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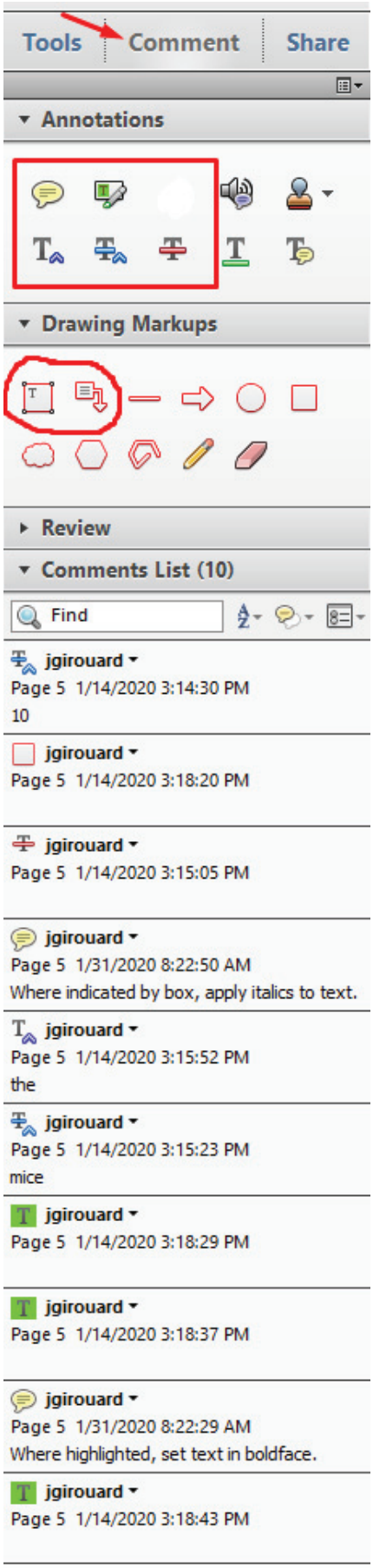

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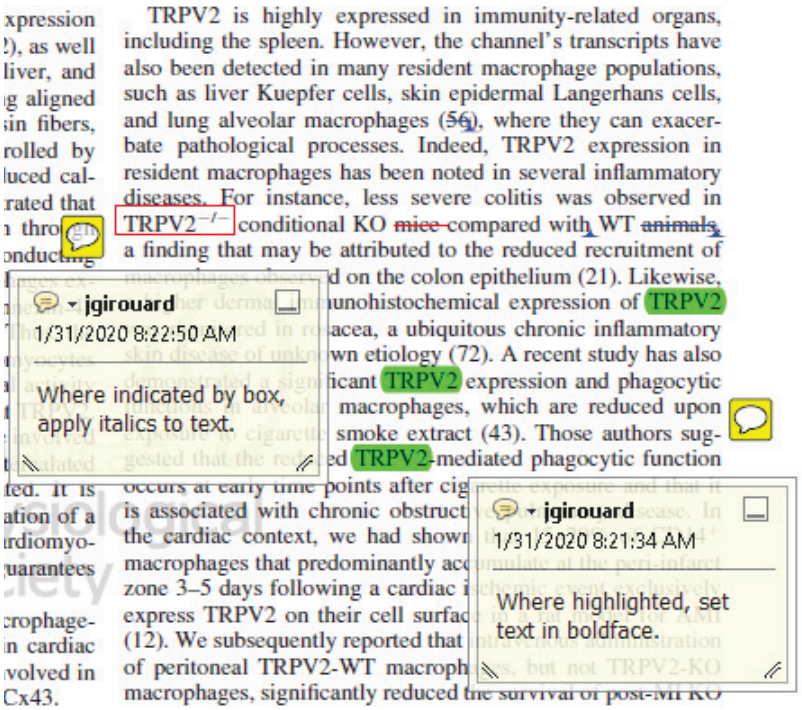

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RESEARCH ARTICLE

Inspiratory flow-resistive breathing, respiratory muscle-induced systemic oxidative stress, and diaphragm fatigue in healthy humans

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Briskey DR, Vogel K, Johnson MA, Sharpe GR, Coombes JS, Mills DE. Inspiratory flow-resistive breathing, respiratory muscle-induced systemic oxidative stress, and diaphragm fatigue in healthy humans. *J Appl Physiol* 129: 000–000, 2020. First published June 18, 2020; doi:10.1152/japplphysiol.00091.2020.—We questioned whether the respiratory muscles of humans contribute to systemic oxidative stress following inspiratory flow-resistive breathing, whether the amount of oxidative stress is influenced by the level of resistive load, and whether the amount of oxidative stress is related to the degree of diaphragm fatigue incurred. Eight young and healthy participants attended the laboratory for four visits on separate days. During the first visit, height, body mass, lung function, and maximal inspiratory mouth and transdiaphragmatic pressure (P_{dimax}) were assessed. During *visits 2–4*, participants undertook inspiratory flow-resistive breathing with either no resistance (control) or resistive loads equivalent to 50 and 70% of their P_{dimax} (P_{dimax}50% and P_{dimax}70%) for 30 min. Participants undertook one resistive load per visit, and the order in which they undertook the loads was randomized. Inspiratory muscle pressures were higher ($P < 0.05$) during the 5th and final min of P_{dimax}50% and P_{dimax}70% compared with control. Plasma F_2-isoprostanes increased ($P < 0.05$) following inspiratory flow-resistive breathing at P_{dimax}70%. There were no increases in plasma protein carbonyls or total antioxidant capacity. Furthermore, although we evidenced small reductions in transdiapragmaic twitch pressures (P_{diTW}) after inspiratory flow-resistive breathing at P_{dimax}50% and P_{dimax}70%, this was not related to the increase in plasma F_2-isoprostanes. Our novel data suggest that it is only when sufficiently strenuous that inspiratory flow-resistive breathing in humans elicits systemic oxidative stress evidenced by elevated plasma F_2-isoprostanes, and based on our data, this is not related to a reduction in P_{diTW}.

NEW & NOTEWORTHY We examined whether the respiratory muscles of humans contribute to systemic oxidative stress following inspiratory flow-resistive breathing, whether the amount of oxidative stress is influenced by the level of resistive load, and whether the amount of oxidative stress is related to the degree of diaphragm fatigue incurred. It is only when sufficiently strenuous that inspiratory flow-resistive breathing elevates plasma F_2-isoprostanes, and our novel data show that this is not related to a reduction in transdiaphragmatic twitch pressure.

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diaphragm fatigue; humans; inspiratory flow resistive breathing; oxidative stress; respiratory muscles

INTRODUCTION

Increased respiratory muscle work is encountered during strenuous whole body exercise, asthma attacks, and exacerbations of chronic obstructive pulmonary disease and during periods of imposed flow-resistive breathing (22, 40, 49). Inspiratory flow-resistive breathing requires inspiration against a variable diameter orifice that results in increased diaphragm and accessory muscle force production to overcome the resistive load imposed.

