITY, AND FIBER CROSS-SECTIONAL AREA, AND OPTICAL DENSITY VALUES, AND ENZYME ACTIVITY IN SHAM- AND CHRONIC INTERMITTENT HYPOXIA-TREATED RATS WITH AND WITHOUT CHRONIC TEMPOL ADMINISTRATION\***

	Sham Male Rats ( <i>n</i> = 7)	CIH Male Rats ( <i>n</i> = 7)	Sham Male Rats + Tempol ( <i>n</i> = 8)	CIH Male Rats + Tempol ( <i>n</i> = 8)
Areal density, %				
MHC 1	0.8 ± 0.1	1.0 ± 0.2	1.2 ± 0.1	0.8 ± 0.2
MHC 2A	16.6 ± 1.1	13.4 ± 0.8	13.1 ± 1.6	11.2 ± 2.4
MHC 2X	6.8 ± 1.2	9.7 ± 1.1	7.0 ± 1.2	9.2 ± 0.3
MHC 2B	74.8 ± 1.4	75.8 ± 2.8	75.8 ± 2.8	75.2 ± 1.3
Cross-sectional area, $\mu\text{m}^2$				
MHC 1	907 ± 31	846 ± 38	974 ± 98	773 ± 91
MHC 2A	987 ± 42	934 ± 53	1,109 ± 54	1,061 ± 100
MHC 2X	1,282 ± 32	1,452 ± 109	1,509 ± 227	1,567 ± 183
MHC 2B	3,848 ± 255	4,432 ± 236	4,542 ± 304	4,252 ± 177
Numerical density, %				
1	1.3 ± 0.3	3.4 ± 0.6	3.3 ± 0.9	2.6 ± 0.9
2A	41.5 ± 4.1	35.5 ± 2.6	37.7 ± 3.2	31.9 ± 3.8
2X	11.4 ± 2.1	13.5 ± 2.1	15.1 ± 1.3	15.7 ± 2.8
2B	52.5 ± 3.6	47.0 ± 2.8	43.5 ± 2.0	46.4 ± 2.3
Enzyme histochemistry (optical density, AU)				
SDH	111 ± 5	103 ± 4	106 ± 5	110 ± 2
GPDH	131 ± 1	132 ± 3	135 ± 2	131 ± 1
NADPH-diaphorase	38 ± 1	41 ± 1	39 ± 1	40 ± 1

*Definition of abbreviations:* AU = arbitrary units; CIH = chronic intermittent hypoxia; GPDH = glycerol-3-phosphate dehydrogenase; NADPH = nicotinamide adenine dinucleotide phosphate; SDH = succinate dehydrogenase.

\*Two-way ANOVA (CIH × tempol) was performed. Statistical commentary is provided in RESULTS.

significantly increased minute ventilation ( $V_E$ ) ( $P = 0.0372$ , two-way [CIH × drug] ANOVA), tidal volume ( $V_T$ ) ( $P = 0.0005$ ), and mean inspiratory flow ( $V_T/T_i$ ) ( $P = 0.033$ ) in male rats. Antioxidant supplementation had no major effect on breathing *per se* (Table 2). The CIH-induced increase in ventilatory parameters was ameliorated by chronic tempol supplementation (Table 2). In female rats, there was no significant effect of CIH on breathing (Table 2).

**Hypoxic and hypercapnic breathing.** Hypoxia significantly increased  $V_E$  in sham and CIH-treated rats ( $P < 0.0001$ ) (Table 1). CIH treatment did not significantly affect the hypoxic ventilatory response (interaction [CIH × acute hypoxia] for  $V_E$ :  $P = 0.4974$ ;  $+70 \pm 12\%$  versus  $+74 \pm 11\%$ , sham versus CIH; % increase from normoxia,  $P > 0.05$ , Student's *t* test). Hypercapnia significantly increased  $V_E$  in sham and CIH-treated rats ( $P < 0.0001$ ) (Table 1). CIH treatment did not significantly affect the hypercapnic ventilatory response (interaction [CIH × acute hypercapnia] for  $V_E$ :  $P = 0.6201$ ;  $+122 \pm 32\%$  versus  $+127 \pm 30\%$ , sham versus CIH,  $P > 0.05$ , Student's *t* test). Tempol supplementation did not significantly affect the hypoxic and hypercapnic ventilatory responses (Table 1).

### Isometric Twitch Force and Contractile Kinetics

**Control (hyperoxia).** Twitch force and contractile kinetics for sham and CIH-treated male and female animals are shown in Table E2 of the online supplement. Twitch force was significantly depressed in CIH-treated male muscle (Table E2) ( $P = 0.0011$ , Mann-Whitney test). Chronic tempol administration caused a significant increase in twitch force in the sham ( $P = 0.0379$ ) and CIH-treated ( $P = 0.007$ ) groups. CIH had no significant effect on female muscle twitch force.

