Respiratory loading intensity and diaphragm oxidative stress: 
N-acetyl-cysteine effects

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Respiratory loading intensity and diaphragm oxidative stress: N-acetyl-cysteine effects. J Appl Physiol 100: 555–563, 2006. First published September 29, 2005; doi:10.1152/japplphysiol.00780.2005.—We hypothesized that resistive breathing of moderate to high intensity might increase diaphragm oxidative stress, which could be partially attenuated by antioxidants. Our objective was to assess the levels of oxidative stress in the dog diaphragm after respiratory muscle training of a wide range of intensities and whether N-acetyl-cysteine (NAC) might act as an antioxidant. Twelve Beagle dogs were anesthetized with 1% propofol, tracheostomized, and subjected to continuous inspiratory resistive breathing (IRB) (2 h/day for 2 wk). They were further divided into two groups (n = 6): NAC group (oral NAC administration/24 h for 14 days) and control group (placebo) and after (contralateral hemidiaphragm) IRB and NAC vs. placebo treatment. Oxidative stress was evaluated in all diaphragm biopsies through determination of 3-nitrotyrosine immunoreactivity, protein carbonylation, hydroxynoneal protein adducts, Mn-SOD, and catalase, using immunoblotting and immunohistochemistry. Both protein tyrosine nitration and protein carbonylation were directly related to the amount of the respiratory loads, and NAC treatment abrogated this proportional rise in these two indexes of oxidative stress in response to increasing inspiratory loads. A post hoc analysis revealed that only the diaphragms of dogs subjected to high-intensity loads showed a significant increase in both protein tyrosine nitration and carbonylation, which were also significantly reduced by NAC treatment. These results suggest that high-intensity respiratory loading-induced oxidative stress may be neutralized by NAC treatment during IRB in the canine diaphragm.

inspiratory loading: respiratory muscles; 3-nitrotyrosine; protein carbonylation; antioxidants

RESPIRATORY LOADING OF A MAGNITUDE similar to that observed in some respiratory diseases, such as chronic obstructive pulmonary disease (COPD) (25, 48), or that used in respiratory training programs induces muscle membrane and sarcomere damage (31), as well as increased production of reactive oxygen species (ROS) in the diaphragm muscle (1, 2, 6, 8). In unfatigued muscles, ROS are usually kept at low levels by intracellular antioxidants and are required for normal force production. Moderate exposure of muscles to ROS increases their ability to generate force, whereas either oxygen free-radical scavenging (17, 33) or excessive ROS production, as occurs during resistive breathing (1, 2, 5, 6, 32, 37), signif-

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effect) and also to assess whether the antioxidant NAC might attenuate the increased oxidant production in the dog diaphragm.

METHODS

Animals and Study Design

Twelve awake male Beagle dogs (12.3 ± 0.3 kg) of identical age were included. This sample size was calculated on the basis of previous studies from our group (11, 48). On arrival, the animals were maintained on a regular light-dark cycle and fed food and water ad libitum for 1 wk before the study protocol. This is an experimental study conducted on a canine model. The study was designed in accordance with both the ethical standards on animal experimentation in our institution, and the Helsinki convention for the use and care of animals. All experiments were approved by the Animal Research Committee in our institution.

NAC Administration

NAC powder (kindly provided by Zambon, Barcelona, Spain) was dissolved in distilled water to obtain a 3 mmol/kg dose. This NAC dose was chosen on the basis of previous studies showing the protective effects of NAC on tissues (7, 14, 36). NAC was administered orally using a 14-mm gauge needle every 24 h for the entire duration of the study protocol (14 days) and after the 7-day recovery period. NAC was administered using the 14-mm gauge needle every 24 h for the entire duration of the study protocol (14 days) and after the 7-day recovery period.

Surgical Procedures

Immediately after the acclimatization period and before NAC/placebo administration, all 12 dogs were anesthetized. Induction was carried out by means of fentanyl, atracurium, and atropine, and anesthesia was achieved by means of the administration of 1% isoflurane. With the use of sterile conditions, the animals were tracheostomized and intubated with a 4 or 6 cuffed endotracheal tube. An incision was made caudally along the linea alba from the xyphoid process. All animals were exposed, and a muscle sample was obtained from the costal part of the diaphragm. In all cases, great care was taken to ensure the absence of pneumothorax. All animals were allowed a 7-day recovery period after the first biopsy at baseline (control biopsy). In all cases, a second biopsy was obtained from the contralateral hemidiaphragm immediately after a 2-wk IRB period (11, 48). Diaphragm biopsies (∼100 mg) were immediately frozen in liquid nitrogen or embedded in paraffin and used for immunohistochemistry.