Reactive oxygen species (ROS) form as products under normal physiological conditions due to the partial reduction of molecular oxygen (42, 43). Oxidative stress is defined as macromolecular oxidative damage along with a disturbance of redox signaling and control and usually results from either excessive ROS production, mitochondrial dysfunction, impaired antioxidant system, or a combination of these factors (42, 43). ROS produced under oxidative stress can damage all cellular biomolecules, including lipids, proteins, carbohydrates, and DNA (42, 43). The measurement of oxidative stress in vivo is difficult, as ROS are highly reactive and/or have a very short half-life (<1 s for some), so they can be estimated from changes in free radicals, radical-mediated damages to lipids, proteins, and nucleic acids, and antioxidant enzyme activity or concentration (39). Therefore, a battery of different markers that are reliable are essential to summarize the effects of oxidative stress (39). Systemic measurements can include protein carbonyls as a marker of protein oxidation, total antioxidant capacity for exogenous antioxidant utilization, and F_2-isoprostanes for lipid peroxidation, which is widely regarded as a gold standard because of their chemical stability and prevalence in all human tissues and biological fluids (35, 38, 64).

Oxidative stress is elevated in the diaphragms of animals exposed to inspiratory flow-resistive breathing, and the amount of oxidative stress is positively associated with the level of resistive load (1, 7, 12, 13, 51). Supplementation with a combination of antioxidants also reduces the response of plasma cytokines in humans following 45 min of inspiratory flow-resistive breathing undertaken at 75% of maximal inspira-

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tory mouth pressure (P_{Imax}) (57). Mild and acute exposure to exogenous ROS generally increase the muscles' ability to generate force (11, 24, 61), whereas stronger or prolonged exposure as occurs during flow resistive breathing (1, 7, 12, 13, 51) significantly reduces respiratory muscle force generation (19, 45). Indeed, in vitro studies have shown that ROS released from diaphragm fibers promotes low-frequency diaphragm fatigue (5, 25, 46, 52), which in humans can be measured objectively using phrenic nerve stimulation (29). Supplementation with the antioxidant *N*-acetylcysteine before inspiratory resistive breathing or heavy exercise may also attenuate respiratory muscle fatigue (21, 56). In patients with severe chronic obstructive pulmonary disease, diaphragm fatigue can contribute to muscle dysfunction (6, 17) and the development of respiratory failure (41). Taken together, these animal in vitro and supplementation studies indicate that resistive breathing leads to increased oxidative stress, that the amount of oxidative stress is associated with the level of resistive load, and that this is related to diaphragm fatigue. The findings for the animal and in vitro studies, however, have not been repeated in humans.

Accordingly, we questioned whether the respiratory muscles of humans contribute to systemic oxidative stress following inspiratory flow-resistive breathing, whether the amount of oxidative stress is influenced by the level of resistive load, and whether the amount of oxidative stress is related to the degree of diaphragm fatigue incurred. We utilized a battery of oxidative stress markers, including plasma F_2-isoprostanes, protein carbonyls, and total antioxidant capacity, and objectively measured low-frequency diaphragm fatigue using phrenic nerve stimulation. We hypothesized that oxidative stress would be increased following exposure to inspiratory flow resistive breathing and greater with increased resistive loads, and the increase in oxidative stress measures would be related to the degree of diaphragm fatigue incurred.

METHODS

Participants. Five males and three females that were free from respiratory disorders and who provided written, informed consent participated in the study (Table 1). A self-reporting medical questionnaire confirmed that participants were free from illness and injury and not taking any medication and/or antioxidant supplements during the study. Each participant completed a 24-h diet record before their first trial, which was then replicated before all subsequent trials. Participants reported that they were recreationally active, which included playing sports and participating in aerobic and resistance exercise 3–4 days/wk. Throughout the study, participants were instructed to adhere to their habitual exercise training regimens and to not increase or decrease their volume of exercise. They were also instructed to not engage in any strenuous exercise the day preceding and the day of a trial. Participants arrived at the laboratory 4 h postprandially, having abstained from alcohol and caffeine in the 24 h before testing. All study procedures were approved by the University of Southern Queensland Research Ethics Committee, which adheres to the Declaration of Helsinki.

Table 1. *Participant anthropometrics and respiratory function*

	Males (n = 5)	Females (n = 3)
Age, yr	26 ± 5	26 ± 4
Height, cm	176 ± 7	164 ± 9
Body mass, kg	91 ± 8	70 ± 9
FVC, L	5.01 ± 0.79	4.30 ± 0.94
FVC, % predicted	101 ± 4	109 ± 17
FEV_1, L	4.11 ± 0.76	3.59 ± 0.73
FEV_1, % predicted	99 ± 12	106 ± 15
FEV_1/FVC, %	79.8 ± 7.0	80.7 ± 1.9
FEV_1/FVC, % predicted	96 ± 8	97 ± 3
P_{Imax}, cmH_2O	101 ± 35	117 ± 48
P_{Imax}, %predicted	92 ± 22	131 ± 14
P_{dimax}, cmH_2O	90 ± 27	98 ± 24

Values are means ± SD. FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; P_{dimax}, maximal transdiaphragmatic pressure; P_{Imax}, maximal inspiratory mouth pressure. Predicted values for pulmonary volumes and capacities are from Quanjer et al. (44) and for P_{Imax} are from Wilson et al. (63).

Experimental design. Participants attended the laboratory for four visits on separate days. Each laboratory visit was separated by a minimum of 48 h and took place at the same time of day. During the first visit, height, body mass, lung function, P_{Imax}, and maximum transdiaphragmatic pressure (P_{dimax}) were assessed according to published guidelines and statements (4, 32). Subsequently, participants were familiarized with all other measurements and inspiratory flow-resistive breathing. During *visits 2–4*, participants undertook inspiratory flow-resistive breathing with either no resistive load (control) or loads equivalent to 50 and 70% of their P_{dimax} (P_{dimax}50% and P_{dimax}70%) for 30 min; these reflected "low," "moderate," and "heavy" flow-resistive loads, respectively. Participants undertook one resistive load per visit, and the order in which they undertook the loads was randomized. Participants were naïve to the prescribed resistive load, and the resistive loading device was hidden from view. The resistive loads were chosen because, through our pilot studies and other work (3, 8, 23), they were sustainable for 30 min and would elicit varying degrees of diaphragm fatigue. Transdiaphragmatic twitch pressures (P_{diTW}) were measured at baseline, at 5 min, at the end, and >30 min after the completion of inspiratory flow-resistive breathing trials. Blood samples for oxidative stress measures, respiratory pressures, cardiorespiratory data, and rating of perceived dyspnea [RPD; Borg modified CR10 scale (9) as a measure of the effort required to overcome the resistance] were measured at rest, during the 5th min, in the final min, and >30 min after the completion of inspiratory flow-resistive breathing trials.