**Hypoxia.** Twitch force was significantly decreased in CIH-treated male muscle ( $P = 0.0499$ ), but contractile kinetics were unaffected (Table E2). Chronic tempol administration had a positive inotropic effect ( $P = 0.0019$ ) in sham animals and reversed CIH-induced decreases in twitch force (Table E2). CIH had no significant effect on female muscle twitch force.

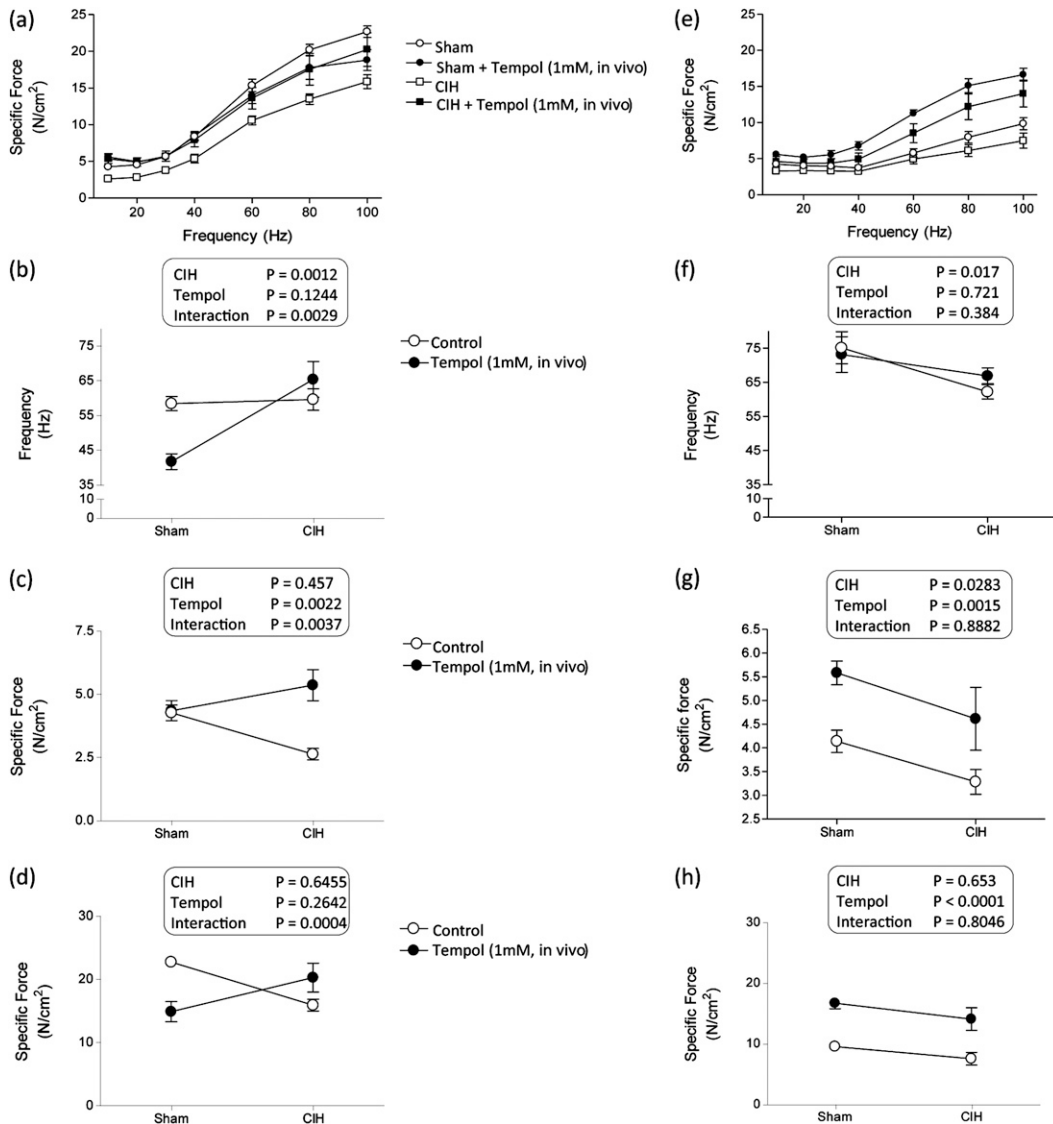
### Force–Frequency Relationship

**Control (hyperoxia).** In male rats, CIH treatment significantly decreased force (Figure 1A) ( $P = 0.021$ , three-way ANOVA). This was ameliorated by chronic tempol administration (Figure 1A) (interaction [CIH × tempol × frequency],  $P = 0.023$ ). The  $EF_{50}$  value was equivalent in sham and CIH-treated muscles (Figure 1B). Chronic tempol decreased  $EF_{50}$  in sham but not CIH-treated muscles (Figure 1B). CIH decreased minimum force production, which was ameliorated by chronic antioxidant treatment (Figure 1C). Likewise, maximum force was decreased by CIH treatment (Figure 1D), and this was partly recovered by chronic tempol administration (Figure 1D). *In vitro* (acute) tempol application significantly increased force production in sham and CIH-treated tissue (Figure 2A) ( $P < 0.001$ , three-way ANOVA) and caused a significant decrease in  $EF_{50}$  (Figure 2B). Female sternohyoid force was unaffected by CIH treatment (Figure 3A).