Experimental Protocol

The dogs were always awake and were subjected to continuous periods of IRB (2 h/day for 2 wk). A two-way valve (Hans Rudolf) was connected to the endotracheal tube, and inspiratory resistive loads were applied to the inspiratory port of the valve. A relatively wide spectrum of inspiratory pressures was administered to the animals (31–45% P_{max}). The force developed by the inspiratory muscles was measured by connecting the inspiratory port to a differential pressure transducer (range ±250 cmH2O, Hellige, Freiburg, Germany) (11, 48). Both the flow and breathing pattern were assessed through a Fleisch pneumotachograph (Poch-Millas, I+D, Madrid, Spain). These variables were continuously monitored, and corresponding signals were subsequently digitalized at a sampling rate of 300 Hz and recorded in a computer for later analysis.

Physiological Assessment

During the IRB sessions, both the inspiratory pressure and the ventilatory pattern were assessed in all cases. The following variables were determined: peak inspiratory pressure (PIP), mean inspiratory pressure (P_{i} P_{max}), inspiratory time (Ti), duty cycle (Tr), and the tension time index of the respiratory muscles (TT_{RM}), according to the equation:

\[ TT_{RM} = \left( \frac{P_{i}}{P_{max}} \right) \times \left( \frac{Ti}{Tr} \right) \]

Immunoblotting

The levels of oxidative stress were assessed as described elsewhere (2, 3). Selective antibodies were used to detect 3-nitrotyrosine immunoreactivity (anti-3-nitrotyrosine antibody; Cayman Chemical, Ann Arbor, MI), carbonyl groups through derivatization (2, 22) to 2,4-dinitrophenylhydrazine (DNP) (anti-DNP moiety antibody; Oxyblot kit, Chemicon International, Temecula, CA), 4-hydroxy-2-nonenal (HNE) protein adducts (10), catalase (anti-HNE and anti-catalase antibodies; Calbiochem, San Diego, CA), and Mn-SOD (anti-Mn-SOD antibody; StressGen, Victoria, BC). Frozen muscle samples were homogenized in a homogenization buffer. Samples were then centrifuged at 1,000 g for 30 min. The pellet was discarded, and the supernatant was designated as a crude homogenate. Total muscle protein level in each sample was spectrophotometrically determined with Bradford technique by using different runs of triplicates in each case and bovine serum albumin as the standard (Bio-Rad protein reagent, Bio-Rad, Hercules, CA). The final protein concentration in each sample was calculated from at least two almost identical Bradford measurements. Equal amounts of total protein from crude muscle homogenates were always loaded (20 µg per sample per lane) onto the gels, as well as identical sample volumes per lane. While the different Western blot analyses were conducted, the same samples were always run together and kept in the same order. Proteins were then separated by electrophoresis, transferred to polyvinylidene difluoride membranes, blocked with nonfat milk, and incubated overnight with the corresponding primary antibodies. Specific proteins from all samples were detected with horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence kit. For each of the antigens, all of the samples were detected in the same film under identical exposure times. Blots were scanned with an imaging densitometer, and optical densities of specific proteins were quantified with Diversity Database 2.1.1 (Bio-Rad). Values of total protein tyrosine nitration and carbonylation and HNE protein adducts in a given sample were calculated by addition of optical density of individual protein bands in each case. Ponceau red staining of crude homogenates on the membranes was used to determine equal loading/transfer across lanes.

Immunohistochemistry

Immunohistochemical analyses were performed as described elsewhere (2, 3). All sections (3 µm) were deparaffinized and incubated with anti-3-nitrotyrosine and anti-DNP primary antibodies, followed by incubation with biotinylated secondary antibodies, and with both horseradish peroxidase-conjugated streptavidin and diaminobenzidine (Dako, Carpenteria, CA). Negative control slides were only exposed to both secondary antibodies and the detection system.