Pulmonary function and maximal inspiratory mouth and transdiaphragmatic pressure. Pulmonary function was assessed using a calibrated testing system (JAEGER Vyntus; CareFusion, San Diego, CA). P_{Imax} and P_{dimax} were assessed using the same experimental equipment used for the inspiratory flow-resistive breathing. Participants inspired through a two-way nonrebreathing valve (Model 2730; Hans Rudolph, Shawnee Mission, KS), with resistance provided by a custom-built variable-sized aperture with a length of 2 mm placed into the inspiratory port. To assess P_{Imax} and P_{dimax}, the aperture was closed and incorporated a 1-mm orifice to prevent glottic closure during inspiratory efforts. Mouth pressure was measured using a calibrated transducer (MLT844; AD Instruments, Dunedin, New Zealand) inserted into the mouth port of the two-way nonrebreathing valve. Inspiratory maneuvers for P_{Imax} and P_{dimax} were performed while seated, initiated from residual volume, and sustained for ≥1 s. Repeat efforts separated by 30 s were performed until three serial measures differed by ≤10% or 10 cmH_2O, whichever was smallest (33). The highest value recorded was used for subsequent analysis.

Respiratory muscle pressures. Respiratory muscle pressures were quantified by measuring esophageal (P_e) and gastric (P_g) pressures, using two 10-cm balloon-tipped latex catheters (Model 47-9005; Ackrad Laboratories, Cranford, NJ) that were attached to calibrated differential pressure transducers (MLT844; AD Instruments, Dunedin, New Zealand) (33, 34). The esophageal and gastric balloons were filled with 1 and 2 mL of air, respectively. During the first experimental trial, the distance from the tip of the nares to the most distal point of the catheters was recorded and replicated in subsequent trials. P_{di} was calculated automatically using LabChart Pro software (AD Instruments, Bella Vista, Australia) by subtracting P_e from P_g. To

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estimate respiratory muscle energy expenditure (16), P_{di} and P_e were integrated over the period of inspiratory flow and multiplied by breathing frequency and labeled the diaphragm pressure-time product (PTP_{di}) and the inspiratory muscle pressure-time product (PTP_e), respectively. Nonphysiological flows and pressures that resulted from swallowing, coughing, and breath holding were visually identified and removed. Raw pressure data were recorded continuously at 200 Hz using a 16-channel analog-to-digital data acquisition system (PowerLab 16/35; AD Instruments).

Cervical magnetic phrenic nerve stimulation. Cervical magnetic phrenic nerve stimulation was applied via a double 70-mm coil connected to a Magstim 200^2 stimulator (Magstim, Dyfed, UK). Participants initially rested for 20 min to minimize postactivation potentiation. Subsequently, while participants were sat upright and the neck flexed, the coil was placed over the midline between the 5th (C5) and 7th (C7) cervical vertebrae (50). The optimal coil position was defined as the vertebral level that when stimulated at 50% of maximum stimulator output evoked the highest P_{diTW}. This location was marked with indelible ink and used for subsequent stimulations. During stimulations, participants wore a noseclip and before stimulation were instructed to hold their breathing effort at functional residual capacity, which was inferred from visual feedback of P_e. To determine supramaximal phrenic nerve stimulation, three single twitches were obtained every 30 s at intensities of 50, 60, 70, 80, 85, 90, 95, and 100% of maximal stimulator output. A plateau in P_{diTW} responses with increasing stimulation intensities indicated maximum depolarization of the phrenic nerves.

Maximum P_{diTW} was assessed at each measurement point every 30 s using three stimuli at 100% of maximal stimulator output. Additionally, P_{diTW} at each measurement point was followed by the assessment of the potentiated P_{diTW} response. Participants performed a 3-s maximal Müeller maneuver, and ~5 s later a single stimuli was delivered. This procedure was repeated six times, with each measure separated by 30 s. The average of the three individual nonpotentiated P_{diTW} responses and the final three potentiated P_{diTW} responses was used for analysis. This procedure was undertaken at baseline, after 5 min of inspiratory flow-resistive breathing, at the end, and >30 min after the completion of inspiratory flow-resistive breathing trials.

Inspiratory flow resistive breathing. Following cervical magnetic phrenic nerve stimulation, participants remained seated and continued to wear a nose clip. Resting measurements were collected for 5 min while participants breathed through a mouthpiece to a two-way nonrebreathing valve. For P_{dimax}50% and P_{dimax}70% trials, the custom-built variable-sized aperture was adjusted to narrow its diameter. This was continued until participants could match the target P_{di}, which was displayed on a screen in front of them and monitored continuously to ensure adequate pressure development. Participants were asked to maintain tidal volumes close to those achieved at rest, and the proportion of P_{di} contributed by P_g and P_e was not controlled. In the event that the partial pressure of end-tidal carbon dioxide fell from resting concentrations, carbon dioxide was added to the inspirate to maintain isocapnia and avoid the deleterious effects of hypocapnia (e.g., light-headedness, confusion, paresthesia, tetany). This occurred in two participants after ~3 min during the P_{dimax}70% trial, when end-tidal carbon dioxide partial pressure fell below 30 mmHg. Once isocapnia was restored, these participants were coached to maintain expired volumes close to that achieved at rest to prevent further episodes of hypocapnia. Participants maintained a breathing frequency of 15 breaths/min and a duty cycle of 0.5 by listening to a computer-generated audio signal with distinct inspiratory and expiratory tones.

Cardiorespiratory responses. Standard ventilatory responses were measured on a breath-by-breath basis using a metabolic cart (JAEGER Vyntus; CareFusion, San Diego, CA), with the flow sensor inserted into the mouth port of the two-way nonrebreathing valve. Cardiac frequency and estimated arterial oxygen saturation were measured using a monitor (Polar T34; Polar Electro, Kempele, Finland) and fingertip pulse oximeter (Radical-7 Pulse CO-Oximeter; Masimo Corporation, Irvine, CA), respectively.

Blood sampling. Ten milliliters of venous blood was sampled at each time point from an antecubital vein via an indwelling 21-G cannula. Blood was transferred into precooled tubes containing K_3E EDTA (BD Vacutainers, Franklin Lakes, NJ). Samples were stored on ice before being centrifuged at 2,500 rpm for 10 min at 4°C. Plasma was then aliquoted and stored at −80°C until biochemical assays were performed.