**Hypoxia.** In male rats, CIH had no significant effect on the force–frequency relationship, but chronic tempol administration increased force (Figure 1E) (CIH:  $P = 0.39$ ; tempol:  $P < 0.001$ , three-way ANOVA). CIH treatment significantly decreased  $EF_{50}$  (Figure 1F), but tempol had no effect (Figure 1F). CIH significantly decreased minimum force (Figure 1G) but had no effect on maximum force (Figure 1H). Chronic tempol administration increased minimum force (Figure 1G) and maximum force (Figure 1H) in sham and CIH-treated muscle. *In vitro* (acute) tempol application significantly increased force production in sham and CIH-treated tissue (Figure 2C) ( $P < 0.001$ , three-way ANOVA). *In vitro* tempol also caused a marked significant decrease in the  $EF_{50}$  of sham muscle (Figure 2D). Female sternohyoid force was unaffected by CIH (Figure 3C).

### Fatigue Index, Potentiation, and Performance Index during Repeated Muscle Stimulation

**Control (hyperoxia).** In male rats, CIH decreased force during the fatigue trial (Figure 4A) ( $P = 0.022$ , three-way ANOVA), and this was prevented by chronic tempol treatment. Tempol had a positive inotropic effect in CIH-treated but not on sham



**Figure 1.** Group data (mean  $\pm$  SEM) for force–frequency relationship in sham and chronic intermittent hypoxia (CIH)-treated rats with and without chronic tempol administration (1 mM in the drinking water) under control (hyperoxic) (a) and hypoxic (e) conditions. Three-way ANOVA (CIH  $\times$  tempol  $\times$  frequency) was performed, and statistical commentary is provided in RESULTS. Group data (mean  $\pm$  SEM) for half-maximal effective frequency (EF<sub>50</sub>) in control (hyperoxia) (b) and hypoxia (f). Group data (mean  $\pm$  SEM) for minimum force in control (hyperoxia) (c) and hypoxia (g). Group data (mean  $\pm$  SEM) for maximum force in control (hyperoxia) (d) and hypoxia (h). Two-way ANOVA (CIH  $\times$  Tempol) results are shown.

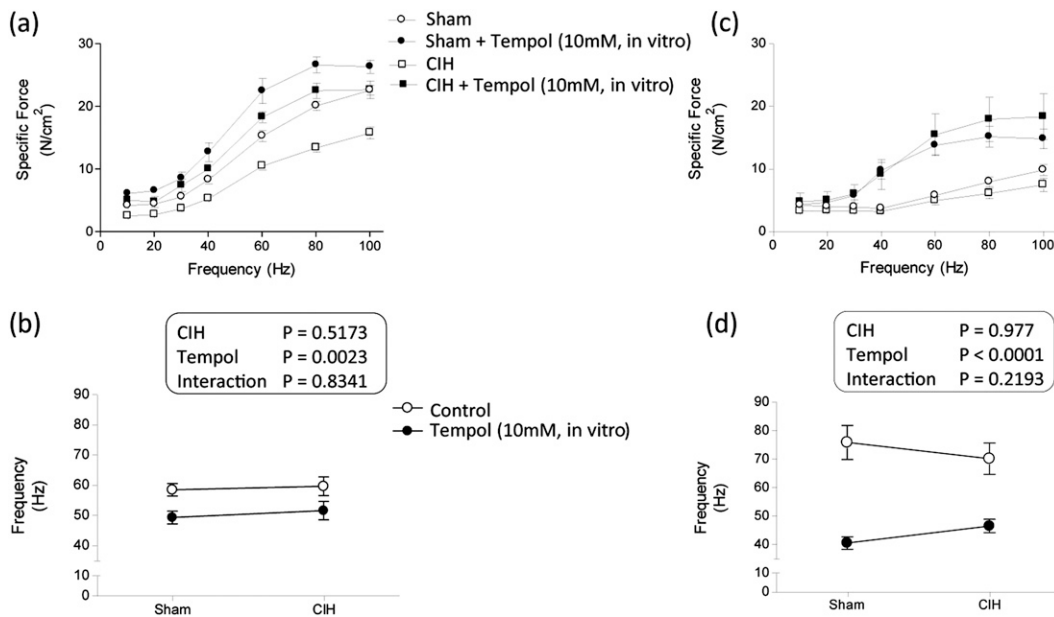
muscle (interaction [CIH  $\times$  tempol]:  $P = 0.03$ ). Fatigue index was unaffected by CIH treatment or chronic tempol administration (Figure 4B). CIH had no significant effect on force potentiation (Figure 4C). Chronic tempol significantly increased potentiation most notably in CIH-treated tissue (Figure 4C). CIH decreased performance index (Figure 4D), an effect that was ameliorated by chronic tempol treatment (Figure 4D). *In vitro* (acute) tempol significantly increased force production during fatigue trials in sham and CIH-treated muscle (Figure 5A) ( $P < 0.001$ ). Acute tempol treatment also increased performance index in CIH-treated but not in sham muscle (Figure 5B). Female sternohyoid fatigue was unaffected by CIH treatment during the repeated stimulation protocol (Figure 3B).