Statistical Analysis

Baseline data for the different biological variables are reported as 100%, whereas data following IRB, in either the control or NAC group of dogs, are reported as a percentage of baseline ± SD. The percentage of change was also used to describe modifications. Wilcoxon and Mann-Whitney nonparametric tests were used for paired and unpaired comparisons, respectively. Spearman’s coefficient was used to assess correlations between critical biological and physiological variables, using either PIP as an index of the maximal contractile effort of the respiratory muscles, or the TT_{RM} as the expression of the relative load in relation to the Tr. Statistical significance was established at P ≤ 0.05.
RESULTS

Physiological Characteristics

Table 1 indicates the main physiological characteristics of both groups of dogs. No significant differences were observed in weight, PIP, P_i, P_{i max}, T_i/T_T, and TTRM, obtained during the IRB period between the control and the NAC group of dogs.

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<th>Physiological Parameters</th>
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<td>PIP, cmH_2O</td>
<td>21.2±2.8</td>
<td>20.5±2.7</td>
</tr>
<tr>
<td>P_i, cmH_2O</td>
<td>10.2±1.6</td>
<td>8.7±0.8</td>
</tr>
<tr>
<td>P_{i max}</td>
<td>0.18±0.06</td>
<td>0.16±0.05</td>
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<tr>
<td>T_i/T_T</td>
<td>0.28±0.04</td>
<td>0.31±0.07</td>
</tr>
<tr>
<td>TTRM</td>
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Values are means ± SD; n = 6. NAC, N-acetyl-cysteine; PIP, peak inspiratory pressure; P_i, mean inspiratory pressure; P_{i max}, maximal inspiratory pressure; T_i, inspiratory time; T_T, duty cycle; TTRM, tension-time index of the respiratory muscles.

Oxidative Stress After IRB

Protein tyrosine nitration. Several tyrosine nitrated protein bands were detected in all of the diaphragms, with apparent masses ranging from 84 to 24 kDa. After IRB, the levels of total muscle carbonyl groups did not significantly differ compared with baseline in the control dogs (Fig. 2A). The individual percentage of change of total protein carbonylation was also heterogeneous in this group of animals and did not significantly correlate with PIP, but nevertheless did correlate with TTRM (Fig. 2B).

High-intensity respiratory loading analysis. In a post hoc analysis, where only those animals that had received IRB of high intensity were studied, both diaphragm protein nitration and carbonylation were significantly higher compared with baseline in the control dogs (Fig. 3, A and B, respectively). A respiratory load of 38% P_{i max} was chosen as the cutoff value in this post hoc analysis, since one-half of the animals in each group breathed against loads that were greater and lower than this load, respectively. It is also worth mentioning that neither diaphragm protein nitration nor carbonylation was significantly modified compared with baseline in the control dogs that had received IRB of low intensity (lower than 38% P_{i max}). Furthermore, within the control group, immunohistochemical analysis revealed positive 3-nitrotyrosine and protein carbonylation staining (Figs. 4 and 5, respectively) of greater intensity in the muscle fibers of the dogs that were subjected to high-intensity IRB, compared with diaphragms obtained at baseline.

Total protein carbonylation. In the diaphragms of all of the animals, anti-DNP antibody detected different positive protein bands, with apparent masses ranging from 145 to 25 kDa. After IRB, the levels of total muscle carbonyl groups did not significantly differ compared with baseline in the control dogs (Fig. 2A). The individual percentage of change of total protein carbonylation was also heterogeneous in this group of animals and did not significantly correlate with PIP, but nevertheless did correlate with TTRM (Fig. 2B).

HNE protein adduct formation. In the diaphragms of all of the animals, anti-HNE protein adduct antibody detected different positive protein bands, with apparent masses ranging from 52 to 15 kDa. IRB did not significantly modify the levels of total HNE protein adducts compared with baseline in the control group of dogs. No significant correlations were found between this index and physiological variables.

Antioxidant enzymes. Both catalase and Mn-SOD were present in all control diaphragms, showing no significant differences or correlations with physiological variables after the IRB period.

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**Table 1. Physiological variables of the two groups of dogs**

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Values are means ± SD; n = 6. NAC, N-acetyl-cysteine; PIP, peak inspiratory pressure; P_i, mean inspiratory pressure; P_{i max}, maximal inspiratory pressure; T_i, inspiratory time; T_T, duty cycle; TTRM, tension-time index of the respiratory muscles.