Plasma F_2-isoprostanes. Samples were analyzed in duplicate using an optimized method for quantification of total F_2-isoprostanes using gas chromatography-tandem mass spectrometry (10). Isoprostanes were extracted from plasma after saponification with methanolic NaOH. Samples were spiked with 8-iso-PGF2α-d4 (Cayman Chemicals, Ann Arbor, MI) as an internal standard and incubated at 42°C for 60 min. Samples were then acidified to pH 3 with hydrochloric acid, and hexane was added and samples were mixed for 10 min before centrifugation. The supernatant was removed and the remaining solution extracted with ethyl acetate and dried under nitrogen. Samples were reconstituted with acetonitrile, transferred into vials with silanized glass inserts, and dried. Derivatization with pentafluorobenzylbromide and diisopropylethylamine and incubation at room temperature for 30 min followed. Samples were then dried under nitrogen before pyridine, 99% bis(trimethylsilyl)trifluoroacetamide, and 1% trimethylchlorosilane were added and incubated at 45°C for 20 min. Finally, hexane was added and samples were mixed, and then 1 mL was injected for analysis using gas chromatography-mass spectrometry (Varian, Belrose, NSW, Australia) in negative chemical ionization mode. The laboratory coefficient of variation for this assay is 4.5%.

Plasma protein carbonyls. Protein carbonyls were analyzed using an adapted version of the methodology from Levine et al. (27). Duplicate plasma samples were incubated with 2,4-dinitrophenylhydrazine in 2.5 M hydrochloric acid (HCl) for 1 h in the dark. Plasma blanks were incubated in 2.5 M HCl only. All samples were then precipitated with 20% trichloroacetic acid (TCA) on ice and centrifuged at 10 000 *g* for 10 min. Supernatants were discarded and the pellets resuspended in 10% TCA and again centrifuged as above. Supernatants were removed, and the pellets resuspended in 1:1 ethanol-ethylacetate solution. After centrifugation as above, the pellets were washed twice more with the ethanol-ethylacetate solution. Pellets were then resuspended in 6 M guanidine hydrochloride solution, and 220 mL of samples and blanks was transferred to microplate wells and absorbance read at 370 nm with correction at 650 nm using a microplate reader (Fluostar Optima; BMG Labtech, Offenburg, Germany). Protein carbonyl concentration was normalized to plasma protein content measured using a Pierce BCA protein assay kit (Thermo Scientific). The laboratory coefficient of variation for this assay is 11.9%.

Plasma total antioxidant capacity. Total antioxidant capacity was measured using a modified version (36) of an assay previously described (47, 62) and adapted for a Cobas Mira autoanalyzer (Cobas Mira; Roche Diagnostica). Briefly, plasma was incubated with metmyoglobin and 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). After incubation, hydrogen peroxide was added, and the sample was incubated again. Absorbance was measured spectrophotometrically to determine total antioxidant capacity. The laboratory coefficient of variation for this assay is 1.9%.

Statistical analysis. Statistical analyses were performed using SPSS for Windows (IBM, Chicago, IL). An initial power calculation was performed on the basis of previous work (36) showing that eight participants would be required to demonstrate a 10% increase in plasma F_2-isoprostanes with an α of 0.05 and power 0.8. All data were confirmed as parametric via a Shapiro-Wilk test for normality. The data from supramaximal phrenic nerve stimulation was analyzed using a one-way ANOVA. The data from the three inspiratory flow-resistive breathing trials were analyzed using a two-way repeat-

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ed-measures ANOVA procedure to determine the effects of "time" (rest/baseline, 5th min, final min/end, and >30 min) and "resistive load" (control, $P_{dimax}50\%$, and $P_{dimax}70\%$). Following significant time × resistive load interaction effects, planned pairwise comparisons were made using the Bonferroni method. Pearson's product moment correlation coefficient was used to examine the relationship between the degree of oxidative stress incurred and *1*) flow-resistive load and *2*) degree of diaphragm fatigue incurred. Reliability was assessed using a coefficient of variation calculated from a pooled mean of all trials. Statistical significance was set at $P < 0.05$. Results are presented as means ± SD.

RESULTS

Cardiorespiratory and perceptual responses. Inspiratory muscle pressures and estimates of respiratory muscle energy expenditure during inspiratory flow-resistive breathing are shown in Table 2 and Fig. 1, respectively. PTP_{di}, PTP_e, P_{Ipeak}, P_{epeak}, and P_{dipeak} were higher during the 5th and final min of $P_{dimax}50\%$ and $P_{dimax}70\%$ compared with control. The relative contribution of the diaphragm to the inspiratory muscle pressure/time product (PTP_{di}/PTP_e) was lower during the 5th min of $P_{dimax}50\%$ compared with control (Fig. 1). RPD was elevated during the 5th and final min of $P_{dimax}70\%$ compared with both $P_{dimax}50\%$ and control (Table 2). Duty cycle was increased during the 5th and final min of $P_{dimax}70\%$ and 5th min of $P_{dimax}50\%$ compared with control. There was a time × resistive load interaction effect ($P = 0.003$) for cardiac frequency (Table 2) but no pairwise differences. There were no differences between control, $P_{dimax}50\%$ and $P_{dimax}70\%$ for

Table 2. *Cardiorespiratory and perceptual responses to inspiratory flow-resistive breathing for control and at $P_{dimax}50\%$ and $P_{dimax}70\%$*