**Hypoxia.** In male rats, there was no significant effect of CIH on force during repeated muscle stimulation (Figure 4E) ( $P = 0.484$ , three-way ANOVA). However, tempol significantly increased force in sham and CIH-treated muscle (Figure 4E) ( $P < 0.001$ ). The fatigue index was unaffected by CIH treatment, and chronic tempol administration had no significant effect on the fatigue index (Figure 4F). Force potentiation was significantly increased by chronic tempol administration (Figure 4G). CIH did not affect performance index (Figure 4H). Chronic tempol increased performance index in sham and CIH-treated muscle

(Figure 4H). *In vitro* (acute) tempol treatment significantly increased force production during the fatigue trials in sham and CIH-treated muscle (Figure 5C) ( $P < 0.001$ , three-way ANOVA) but had no significant effect on performance index in both groups (Figure 5D). Female sternohyoid fatigue was unaffected by CIH treatment (Figure 3D).

#### Myosin Heavy Chain Composition and Enzyme Activities

Representative immunofluorescence images from male sternohyoid muscle are shown in Figures 6A and 6B. Table 2 shows group data for fiber areal and numerical density and CSA. CIH caused a significant increase in the areal density of type 2X fibers ( $P = 0.0217$ , two-way ANOVA). Hypertrophy of 2X fibers was evident in muscle from chronic tempol-treated animals ( $P = 0.0193$ , two-way ANOVA). SDH activity was unaffected by CIH or tempol treatment (CIH:  $P = 0.6751$ ; tempol:  $P = 0.9124$ ; interaction,  $P = 0.1965$ , two-way ANOVA). Similarly, GPDH activity was unaffected by CIH or tempol treatment (CIH:  $P = 0.4806$ ; tempol:  $P = 0.5303$ ; interaction:  $P = 0.2338$ ). NADPH diaphorase activity was increased by CIH ( $P = 0.0614$ ) but not by tempol treatment ( $P = 0.7634$ ). The interaction was not significant ( $P = 0.2705$ ).



**Figure 2.** Group data (mean  $\pm$  SEM) for force-frequency relationship in sham and CIH-treated rats with and without 10 mM *in vitro* tempol in control (hyperoxia) (a) and hypoxia (c). Three-way ANOVA (CIH  $\times$  tempol  $\times$  frequency) was performed, and statistical commentary is provided in RESULTS. Group data (mean  $\pm$  SEM) is shown for EF<sub>50</sub> in sham- and CIH-treated rats with and without 10 mM *in vitro* tempol in control (hyperoxia) (b) and hypoxia (d). Two-way ANOVA (CIH  $\times$  tempol) results are shown.

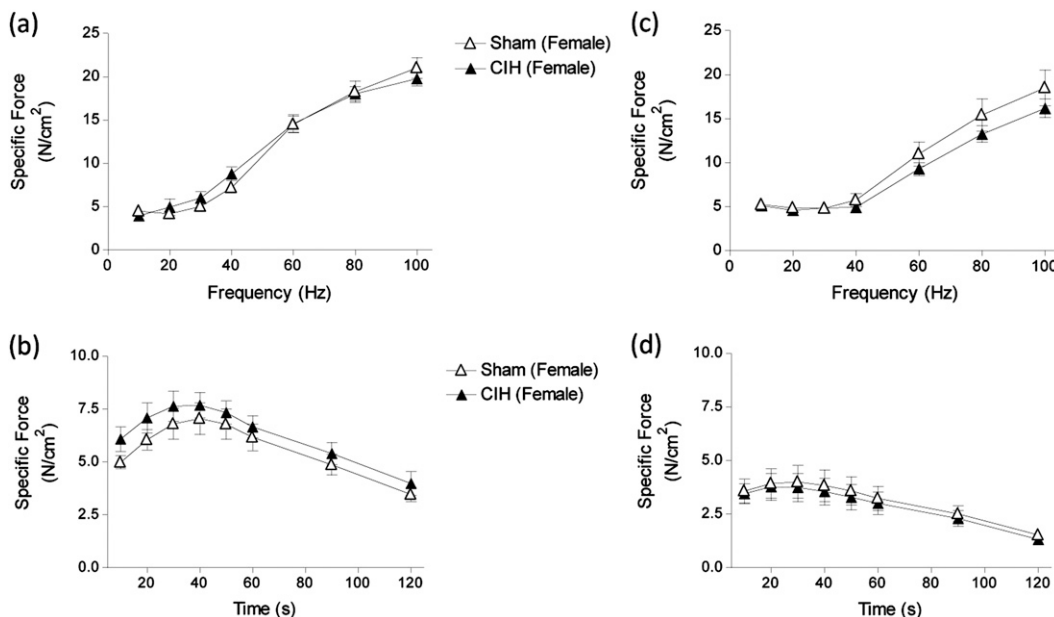
## DISCUSSION

The main findings of the present study are: (1) CIH causes ventilatory adaptation in adult male but not female rats; (2) CIH does not affect hypoxic or hypercapnic ventilatory responses; (3) CIH causes upper airway muscle weakness, with sex differences in the effects of CIH on respiratory muscle function; (4) chronic antioxidant treatment attenuates CIH-induced respiratory plasticity and ameliorates CIH-induced deficits in respiratory muscle function; and (5) tempol is a powerful inotrope that reverses CIH-induced respiratory muscle impairment and improves muscle hypoxic tolerance.