**Fig. 1.** A: values are expressed as a percentage of baseline ± SD. Total 3-nitrotyrosine formation was not significantly modified [nonsignificant (ns)] in the dog diaphragms after the administration of inspiratory resistive breathing (IRB) in either the control or the N-acetyl-cysteine (NAC) group of animals. B: within the control group of dogs, the individual percentage of change of 3-nitrotyrosine formation directly and significantly correlated with the respiratory loading as expressed by peak inspiratory pressure (PIP) received by each animal. MIP, maximal inspiratory pressure.
NAC Effects on Oxidative Stress Indexes After IRB

Protein tyrosine nitration. The diaphragm levels of 3-nitrotyrosine were not significantly modified after IRB by NAC administration in the NAC group of dogs (Fig. 1A). No significant correlations were found between nitrotyrosine formation and PIP (Fig. 6A) or any other physiological variables.

Total protein carbonylation. After IRB, muscle levels of total protein carbonylation did not significantly change as a result of NAC treatment in the NAC group of animals (Fig. 2A). Furthermore, no significant relationship was observed between protein carbonylation and $T_{TRM}$ (Fig. 6B) or other physiological parameters.

High-intensity respiratory loading analysis. In the post hoc analysis where only those animals from the NAC group, which had received IRB of high intensity, were studied, no significant differences were observed in weight, PIP, $P_i/P_{Tmax}$, $T_i/T_{Tr}$, and $T_{TRM}$, obtained during the IRB period compared with the control animals subjected to higher inspiratory loads (Table 2). NAC administration significantly reduced the increased levels of both diaphragm 3-nitrotyrosine immunoreactivity (33%)
and total protein carbonylation (39%) (Fig. 3, A and B, respectively) in the NAC dogs, which had received higher resistive loading. Furthermore, within the NAC group, immunohistochemical analysis revealed positive 3-nitrotyrosine and protein carbonylation staining (Figs. 4 and 5, respectively) of weaker intensity in the muscle fibers of those dogs subjected to high-intensity IRB, compared with diaphragms from the control animals.

Fig. 4. Immunohistochemical localization (×200) of 3-nitrotyrosine in diaphragm muscles of one control dog both at baseline (A) and after administration of IRB of high intensity (B), and also in a NAC dog after breathing against high-intensity inspiratory loads (C). Anti-3-nitrotyrosine antibody detected positive staining diffusely localized within the muscle fibers (A, B, and C). Replacement of the primary anti-nitrotyrosine antibody with nonspecific secondary antibodies completely eliminated positive staining (D, E, and F, respectively). It is worth noting that the staining was less intense in the fibers of dogs treated with NAC.

Fig. 5. Immunohistochemical localization (×200) of protein carbonylation in diaphragm muscles of one control dog both at baseline (A) and after administration of IRB of high intensity (B), and also in a NAC dog after breathing against high-intensity inspiratory loads (C). Anti-2,4-dinitrophenylhydrazone (DNP) antibody detected positive staining diffusely localized within the muscle fibers (A, B, and C). Replacement of the primary anti-DNP antibody with nonspecific secondary antibodies completely eliminated positive staining (D, E, and F, respectively). It is worth noting that the staining was less intense in the fibers of dogs treated with NAC.
**HNE protein adduct formation.** NAC treatment did not significantly modify the levels of total HNE protein adducts after the IRB period in the NAC group of dogs.

**Antioxidant enzymes.** Both catalase and Mn-SOD were also present in all diaphragms of animals treated with NAC, showing no significant differences after the IRB period.

**DISCUSSION**

The main findings in our study are that, in the diaphragms of dogs subjected to an IRB period of a wide range of inspiratory pressure loads above 30% PImax compared with baseline:

1) both protein tyrosine nitration and protein carbonylation were directly related to the amount of the respiratory loads;
2) NAC administration to the animals abrogated this proportional rise in both 3-nitrotyrosine and carbonyl group formation in response to increasing inspiratory loads; and
3) a post hoc analysis revealed that only the diaphragms of dogs subjected to high-intensity loads showed a significant increase in both protein tyrosine nitration and carbonylation levels, which were also significantly reduced by NAC administration.