Variable (Resistive Load)	Rest	5th Min	Final Minute	>30 Min
P_{Ipeak}, cmH_2O				
Control	−1.2 ± 0.2	−1.2 ± 0.2	−1.2 ± 0.2	−1.2 ± 0.2
$P_{dimax}50\%$	−1.1 ± 0.4	−24.7 ± 14.1*	−34.5 ± 22.0*	−1.1 ± 0.4
$P_{dimax}70\%$	−1.3 ± 0.3	−35.2 ± 22.1*	−42.1 ± 25.7*	−1.3 ± 0.4
P_{epeak}, cmH_2O				
Control	−10.0 ± 2.0	−9.5 ± 1.5	−9.3 ± 1.3	−10.0 ± 2.0
$P_{dimax}50\%$	−9.6 ± 2.5	−28.9 ± 12.2*	−37.3 ± 19.9*	−9.8 ± 3.5
$P_{dimax}70\%$	−9.4 ± 2.4	−37.4 ± 19.8*	−42.6 ± 20.2*	−9.2 ± 1.4
P_{dipeak}, cmH_2O				
Control	28.5 ± 5.3	27.6 ± 5.4	26.6 ± 4.6	30.3 ± 5.9
$P_{dimax}50\%$	27.1 ± 7.8	47.4 ± 11.5*	48.5 ± 11.4*	26.6 ± 6.6
$P_{dimax}70\%$	32.4 ± 6.2	63.2 ± 17.1*	60.2 ± 11.6*	30.4 ± 5.0
RPD				
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
$P_{dimax}50\%$	0.0 ± 0.0	1.8 ± 1.3	2.8 ± 1.4*	0.1 ± 0.2
$P_{dimax}70\%$	0.0 ± 0.0	4.7 ± 2.4*†	6.5 ± 2.6*†	0.3 ± 0.4*
$\dot{V}_E$, L/min				
Control	9.0 ± 1.6	11.0 ± 7.3	9.9 ± 4.0	9.3 ± 2.5
$P_{dimax}50\%$	10.2 ± 2.8	10.8 ± 2.9	11.8 ± 4.2	9.8 ± 2.4
$P_{dimax}70\%$	8.6 ± 2.2	11.1 ± 3.9	9.6 ± 1.6	9.8 ± 2.7
f_B, breaths/min				
Control	16 ± 5	15 ± 0	15 ± 1	14 ± 4
$P_{dimax}50\%$	14 ± 3	15 ± 0	15 ± 0	15 ± 5
$P_{dimax}70\%$	16 ± 7	14 ± 1	15 ± 0	15 ± 5
V_T, L				
Control	0.68 ± 0.11	0.88 ± 0.58	0.80 ± 0.32	0.88 ± 0.23
$P_{dimax}50\%$	0.95 ± 0.31	0.88 ± 0.24	0.94 ± 0.34	0.89 ± 0.41
$P_{dimax}70\%$	0.71 ± 0.25	0.98 ± 0.29	0.78 ± 0.13	0.86 ± 0.32
T_I/T_{TOT}				
Control	0.44 ± 0.04	0.45 ± 0.04	0.44 ± 0.04	0.44 ± 0.04
$P_{dimax}50\%$	0.43 ± 0.04	0.52 ± 0.06*	0.50 ± 0.08	0.43 ± 0.05
$P_{dimax}70\%$	0.42 ± 0.06	0.54 ± 0.06*	0.55 ± 0.07*	0.43 ± 0.03
f_C, beats/min				
Control	65 ± 9	66 ± 9	64 ± 11	65 ± 11
$P_{dimax}50\%$	70 ± 16	75 ± 14	75 ± 14	67 ± 17
$P_{dimax}70\%$	68 ± 13	77 ± 12	80 ± 13	66 ± 11
Sp_{O_2}, %				
Control	97.1 ± 1.2	97.6 ± 1.1	97.4 ± 1.2	98.0 ± 0.9
$P_{dimax}50\%$	97.6 ± 0.9	97.6 ± 1.1	98.1 ± 0.7	97.9 ± 0.7
$P_{dimax}70\%$	97.5 ± 1.1	98.0 ± 0.6	97.1 ± 1.4	98.2 ± 0.7
$P_{ET_{CO_2}}$, mmHg				
Control	36.3 ± 5.4	34.1 ± 8.0	34.5 ± 7.4	35.2 ± 5.4
$P_{dimax}50\%$	34.5 ± 4.5	34.0 ± 5.4	35.3 ± 5.1	34.8 ± 4.7
$P_{dimax}70\%$	34.9 ± 4.9	35.7 ± 9.4	35.0 ± 6.1	34.1 ± 3.6

Values are means ± SD. f_B, breathing frequency; f_C, cardiac frequency; $P_{dimax}50\%$, 50% of peak transdiaphragmatic pressure; $P_{dimax}70\%$, 70% of peak transdiaphragmatic pressure; P_{dipeak}, peak transdiaphragmatic pressure; P_{epeak}, peak esophageal pressure; $P_{ET_{CO_2}}$, end-tidal carbon dioxide pressure; P_{Ipeak}, peak inspiratory mouth pressure; RPD, rating of perceived dyspnea; Sp_{O_2}, estimated arterial oxygen saturation; T_I/T_{TOT}, duty cycle; $\dot{V}_E$, minute ventilation; V_T, tidal volume. *Significantly different from control at the same time point ($P < 0.05$); †significantly different from $P_{dimax}50\%$ at the same time point ($P < 0.05$).

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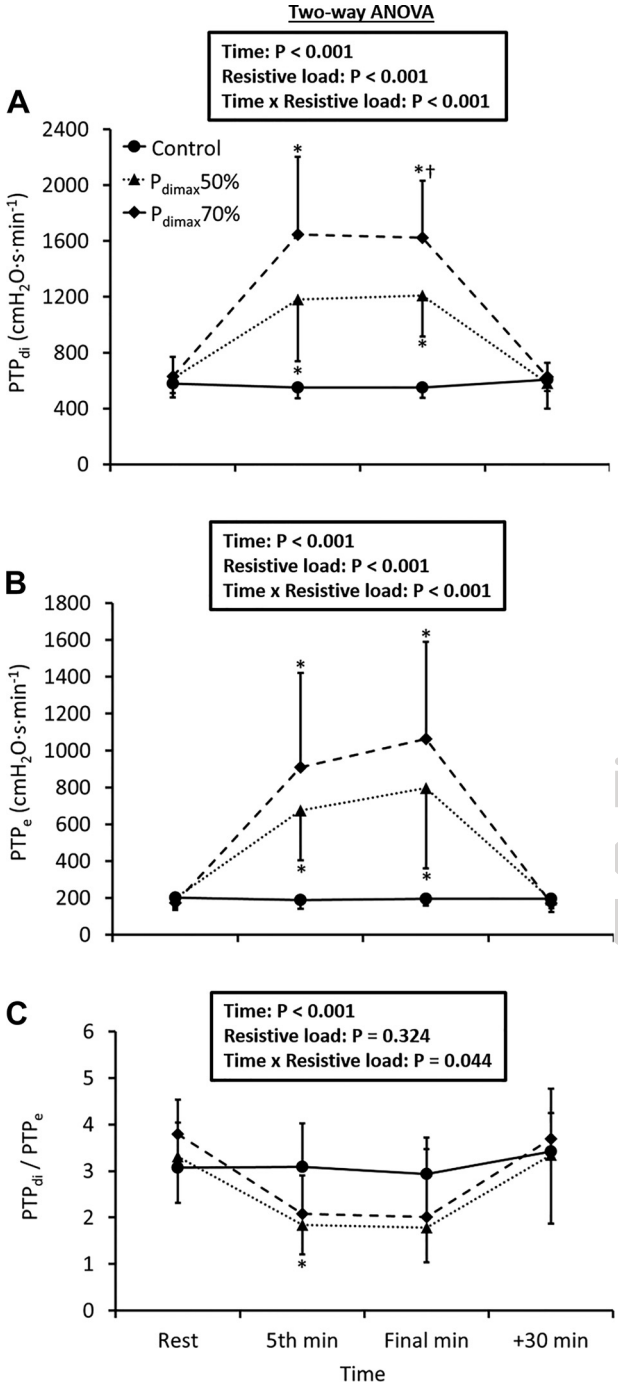

Fig. 1. Diaphragm pressure/time product (PTP_{di}; *A*), inspiratory muscle pressure/time product (PTP_e; *B*), and the relative contribution of diaphragm to the inspiratory muscle pressure/time product (PTP_{di}/PTP_e; *C*) responses to inspiratory flow-resistive breathing for control and at 50 ($P_{dimax}50\%$) and 70% of peak transdiaphragmatic pressure ($P_{dimax}70\%$). Values are means ± SD. *Significantly different from control ($P < 0.05$); †significantly different from $P_{dimax}50\%$ ($P < 0.05$).

minute ventilation, breathing frequency, tidal volume, estimated arterial oxygen saturation, and end-tidal carbon dioxide pressure (Table 2).