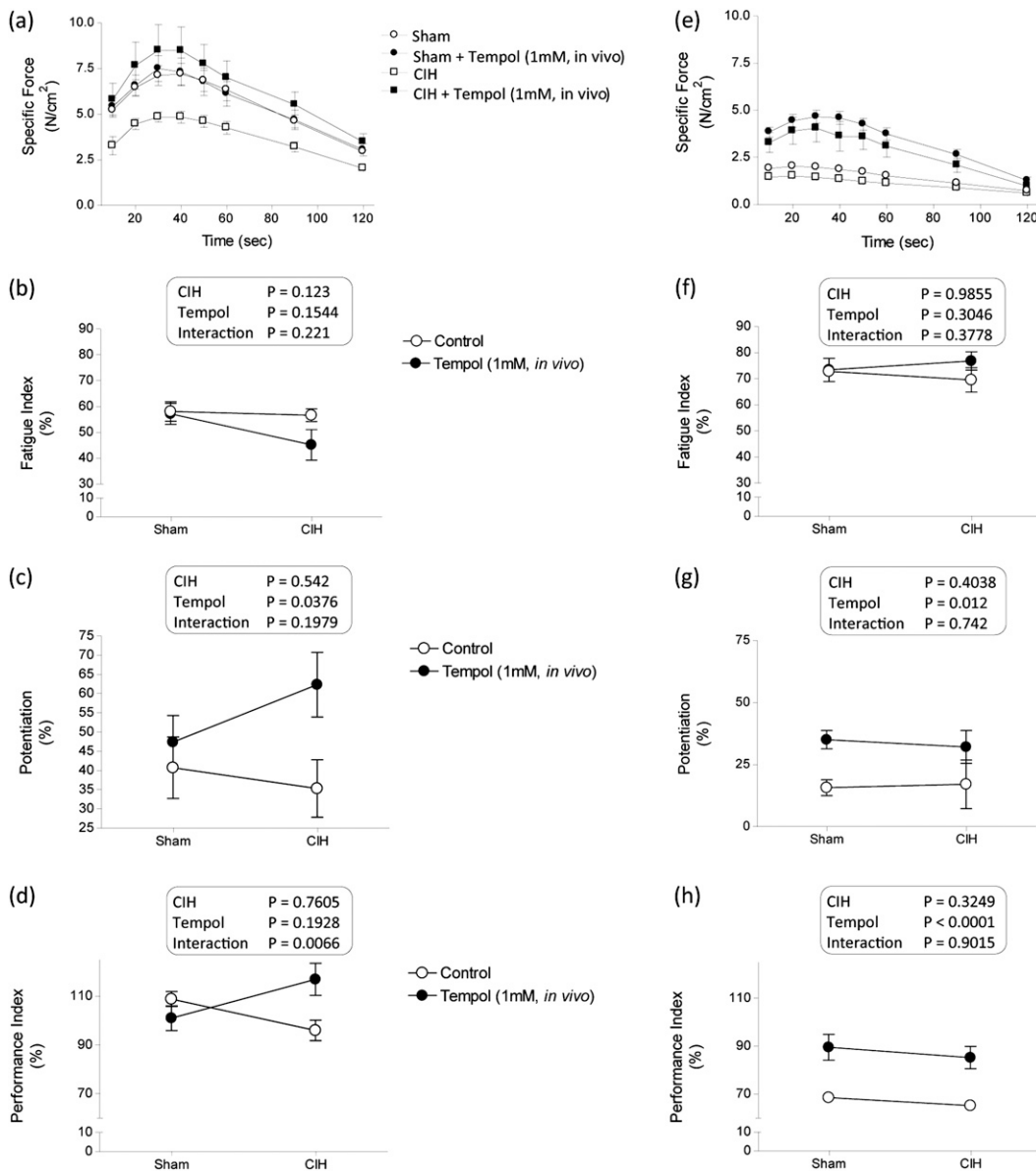
### CIH and Breathing

CIH increased normoxic ventilation in male rats, consistent with previous reports in rats (33) and mice (34). Gozal and colleagues coined the term “ventilatory adaptation” to describe this form of respiratory plasticity. Ventilatory adaptation in

unanesthetized freely behaving rats may be a consequence of carotid body remodeling after CIH (36) or may represent a form of ventilatory long-term facilitation (LTF) that manifests after episodic hypoxia exposure (18, 19). ROS are implicated in sensory (35) and motor (36) LTF in animal models, and recently antioxidant treatment was shown to mitigate CIH-induced LTF in human subjects (37). Our findings are consistent with these reports because chronic tempol supplementation attenuated CIH-induced ventilatory facilitation. The functional significance of CIH-induced facilitation of breathing is unclear, but it is plausible to suggest that it is maladaptive for respiratory homeostasis because CIH-induced hyperventilation could decrease the CO<sub>2</sub> reserve, destabilizing breathing during sleep (20) and thereby increasing the propensity for apnea (43). Sex differences in the control of breathing are well described (21–23). We reasoned that CIH-induced respiratory plasticity would show significant sex differences. Consistent with our hypothesis, our study demonstrates that CIH affects respiratory parameters