**Oxidative Stress After IRB**

Aerobic exercise training of an adequate intensity produces a physiological training effect in patients with COPD (19, 20), along with underlying adaptive changes in the muscle structure (31). Abnormal ventilatory mechanics and dyspnea are the most common limiting factors of the effects of a training program. The magnitude of the pressure load is a significant variable in inspiratory muscle training, since loads <30% of the patient PImax have been shown not to have any training effect on COPD patients (19, 20). Indeed, in several studies, a training-load intensity equal to 60% PImax has proven effective (19, 20, 31). These beneficial effects, however, come with a high price tag, since high-intensity exercise also leads to oxidative stress, as a result either of increasing ROS production within the muscle fibers or of altering the antioxidant defenses (1, 4, 6, 8, 26, 34, 39). The enzyme xanthine oxidase (13) and a relative inefficiency of the mitochondrial respiratory chain (23, 38) have been proposed as the molecular sources that contribute most to free-oxygen radical generation within the muscle fibers in response to strenuous exercise.

To the best of our knowledge, this report is the first to show that only dogs breathing against higher inspiratory loads (PIP), and not those breathing against lower pressure values, showed significantly increased levels of both muscle protein nitration and carbonylation. This finding is also consistent with the positive relationships found between the diaphragm levels of these two indexes of oxidative stress and the increasing levels of the inspiratory loads. It is worth mentioning that dogs showing either a decrease or no change in protein nitration or carbonylation in their diaphragms compared with the baseline were those breathing against loads equivalent to 38% PImax or under. These findings suggest that a “threshold” effect might exist between the degree of the inspiratory loads and ROS-mediated effects on diaphragm proteins may be crucial for the design of specific high-intensity respiratory muscle training programs in patients with chronic lung diseases.

**Table 2. Physiological variables of dogs breathing against high loads from both dog groups**

<table>
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<tr>
<td>PIP, cmH2O</td>
<td>23.4±0.8</td>
<td>22.7±1.4</td>
</tr>
<tr>
<td>Pt, cmH2O</td>
<td>11.3±1.4</td>
<td>9.9±0.4</td>
</tr>
<tr>
<td>P0/PImax</td>
<td>0.20±0.02</td>
<td>0.18±0</td>
</tr>
<tr>
<td>Ti/TT</td>
<td>0.27±0.02</td>
<td>0.28±0.1</td>
</tr>
<tr>
<td>TTRM</td>
<td>0.06±0.01</td>
<td>0.05±0.01</td>
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Values are means ± SD; n = 3.
On the other hand, as previously shown (12), it is likely that even more significant results, in terms of respiratory loading-induced oxidative stress, might have been observed in the external intercostal muscles or other rib cage muscles, which are known to be recruited during IRB. In line with this, Supinski et al. (39) demonstrated that, among several inspiratory muscles of rats subjected to high-intensity resistive loads, the diaphragm was the only muscle showing significant modifications of both glutathione fractions, GSH and GSSG, and also of the GSSG-to-GSH ratio. Hence, the authors concluded from these findings that the diaphragm muscle shows a greater predisposition than other inspiratory muscles to generate free radicals during inspiratory resistive loading (39). Actually, recent data from our group have also shown that the levels of oxidative stress were increased in the diaphragm of patients with stable, severe COPD at rest (2).

Chronic exercise training seems to improve antioxidant capacity in skeletal muscles. For instance, Powers et al. (28) and others (15) demonstrated that training induced an increase in SOD and glutathione peroxidase activities, which followed a muscle-type distribution in rat limb muscles. In quadriceps muscles of healthy subjects, GSH was also shown to be increased as a result of a short-term training period (42). In another study in which rats were subjected to endurance exercise training, the diaphragm also showed increased levels of SOD activity and reduced lipid peroxidation (46, 47). In line with this, Oh-Ishi et al. (24) demonstrated that 2 mo of endurance training improved the resistance of the rat diaphragm to acute exercise-induced oxidative stress by increasing both the protein content and activity of Mn-SOD.