Markers of oxidative stress. Markers of oxidative stress during inspiratory flow resistive breathing are shown in Fig. 3. Plasma F_2-isoprostanes were higher during the final min and at >30 min of inspiratory flow-resistive breathing at $P_{dimax}70\%$ compared with control and $P_{dimax}50\%$ (Fig. 2). There was a main effect of time ($P = 0.048$) for total antioxidant capacity but no main effect of resistive load. There were no differences between control, $P_{dimax}50\%$, and $P_{dimax}70\%$ for plasma protein carbonyls and total antioxidant capacity.

Transdiaphragmatic twitch pressures. A plateau (i.e., no significant increase in amplitude with increasing stimulation

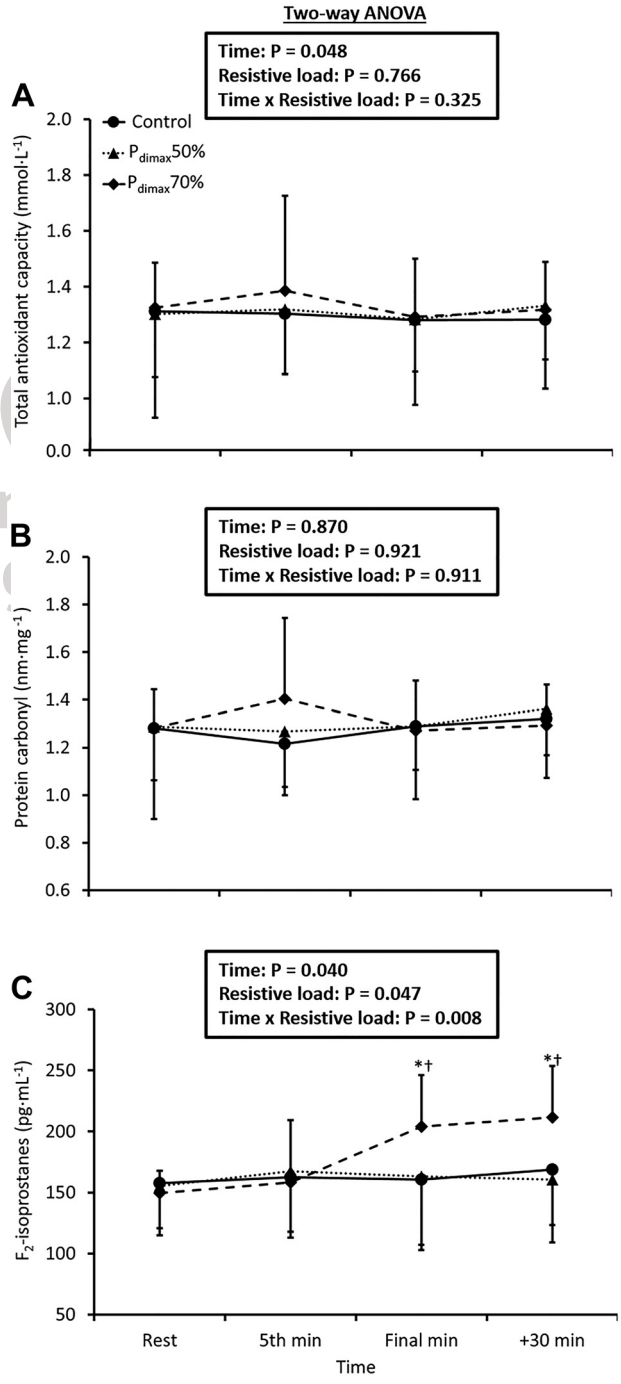

Fig. 2. Plasma total antioxidant capacity (*A*), protein carbonyl (*B*), and F_2-isoprostane (*C*) responses to inspiratory flow-resistive breathing for control and at 50 ($P_{dimax}50\%$) and 70% of peak transdiaphragmatic pressure ($P_{dimax}70\%$). Values are means ± SD. *Significantly different from control ($P < 0.05$); †significantly different from $P_{dimax}50\%$ ($P < 0.05$).

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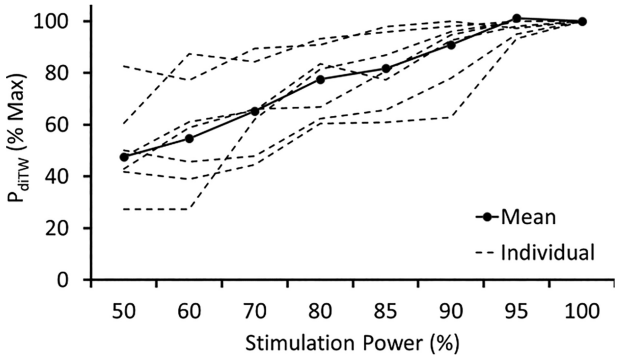

Fig. 3. Individual and group mean transdiaphragmatic twitch pressure (P_{diTW}) in response to cervical magnetic stimulation of increasing stimulation intensity.

intensity) in P_{diTW} amplitude (Fig. 3) was observed in response to supramaximal cervical magnetic phrenic nerve stimulation, indicating maximal depolarization of the phrenic nerves. The within- and between-coefficient of variation for P_{diTW} and potentiated P_{diTW} at rest was <5%. Absolute ($P = 0.03$) and relative potentiated P_{diTW} decreased ($P = 0.02$) following inspiratory flow-resistive breathing at $P_{dimax}50\%$ and $P_{dimax}70\%$. Compared with baseline, $P_{dimax}50\%$ and $P_{dimax}70\%$ were reduced at the end and at >30 min after inspiratory flow-resistive breathing (Fig. 4). There were no main effects of resistive load or time × resistive load interactions (Fig. 4).

Time course and relationship between markers of oxidative stress and diaphragm fatigue. Although the time course of the increase in plasma F_2-isoprostanes during inspiratory flow-resistive breathing at $P_{dimax}70\%$ corresponded with the decrease P_{diTW} (Fig. 5), there were no significant relationships between the individual percentage change from rest for plasma F_2-isoprostanes and percentage change from baseline for potentiated P_{diTW} after $P_{dimax}70\%$ (Fig. 6).