**Figure 3.** Values (mean  $\pm$  SEM) for sternohyoid muscle force-frequency relationship in sham and CIH-treated female rats under control (hyperoxic) (a) and hypoxic (c) conditions. Data (mean  $\pm$  SEM) for repeated stimulation in control (hyperoxia) (b) and hypoxia (d). Three-way ANOVA revealed no significant effects of CIH treatment.



**Figure 4.** Group data (mean ± SEM) for repeated stimulation in sham and CIH-treated rats with and without chronic tempol administration (1 mM in the drinking water) under control (hyperoxic) (a) and hypoxic (e) conditions. Three-way ANOVA (CIH × tempol × time) was performed, and statistical commentary is provided in RESULTS. Group data (mean ± SEM) for fatigue index in control (hyperoxia) (b) and hypoxia (f). Group data (mean ± SEM) for potentiation during repeated stimulation in control (hyperoxia) (c) and hypoxia (g). Group data (mean ± SEM) for performance index in control (hyperoxia) (d) and hypoxia (h). Two-way ANOVA (CIH × tempol) results are shown.

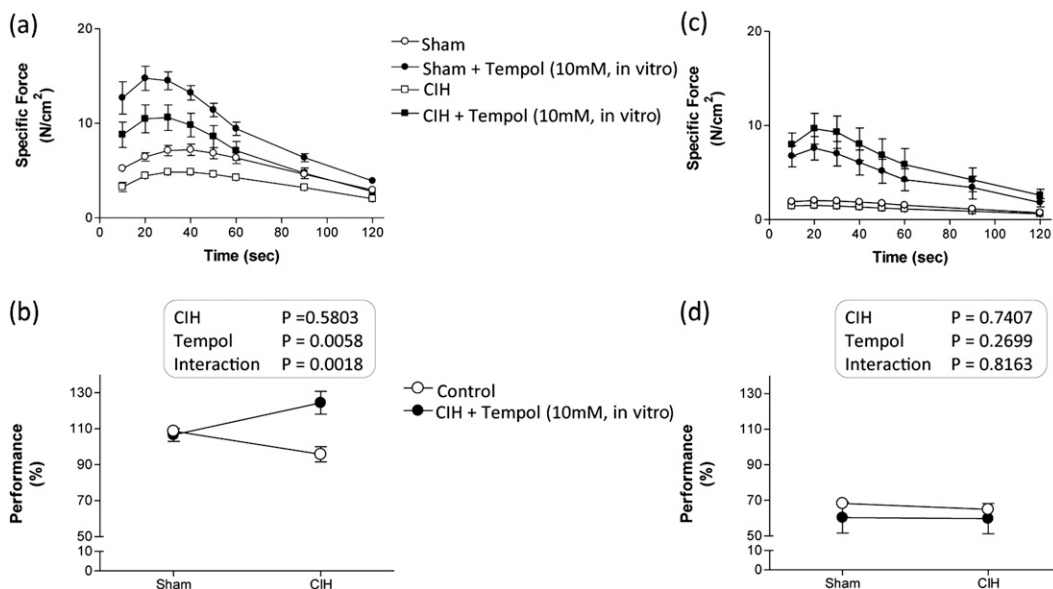
in male rats only. It is tempting to speculate that the antioxidant effects of the female sex hormones (44) ameliorated ventilatory adaptation to CIH, especially in light of the observations in male rats with supplemental antioxidant treatment. Of interest, sex differences in neuronal susceptibility to CIH-induced oxidative injury have been reported (45). Furthermore, sex differences in ventilatory responses to CIH have been reported in neonatal rat pups (25). If CIH-induced respiratory plasticity is maladaptive, then sex differences in the effects of CIH on breathing may be relevant to OSAS, which is more prevalent in male than in female subjects. CIH treatment did not affect the ventilatory responses to acute hypoxia or hypercapnia in sleeping rats. Others have shown that the hypoxic ventilatory response is increased (18, 46–50) or unchanged (51, 52) after CIH exposure, although blunted hypoxic responsiveness has also been reported in animal models of sleep-disordered breathing (53–55). These differences are most likely attributable to varying experimental approaches and treatment paradigms (especially differences in the pattern, intensity, and duration of hypoxia or reoxygenation); together, the studies highlight the complexity of carotid body and brainstem plasticity after CIH

exposure. There are fewer studies examining the effects of CIH on ventilatory responses to CO<sub>2</sub> challenges. Katayama and colleagues (51) reported an increased ventilatory response to 7% CO<sub>2</sub> in sleeping dogs after 2 but not 3 weeks of CIH exposure. The lack of effect of CIH on hypercapnic sensitivity in the present study is consistent with a previous report in a rodent model (52).