On the other hand, other reports have also shown that chronic exercise of moderate intensity may reduce either the systemic or tissue antioxidant capacity (16), contributing to enhancing oxidative stress levels within the muscles. For instance, endurance training induced a reduction in vitamin E concentration in skeletal muscle and heart in rats (43). The same investigators (44) also showed that short-term aerobic training did not have a significant effect on the levels of SOD, catalase, glutathione peroxidase, or glutathione in the vastus lateralis muscles of healthy individuals. Moreover, levels of the reduced fraction of glutathione were lower in rat muscles after endurance training of different types (30) and also in the quadriceps muscles of COPD patients after an 8-wk training program (29). In another study (26) conducted on athletes, overloaded training increased blood glutathione peroxidase activity, while reducing their nonenzymatic antioxidant capacity. In our study, the diaphragmatic content of the two antioxidant enzymes Mn-SOD and catalase remained unmodified by the 15-day training period administered to the dogs. Indeed, no changes in muscle catalase after training have been reported (28), or, if anything, a decrease occurred (21).

NAC Effects on Oxidative Stress Indexes After IRB

In the present study, NAC administration to the animals over the IRB period led to a loss of the relationships found between biological variables, expressing both oxidative and nitrosative stress in the dog diaphragm and physiological parameters representing the amount of the respiratory loading. Furthermore, the post hoc analysis revealed that three dogs breathing against high-intensity loads (from 38 to 45% \( P_{\text{Imax}} \)) showed a clear reduction in both indexes of oxidative stress after the combination of the training period and NAC supplementation. These findings led us to the conclusion that, in our canine IRB model, NAC have beneficial antioxidant effects by reducing the high-intensity inspiratory loading-induced diaphragmatic oxidative stress. In line with this, Travaline et al. (45) already showed that, in healthy humans, NAC treatment significantly attenuated the development of resistive loading-induced diaphragm low-frequency fatigue. The reported reductions in transdiaphragmatic pressure after diaphragm fatigue were also partially blunted by NAC administration in the model in question (45). These observations, along with the findings encountered in our study, suggest that, in these specific models, NAC effects on the contracting diaphragm are probably two-fold: improvements in both diaphragm strength and endurance are likely to be partly due to NAC-induced reductions in ROS-mediated effects on key muscle proteins probably involved in contractility.

As an antioxidant, NAC can directly inactivate electrophils through either reduction or conjugation reactions. Furthermore, as a sulfhydryl-containing molecule, it can serve as a source of cysteine for the de novo synthesis of glutathione, which is the main mechanism of action in the liver toxicity induced by acetaminophen overdose (9). Numerous studies have focused their attention on the assessment of NAC effects on skeletal muscles under various conditions. For instance, Supinski et al. (40, 41) showed that NAC administration attenuated the inspiratory loading-induced reduction in diaphragmatic GSH, as well as the rate of respiratory failure development in rats. Furthermore, the same investigators (40) also demonstrated that NAC administration to rats subjected to loaded breathing reduced the in vitro fatigability of diaphragm fibers. Other studies have also reported that the rate of development of muscle fatigue during repetitive isometric contractions was attenuated by NAC administration in both experimental animal (17, 36) and human studies (34), and it also improved the endurance time of the quadriceps muscles in patients with severe COPD (18). In another study, Sen et al. (35) demonstrated that oral administration of NAC 2 days before and on the same day of the exercise test clearly induced a significant decrease in oxidized glutathione and lipid peroxidation in rat blood.

On the grounds that high-intensity exercise training may lead to the development of increased levels of oxidative stress in muscles, antioxidants other than NAC, such as selenium, ascorbic acid, and \( \alpha \)-tocopherol, were also shown to abrogate both the downregulation of nonenzymatic antioxidants and systemic oxidative stress development (27). Based on the existing literature and on our present findings, it can be stated that the intensity of the exercise training may have a wide range of effects on muscles: from a nontraining effect, with no significant oxidative stress occurring, to cases where a clear training effect is achieved along with oxidative stress-induced cell and tissue damage. Clearly, further studies are required to determine the exact level of loading after which oxidative stress might eventually develop in the respiratory muscles of COPD patients undergoing a high-intensity inspiratory training program, where antioxidant supplementation might prove beneficial.

In conclusion, the development of diaphragm oxidative stress in response to high-intensity inspiratory loads in dogs...
can be neutralized by NAC administration during the IRB period. This might have future therapeutic implications in the management of COPD patients, who might benefit from a combined therapy involving high-intensity respiratory muscle training along with antioxidant supplementation.

ACKNOWLEDGMENTS

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GRANTS

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