DISCUSSION

Main findings. The aim of this study was to examine whether the respiratory muscles of humans contribute to systemic oxidative stress following inspiratory flow-resistive breathing, whether the amount of oxidative stress is influenced by the level of resistive load, and whether the amount of oxidative stress is related to the degree of diaphragm fatigue incurred. The main finding was that the only measured marker of oxidative stress to increase was plasma F_2-isoprostanes following inspiratory flow-resistive breathing at $P_{dimax}70\%$. There were no increases in plasma protein carbonyls or total antioxidant capacity. Furthermore, although we evidenced small reductions in P_{diTW} after inspiratory flow-resistive breathing at $P_{dimax}50\%$ and $P_{dimax}70\%$, this was not related to the increase in plasma F_2-isoprostanes.

Markers of oxidative stress. We observed an increase in plasma F_2-isoprostanes following inspiratory flow-resistive breathing at $P_{dimax}70\%$ but not at $P_{dimax}50\%$. We chose to measure F_2-isoprostanes in blood, and because of their chemical stability and prevalence in all human tissues and biological fluids, this measurement is widely regarded as the gold standard for the assessment of oxidative stress (35, 38, 64). F_2-isoprostanes represent a marker of lipid peroxidation, and acute exercise and muscle contractions generally increase concentrations in skeletal muscle and plasma (37). Furthermore, F_2-isoprostanes are elevated in the diaphragms of rats exposed to prolonged periods of inspiratory flow-resistive breathing (51). Thus, we infer that the increase in plasma F_2-isoprostanes that we observed following inspiratory flow-resistive breathing at $P_{dimax}70\%$ are released from the contracting respiratory muscles into the systemic circulation. In contrary to our hypothesis, we did not see an elevation of plasma F_2-isoprostanes following inspiratory flow-resistive breathing at $P_{dimax}50\%$. This may be due to the intensity of the loading that was insufficient to observe increased appearance rates of ROS to exceed the ability of antioxidants to counteract their effects. Indeed, it has been reported previously that F_2-isoprostane concentrations are higher following high-intensity intermittent rather than constant load cycling exercise (14).

We did not observe an increase in plasma protein carbonyl concentration and total antioxidant capacity. Plasma protein carbonyl concentrations are a marker of protein oxidation. They are elevated in the diaphragms of rats when they are exposed to inspiratory flow-resistive breathing, and concentrations are higher after 8 and 12 days compared with 4 days (51). However, certain exercise conditions can result in a net de-

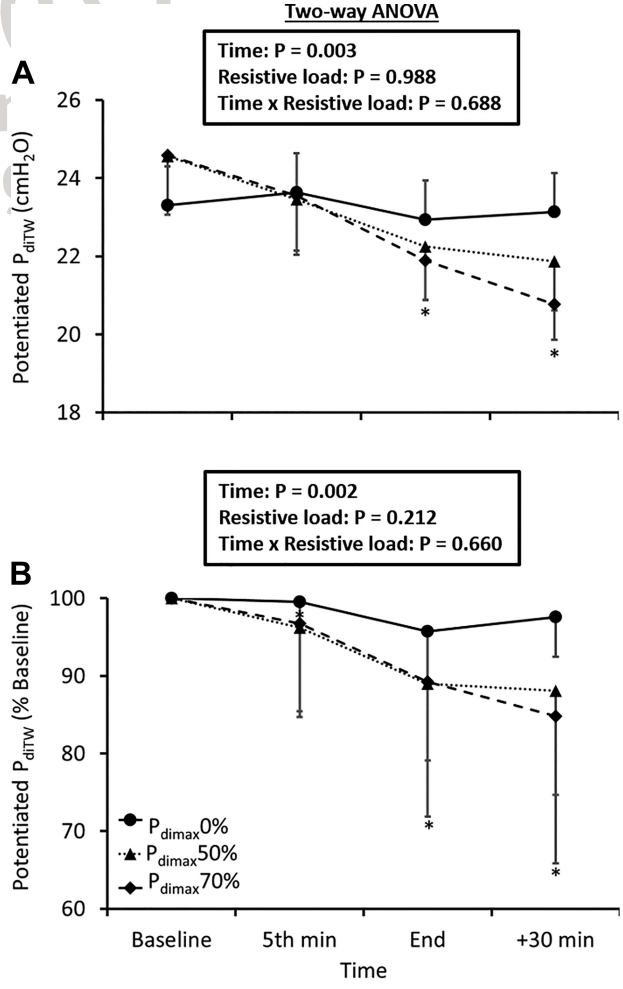

Fig. 4. Absolute (*A*) and relative (*B*) and potentiated transdiaphragmatic twitch pressure (P_{diTW}) responses to inspiratory flow-resistive breathing for control and at 50 ($P_{dimax}50\%$) and 70% of peak transdiaphragmatic pressure ($P_{dimax}70\%$). Values are means ± SD. *Significantly different from baseline for $P_{dimax}50\%$ and $P_{dimax}70\%$ ($P < 0.05$).

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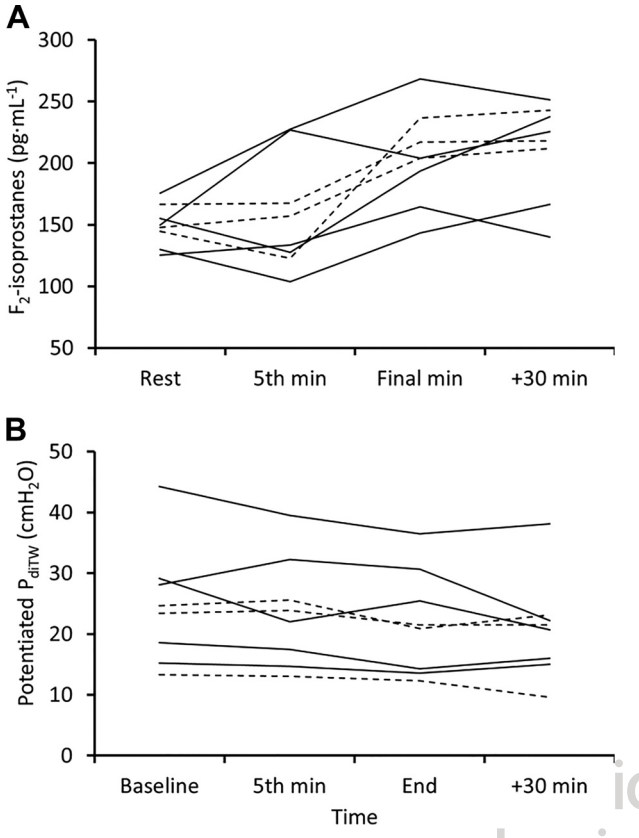

Fig. 5. Individual male (solid line) and female (dashed line) plasma F_2-isoprostanes (*A*) and absolute potentiated transdiaphragmatic twitch pressure (P_{diTW}; *B*) responses to inspiratory flow-resistive breathing at 70% of peak transdiaphragmatic pressure.

crease in plasma protein carbonyl concentrations, which occurs in parallel with increases in other biomarkers of oxidative stress. Greater inspiratory flow-resistive intensities and/or durations may be required to elicit increases in markers of oxidative stress. Exercise intensity (>70% maximal oxygen uptake) and prolonged duration (>60 min) appear to be the main contributing factors in the observed postexercise increases in plasma protein carbonyl concentration (59). However, it must be noted that whole body exercise engages a significantly greater muscle mass than inspiratory flow-resistive breathing. The factors influencing decreases in protein carbonyls are more difficult to interpret but likely involve the clearance of oxidized proteins from plasma, potentially by plasma proteasomes, excretion, or uptake into active tissues (59).