### CIH and Upper Airway Muscle Function

Upper airway dilator muscles play a pivotal role in the regulation of airway patency. Impaired function of the upper airway muscles increases the susceptibility to collapse and may trigger a vicious cycle perpetuating obstructive airway conditions (2, 3). Upper airway muscle structure and function are impaired in patients with OSAS (4–6), and functional deficits correlate with upper airway collapsibility in patients with OSAS (4). The mechanisms driving aberrant muscle remodeling in OSAS are likely multifactorial, but CIH is implicated as a major factor. Although sex hormones can increase respiratory muscle strength (56), we found that isolated sternohyoid muscle



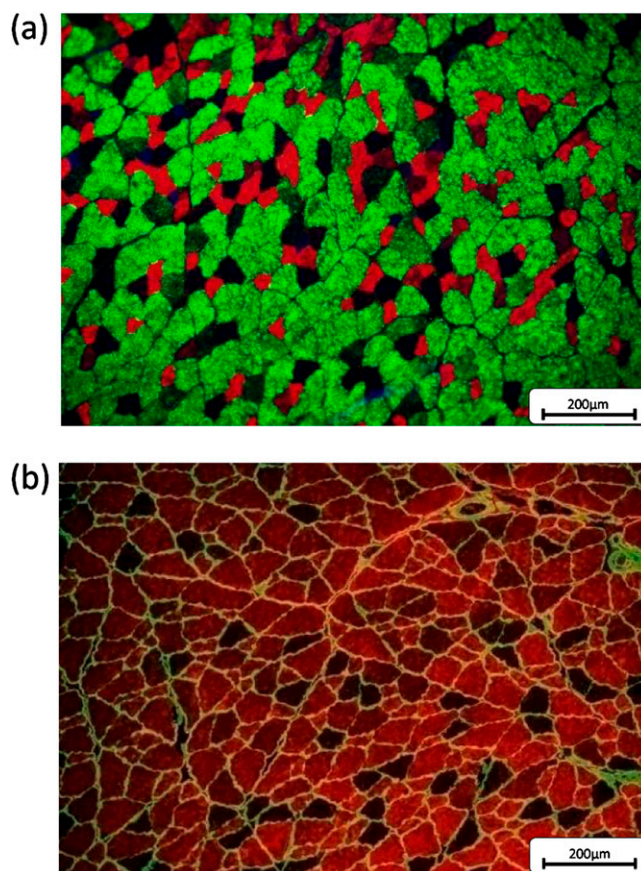


**Figure 5.** Group data (mean  $\pm$  SEM) for repeated stimulation in sham and CIH-treated rats with and without 10 mM *in vitro* tempol in control (hyperoxia) (a) and hypoxia (c). Three-way ANOVA (CIH  $\times$  tempol  $\times$  time) was performed, and statistical commentary is provided in RESULTS. Group data (mean  $\pm$  SEM) are shown for performance index in sham- and CIH-treated rats with and without 10 mM *in vitro* tempol in control (hyperoxia) (b) and hypoxia (d). Two-way ANOVA (CIH  $\times$  Tempol) results are shown.

contractile and endurance properties were similar in sham male and female animals, consistent with previous observations (57, 58) showing no sex difference in upper airway muscle specific force. However, CIH treatment significantly impaired sternohyoid function in male rats only. Furthermore, hypoxic tolerance of female sternohyoid muscle assessed *in vitro* was considerably greater than in male muscle. Sex differences in human limb muscle responses to chronic hypoxia have been described (26). Although the implications of this apparent tolerance in female respiratory muscle are obvious in the context of OSAS, the underlying reasons remain unclear, although ovarian hormones are implicated; indeed, estrogen therapy improves upper airway muscle function in CIH-treated ovariectomized rats (9).

We assume that our observations in isolated airway muscle translate to the *in vivo* setting (i.e., that CIH impairs the control of airway caliber). Although we are not of the opinion that upper airway muscle weakness leads to airway obstruction in the rat, given that the upper airway is relatively well supported such that rodents are not likely to develop obstructive apneas, the study nevertheless illustrates that CIH causes muscle weakness in mammalian striated muscle, which one could argue puts the intrinsically collapsible human airway at risk and in particular may put a patient with OSAS at risk of protracted apnoea. Whereas state-dependent reductions in neural traffic to the muscles of the upper airway essentially predispose to airway narrowing in humans (and occlusions in patients with OSAS; i.e., sleep apnea is predominantly a neurogenic phenomenon), resolving the obstructive event requires forceful muscle activation to reopen the airway such that weak dilator muscles may predispose to prolonged obstruction and perhaps increased frequency of obstructions. In this regard, it is interesting to note that apnea-hypopnea index and the duration of apneic events increases throughout the night in patients with severe OSAS (59). CIH-induced alterations in neural control could potentially compensate for upper airway muscle weakness (e.g., facilitation of cranial motor outflow [15]), but several studies point to the fact that CIH exposure impairs motor control of the upper airway in male rats (15–17). The combination of motor neuron and muscle impairment could prove especially detrimental for the control of airway patency in humans. If CIH-induced respiratory muscle dysfunction contributes to OSAS, then a protective effect of the female sex hormones on upper airway muscle performance may in part underlie the sex

difference in the prevalence of OSAS. The incidence of OSAS increases significantly postmenopause, and this is ameliorated by hormone replacement therapy (60).