Total antioxidant capacity is a marker of exogenous antioxidant utilization (30). Other studies using maximal treadmill exercise have also found no changes to plasma total antioxidant capacity immediately postexercise (2, 15). However, others have observed significant increases at 30 min (58) and 1 h (60). Therefore, the timing of measurements may be important for total antioxidant capacity and plasma protein carbonyl measurements. For example, ~50 min of exercise resulted in a 32% increase in protein carbonyls 30 min postexercise and 94% 4 h later (31). Unfortunately, our experimental design did not allow us to take measurements beyond 30 min after inspiratory flow-resistive breathing, as we wanted to mirror the time course of the reduction in P_{diTW}. We acknowledge that this is a limitation of our study design, and future research would aim to undertake blood sampling at later time points. We must also note that whole body exercise engages a significantly greater muscle mass than inspiratory flow-resistive breathing.

Diaphragm fatigue and relationship between markers of oxidative stress. Similarly to others (19), we observed a reduction in potentiated and nonpotentiated P_{diTW} following inspiratory-resistive breathing that is indicative of low-frequency peripheral fatigue. The underlying mechanisms are thought to be reduced Ca^{2+} release from the sarcoplasmic reticulum, reduced Ca^{2+} sensitivity of the myofibrils, and/or damaged sarcomeres caused by overextension of the muscle fiber (20). Mild and acute exposure to exogenous ROS generally increases the muscles' ability to generate force (11, 24, 61), whereas stronger or prolonged exposure, as occurs during flow-resistive breathing (1, 7, 12, 13, 51), significantly reduces respiratory muscle force generation (19, 45). Indeed, in vitro studies have shown that ROS released from diaphragm fibers promotes low-frequency diaphragm fatigue (5, 25, 26, 46, 52). Supplementation with the antioxidant *N*-acetylcysteine before inspiratory-resistive breathing or heavy exercise may also attenuate respiratory muscle fatigue (21, 56). Therefore, we hypothesized that the amount of oxidative stress that we observed would be related to the degree of diaphragm fatigue incurred. However, although the time course of the increase in plasma F_2-isoprostanes during inspiratory flow-resistive breathing at P_{dimax}70% corresponded with the decrease P_{diTW}, there were no significant relationships between the absolute and relative changes in potentiated and nonpotentiated P_{diTW}. These indirect measures of lipid peroxidation and respiratory muscle force generation in our systemic in vivo experiment may not be strong enough to demonstrate significant relationships and warrant further experimentation. The source of the increase in plasma F_2-isoprostanes could also be the lung, as previous research had demonstrated that inspiratory-resistive breathing in animal models can lead to lung injury and oxidative stress (18, 53–55). This may also explain the lack of relationship between the increases in plasma F_2-isoprostanes and the reduction in P_{diTW}.

Methodological limitations. There are several methodological limitations to our study that need to be acknowledged. First,

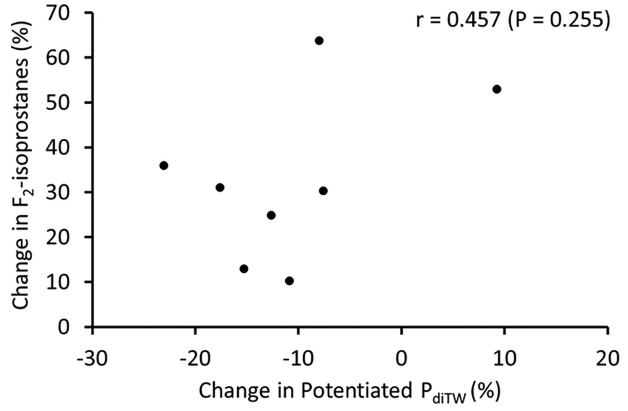

Fig. 6. %Change from rest to final minute for plasma F_2-isoprostanes vs. %change from baseline to end during inspiratory flow-resistive breathing at 70% of peak transdiaphragmatic twitch pressure (P_{diTW}).

sex differences occur in respiratory physiology (28, 48), and we acknowledge that our data may be confounded by including both male and female participants, although in a small sample size, the individual responses presented in Fig. 5 do not indicate that there are any sex differences, but this warrants further investigation. Second, we did not control the contributions of P_e to P_{di}, which allowed participants to possibly preferentially use their rib cage muscles rather than the diaphragm and to alternate between these muscle groups. Third, the outcome assessor was not blinded to the level of inspiratory resistance or other participant information, as they undertook both the experimental testing and analyses. Finally, as our oxidative stress markers are indirect measurements, they may have contributed to the lack of association with P_{diTW}.

Conclusion. In conclusion, inspiratory flow-resistive breathing undertaken at $P_{dimax}70\%$ induces significant increases in the gold standard oxidative stress biomarker plasma F_2-isoprostanes. However, there were no increases in plasma protein carbonyls or total antioxidant capacity, and although we evidenced small reductions in P_{diTW} after inspiratory flow-resistive breathing at $P_{dimax}50\%$ and $P_{dimax}70\%$, this was not related to the increase in plasma F_2-isoprostanes. Our novel data suggest that only when sufficiently strenuous does inspiratory flow-resistive breathing in humans elicit systemic oxidative stress, and based on our data, this is not related to diaphragm fatigue.

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GRANTS

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DISCLOSURES

AQ: 6 No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

D.B., M.A.J., G.R.S., J.S.C., and D.E.M. conceived and designed research; D.B., K.V., and D.E.M. performed experiments; D.B. and D.E.M. analyzed data; M.A.J., G.R.S., and D.E.M. interpreted results of experiments; D.E.M. prepared figures; M.A.J., G.R.S., and D.E.M. drafted manuscript; K.V., M.A.J., G.R.S., J.S.C., and D.E.M. edited and revised manuscript; K.V., AQ: 7 M.A.J., G.R.S., J.S.C., and D.E.M. approved final version of manuscript.

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