**Figure 6.** (a) Triple-labeled immunohistochemistry (merged image) of sternohyoid muscle showing MHC type 1 (blue), type 2A (red), and type 2B (green) immunolabeled myocytes. (b) Double-labeled immunohistochemistry (merged image) of sternohyoid muscle showing positive labeling for MHC 1, 2A, and 2B fibers (red) with MHC2X fibers untagged (black). The perimeter of each fiber is positively labeled for laminin (green).

### CIH and Upper Airway Muscle Structure

We examined sternohyoid muscle MHC fiber types and the activities of key metabolic enzymes after CIH treatment to establish whether the functional impairment seen in male sternohyoid was the result of a shift in the structural phenotype of the respiratory muscle, as is the case for upper airway muscles in patients with OSAS (4, 7) and in some animal models (10, 13). There was some evidence of structural reorganization in CIH muscles, with a significant increase in type 2X areal density. Our findings are consistent with previous reports in other CIH models showing little or no change in upper airway muscle fiber type composition (3, 12, 17), although a slow-to fast transition was observed in response to short-term IH in one study (13). The results indicate that functional remodeling in our model is likely not driven by fiber-type transitions, but we acknowledge that long-term exposure to CIH may induce structural remodeling in respiratory muscles and that putative CIH-induced fiber changes in human OSAS may take months or years to develop. There was no significant effect of CIH treatment on SDH or GPDH activities (indices of oxidative and glycolytic capacity respectively). However, a small increase ( $\sim 8\%$ ) in NADPH diaphorase activity was noted, suggesting that nitric oxide synthase activity may be increased in CIH-treated upper airway muscle. NO is implicated in respiratory muscle remodeling after chronic sustained hypoxia (61). The putative role of NO in CIH-induced respiratory muscle remodeling warrants investigation.

### Antioxidant Treatment Prevents CIH-Induced Muscle Dysfunction

We reasoned that oxidative stress associated with the recurrent hypoxia/reoxygenation cycles that are characteristic of CIH and OSAS are responsible for CIH-induced respiratory muscle impairment. Free radical-mediated respiratory muscle dysfunction is well described in animal models and human diseases, and several studies have documented free radical-induced brain injury in animal models of sleep-disordered breathing (62–66). Moreover, antioxidant strategies can often prevent the deleterious effects of CIH on brain structure and function (62–66). It is also established that antioxidant treatment prevents CIH-induced respiratory motor neuron dysfunction (16). In the present study, chronic treatment with tempol, a superoxide dismutase mimetic, ameliorated CIH-induced decreases in sternohyoid muscle force and performance. This implicates ROS and oxidative stress in the mechanism of CIH-induced muscle weakness in our model. Moreover, chronic antioxidant supplementation improved male sternohyoid muscle hypoxic tolerance, a finding that may be relevant to respiratory disorders characterized by hypoxia.

Improved upper airway muscle performance during sleep in patients with OSAS may help reduce the incidence and severity of occlusive events. In a recent study, we demonstrated that superoxide scavengers improve sternohyoid muscle performance (32). Data from the present study in sham animals confirms our previous findings that tempol has a powerful positive inotropic effect on sternohyoid muscle under control and hypoxic conditions. In addition, in the present study we demonstrated that acute *in vitro* application of tempol rescued force in CIH-treated muscles. This is important in the context of antioxidant therapy for OSAS because it suggests that respiratory muscle weakness after CIH can be reversed by treatment. Our data suggest that CIH-induced functional remodeling in sternohyoid muscle is not a result of irreversible oxidative damage *per se*. We speculate that increased ROS in CIH-treated muscle depresses force by decreasing calcium sensitivity of the myofibrillar contractile proteins (67) or by decreasing membrane

excitability (68). Tempol most likely prevents these effects by scavenging superoxide anions (68) and in this way acts to recover force in isolated muscle preparations. Indeed, in separate studies we observed no effect of *in vitro* tempol on sternohyoid muscle preparations studied at 25°C (Fig. E2). Because superoxide production in muscle increases significantly at temperatures above 32°C (67), this suggests that the positive inotropic effect of tempol relates entirely to its antioxidant (superoxide scavenging) properties. When viewed together, our data suggest that antioxidant therapy may be beneficial in the treatment of OSAS and other respiratory muscle weakness disorders.

**Author disclosures** are available with the text of this article at [www.atsjournals.org](http://www.atsjournals.org).

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